Prevalence of *Listeria* pathogens in effluents of some wastewater treatment facilities in the Eastern Cape Province of South Africa

By

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DECLARATION

I, the undersigned, declared that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in th Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my orignal work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

Name:_____

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Date:_____

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Dedication

This thesis is

Dedicated to my beloved wife

Diana Oghoifo Odjadjare.

LIST OF ABBREVIATIONS

AEMREG	-	Applied and Environmental Microbiology Research Group
AIDS	-	Acquired Immune Deficiency Syndrome
ANOVA	-	Analysis of Variance
ATCC	-	American Typed Culture Collection
BOD	-	Biological Oxygen Demand
CAMP	-	Christie Atkins Munch Petersen
CCME	-	Canadian Council of Ministers of the Environment
CFU	-	Colony Forming Unit
CLSI	-	Clinical and Laboratory Standard Institute
COD	-	Chemical Oxygen Demand
DNA	-	Deoxyribonucleic Acid
DO	-	Dissolved Oxygen
DP	-	Discharge Point
DW	-	500 m Downstream of Discharge Point
DWAF	-	Department of Water Affairs and Forestry
EC	-	Electrical Conductivity
EPA	-	Environmental Protection Agency
ELISA	-	Enzyme-Linked Immunosorbent Assay
et al	-	(*et alii*) and others
FISH	-	Fluorescent *In Situ* Hybridization
FAO	-	Food and Agricultural Organisation
FE	-	Final Effluent
HIV	-	Human Immunodeficiency Virus
LCA	-	*Listeria* Chromogenic Agar

MCL	-	Maximum Contaminat Levels
MLST	-	Multi-Locus Sequence Typing
MPN	-	Most Probable Number
No	-	Number
NPDES	-	National Pollutant Discharge Elimination System
NRC	-	National Research Council
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PFGE	-	Pulse –Field Gel Electrophoresis
RBS	-	Rotating Biological Contractors
S.D.	-	Standard Deviation
SANCOR	-	South African National Committee for Oceanographic Research
SPSS	-	Statistical Package for the Social Sciences
TDS	-	Total Dissolved Oxygen
TTC	-	Thermotolerant Coliforms
UNCSD	-	United Nations Commission on Sustainable Development
UNEP	-	United Nations Environmental Programme
UP	-	500 m Upstream of Discharge Point
USA	-	United States of America
USEPA	-	United States Environmental Protection Agency
VBNC	-	Viable But Non-Culturable
WHO	-	World Health Organisation

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GENERAL ABSTRACT

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Wastewater discharges may contain health compromising pathogens and carcinogenic and/or chemical substances that could compromise the public health and impact negatively on the environment. The present study was conducted between August 2007 and July 2008 to evaluate the *Listeria* abundance (as free-living and plankton associated species) and physicochemical qualities of the final effluents of three wastewater treatment facilities in the Eastern Cape Province of South Africa selected to represent typical urban, peri-urban and rural communities and the impact of the discharged final effluents on their respective receiving watershed, as well as to elucidated the *in vitro* antibiotic susceptibilities and resistance genes profile of *Listeria* species isolated from the final effluents. The suitability of the secondary effluent of the urban treatment facility (as a case study) for use in agriculture and aquaculture with reference to recommended standards was also determined. Wastewater samples were collected from the raw sewage, secondary effluent, final treated effluent, discharge point, 500 m upstream discharge point, and 500 m downstream discharge point from all three locations on a monthly basis throughout the study period.

Listeria abundance in the final effluents and the receiving watersheds varied between 2.9×10^{0} and 3.52×10^{5}cfu/ml across the sampled locations. Free-living listerial density across the sampled locations ranged between 0 and 3.2×10^{3}cfu/ml while counts of *Listeria* species attached to large (180 µm) planktons varied from 0 to 1.58×10^{5} cfu/ml and those of the 60 and 20 µm categories were in the range of 0 to 1.32×10^{3} cfu/ml and 0 to 2.82×10^{5} cfu/ml respectively. *Listeria* abundance did not vary significantly with location and season; there was however, significant ($P < 0.05$; $P < 0.01$) variance in *Listeria* abundance with plankton sizes across the locations. Free-living *Listeria* species were more abundant in the rural and urban

communities than plankton attached *Listeria* species; whereas the reverse was the case in the peri-urban community.

Prevalence of *Listeria* in terms of total counts was 100% across all sampled locations. Free-living *Listeria* species showed prevalence ranging from 84-96% across the sampling locations; while *Listeria* species attached to large (180 µm) planktons exhibited prevalence ranging from 75% to 90%. The prevalence of medium-sized (60 µm) plankton associated *Listeria* species varied between 58% and 92.5%; whereas those of *Listeria* species attached to small (20 µm) planktons ranged from 65-100% across all three communities. *Listeria* prevalence was generally a reflection of the turbidity of the water system, with free-living *Listeria* species being more prevalent than plankton associated cells in the relatively less turbid rural and urban waters compared to the more turbid peri-urban waters where plankton attached cells were more prevalent in comparison with their free living counterparts.

The final treated effluent quality fell short of recommended standards for turbidity, chemical oxygen demand and phosphate across all three communities. In addition, the final effluent of the rural treatment plant also fell short of recommended standard for $NO_{3,}$ while that of the urban treatment plant did not comply with acceptable limits for dissolved oxygen and nitrite. Other physicochemical parameters were compliant with set standards after treatment. An inverse relationship was observed between chlorine residual and listerial density across the sampled facilities; the effect of chlorine was however not enough to eliminate the pathogen from the water systems.

At the urban treatment plant and its receiving watershed, pH, temperature, EC, turbidity, TDS, DO, and nitrate varied significantly with season and sampling point ($P < 0.05$; $P < 0.01$).

Salinity also varied significantly with sampling point ($P < 0.01$), while COD and nitrite varied significantly with season ($P < 0.05$). Although, the treated effluent fell within recommended water quality standard for pH, TDS, nitrate and nitrite, it fell short of stipulated standards for other parameters. Whereas the microbial quality of the secondary treated effluent at this (urban) facility fell short of recommended standard after secondary treatment, its physicochemical quality were generally compliant with recommended standards for reuse wastewater in agriculture and aquaculture.

Listeria pathogens isolated from effluents of the rural wastewater facility were sensitive to 11 (55%) of the 20 test antibiotics, and showed varying (7-71%) levels of resistance to 8 antibiotics; whereas those isolated from the peri-urban community showed sensitivity to 6 (30%) of the 20 test antibiotics, and varying (6-94%) levels of resistance to 12 antibiotics; while the urban effluent isolates were sensitive to 3 (15%) of the 20 test antibiotics, and showed varying (4.5-91%) levels of resistance to 17 antibiotics. Multiple antibiotic resistances involving 78.5-100% of isolates and antibiotics combination ranging from 2-10 antibiotics was observed across the sampled locations. Penicillin G and ampicillin showed remarkably high (64-91%) phenotypic resistance across the three sampled facilities. Other antibiotics, to which isolates showed significant resistance, were linezolid (22-88%); erythromycin (43-94%) and sulphamethoxazole (7-94%).

Two of the 14 *Listeria* strains isolated from the rural effluents were positive for *ereA* and *sul1* antibiotic resistance genes; while *sulII* genes were detected in five of the 23 *Listeria* isolates from the urban effluent and none was detected in isolates from the peri-urban community. The presence of antimicrobial resistance genes in the isolates did not correlate with phenotypic antibiotic resistance.

The current study demonstrated that *Listeria* pathogens easily survived the activated sludge treatment process as free-living and plankton attached entities and suggests that municipal wastewater treatment plants are a significant source of multiple resistant *Listeria* pathogens in the South African aquatic milieu. While the physicochemical quality of the urban final effluent suggests that it is a major source of pollution to the receiving watershed, the secondary effluent quality demonstrated a great potential for use in agriculture and aquaculture.

CHAPTER 1

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Listeriosis is a disease condition commonly associated with food and caused by pathogenic bacteria of the genus *Listeria.* Although seven species are recognized (*L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri, L. grayii* and *L. murrayi*), only two, *L. monocytogenes* and *L. ivanovii,* are pathogenic; the former is responsible for disease in both humans and animals, while the latter causes disease mostly in ruminants but also in other animals (Roberts and Wiedmann, 2003; Brugere-Picoux, 2008). There are reports however of *L. seeligeri* and *L. ivanovii* causing illness in humans (Cummins *et al*., 1994; Cocolin *et al*., 2002), and *L. innocua* occasionally associated with encephalitis in ruminants (Walker *et al*., 1994). Other species are generally regarded as non-pathogenic (Brugere-Picoux, 2008).

Hülphers (1911) described a bacterium that caused necrosis of the liver in rabbits, and because of its characteristic affinity to liver, it was named *Bacillus hepatis*. The same bacterium was isolated by Murray, Webb and Swann in 1924 from diseased guinea pigs and rabbits in their laboratory at Cambridge, England and named *Bacterium monocytogenes*; giving rise to the official discovery of *Listeria* (Murray *et al*., 1926). Pirie (1940) isolated the organism from gerbilles (*Tetra lobengulae*) near the Tiger River in South Africa and named his isolate *Listerella hepatolytica*, the generic name being dedicated in honour of a British Surgeon, Sir Joseph Lister. Pirie changed the name of the bacterium to *Listeria monocytogenes* in 1940, and in 1948 the organism was recognized by the same name in the 6th edition of Bergey's Manual of Determinative Bacteriology (Breed *et al*., 1948). *Listeria monocytogenes* was first isolated from sheep in 1929 (Gill, 1933) and the first human case was reported in the same year by Nyfeldt (1929). By 1961 *Listeria monocytogenes* was the only known species in the genus *Listeria*; however, to date six other species have been identified as mentioned earlier (Roberts and Wiedmann, 2003; Brugere-Picoux, 2008).

Listeria species are small, Gram-positive rods, 1-2 µm long and 0.5 µm wide. The bacteria are aerobic and facultative anaerobic, non-spore and non-capsule forming, with optimal growth temperature of between 30^{o} and 37^{o}C. They can however, grow and reproduce at temperatures between - 0.4^{o} and 45^{o}C and pH 4.5-9.6 (Brugere-Picoux, 2008); the bacteria exhibits a characteristic tumbling motility using peritrichous flagella at 20^{o}-25^{o}C (Peiris, 2005).

On nutrient agar (24 h culture) *Listeria* colonies are round, 0.5-1.5 mm in diameter, exhibiting bluish-gray colour by normal illumination, but a blue-green sheen under oblique light. *Listeria* species are catalase positive, oxidase negative, methyl red positive, and Voges-Proskauer positive. *L. monocytogens* is β-haemolytic on blood agar forming a narrow zone of haemolysis around colonies, while *L. ivanovii* forms double or triple haemolytic zones on sheep or horse blood agar, whereas other *Listeria* species are non haemolytic (Schuchat *et al*., 1991). Table 1.1 summarizes some characteristic features of *Listeria* species.

Listeria is widely distributed in nature. The bacteria is an ubiquitous saprophyte that lives in plant-soil environments and has been isolated from about 42 species of domestic and wild mammals and 22 species of birds, as well as fish, crustaceans, insects, sewage, water, feedstuffs, milk, cheese, meconium, feces and soil (Kirkan *et al*., 2006). *Listeria* survives wide ranges of temperature (-7^{o}-45^{o}C), pH (4.3-9.6), and salt concentrations (up to 10%) (Robert and Wiedmann, 2003); the ability to survive and multiply under conditions frequently used for food preservation makes the bacteria particularly problematic to the food industry (Roberts and Wiedmann, 2003).

Table 1.1. Characteristics of *Listeria* species.

Species	**Haemolysis**	**Acid production from:**				**Nitrate reduction**	**CAMP test with:**	
		D-Glucose	D-Xylose	D-Mannitol	L-rhamnose		*Staphylococcus aureus*	*Rhodococcus equi*
L. monocytogenes	+	+	-	-	+	-	+	+ or -
L. innocua	-	+	-	-	V	-	-	-
L. ivanovii	+	+	+	-	-	-	-	+
L. seeligeri	+	+	+	-	-	-	+	-
L. welshimeri	-	+	+	-	V	-	-	-
L. grayi	-	+	-	+	-	-	-	-
L. murrayi	-	+	-	+	V	+	-	-

Source: Schuchat *et al*. (1991)

Key: + = ≥ 90% of strains were positive; - = ≥ 90% of strains were negative; V = 11 to 89% were positive.

Listeriosis is reported to be largely foodborne (Mead *et al*., 1999); however, nosocomial infections (Elsner *et al*., 1997; Graham *et al*., 2002) and person-to-person transmission (Jacobs and Murray, 1986) have been reported. The clinical syndromes of the disease include invasive listeriosis, non-invasive gastrointestinal disease, as well as local skin and eye symptoms (Maijala *et al*., 2001). Invasive listeriosis causes meningoencephalitis, encephalitis, sepsis, and abortions and has a high mortality rate (20 to 30%). It occurs mainly in high risk individuals, including the young, old, pregnant, and immunocompromized persons. On the other hand non-invasive listeriosis causes fever, diarrhea, muscle pain, headache, nausea, vomiting, and abdominal pain in healthy adults (Lunden *et al*., 2004). Despite its rare incidence, listeriosis remains of great public health concern due to its high fatality rate (up to 51%) and common-source epidemic potential (de Valk *et al*., 2001; Rocourt *et al*., 2000).

Although listeriosis is cosmopolitan in nature, it is mainly reported in industrialized nations compared to their developing counterparts (Rocourt, 1996; Low and Donachie, 1997). Rocourt (1996) reported a low to non-existent prevalence of the disease in Africa, Asia and South America, and according to the author the observation is likely a reflection of different consumption patterns, dietary habits, different host susceptibility, or availability of testing facilities that exists between these two blocks of civilization.

An estimated 2,500 cases of listeriosis occur annually in the United States (Mead *et al*., 1999), with an overall annual incidence of approximately 4.4 per million (Tappero *et al*., 1995). In September and October, 1979, 20 patients suspected to have consumed raw vegetables were hospitalized in Boston due to listeriosis (Schlech *et al*., 1983); while a second outbreak involving immunocompromised non-pregnant adults suspected to have drunk a specific brand of pasteurized or 2% whole milk was reported in Massachusetts in 1983 with a case fatality of 29%

(Fleming *et al*., 1985). A relatively large outbreak occurred in 1985 in Los Angeles, California resulting in a case fatality rate of 63% for early neonatal or foetal infections and 37% for non-neonatal infections involving pregnant women and their offspring (Linnan *et al*., 1988). Between 2000 and 2003 over 109 other cases of invasive listeriosis were reported in the United States involving several food products including delicatessen turkey, ready-to-eat meats and home-made Mexican-style cheese (Swaminathan and Gerner-Smidt, 2007).

In Canada, the first human listeriosis outbreak directly linked to the consumption of *Listeria* contaminated food (coleslaw) was reported by Schlech *et al*. (1983). Since then at least two other cases of listeriosis have been reported in Canada. Farber *et al*. (2000) reported a small outbreak in Ontario involving two previously healthy adults who went down with the disease after consuming imported imitation crab meat; while another outbreak involving 17 individuals was reported in Quebec in 2002, this time the culprit was cheese made from raw milk (Swaminathan and Gerner-Smidt, 2007).

Incidence of listeriosis outbreaks have also been widely reported in Europe. In Finland annual cases of invasive listeriosis were reported to vary between 20 and 50 from 1990 to 2001 (Lukinmaa *et al*., 2003); while Maijala *et al*. (2001) reported a foodborne outbreak involving the consumption of butter, between June, 1998 and April, 1999. Two hundred and ninety nine cases of invasive listeriosis resulting in 21% mortality was reported in Denmark between 1994 and 2003 (Gerner-Smidt *et al*., 2005); and Kiss *et al*. (2006) reported 17 cases in Hungary resulting in 2 fatalities and associated with the consumption of especially milk and dairy products. Over 108 deaths were reported in France from about 657 cases of listeriosis involving ready-to-eat-foods like rilletes, pork tongue in jelly and pork tongue in aspic, between 1992 and 2001(de Valk *et al*., 2001; Hong *et al*., 2007; Swaminathan and Gerner-Smidt, 2007); while 283 cases were

reported in the Netherlands resulting in 18% mortality rate between 1995 and 2003. Reports elsewhere in Europe indicated listeriosis incidence is in the region of 3.5 per million persons in Bristol, England (hospital-based surveillance); 1.8 per million persons, England, Wales and Northern Ireland (passive surveillance); 6-7 per million persons, Denmark (laboratory-based surveillance) (Slutsker and Schuchat, 1999).

A surveillance of the disease conducted in Los Angeles County (USA) between 1985 and 1986 reveals that there was no difference in listeriosis incidence between race and ethnic groups (Mascola *et al*., 1989). Most authors also described seasonal variation, with a peak incidence in summer possibly related to seasonal consumption of specific food products (McLauchlin *et al*., 1990) or to more frequent breakdowns in food handling at higher temperatures (Siegman-Igra *et al*., 2002).

The Eastern Cape Province of South Africa is mostly non-urban, poor and without adequate infrastructure, with a significant proportion of the rural communities lacking pipe-borne water, and as such depend on streams, rivers, groundwater and other available water bodies for drinking and domestic purposes (Okoh *et al*., 2007). Many of these water bodies are often impacted by inadequately treated effluents from municipal wastewater treatment plants as receiving water bodies (Fatoki *et al*., 2003), thus creating increased pressure on this scarce resource. The attendant consequence of such negative practice is the compromising of the primary health of the people resulting in death threatening diarrhoeal diseases (Bourne and Coetzee, 1996), caused by waterborne pathogens, especially in the age group 1-5 years (Mackintosh and Colvin, 2003) and in immunocompromised individuals, leading to tens of thousands of deaths annually (Pelgrum *et al*., 1998).

In line with the spirit and letter of the South African Constitution under the Bill of Rights which states that "*everyone has the rights to have access to sufficient food and water*" (Constitution of South Africa, 1996 s27b); every South African deserves clean, safe and affordable water. To consistently comply with specific sanitation and wastewater standards set by relevant legislation and regulations, and consistent with the broader environmental policy, there is need for regular check up of the working efficiency of wastewater treatment plants. This is more so as population and industrial growth across the Eastern Cape Province over the years is posing a serious challenge at the capacity of existing wastewater treatment plants to adequately handle and treat current wastewater influents in terms of their volumes and complexity (Welgens, 2006; Okoh *et al*., 2007).

Wastewater discharges may contain health compromising pathogens, carcinogenic substances (e.g. heavy metals, trihalomethanes, etc), and/or chemical substances which may cause adverse environmental impact such as changes in aquatic habitats and species composition, decrease in biodiversity, impaired use of recreational waters and shellfish harvesting areas, and contaminated drinking water (Environment Canada, 2001; CCME, 2006). All of these impact leads to a less valuable environment, poor health, a less prosperous economy, and ultimately, a diminished quality of life (Environment Canada, 2001). Physicochemical parameters such as temperature, pH, DO, salinity, and nutrient loads have been reported to influence biochemical reactions within water systems. Such changes in the concentration of these parameters are indicative of changes in the condition of the water system (Hacioglu and Dulger, 2009); the consequence of such is the compromise of the water quality for beneficial uses.

Growing economic and physical scarcity of water, made worse by global climatic changes and increasing demands for freshwater, calls for innovative ways of water use and

development (Inocencio *et al*., 2003). The Southern African region is predicted to experience more and longer droughts over the next 70 years (Palitza, 2009); according to the report the impending water-shortage will result in more strain on available freshwater resources and in turn lead to increased crop failures, less pasture for livestock and ultimately less food for the growing population. The United Nations Environmental Program (UNEP, 2009) also predicted that the situation may get so bad in the coming years that wastewater may account for 25-75% of the total available irrigation water in the region, especially in the very dry zones. The bleak future of freshwater availability is thus forcing planners and stakeholders to consider any sources of water which might be useful economically to effectively promote food security and further development (FAO, 1992). Hence reuse of wastewater may be an inevitable option for most farmers in South Africa and neighbouring States in the near future for obvious reasons.

While it is necessary to encourage the reuse of wastewater especially in the very dry zones of the Southern African region, conscious steps needs be taken to ensure acceptable reuse wastewater quality in order to preserve the public health and protect the environment. Unfortunately, there is a dearth of information on the quality of treated wastewater effluent used for agricultural purposes in South Africa. Information on the quality of wastewater for reuse in agriculture will enable farmers and other stakeholders to make adequate plans with regards to optimal effluent utilization potential. Effluent quality information will also enable planners to determine the best measure to take at improving the quality of the irrigation water for intended purposes.

Although food and food products were widely reported to be the route of transmission of *Listeria* pathogens, recent reports (Watkins and Sleath, 1981; Al-Ghazali and Al-Azawi, 1986, 1988; Czeszejko *et al*. 2003; Paillard *et al*. 2005) indicate that *Listeria* species very easily

survive conventional wastewater treatment processes and suggests that wastewater effluent could play a significant role in the epidemiology of the pathogen in the population. With reports of inadequate removal of *Listeria* pathogens in wastewater coming from the developed world (Czeszejko *et al*. 2003; Paillard *et al*. 2005), it can be safely presumed that the scenario would be worse in developing countries such as South Africa for obvious reasons.

Listeria infections are reported to have the highest (up to 51%) mortality rate amongst foodborne pathogens (Rocourt *et al*., 2000), making the South African public particularly vulnerable in the event of an outbreak due to the high HIV/AIDS prevalence level and rate of drug and alcohol abuse in the country (Obi *et al*., 2006).

There is a general belief that the larger population of bacteria species grow as adherent to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ profoundly from their planktonic (free-living) counterpart (Costerton *et al*., 1978). It has also been reported that the existence of pathogens as free-living or plankton-associated cells, is critical to their survival in the environment as well as their transmission from one host to another (Donlan and Costerton, 2002). Several studies have revealed the preponderance of *Listeria* species to exist as biofilms attached to surfaces such as stainless steel, glass and propylene (Mafu *et al*., 1990), PVC (Djordjevic *et al*., 2002), and food and food processing environments (Lunden *et al*., 2000).

There is however little or no report in the literature on *Listerio*-plankton association in the natural environment. Understanding the distribution of *Listeria* cells as free-living or plankton-associated niches may provide clues on how best to reduce the survival potentials of these

pathogens in the environment and during wastewater treatment, and consequently reduce their ability to interact with human and animal populations.

Listeria species were generally reported to be susceptible to most antibiotics (Li *et al*., 2007). This assertion however, may be borne out of the fact that most study on the antimicrobial susceptibility profiles of *Listeria* species focused almost exclusively on clinical and/or food isolates with little or no information in the literature on antibiotic susceptibility profiles for *Listeria* strains isolated from treated municipal wastewater effluent. Recent studies (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004) however indicated that wastewater effluents normally contain considerable concentration of antibiotics after treatment, thereby raising chances of antibiotic contaminants perturbing the microbial ecology; increasing proliferation of antibiotic resistant pathogens; and posing threats to human health as well as create challenges for the water industry on issues of water reuse and water resource planning (Daughton and Ternes, 1999). Listerial resistance to antimicrobial therapy was also reported (Srinivasan *et al*., 2005; Davis and Jackson, 2009) to be mediated by certain resistance genes which code for proteins that function in ways that inhibits or reduce the effects of antimicrobials on the pathogen.

While other pathogens (*Shigella*, *Salmonella* and *Vibrio* spp.) were implicated in the waterborne disease outbreaks mentioned earlier, there has been no report in the literature of waterborne listeriosis in South Africa. Of considerable interest however, is the fact that in most cases the identity of the pathogens responsible for these outbreaks was unknown. A case in point was seen in the report of the Daily Dispatch (2003) where out of 446 cases of water related diseases reported to the Eastern Cape health authorities, only 25 (5.6 %) were confirmed to be cholera and yet the disease was termed a 'cholera outbreak' without ascertaining the true identities of the pathogens responsible for over 84% of reported cases.

Reports of waterborne disease outbreaks in South Africa in general and the Eastern Cape Province in particular (WHO, 2003) suggests that there is need to re-evaluate the working efficiency of wastewater treatment plants at removing pathogens such as *Listeria* species from wastewater effluents, prior to their discharge into the receiving watersheds. This is further justified by reports in the literature (Paillard *et al*., 2005) which suggests that the dependence on classical pollution indicators like *Escherichia coli* and culturable total and faecal coliforms may be misleading, as some of these indicators are reported to be more susceptible to disinfection than more resistant bacteria like *Listeria*. The implication of this is that a water supply may be adjudged fit and safe for human consumption based on the *E. coli* and coliform standards whereas in actual sense it may contain more deadly pathogens like *Listeria*. In addition, due to emphasis on the monitoring of classical pollution indicators as stated earlier, not much is being done with regards to the survival and molecular epidemiology of resistant strains of *Listeria* species in wastewater effluents, either as free or attached cells (Karlowsky *et al*., 2004).

The foregoing therefore gave rise to the following research questions:

1. Does the activated sludge system of wastewater treatment plants in the Eastern Cape Province of South Africa adequately remove *Listeria* pathogens from wastewater influent prior to discharge?
2. Do *Listeria* species exist as free-living and/or plankton associated entities?
3. What is the profile of antibiotic susceptibilities of *Listeria* isolates from chlorinated final effluents from the study communities, and how does this profile correlate with established antibiotic resistance genes?
4. How do *Listeria* isolates from chlorinated effluents compare generally with isolates from other sources?

With these questions in mind, this study hypothesizes that *Listeria* bacteria very easily survive the treatment processes of the activated sludge system either as free-living and/or plankton attached biofilms; and that wastewater treatment facilities in the Eastern Cape Province are veritable sources of pollution in the South African aquatic milieu.

To achieve the broad aim of this study, the following specific objectives were set out:

(i) Ascertain the prevalence and distribution of *Listeria* pathogens in the final effluents of the wastewater treatment plants in some communities of the Eastern Cape Province and their receiving watershed.

(ii) Investigate the occurrence of *Listeria* spp. as free living and plankton attached cells in the final effluents of the wastewater treatment plants and their receiving watersheds.

(iii) Assess the effect of season on the prevalence and distribution of *Listeria* spp. in the final effluents and their receiving watersheds.

(iv) Determine the suitability of secondary treated effluents for use in agriculture and aquaculture using the urban treatment plant as a case study.

(v) Carry out culture based isolation of *Listeria* pathogens from chlorinated wastewater final effluents and confirm their identities.

(vi) Elucidate the antibiotic susceptibility profiles of the *Listeria* isolates as well as their antibiotic resistance genes.

(vii) Compare data obtained from typical urban, peri-urban, and rural communities, and in relation to the physicochemical qualities of the effluents.

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CHAPTER 2

Wastewater treatment plants as a source of microbial pathogens in receiving watersheds

(Published in African Journal of Biotechnology)

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Abstract

Wastewater treatment facilities have become *sin quo non* in ensuring the discharges of high quality wastewater effluents into receiving waterbodies and as consequence, a healthier environment. Due to massive worldwide increases in the human population, water has been predicted to become one of the scarcest resources in the 21st century, and despite large advances in water and wastewater treatments, waterborne diseases still pose a major threat to public health worldwide. Several questions have been raised on the capacity of current wastewater treatment regimes to remove pathogens from wastewater with many waterborne diseases linked to supposedly treated water supplies. One of the major gaps in the knowledge of pathogenic microorganisms in wastewater is a thorough understanding of the survival and persistence of the different microbial types in different conditions and environments; this therefore brings to the fore the need for a thorough research into the movement and behavior of these microorganisms in wastewaters. In this review paper we give an overview of wastewater treatment practices with particular emphasis on the removal of microbial pathogens.

Key words: Wastewater, treatment plants, microbial pathogens, watershed.

2.1 Introduction

It has been predicted that, due to massive worldwide increases in the human population, water will become one of the scarcest resources in the 21st century (Day, 1996). As human numbers increase, greater strains will be placed on available resources and pose even greater threat to environmental sources. A report by the Secretary-General of the United Nations Commission on Sustainable Development (UNCSD, 1997) concluded that there is no sustainability in the current uses of fresh water by either developing or developed nations, and that worldwide, water usage has been growing at more than three times the world's population increase, consequently leading to widespread public health problems, limiting economic and agricultural development and adversely affecting a wide range of ecosystems.

Much of the wastes of civilization enter water bodies through the discharge of waterborne waste from domestic, industrial and nonpoint sources carrying unwanted and unrecovered substances (Welch, 1992). Although the collection of wastewater dates back to ancient times, its treatment is a relatively recent development dating from the late 1800s and early 1900s (Chow *et al*., 1972). Modern knowledge of the need for sanitation and treatment of polluted waters however, started with the frequently cited case of John Snow in 1855, in which he proved that a cholera outbreak in London was due to sewage contaminated water obtained from the Thames River (Coopers, 2001).

Wastewater treatment practices vary from country to country across the globe. In developed nations, treatment and discharge systems can sharply differ between countries and between rural and urban users, with respect to urban high income and urban low-income users (Doorn *et al*., 2006). The authors further reported that the most common wastewater treatment methods in developed countries are centralized aerobic wastewater treatment plants and lagoons

for both domestic and industrial wastewater. Domestic wastewater may also be treated in *on-site* septic systems involving wastewater from one or several households consisting of an anaerobic underground tank and a drainage field for the treatment of effluent from the tank (UNEP, 2002). However, there are still communities without wastewater treatment facilities in developed countries, and in some cases existing infrastructure is faltering in many countries; even in areas with a high degree of wastewater treatment, pathogens and some chemicals, many with unknown ecological consequences, may still be released into the environment (LeChevallier and Au, 2004; Paillard *et al*., 2005).

The degrees of wastewater treatment vary in most developing countries. Domestic wastewater may be treated in centralized plants, pit latrines, septic systems or disposed of in unmanaged lagoons or waterways, via open or closed sewers (UNEP, 2002). In some cases industrial wastewater is discharged directly into bodies of water, while major industrial facilities may have comprehensive in-plant treatment (Carter *et al*., 1999; Doorn *et al*., 2006). In many developing countries the bulk of domestic and industrial wastewater is discharged without any treatment or after primary treatment only. In Latin America about 15% of collected wastewater passes through treatment plants (with varying levels of actual treatment). In Venezuela, 97 percent of the country's sewage is discharged raw into the environment (Caribbean Environment Programme Technical Report #40 1998). Even a highly industrialized country such as China discharges about 55 percent of all sewage without treatment of any kind (The People's Daily, Friday, November 30, 2001). In a relatively developed Middle Eastern country such as Iran, the majority of Tehran's population has totally untreated sewage injected into the city's groundwater (Tajrishy and Abrishamchi, 2005). In South Africa where some level of wastewater treatment is observed, Momba *et al*. (2006) reported the poor operational state and inadequate maintenance

of most of the municipalities' sewage treatment works as leading to the pollution of various water bodies thereby posing very serious health and socio-economic threats to the dependants of such water bodies. Most of sub-Saharan Africa is without wastewater treatment (Sci-Tech. Encyclopaedia, 2007).

Despite advances in water and wastewater treatments, waterborne diseases still pose a major threat to public health worldwide (Zhou and Smith, 2002). Many of these infections occur in developing countries which have lower levels of sanitation, problems associated with low socio-economic conditions, and less public health awareness than in developed countries (Toze, 1997; Elimelech, 2006). The health risks for the public from wastewater can come from microbial pathogens, toxic chemicals, and heavy metals. This review addresses the common practices of wastewater treatment with emphasis on the consequences of inadequate treatment regimes resulting in the pollution of the receiving aquatic milieu with microbial pathogens as is common in developing countries.

2.2 Types of wastewater treatment facilities

2.2.1. Oxidation ponds

Oxidation ponds are effective, low-cost, and simple technology for reducing the biological oxygen demand (BOD) of a wastewater before it is discharged to an aquatic ecosystem. It consists of ring or oval shaped channel equipped with mechanical aeration devices. Screened wastewater entering the pond, is aerated by mechanical devices which circulates at about 0.25 to 0.35 ms^{-1}. Oxidation ponds typically operate in an extended aeration mode with long detention and solids retention times (von Sperling and de Lemos Chernicharo, 2005).

2.2.2. Anaerobic ponds

Anaerobic pond as a wastewater treatment facility is a biological process ideally suited for the pre-treatment of high-strength wastewaters. The anaerobic process utilizes naturally-occurring bacteria to break down biodegradable material in wastewater. Because the bacteria are anaerobic they do not require oxygen like the organisms in an aerobic process. Used prior to aerobic treatment, an anaerobic system can be very effective and economical for removing high concentrations of oxygen demanding substances prior to final treatment by an aerobic process (Dewil *et al*., 2006).The pond is relatively deep, 3 m to 4 m, as this concentrates the biological action and reduces heat loss. Anaerobic ponds contain an organic loading that is very high relative to the amount of oxygen entering the pond. This maintains anaerobic conditions to the pond surface. Anaerobic bacteria break down the organic matter in the effluent, releasing methane and carbon dioxide. Sludge is deposited at the bottom and a crust may form on the surface (Doorn *et al*., 2006). They work extremely well in warm climates; a properly designed and not significantly under loaded anaerobic pond will achieve around 60% BOD removal at 20 ^{o}C and as much as 75% at 25^{o}C.

2.2.3. Aerobic ponds

This is another wastewater treatment facility which contains bacteria and algae in suspension and maintains aerobic conditions throughout its depth. There are two types of aerobic ponds: shallow ponds and aerated ponds (Vijayaraghavan *et al*., 2007).

2.2.3.1. Shallow pond

Shallow aerobic ponds obtain their dissolved oxygen via two processes: oxygen transfer between air and water surface, and oxygen produced by photosynthetic algae. Although the efficiency of soluble biochemical oxygen demand removal can be as high as 95 percent, the pond effluent will contain a large amount of algae which will contribute to the measured total biochemical oxygen demand of the effluent. To achieve removal of both soluble and insoluble biochemical oxygen demand, the suspended algae and microorganisms have to be separated from the pond effluent (George and Andrew, 2003).

2*.2.3.2. Aerated ponds*

An aerated pond is similar to an oxidation pond except that it is deeper and mechanical aeration devices are used to transfer oxygen into the deeper portions of the pond. The aeration device also facilitates a proper mix of the wastewater and bacteria. The main advantage of the aerated pond is that they require less area than oxidation ponds. The disadvantage is that the mechanical aeration devices require maintenance and use energy (Craggs *et al*., 2003; Elimelech, 2006).

2.2.4. Facultative pond

Facultative ponds are generally aerobic; however, these ponds do operate in a facultative manner and have an anaerobic zone. The depth of natural-aeration facultative ponds usually 1.0 to 1.5 m is too deep for oxygen to penetrate to the bottom of the pond, and an anaerobic zone develops there. Solids from the incoming waste settle into the anaerobic sludge near the bottom of the

pond and degrade anaerobically releasing soluble degradable organic material and nutrients which diffuse upwards in the pond (Sharman, 2004). Near the top of the pond oxygen is supplied by algal photosynthesis and to a limited extent by diffusion from the air. There is dissolved oxygen present to only a few centimetres depth at night, but dissolved oxygen diffuses deeper during daylight (Tchobanoglous and Angelaki, 1996; Al-Sa'ed, 2001). Thus there exists a fully aerobic zone at the top of the pond, and between this and the anaerobic zone at the bottom there is a middle zone where oxygen is cyclically present and bacterial respiration is "facultatively" aerobic-anaerobic (Tanik *et al*., 1996; von Sperling and de Lemos Chernicharo, 2005). A facultative oxidation pond receiving sewage typically achieves between 70 to 95 percent removal of BOD_5 (non-filtered) at a loading rate to the pond of 2.2 to 3.5 g $BOD_5\ m^{-2}day^{-1}$ depending on temperature. An effluent quality standard of 30 g $BOD_5\ m^{-3}$ is typically set. Facultative oxidation ponds are directed at reduction of BOD_5 and to a lesser extent suspended solids in wastewater (Al-Sa'ed, 2001).

2.2.5. Trickling filter

A trickling filter (TF) is a wastewater treatment system that is used to reduce BOD_5, pathogens, and Nitrogen levels. It is composed of a bed of porous material (rocks, slag, plastic media, or any other medium with a high surface area and permeability). The microorganisms in the wastewater attach themselves to the bed (also known as the filter media), which is covered with bacteria. Wastewater is first distributed over the surface of the media where it flows downward as a thin film over the media surface for aerobic treatment and is then collected at the bottom through an under drain system. The effluent is then settled by gravity to remove biological solids prior to

being discharged (Al-Sa'ed, 2001). Like the activated sludge, trickling filter is used in both large and small communities (Kornaros and Lyberatosa, 2006).

2.2.6. Rotating biological contractors

In Rotating Biological Contractors (RBCs) a number of circular plastic disc are mounted on a central shaft. These discs are submerged and rotated in a tank containing the wastewater to be treated. The microorganisms responsible for treatment become attached to the disc and rotate into and out of the wastewater. The oxygen necessary for the conversion of organic matter adsorbed from the liquid is obtained from the air as a certain area of the disc is rotated out of the liquid. In some designs, air is added to the bottom of the tank to provide oxygen and to rotate the disc when those are provided with air capture cups. It is a very useful system in small communities instead of the conventional secondary treatment, obtaining similar quality in the effluent. RBCs have also been developed for the biological treatment of odours (Smeets *et al*., 2006). It is flexible enough to undergo fluctuating organic loads, requires little personal attention, cheap to run and does not require too much land. The RBCs have been used in treating winery wastewater and has also been used in the treatment of effluents produced by various industries such as gold mining and domestic sewage treatment (Tawfit *et al*., 2002).

2.3 Activated sludge system

The activated sludge process is the most widely applied biological wastewater treatment process in the world. The primary objective of the activated sludge system is the removal of soluble

biodegradable compounds. It also removes pathogenic microorganisms from wastewaters. It is capable of achieving equal reductions in soluble substrate in reactors of much smaller volume while producing an effluent relatively free of suspended solids (Mara, 2004; Dewil *et al*., 2006). The removal efficiency of pathogenic and indicator microorganisms in these wastewater treatment plants vary according to the treatment process type, retention time, other biological flora present in activated sludge, oxygen concentration, pH, temperature and the efficiency in removing suspended solids (Doorn *et al*., 2006).

2.3.1. Components of the Activated Sludge Treatment Process

2.3.1.1. Anaerobic zone

The anaerobic zone is considered to be one in which both dissolved oxygen and oxidized nitrogen are absent (Eikelboom and Draaijer, 1999). In this zone, sludge from the clarifier flows in jointly with the influent wastewater. It has been reported that for this zone to operate efficiently, oxygen and nitrates must be absent. This is responsible for the release of phosphate (Tanaka *et al*., 2007).

2.3.1.2. Primary anoxic zone

The primary anoxic zone is the main denitrification reactor in the process; it is fed by the effluent from the anaerobic zone and mixed liquor recycled from the aerobic zone. The presence of nitrate or nitrite and absence of oxygen leads to the enrichment of denitrifying bacteria, which

reduces nitrate or nitrite to molecular nitrogen. Thus soluble and colloidal biodegradable matters are readily removed in this zone (Metcalf and Eddy, 2003).

2.3.1.3. Primary aerobic zone

The primary aerobic zone functions mainly to oxidize organic material in wastewater, ammonia into nitrate and also provides an environment to take up all the phosphate released in the anaerobic zone (Torpak, 2006). For the removal of ammonia, it must first be oxidized to nitrites by nitrifying bacteria such as *Nitrosomonas*, *Nitrospria* and *Nitrosolobus* spp. Nitrites are then oxidized to nitrates by *Nitrobacter*, *Nitrospira* and *Nitrococcus* spp. These nitrates are then removed in the primary anoxic zone by denitrifying bacteria. Phosphates uptake is based on the enrichment of the activated sludge with bacteria capable of taking orthophosphate and *E. coli* which akso have been associated with the enhanced phosphate removal in activated sludge (Sci-Tech. Encyclopaedia, 2007).

2.3.1.4. Secondary anoxic zone

This zone further converts an excess nitrate which was not removed in the zone preceding it into nitrogen. Because of the very slow denitrification rate in this zone, the quantity of nitrate removed is very small. The retention time in the anoxic zone is relatively long because of the lower chemical oxygen demand (Torpak, 2006).

2.3.1.5. Secondary aerobic zone and clarifier

This zone removes additional phosphate, which was not removed in the primary aerobic zone. Residual ammonia is also oxidized in this zone. The secondary aerobic zone increases the level of the dissolved oxygen between 2 and 4 mg^{-l} in the mixed liquor before it enters the clarifier. Aeration should be more to promote phosphate uptake and maintain good aerobic conditions. Phosphorus is retained in the biomass as long as aerobic condition prevails (von Sperling and de Lemos Chernicharo, 2005). This zone prevents the development of anaerobic condition in the clarifier and phosphate release before clarification. In the clarifier, treated wastewater, free of organic matter and dissolved solid is released (Zhou and Smith, 2002; Smeets *et al*., 2006).

2.4 Microbiology of activated sludge

The activated sludge process is a biological method of wastewater treatment that is performed by a variable and mixed community of microorganisms in an aerobic aquatic environment (Jenkins *et al*., 2003; Richard, 2003). These microorganisms derive energy from carbonaceous organic matter in aerated wastewater for the production of new cells in a process known as synthesis, while simultaneously releasing energy through the conversion of this organic matter into compounds that contain lower energy, such as carbon dioxide and water, in a process called respiration. A variable number of microorganisms in the system also obtain energy by converting ammonia nitrogen to nitrate nitrogen in a process termed nitrification. This consortium of microorganisms, the biological component of the process, is known collectively as activated sludge (Ottoson *et al*., 2005; Norstrom, 2005). Bacteria, fungi, protozoa, and rotifers constitute the biological mass, of activated sludge. In addition, some metazoa, such as nematode worms,

may be present. Cell makeup depends on both the chemical composition of the wastewater and the specific characteristics of the organisms in the biological community. However, the constant agitation in the aeration tanks and sludge recirculation are deterrents to the growth of higher organisms (Lardotter, 2006).

2.5 Stages of treatment of wastewater

2.5.1. Preliminary treatment

As wastewater enters a treatment facility, it usually undergoes preliminary treatment. This treatment typically involves screening to remove large floating objects, such as rags, cans, bottles and sticks that may clog pumps, small pipes, and down stream processes (USEPA, 2004). Screens are generally placed in a chamber or channel and inclined towards the flow of the wastewater. The inclined screen allows debris to be caught on the upstream surface of the screen, and allows access for manual or mechanical cleaning. Some plants use devices known as comminutors or barminutors which combine the functions of a screen and a grinder. These devices catch and cut or shred the heavy solid and floating materials. In the process, the pulverized matter remains in the wastewater flow in smaller pieces to be removed later in a primary settling tank (Mara, 2004).

2.5.2. Primary treatment

Primary treatment is the second step in wastewater treatment and this step helps to separate suspended solids and grease from wastewater (USEPA, 2004). In some treatment plants, primary

and secondary stages may be combined into one basic operation (Environment Canada, 2003). At many wastewater treatment facilities, influent passes through preliminary treatment units before primary and secondary treatments begin. With the screening completed and the grit removed, wastewater still contains dissolved organic and inorganic constituents along with suspended solids. The suspended solids consist of minute particles of matter that can be removed from the wastewater with further treatment such as sedimentation or gravity settling, chemical coagulation, or filtration. Pollutants that are dissolved or are very fine and remain suspended in the wastewater are not removed effectively by gravity settling. When the wastewater enters a sedimentation tank, it slows down and the suspended solids gradually sink to the bottom, as primary sludge which can then be removed from the tank by various methods (Environment Canada, 2003).

2.5.3. Secondary treatment

This is a biological treatment process that removes dissolved organic matter from wastewater. Ninety percent of the organic matter in wastewater could be removed by this treatment processes. Sewage microorganisms are cultivated and added to the wastewater. The microorganisms absorb organic matter from sewage as their food supply in the process removing such organic matters from circulation (USEPA, 2004). The three most common conventional methods used to achieve secondary treatment are attached growth processes, suspended growth processes and lagoon systems (Upadhyaya *et al*., 2007). Attached growth processes involve microbial growth in surfaces such as stone or plastic media. Wastewater passes over the media along with air to provide oxygen. Attached growth process units include trickling filter,

biotowers and rotating biological contractors. The growth processes are effective at removing biodegradable organic material from the wastewater (Environment Canada, 2003). Suspended growth processes are designed to remove biodegradable organic material and organic nitrogen-containing material by converting ammonia nitrogen to nitrate. In this growth processes the microbial growth is suspended in an aerated water mixture where the 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).

2.5.4. Advanced or tertiary treatment

Tertiary treatment is the term applied to additional treatment that is needed to remove suspended and dissolved substances remaining after conventional secondary treatment. This may be accomplished using a variety of physical, chemical or biological treatment processes to remove the target pollutants (Environment Canada, 2003). Tertiary treatment may include: Filtration, Removal of Ammonia and other specific contaminants and Disinfection to destroy pathogens (Hijnen *et al*., 2006).

2.5.5. Disinfection

Untreated or inadequately treated wastewaters may contain pathogens. Processes used to kill or deactivate these harmful organisms are called disinfection. Chlorine is the most widely used disinfectant but ozone and ultraviolet radiation are also frequently used for wastewater effluent disinfection (Hijnen *et al*., 2006). Chlorine kills microorganisms by destroying cellular materials and can be applied to wastewater as a gas, liquid or in a solid form. However, any free (uncombined) 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 also used for disinfection, and it is produced from oxygen exposed to a high voltage current. Ozone is very effective at destroying viruses and bacteria and decomposes back to oxygen rapidly without leaving harmful by-products. The setback in the use of ozone however, is its high energy costs (Hijnen *et al*., 2004). 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).

2.6 Regulatory standards for wastewater effluent quality

Wastewater treatment aims at producing effluent suitable for agricultural or aquacultural reuse (or both), or to produce an effluent that can be safely discharged into inland or coastal waters. Effluent quality requirements often termed effluent quality standards are set by regulatory agencies that are empowered by legislation to make such regulations. These agencies have

duties, either explicitly defined in the governing legislation or at any rate implicitly, to set sensible regulations. Unfortunately, in many countries not all such regulations are sensible as they should be (Mara, 2004; von Sperling and de Lemos Chernicharo, 2005). Permits for wastewater treatment systems must by obtained from appropriate authorities (WHO, 2006). In the US for example, if the discharge from a treatment plant enters a stream, a National Pollutant Discharge Elimination System (NPDES) permit is required. The NPDES permit specifies the maximum allowable level of total suspended solids, biochemical oxygen demand, nutrients and bacteria that can be discharged to a stream as well as the minimum level of dissolved oxygen that must be present in the discharge. The levels specified in the NPDES permit are determined by the condition of the receiving stream. Therefore, NPDES permits are subject to change every 5 years as water quality concerns change throughout (WHO, 2006).

Wastewater poses a significant pollution threat to water-bodies and soil and hence the quality of the effluents must be controlled, especially with regards to the two variables- (i) polluting power (BOD, TOC, suspended solids and COD) (ii) nutrients (phosphate, nitrate, and ammonium). Toxins are also controlled depending on the industry type, and these would include solvents, heavy metals, phenols, chlorinated compounds and such like (WHO, 2006). In South Africa, municipal wastewater according to the water Act No. 36 of 1998 stipulates limits for certain parameters especially effluent disposal in catchment areas as shown in Table 2.1. The effluent must not contain any substance capable of producing colour, odour or taste. In South Africa, sewage articulations exist in nearly all urban areas. However, rural areas as well as most semi-urban areas are generally devoid of such facilities (Momba *et al*., 2006).

Table 2.1: Wastewater limit values applicable to discharge of wastewater into a water source according to amended Act of 1956 (South Africa water service Act No 54 of 1956).

Parameter	**General Limit**	**Special Limit**
Faecal Coliform per 100 mL	0	0
Chemical Oxygen Demand (COD) mgL^{-1}	75	30
Maximum Temperature (^{o}C)	35	25
Chlorine as free Chlorine (mgL^{-1})	0.25	0
Orthophosphate as Phosphorus (mgL^{-1})	10	1
Fluoride (mgL^{-1})	1	1
Soap, Oil or grease (mgL^{-1})	2.5	2.5
pH	5.5-9.5	5.5-7.5

Source: Veenstra *et al*. (1997)

2.7 Effectiveness of wastewater treatment

The effectiveness of conventional wastewater treatment processes has become limited over the last two decades because of new challenges (Smeets *et al*., 2006). Zhou and Smith (2002) observed that increased knowledge about the consequences from water pollution and the public desire for better quality water has promoted the implementation of much stricter regulations by expanding the scope of regulated contaminants and lowering their maximum contaminant levels(MCLs). Another factor is the diminishing water resources and rapid population growth and industrial development (USEPA, 2004). Some of the key challenges faced by the wastewater treatment sector today include: old and worn-out collection facilities requiring further improvement, repair or replacement to maintain their useful life; the character and quantity of contaminants presenting problems today are far more complex than those that presented challenges in the past; population growth is taxing many existing wastewater treatment systems and creating a need for new plants; farm runoff and increasing urbanization provide additional sources of pollution not controlled by conventional wastewater treatment; and one third of new development is served by decentralized systems (e.g., septic systems) as population migrates further from metropolitan areas (Mara, 2004).

Treatment plants remove varying amounts of contaminants from wastewater; depending on the level of treatment they provide (Environment Canada, 2003). Chlorination, UV irradiation and ozonation are three common disinfection techniques among others that have shown various degree of success in the removal of pathogens from wastewater over the years (EPA, 2002). Recent literature however, points to the inadequacies of these techniques in the removal of some pathogens from wastewater. For example, UV and chemical disinfection with chlorine has been reported to be ineffective against some viruses and bacterial spores, *Acanthamoeba*,

Cryptosporidium and *Giardia* spp. (Tree *et al*., 2003; Gomez *et al*., 2006). Ozone applied at low CT (concentration and contact time) values to limit formation of bromate was also reported to have relatively little effect on the infectivity of the protozoan (oo)cysts (Hijnen *et al*., 2006). Hoch *et al*. (1996) reported that heterotrophic bacterial community was not significantly affected by the input of treated sewage, as faecal contamination was readily detected over a comparatively long stretch of 30 km in the receiving watershed (Danube River, Vienna, Austria)

following the point of sewage discharge. Factors that influence microbial sensitivity to disinfection include attachment to surfaces, encapsulation, aggregation and low-nutrient growth (LeChevallier and Au, 2004). Waste-Activated Sludge (WAS) processes which are key technologies to treat wastewater have been shown to also have presence of heavy metals in the excess sludge which are difficult to remove by common sludge treatment methods such as aerobic or anaerobic digestion (Dewil *et al*., 2006). It was opined that the advancement of wastewater treatment technology notwithstanding, treated sewage may still contain some harmful substances irrespective of thoroughness and sophistication of treatment process, albeit in smaller quantities than in raw sewage (Environment Canada, 2003). The authors further reported that in many cases, the concentrations of the remaining pollutants may still be high enough to cause serious environmental damage.

2.8 Consequences of inadequate wastewater treatment

The consequences of discharging untreated or inadequately treated wastewater into the environment are as diverse as they are many. Municipal wastewater can result in increased nutrient levels (eutrophication), often leading to algal blooms; depleted dissolved oxygen,

sometimes resulting in fish kills; destruction of aquatic habitats with sedimentation, debris, and increased water flow; and acute and chronic toxicity to aquatic life from chemical contaminants, as well as bioaccumulation and biomagnification of chemicals in the food chain (Kapitain, 1995; Boesch *et al*., 2001).

The release of untreated or inadequately treated municipal wastewater effluents may put public health at risk from drinking water contaminated with pathogenic bacteria, protozoans (such as *Giardia* and *Cryptosporidium* spp.) and several toxic substances (Paillard *et al*., 2005). The masses are also put at risk from consuming contaminated fish and shellfish and engaging in recreational activities in contaminated waters (Kapitain, 1995). Carcinogenic and endocrine disrupting substances as well as pharmaceuticals can pass through even the most advanced wastewater treatment systems (Heberer, 2002). Endocrine disrupting substances are known to disrupt or mimic naturally occurring hormones and may have an impact on the growth, reproduction, or development of many species of wildlife (Furuichi *et al*., 2004).

Wastewater pollution also has its socio-economic impacts on the teeming populace. Goodland and Daly (1996) states that the natural capital is comprised of intact ecosystems and ecosystem services (structurally and functionally). Wastewater pollution negatively affects the ecosystem; with the high rate of wetland destruction, depletion of plant biomass, effects on aquatic wildlife habitat, and the decrease in fresh water access, the ecosystem services provided by these components will continue to degrade (Boesch *et al*., 2001). The natural capital of the earth is thus depleted where the ecosystem looses its capacity to provide the usual vital services. The main objective therefore of championing the course of a high wastewater effluent standard, is to maintain the natural capital so as to ensure that adequate resources are available for natural benefits (Smith *et al*., 2005).

2.9 Microbial pathogens in wastewater

Microbial pathogens which can be potentially present in wastewater can be divided into three separate groups: viruses, bacteria, and the protozoans/helmiths (LeChevallier and Au, 2004).

2.9.1. Viruses

Viruses are among the most important and potentially most hazardous pathogens in wastewater (Tree, 2003). According to Toze (1997), untreated wastewater can contain a range of viruses with their numbers in excess of 10^3 to 10^4 viral particles per litre of wastewater. Viruses are generally more resistant to treatment, more infectious, more difficult to detect in environmental samples such as wastewater and require smaller doses to cause infection than most of the other pathogens (Gomez *et al*., 2006). The common viruses found in wastewater enter the environment through faecal contamination from infected host or carriers (Leclerc *et al*., 2000). Most of the commonly detected pathogenic viruses in wastewater are the enteroviruses; they are small, single-stranded RNA viruses and include the poliovirus types 1 and 2. Others are multiple strains of echovirus, enterovirus and coxsackievirus (Tanji *et al*., 2002). While most members of the general population are susceptible to enteric viral infection, children, the elderly and the immunocompromised are the most at risk and have highest infection rate (Toze, 1997).

2.9.2. Bacteria

Bacteria are the most common of microbial pathogens found in wastewater. A wide range of bacterial pathogens and opportunistic pathogens associated with wastewater are enteric in origin

and have been reported in literature (Simson and Charles, 2000). Gastrointestinal infections are amongst the most common diseases caused by bacterial pathogens in wastewater (LeChevailler and Au, 2004). Wastewater associated infections generally include diarrhoea, dysentery, dysentery-like infections, *Leptospira interrogans* infections, typhoid, human enteritis, legionellosis, melioidosis, stomach ulcer and cancer (Liang *et al*., 2006). The contamination of food by water containing known toxin producing organisms such as *Staphylococcus aureus, Salmonella* spp., *Escherichia coli,* or *Clostridium perferinges* can cause outbreaks of food poisoning (often severe and widespread) (Toze, 1997). One of the emerging wastewater bacterial pathogens of grave public health concern in recent times is *Listeria monocytogenes* otherwise known as invasive *Listeria*. Several cases of Listeriosis outbreaks associated with wastewater have been reported around the globe (Paillard *et al*., 2005).

2.9.3. Protozoa

Pathogenic protozoa are more prevalent in wastewater than any other environmental source (Toze, 1997). Pathogenic protozoans associated with wastewater include, *Entamoeba histolytica, Giadia intestinalis* (formerly *Giadia lamblia*) and *Cryptosporidium parvum, and* these organisms have been frequently isolated from wastewater sources with faecal contamination (Caccio *et al*., 2003; Toze, 1997).

2.9.4. Helminths

Helminths (nematodes and tape worms) are common intestinal parasites which, like the enteric protozoan pathogens, are usually transmitted by faecal route in humans (Feenstra *et al*., 2000). Helminth parasites commonly detected in wastewaters include the round worm (*Ascaris lumbricoides*), the hook worm (*Ascaris duodenale* or *Nector americanus*), the whip worm (*Trichuris trichiura*) and *Strongloides stercolaris* the causative agent of strongyloidiasis (Feenstra *et al*., 2000). It has been estimated that approximately 25% of the world human population is infected with the round worm, *Ascaris lumbricoides* (WHO, 1989). The prevalence of Ascaris infection is influenced by population density, education standards, sanitation levels, degree of agricultural development, and cultural dietary habits (Smith *et al*., 2001). The World Health Organisation lists intestinal nematodes to be of greatest health risk in the use of untreated excreta as well as wastewater for agricultural/aquacultural purposes (WHO, 1989). Children under the age of 19 were reported to be the most affected by nematode infection (Feenstra *et al*., 2000).

2.10 Microbial indicators of wastewater pollution

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. To avoid the necessity of undertaking such huge ventures, indicator microorganisms are used to determine the relative risk of the possible presence of pathogenic microorganisms in a sample (Ashbolt *et al*., 2001). To function effectively as indicators, such microorganisms should be a member of the intestinal microflora

of warm-blooded animals; should be present when pathogens are present, and absent in uncontaminated samples; it should be present in greater numbers than the pathogen(s); should be at least equally resistant as the pathogen to environmental factors and to disinfection in water and wastewater treatment plants; it should not multiply in the environment; It should be detectable by means of easy, rapid, and inexpensive methods and the indicator organism should be non pathogenic (Bitton, 2005).

Escherichia coli have for a very long time been used as indicators of faecal contamination of water sources, and its growth characteristics and behaviour in the environment is relatively well known (Ashbolt *et al*., 2001). The ability of *E coli* to be cultured at elevated temperatures (44.5 ^{o}C) has earned them the name of thermotolerant coliforms (TTC) and they have become the mainstay indicator for the water industry (Leclerc *et al*., 2000). Thermotolerant coliforms are however disadvantaged in that they are more sensitive to environmental changes and treatment processes than a number of more resistant bacterial pathogens and almost all of the viruses, protozoan cyst and helminth eggs (Ashbolt *et al*., 2001). Another drawback with the use of TTC as an indicator of faecal pollution is that coliform bacteria reside in the gut of many different warm blooded animals. Thus, the detection of TTC in a water source does not necessarily confirm the contamination of that water body with human excrement or the presence of human pathogens. The inappropriateness of faecal coliforms (or TTC) as indicators of human faecal contamination of water sources and of the effectiveness of treatment processes has led to the search for more appropriate indicator microorganisms. A number of bacteria and bacteriophages have been studied for their suitability as indicators.

Clostridium perfringes according to (Ferguson *et al*., 1996) were most useful as indicators of human faecal pollution and the only reliable indicator for the presence of *Giardia*

intestinalis when compared with faecal streptococci and F-RNA bacteriophages. Other potential bacterial indicators for the presence of microbial pathogens in water are the enterococci, bifidobacteria, and bacteroides (Leclerc *et al*., 2000). Anaerobic indicator bacteria such as bacteroides and bifidobacteria are however difficult to apply as indicators of faecal contamination on a large scale due to handling difficulties associated with strict anaerobes. This difficulty notwithstanding, recent development of DNA probes for polymerase chain reaction (PCR) detection alleviates the requirement of culturing and improves the potentials of anaerobes as indicators of faecal pollution (Kreader, 1995). One of the problems associated with the use of bacteria as indicator for the presence of microbial pathogens in water is the greater resistance of protozoan cysts and viruses to environmental factors and treatment processes (Tree *et al*., 2003; Hijnen *et al*., 2006; Gomez *et al*., 2006).

Viruses in particular are difficult to detect in many water sources due to low numbers, and the difficulty and expense of culturing (Tanji *et al*., 2002). To overcome these problems, bacterial viruses (bacteriophages) have been examined for use in faecal pollution and the effectiveness of treatment processes to remove enteric viruses (Ashbolt *et al*., 2001). The most common bacteriophage studied is male-specific (F-RNA) bacteriophage (in particular MS2 and PRD-1) which infect gram negative bacteria containing the F^+ sex plasmid; somatic coliphages (bacteriophage which infects coliforms); and *Bacteroides fragilis* specific bacteriophage (Leclerc *et al*., 2000; Hijnen *et al*., 2006). Somatic coliphage and F-RNA bacteriophage have been shown to survive but not replicate for long periods in tropical pristine rivers (Hernandez-Delgado and Toranzos, 1995), indicating that they could be useful as indicators in environmental waters. One of the main interests in the use of bacteriophage is their potential of indicating the effect treatment processes have on the survival of pathogenic viruses. Jofre *et al*. (1995) examined the

efficiency of three different water treatment systems to remove bacteriophage from water and found that *B. fragilis* bacteriophages were more resistant to treatment processes than F-specific bacteriophage and somatic coliphage and enteroviruses.

While a number of potential replacement for faecal coliforms have been studied for their possible use, none have been found to be completely suitable. All of the potential indicators studied till date has one or more characteristics which prevent their implementation as replacement for faecal coliforms (Ashbolt *et al*., 2001; Bitton, 2005). Thus, despite their drawbacks, faecal coliforms still remain the major organisms used to indicate faecal pollution and the effectiveness of treatment processes (Toze, 1997). However, the improvements in the detection of microorganisms by molecular techniques which have occurred in the last 10 years may mean that the use of indicators may no longer be required (Bitton, 2005).

2.11 Isolation and detection of wastewater pathogens

Methods used to identify and quantify microbial populations in wastewater can be divided into three main groups: culture, immunology and nucleic acid–based.

2.11.1. Culture-based methods

This method employs selective and/or differential media, which provide a 'presumptive identification' and may be followed by a number of other tests. The tests provide confirmation of the identity of isolates by biochemical, immunological or molecular methods. Abundance is either inferred from the number of colony forming units (CFUs) on culture plates or by Most Probable Number (MPN) dilutions of wastewater samples. For accurate quantification,

representative presumptively positive strains must be corroborated by more extensive characterization with biochemical tests or molecular assays. The dilution or concentration (by filtration) of samples prior to culture-based enumeration can accommodate a wide dynamic range of wastewater microbial population sizes (Thompson *et al*., 2004).

One of the disadvantages of culture-based techniques in wastewater sample is that they depend on how reproducibly and quantitatively the target pathogen population will grow on culture media. This is quite limiting as certain pathogens can enter a viable but non-culturable state (VBNC) in response to shifts in environmental conditions possibly complicating interpretation of population dynamics observed in culture-based studies (Besnard *et al*., 2000). Another disadvantage is that since culture-based techniques inherently rely on growth, they are limited by how fast the target population grows to detectable levels; otherwise they may be outgrown by nontarget populations (Toze, 1997). With notable exceptions, most culture-based identification schemes for specific populations are time and labour-intensive, and may require preliminary enrichment or decontamination steps that confound enumeration (Besnard *et al*., 2000). Despite the above-mentioned limitations of culture-based methods, significant benefits remain. Most notably, the cost of materials needed for culture-based assays in wastewater are relatively cheap and does not require extensive training, and highly specialized materials and equipment. In addition, cultured isolates allow subsequent investigations into the virulence and/or clinical significance of environmental pathogen populations (Thompson *et al*., 2004).

2.11.2. Immunological methods

Immunological detection has been used to identify and in some cases, enumerate pathogenic populations in wastewater samples. These methods rely on the inherently high specificity of

immune reactions and typically target pathogen-specific antigens such as cell-wall lipopolysaccharides (LPS), membrane and flagellar proteins or toxins. Immuno-assays can be categorized into three main groups: enzyme-linked immunosorbent assay (ELISA), immunofluorescent microscopy, and agglutination assays (Besnard *et al*., 2000; Bitton, 2005).

There are several notable challenges for the implementation of immunological methods to detection of pathogens in wastewater samples, which contain a large diversity of unknown bacteria. First, the sensitivity of many current methods is not high enough for detection of pathogens at low, environmentally relevant, concentrations. Second, false positive results can be generated by cross-reaction of antibodies with antigens of similar but non-target organisms. This is particularly problematic when polyclonal antibodies are used since these are complex mixtures of antibodies against multiple, mostly uncharacterized cell structures (Thompson *et al*., 2004; Bitton, 2005). Finally, design and production of specific antibodies generally requires growth of target microorganisms, constraining the applicability of the methods to culturable populations (Bitton, 2005). Despite these limitations, immunological methods have many potential applications for detection of pathogens in wastewater environment (Bitton, 2005).

2.11.3. Nucleic acid based methods

Advances in molecular biology have revolutionized wastewater microbiology by facilitating the identification of emerging pathogens, the detection of environmental populations, and the discrimination between closely related pathogenic and non-pathogenic bacteria (Persing *et al*., 2003). Discrimination of nucleotide variation among genes, whose occurrence is specific to an organism or whose sequence differentiates organisms, is often achieved by nucleic acid hybridization; other methods rely on restriction cutting of the chromosome. Hybridization-based

methods include fluorescence *in situ* hybridization (FISH) (Loge *et al*., 1999; Moter and Gobel, 2000; Baudart *et al*., 2002; Rompre *et al*., 2002) and filter hybridization (colony and dot-blot hybridization) (Polz and Cavanaugh, 1997; Jiang and Fu, 2001), and the polymerase chain reaction (PCR) (von Wintzingerode *et al*., 1997; Polz and Cavanaugh, 1998). The PCR couples hybridization of short DNA molecules (primers) to template molecules followed by amplification with a polymerase. Molecular typing methods have used PCR [multi-locus sequence typing (MLST)] or restriction cutting [pulsed field gel electrophoresis (PFGE)] for analyzing genomic signatures (Maiden *et al*., 1998; van Belkum, 2003). The general principles of hybridization-based, PCR-based, and molecular typing methods have been reviewed in widely available protocol books (Sambrock and Russell, 2001; Persing *et al*, 2003).

Nucleic acid-based detection techniques have the advantages of being very target specific, relatively more sensitive and less time consuming. They also have the advantage of detecting viable but non-culturable organisms (Toze, 1997). However, due to their sensitivity, nucleic acid-based methods for detecting wastewater pathogens as in other microorganisms are unable to differentiate between viable and nonviable pathogens (or their resting stages). There is also the issue of false positive reactions due to contamination by extraneous nucleic acids, often through contact with laboratory equipment. Further, there is the need to concentrate large volumes of water in order to get a significant amount of total genomic DNA and this might lead to loss of significant population of the target organism(s) (Toze, 1997). Thus, while the use of nucleic acid-based detection techniques show great promise for the detection of pathogens in wastewater, a number of issues need to be resolved before these techniques could be fully deployed as standard detection methods for the wastewater industry.

2.12 Conclusions

The advancement of wastewater treatment technology notwithstanding, treated sewage may still contain some harmful substances (including microbial pathogens) irrespective of thoroughness and sophistication of the treatment process. There is a wide range of microbial pathogen types which can occur in wastewater, with the type and number present being highly dependent on the socioeconomic conditions and customs of the communities creating the wastewater. In order to propose an efficient way of treating wastewater, there is need to understand the negative environmental impacts posed by the untreated or inadequately treated wastewater entering the nearby ecosystems, especially on the lives that depend on the ecosystem for sustenance. Survival and persistence of such microbial pathogens especially in conventional wastewater treatment facilities is increasingly becoming of interest and is a subject of ongoing investigation in our laboratory.

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CHAPTER 3

Physicochemical quality of an urban municipal wastewater effluent and its impact on the receiving environment

(Published in Environmental Monitoring and Assessment)

CHAPTER 3

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Abstract

The physicochemical qualities of the final effluents of an urban (East London) wastewater treatment plant in South Africa were assessed between August 2007 and July 2008 as well as their impact on the receiving watershed. The pH values across all sampling points ranged between 6.8 and 8.3, while the temperature varied from 18 to 25°C. Electrical conductivity (EC) of the samples was in the range of 29 - 1015 µS/cm, and turbidity varied between 2.7 and 35 NTU. Salinity and total dissolved solids (TDS) varied from 0.36 - 35 psu and 16 - 470 mg/l respectively. The concentrations of the other physicochemical parameters are as follows: chemical oxygen demand [COD] (48 - 1180 mg/l); dissolved oxygen [DO] (3.9 - 6.6 mg/l); nitrate (0.32 - 6.5 mg NO_3^- as N/l); nitrite (0.06 - 2.4 mg NO_2^- as N/l); phosphate (0.29 - 0.54 mg PO_4^{3-} as P/l). pH, temperature, EC, turbidity, TDS, DO, and nitrate varied significantly with season and sampling point ($P < 0.05$; $P < 0.01$), while salinity varied significantly with sampling point ($P < 0.01$) and COD and nitrite varied significantly with season ($P < 0.05$). Although, the treated effluent fell within recommended water quality standard for pH, TDS, nitrate and nitrite, it fell short of stipulated standards for other parameters. The result generally showed a negative impact of the discharged effluent on the receiving watershed and calls for a regular and consistent monitoring programme by the relevant authorities to ensure best practices with regard to treatment and discharge of wastewater into the receiving aquatic milieu in South Africa.

Keywords: physicochemical qualities; wastewater effluent; receiving watershed.

3.1 Introduction

Water forms the backbone of the world's economy and it is critical to the development of all sphere of human endeavor (Obi *et al*., 2006). It is essential for living systems, industrial processes, agricultural production and domestic uses (Hu, 2009). The quality of water available and accessible to a people has tremendous impact on their living standard and well being; hence global and local efforts are rife at ensuring adequate provision of clean and safe water to the world's growing population. As a semi-arid country, South Africa has a peculiar challenge of meeting her ever increasing water demand occasioned by industrial and population growth. This has inspired the government to set up a 'Strategic Framework for Water Services' (DWAF, 2003) aimed at ensuring basic water supply (at least 25 liters of potable water *per capita* per day) to all South Africans. As laudable as this program might be, it may create its own challenges; increasing water supply will most likely translate to increase in wastewater output. The implication therefore is that additional wastewater output without due diligence of the working efficiency of existing wastewater treatment plants might end up compounding an already bad situation.

There are reports in the literature about the inability of existing wastewater treatment plants in South Africa to adequately treat wastewater effluent prior to discharge into the receiving environment (Morrisson *et al*., 2001; Fatoki *et al*., 2003). This has seriously compromised the quality of receiving water systems by altering the interrelationship and interactions of parameters that govern the stability of the ecosystem. Physicochemical parameters such as temperature, pH, DO, salinity, and nutrient loads have been reported to influence biochemical reactions within water systems. Such changes in the concentration of these

parameters are indicative of changes in the condition of the water system (Hacioglu and Dulger, 2009), the consequence of such is the compromise of the water quality for beneficial uses.

Wastewater discharges may contain health compromising pathogens, carcinogenic substances (e.g. heavy metals, trihalomethanes, etc), and/or chemical substances which may cause adverse environmental impact such as changes in aquatic habitats and species composition, decrease in biodiversity, impaired use of recreational waters and shellfish harvesting areas, and contaminated drinking water (Environment Canada, 2001; CCME, 2006). All of these impact leads to a less valuable environment, poor health, a less prosperous economy, and ultimately, a diminished quality of life (Environment Canada, 2001).

Many South Africans live in rural areas and lack potable water supply, thus relying on surface waters that are negatively impacted by untreated or inadequately treated wastewater for their daily subsistence (Pearson and Idema, 1998; Mackintosh and Colvin, 2003). Furthermore, the environmental implications of inadequately treated effluent may take a serious toll on the socio-economic status of South Africa as a leading tourist destination in the world. To preserve the health of unsuspecting South Africans and maintain the integrity of the environment, it is imperative to regularly and consistently monitor the quality of municipal wastewater effluent prior to discharge into the receiving environment. In this study, we evaluate the physicochemical quality of the final treated effluent of a typical urban wastewater treatment facility in South Africa and its impact on the receiving environment.

3.2 Materials and methods

3.2.1 Plant description

The wastewater treatment plant under study is located in East London, an urban settlement in the Buffalo City municipality of the Eastern Cape Province of South Africa and situated in the geographical coordinates 32.97^{o}S and 27.87^{o}E. The plant receives domestic and industrial sewage. It is an activated sludge treatment plant comprising four screens, a grit channel, two aerobic tanks, six sedimentation tanks, two anaerobic tanks and two anoxic tanks. Disinfection of effluent is done by chlorination via a water pressure operated, wall mounted, gas chlorinator in a baffled reinforced concrete contact tank. The final effluent is discharged into the Indian Ocean between Nahoon and Eastern Beach at Bats cave. The average daily inflow during the period of study was 32 000 m^3/day, while the plant has a built in capacity of 40 000 m^3/day.

3.2.2 Sample collection

Wastewater samples were collected on a monthly basis from the final treated effluent (FE), discharge point (DP), five hundred meters (500 m) upstream (UP) and five hundred meters (500 m) downstream (DW) of the discharge point between August 2007 and July 2008. Samples were collected in duplicates in one litre Nalgene bottles previously cleaned by washing in non-ionic detergent, rinsed with tap water and later soaked in 10% HNO_3 for 24 hours and finally rinsed with deionised water prior to usage. During sampling, sample bottles were rinsed three times with sampled water before filling the bottles to the brim at depths of one meter below the surface of each designated sampling point. Samples were then transported in cooler boxes containing ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory

at the University of Fort Hare, Alice, South Africa for analyses. Samples were processed within six hours of sample collection.

3.2.3 Physicochemical analysis

All field meters and equipment were checked and appropriately calibrated according to the manufacturers' instructions. pH, temperature, electrical conductivity (EC), salinity, total dissolve solid (TDS), and dissolved oxygen (DO), were all determined on site using the multi-parameter ion specific meter (Hanna-BDH laboratory supplies). Turbidity was also determined on site using a microprocessor turbidity meter (HACH Company, model 2100P). The concentrations of orthophosphate as P, nitrate, nitrite, and chemical oxygen demand (COD) were determined in the laboratory by the standard photometric method (DWAF, 1992) using the spectroquant NOVA 60 photometer (Merck Pty Ltd). Samples for COD analyses were digested with a thermoreactor model TR 300 (Merck Pty Ltd) prior to analysis using the spectroquant NOVA 60 photometer.

3.2.4 Statistical analysis

Calculation of means and standard deviations were performed using Microsoft Excel office 2007 version. Correlations (paired T-test) and test of significance (two-way ANOVA) were performed using SPSS 17.0 version for Windows program (SPSS, Inc.). All tests of significance and correlations were considered statistically significant at P values of < 0.05 or < 0.01.

3.3 Results and discussion

Mean seasonal values and standard deviation (S.D.) for the different water quality parameters are given in Table 3.1. The pH values (6.8 - 8.3) varied significantly with season ($P < 0.01$) and sampling points ($P < 0.01$) and the interaction effect of both season and sampling point was also significant ($P < 0.01$) on the pH. The seasonal variation was likely caused by the significant difference in pH values observed in spring against those of summer ($P < 0.01$) and autumn ($P < 0.01$) and between autumn and winter ($P < 0.05$); while the variation in pH with sampling point must have been a function of the significant ($P < 0.01$) lower pH values (6.8 - 7.5) observed in the FE compared to DP, UP and DW (8.0 - 8.3) and between DP (8.0) and UP/DW (8.2 – 8.3) ($P < 0.01$). The significant interaction effect of season and sampling point on pH indicates that the variation of pH with season was dependent on the sampling point and the observation is corroborated by the fact that FE and to some extent DP were mainly responsible for the observed differences in pH during this study (Table 3.1).

pH ranges similar to those observed in this study have been reported in the literature for final effluents and their receiving waters (Manios *et al*., 2006). Conversely, Ogunfowokan *et al*. (2005) reported lower pH ranges (5.23 - 6.32) and Akan *et al*. (2008) reported higher pH values (8.94 - 10.34) for wastewater effluents and their receiving watersheds in Ile-Ife and Jakara (both in Nigeria) respectively. The composition of wastewater effluent varies from facility to facility according to level of treatment, type of households, businesses, industries, and public facilities discharging into the system (Environment Canada, 2001) and this could be an important contributory factor to the observed differences in pH. The pH level of a water system determines its usefulness for a variety of purposes. Very high or low pH has been reported (Morrison *et al*. 2001; DWAF, 1996c) to be toxic to aquatic life and alter the solubility of other chemical

Table 3.1 Seasonal distribution of physicochemical parameters of the treated final effluents and its receiving waters

Seasons	Sample points	Parameters [a] (Mean ± SD)										
		pH	Temperature	EC	Turbidity	Salinity	TDS	COD	DO	NO_3^-	NO_2^-	PO_4^{3-}
Spring	FE	7.1±0.20	20±1.17	749±44	13±2.53	0.36±0.09	372±26	68±0	5.4±0.13	3.8±3.2	2.4±3.8	0.38±0.08
	DP	8.0±0.17	20±0.69	33±9	8.0±2.78	22.9±7.47	16±5	75±0	6.6±0.7	1.28±1.1	0.91±1.1	0.54±0.12
	UP	8.0±0.18	20±1.38	47±1.3	3.3±0.77	34.4±1.0	24±0.58	82±0	6.4±0.36	0.65±0.95	0.7±1.1	0.31±0.1
	DW	8.0±0.16	20±1.24	46±0.52	34±1.26	33.8±1.26	23±0.71	83±0	6.5±0.77	0.32±0.37	0.78±1.22	0.39±0.04
Summer	FE	7.2±0.19	24±0.95	789±172	4.1±1.75	0.38±0.09	367±80	462±599	4.2±0.19	6.5±0.28	0.2±0.15	0.32±0.13
	DP	8.0±0.15	23±1.34	41±8	4.3±1.81	27.8±5.63	20±4	887±1442	6.1±0.99	3.7±1.8	0.11±0.05	0.34±0.18
	UP	8.3±0.02	21±1.8	48±2.6	2.7±1.30	34.2±0.81	24±0.55	865±1409	5.7±0.57	2.4±0.67	0.06±0.01	0.29±0.21
	DW	8.2±0.04	21±1.96	47±2.6	34±0.52	33.5±0.52	23±0.42	49±4	6.2±0.80	2.8±2.9	0.07±0.01	0.29±0.20
Autumn	FE	7.5±0.17	25±1.77	1015±472	3.8±1.10	0.51±0.27	470±232	48±29	3.9±0.99	3.4±3.0	0.23±0.05	0.37±0.31
	DP	8.0±0.18	22±0.67	29±17	6.3±1.74	19.4±12.3	19±4.44	379±445	6.3±0.72	2.3±0.64	0.22±0.18	0.37±0.24
	UP	8.2±0.15	21±1.51	47±0.76	3.8±2.44	33.8±1.55	23±0.88	457±389	6.0±0.49	1.8±0.37	0.10±0.07	0.44±0.39
	DW	8.2±0.18	20±1.52	47±1.84	34±0.36	33.8±0.36	23±0.28	460±382	6.0±0.24	1.3±0.37	0.11±0.05	0.30±0.21
Winter	FE	6.8±0.10	20±2.03	776±42	5.6±0.42	0.42±0.02	387±17	53±31	4.3±0.5	5.6±1.85	0.73±0.40	0.29±0.13
	DP	8.0±0.35	19±0.85	35±12	7.3±3.2	25.4±9.74	19±3.93	1128±923	5.9±0.99	3.0±0.21	0.2±0.15	0.47±0.44
	UP	8.2±0.13	18±1.15	45±1.33	4.3±3.35	35±0.22	24±0.18	1180±971	6.1±1.48	3.2±0.64	0.09±0.04	0.42±0.36
	DW	8.3±0.08	18±1.17	45±1.52	35±0.42	34.5±0.42	24±0.3	1123±919	5.6±0.85	2.3±1.31	0.10±0.05	0.37±0.30
[b]F-values		10.32	59.39	2.696	13.973	1.961	4.204	3.616	7.695	9.86	6.276	.838
[c]*P*-values		.000**	.000**	.049*	.000**	.123	.007*	.018*	.000**	.000**	.001*	.477
[d]F-values		311.37	31.88	465.104	28.535	546.92	4.072	1.564	41.84	16.151	2.221	.876
[e]*P*-values		.000**	.000**	.000**	.000**	.000**	.008*	0.207	.000**	.000**	.092	.457
[f]F-values		7.327	3.101	2.863	7.94	1.733	3.712	.999	1.372	.517	.723	.467
[g]*P*-values		.000**	.002*	.004*	.000**	.088	.000**	.452	.207	.857	.686	.893

FE final effluent, *DP* discharge point, *UP* 500 m upstream, *DW* 500 m downstream

* $P < 0.05$; ** $P < 0.01$; significant variation

[a] Values are expressed in mg/l except in pH, Temperature (in degrees Celsius), turbidity (in nephelometric turbidity unit), salinity (in practical salinity unit), and EC (in microsiemens per centimeter)

[b] F-values for parameters and season [c] *P*-values for parameters and season [d] F-values for parameters and sampling point [e] *P*-values for parameters and sampling point [f] F-values for combined effect of season and sampling point on parameters [g] *P*-values for combined effect of season and sampling point on parameters

pollutants as well as some essential elements in water systems (DWAF, 1996c), thereby causing adverse effects on the ecosystem and those who depend on it. The South African target water quality for pH in water for domestic use is 6 to 9 (DWAF, 1996b), and the European Union tolerance limit for pH in water for the support of fisheries and aquatic life is also set at 6 - 9 pH units (Chapman, 1996). The pH values observed in this study across all sampled points fell within the recommended standards irrespective of season. This suggests that the effluent may not have negative impact on the usefulness of the receiving watershed for domestic, fishery and recreational purposes with reference to pH standards.

Temperature is an important water quality parameter due to its influence on other parameters. Temperature affects the solubility and consequently the availability of oxygen in water (Akan *et al*. 2008); it also affects the toxicity of some chemicals in water systems as well as the sensitivity of living organisms to toxic substances (Dojlido and Best, 1993; Mayer and Ellersieck, 1988). The temperature observed in this study ranged from 18^{o} to 25^{o}C and varied significantly with season and sampling point ($P < 0.01$).The highest temperature was observed in autumn in FE and the lowest observed in winter in the receiving watershed (UP and DW). Temperature was significantly ($P < 0.01$) higher in the FE and DP compared to other sampling points irrespective of season except in spring where the temperature (20^{o}C) was the same for all sampled points. This explains the significant ($P < 0.05$) interaction effect of season and sampling point on temperature (Table 3.1) and indicates that temperature was not only a function of season but also dependent on sampling point. Our values for temperature fell within the acceptable limit of no risk ($\leq 25^{o}$C) for domestic water uses in South Africa (DWAF,WRC, 1995). This observation implies that the discharged effluent was of standard quality with respect to temperature and may not significantly offset the homeostatic balance of the receiving

ecosystems; neither will it adversely affect the use of the receiving watershed for domestic purposes.

The values for EC in this study ranged between 29 and 1015 µS/cm across the sampling points and varied significantly with season ($P < 0.05$) and sampling point ($P < 0.01$). The weak significant variation ($P < 0.49$) in EC with season indicates that season only marginally affected EC values (Table 3.1); while the wide diference in EC values between FE (749 - 1015 µS/cm) and other points (29 - 48 µS/cm) is likely responsible for the the strong significant variation ($P < 0.01$) observed for EC with sampling point. The significantly higher EC values consistently observed at FE compared to other sampled points may suggest that chlorine concentration contributed to the high EC levels at FE (Mamba *et al*., 2009). The similar EC values observed upstream (UP) and downstream (DW) of the discharge point (DP) showed that the effluent quality normalized with that of the receiving watershed 500 m downstream (Table 3.1), and generally alludes to the self-cleaning capacity of the receiving watershed as expected of a massive ocean. Electrical conductivity (EC) is a measure of dissolved ions in water systems; it has also been reported to be a useful and easy indicator of salinity or total salt content of water systems (Oluyemi *et al*., 2006; Morrison *et al*., 2001). EC concentrations for FE (749 - 1015 µS/cm) fell short of the target water quality limit (70 mS/m or 700 µS/cm) of no risk for domestic water uses (DWAF, 1996b), while values for the receiving watershed (29 - 48 µS/cm) largely fell within the acceptable limits and suggest that the receiving watershed is safe and fit for domestic uses with respect to EC.

The turbidity of the water systems under study (Table 3.1) varied from 2.7 NTU (UP, summer) to 35 NTU (DW, winter). The values were similar to those observed by Igbinosa and Okoh (2009) but relatively higher than those reported by Fatoki *et al*. (2003). Turbidity

throughout the study fell short of the target water quality limit (0 - 1 NTU) of no risk for domestic water uses in South Africa (DWAF, 1996b); implying that the water system under study is not suitable for domestic uses with reference to turbidity. Turbidity however, fell within acceptable limits by World Health Organization [WHO] (WHO, 2004) standard (≤ 5 NTU) for effluents to be discharged into the environment in spring (UP), summer (FE, DP and UP), autumn (FE and UP), and winter (UP; Table 3.1). Turbidity is a measure of suspended particles (inorganic and/or organic matters) in water systems and usually correlates significantly with microbial load; hence high turbidity will more often than not support the growth of pathogens and increase the chances of infection (Obi *et al*., 2007). The presence of suspended particles in a water body could also render it unfit for full-contact recreational uses (DWAF, 1996a). There was significant variation in turbidity with season ($P < 0.05$) and sampling point ($P < 0.05$) in this study. The significant difference in turbidity in spring compared to those of summer and autumn ($P < 0.01$) and winter ($P < 0.05$) might be caused by the seasonal variation (Table 3.1). The relatively higher turbidity values in spring could be attributed to surface runoff and erosion occasioned by rainfall, carrying soil and silt into the water system (Morokov, 1987). The significantly ($P < 0.01$) higher turbidity values observed at DW in relation to other sampling points may be responsible for the variation in turbidity with sampling point (Table 3.1) and suggests that factors other than effluent quality contributed to the turbidity of the receiving water downstream. The relatively high turbidity levels observed at FE gives cause for concern as high turbidity is reported to affect the effectiveness of chlorination as a means of disinfection (Obi *et al*., 2007) and increase chances of trihalomethane (THM) precursor formation in the effluent (Fatoki *et al*., 2003). THM is a carcinogenic compound formed as a by-product of chlorine and organic matter reaction in water systems and has serious health implications for aquatic life and

humans exposed to it (Environment Canada, 2001).

Salinity in this study ranged from 0.36 psu (FE, spring) to 35 psu (UP, winter). The values for salinity in FE (0.36 - 0.51 psu) and DP (19.4 - 27 psu) fell short of the acceptable limits (33 - 35 psu) of no risk for all biological activities in the marine ecosystems (SANCOR 1984; Whitfield and Bate, 2007) and may adversely affect the aquatic biota of the receiving watershed. However, salinity levels (33 – 35 psu) at UP and DW fell within the acceptable limits; indicating the self-recovery capacity of the ocean. Salinity varied significantly with sampling point ($P < 0.01$) but not with season during this study. Salinity at FE was consistently and significantly ($P < 0.01$) lower than values recorded in the receiving watershed (DP, UP, DW) which may be responsible for the observed difference in salinity with sampling point (Table 3.1). Salinity is the saltiness of a water body and high salt content in effluents discharged into a receiving watershed could cause serious ecological disturbance that may result in adverse effects on the aquatic biota (Morrison *et al*., 2001; Oluyemi *et al*., 2006).

TDS values in this study varied between 16 mg/l (DP, spring) and 470 mg/l (FE, autumn). The values fell within acceptable limits (≤ 2000 mg/l) for effluents discharged into surface waters by WHO standards (Akan *et al*., 2008). It also fell within acceptable limits (0 - 450 mg/l) for South African water systems applied in domestic uses (DWAF, 1996b) except in autumn when the TDS value (470 mg/l) in the final effluent (FE) exceeded the target water quality limit of no risk (Table 3.1). TDS like EC is a measure of salinity in water systems. The relevance of this parameter to water quality is similar to those discussed under EC and salinity. In addition, TDS as a measure of salinity is an important agricultural water quality parameter with respect to soil salinity. Salinity of soil has been reported to be related to and often determined by the salinity of the irrigation water (FAO, 1992); while plant growth, crop yield,

and quality of produce are affected by the TDS concentration in irrigation water (FAO, 1992). This is worthy of note as the effluent from the wastewater facility under study is used as water resource for a fish pond as well as to irrigate a nearby golf course. TDS varied significantly with season ($P < 0.05$) and sampling point ($P < 0.01$). The significant difference ($P < 0.05$) in TDS values observed in autumn compared to those of other seasons may be responsible for the observed seasonal variation; while the relatively high TDS concentration (367 – 470 mg/l) observed in FE compared to other sampled points (16 – 24 mg/l) is likely the reason for the observed difference in TDS with sampling point (Table 3.1). The TDS values at FE during this study were higher than those reported by Igbinosa and Okoh (2009); conversely, Akan *et al*. (2008) reported higher TDS values (2210 - 2655 mg/l) for the receiving watershed compared to those (16 - 36 mg/l) observed in this study.

COD is a measure of the amount of oxygen required by a strong oxidant (e.g. H_2SO_4) to breakdown both organic and inorganic matters in a water system (Akan *et al*., 2008). Elevated levels of COD in water systems lead to drastic oxygen depletion which adversely affects the aquatic biota (Fatoki *et al*., 2003). COD concentrations in this study ranged between 48 and 1180 mg/l with the highest value recorded upstream of the urban effluent discharge (UP) in winter and the lowest value observed at FE in autumn. The values fell short of the acceptable target limit (30 mg/l) recommended by the South African government for effluents to be discharged into surface waters (Government Gazette, 1984) and suggests that the effluent may negatively impact on the receiving environment. COD concentrations however, fell within acceptable limits (≤1000 mg/l) of no risk by WHO standard for effluents to be discharged into surface waters (Akan *et al*., 2008) except in winter where COD values for DP, UP and DW were higher than the recommended limit. COD significantly varied with season ($P < 0.05$) and sampling point ($P < 0.01$); and

values were generally highest in winter followed by summer, autumn and spring respectively. The higher COD values in winter compared to other seasons could be attributed to the lesser rate of organic matter breakdown (occasioned by lower microbial activity) during the cold (winter) season compared to the warmer seasons (Tomida *et al*., 1999). The higher COD values observed in the receiving watershed (UP, DW, and DP respectively) compared to FE suggested that unidentified sources contributed more COD to the watershed than the final effluent. Several authors have reported the pollution of surface water bodies by non-point sources such as domestic, municipal, and/or agricultural run-offs (Hacioglu and Dulger, 2009; Pradhan *et al*., 2009; Shirodkar *et al*., 2009). Contrary to the observation of this study Morrison *et al*. (2001) reported higher COD values for final effluents compared to the receiving watershed in their study of the Keiskammahoek sewage treatment facility and its receiving river.

The DO levels in this study varied from 3.9 to 6.6 mg/l across the sampled points, and were similar to those reported previously (Oluyemi *et al*., 2006; Akan *et al*., 2007). DO varied significantly with season ($P < 0.01$) and sampling point ($P < 0.01$). The significantly higher DO values recorded in spring versus summer and autumn ($P < 0.05$) and winter ($P < 0.01$) may be responsible for the observed seasonal variation (Table 3.1); while the observed difference in DO with sampling points must have been occasioned by the significant ($P < 0.01$) lower DO values (3.9 – 5.4 mg/l) observed in FE compared to the other sampling points (5.7 – 6.6) (Table 3.1). This indicates that the nutrient load of the final effluent was generally higher than those of the receiving watershed (Akan *et al*., 2008; CCME, 2006) and implies that the treated effluent is a contributing source of nutrient to the receiving watershed. The DO levels in this study fell short of the acceptable limit (≥ 5 mg/l) of no risk for the support of aquatic life (Fatoki *et al*., 2003) in the final effluent except in spring 2007 where FE was compliant with the stipulated standard

(Table 3.1). DO levels in the receiving watershed were however, within the recommended standard throughout the period of study; indicating that the receiving watershed supports the survival of the aquatic biota. Dissolved oxygen is essential in maintaining the oxygen balance in an aquatic ecosystem; low dissolved oxygen level in water system is reported to have adverse effects on the aquatic life (Fatoki *et al*., 2003). It affects the survival of fish by increasing their susceptibility to disease, hampering swimming ability, altering feeding, migration, reproductive behaviour, and ultimately leads to death of aquatic life (Environment Canada, 2001).

Nitrate concentration in this study varied between 0.32 mg NO_3^- as N/l and 6.5 mg NO_3^- as N/l and generally fell short of the acceptable safety limit (1.5 mg NO_3^- as N/l) for effluent to be discharged into surface waters in South Africa (Government Gazette, 1984). The new South African target water quality standard for nitrate considers the effect of this compound on the health of infants and pregnant women and thus set the safety limit for domestic water supply at 6 mg NO_3^- as N/l (DWAF, 1996b). Based on this new standard, the nitrate concentrations in this study were mostly within acceptable limits (Table 3.1) and suggest that the water system under study is fit and safe for domestic applications. Nitrate concentration however, slightly exceeded the safety limit in the final effluent during summer (6.5 mg NO_3^- as N/l) (Table 3.1). Nitrate concentrations varied significantly ($P < 0.01$) with season and sampling point. The significant difference in nitrate concentrations recorded in spring against summer ($P < 0.01$) and winter ($P < 0.05$) and for autumn against summer ($P < 0.05$) and winter ($P < 0.05$) may be responsible for the observed seasonal variation. The significant ($P < 0.01$) higher nitrate values (3.4 – 6.5 mg NO_3^- as N/l) in the FE compared to other sampled points (0.32 – 3.7) is likely the cause of the observed difference in nitrate with sampling point (Table 3.1). The observation suggests that the final effluent was a significant contributor of nitrate to the receiving watershed in agreement with

the report of Morrison *et al*. (2001) but contrary to the observation of Ogunfowokan *et al*. (2005). Nitrates are inorganic sources of nitrogen that support the growth and development of living organisms at appropriate concentrations. However, high nitrate levels may result in excessive nutrient enrichment in water systems (eutrophication) leading to loss of diversity in the aquatic biota and overall ecosystem degradation through algal blooms, excessive plant growth, oxygen depletion, and reduced sunlight penetration (CCME, 2006). It has also been reported that nitrate concentration above 45 mg/l may result in anaemia in infants and pregnant women and formation of carcinogenic nitrosomines (Akan *et al*., 2007).

Nitrite like nitrate is a source of nutrient that could have adverse effects on aquatic ecosystems at elevated concentrations. Their effects on water systems are generally similar to those described for nitrate. The South African limit (0 – 6 NO_2^- as N/l) of no adverse effect for nitrite in domestic water supply is the same as in nitrate (DWAF, 1996b) and suggests that the entire water system under study was fit and safe for domestic uses based on their nitrite concentrations (0.06 – 2.4 NO_2^- as N/l). The nitrite levels recorded in the entire water system in spring and in the final effluent in winter however, fell short of the South African standard (< 0.5 NO_2^- as N/l) for the preservation of the aquatic ecosystem (DWAF, 1996c) and therefore put the aquatic ecosystem at risk of eutrophication. The nitrite levels during the other seasons do not pose any serious threat to the integrity of the aquatic ecosystem by reason of this standard. Nitrite significantly varied with season ($P < 0.05$) but not with sampling point (Table 3.1). Nitrite concentration was highest in spring followed by winter, autumn and summer respectively. The significant ($P < 0.05$) difference in nitrite concentration in spring compared to other seasons may be responsible for the observed seasonal variation and suggests that surface runoff and erosion occasioned by rainfall during this (spring) season may be a significant factor in the

observation (Morokov, 1987). Although nitrite did not vary significantly with sampling point, the nitrite concentration downstream (DW) generally reflected nitrite levels in the final effluent throughout the sampling period (Table 3.1) and suggests that the final effluent was the major contributor of nitrite to the receiving watershed.

Orthophosphate (as P) levels in this study varied from 0.29 mg PO_4^{3-} as P/l to 0.54 mg PO_4^{3-} as P/l across seasons and sampling points. The P levels observed in this study exceeded the South African target limit of 5 µg/l (0.005 mg PO_4^{3-} as P/l) for P in water systems that will reduce the growth of algae and other plants; and suggests that the water is polluted and pose serious threat to the aquatic biota in particular and the ecosystem in general. Phosphorus did not vary significantly with season or sampling point. The higher P levels sometimes observed in the receiving watershed compared to the final effluent suggests that there were other non-identified sources of P in the water system. This could be as a result of agricultural, municipal or domestic runoffs (non-point sources) that flowed into the receiving watershed from diverse sources in the catchment area under study (Correl, 1998). Similar P levels as observed in this study had been previously reported (Morrison *et al*., 2001; Fatoki *et al*., 2003); higher P levels were however reported by other workers (Ogunfowokan *et al*., 2005; Akan *et al*., 2008). Phosphates are reported to be the most important growth-limiting factor in eutrophication and results in a number of undesirable ecological effects in the water system (CCME, 2006). Common sources of phosphate in water systems are domestic wastes (e.g phosphate-based detergents) and agro-allied chemicals such as fertilizers (Ogunfowokan *et al*., 2005).

Conventional approaches to water quality assessment are based on comparison of experimentally determined parameter values with existing guidelines. While this methodology is appropriate for checking legal compliance and allows proper identification of contamination

sources, it does not give a holistic picture of the spatial and temporal trend of the overall quality of the water system (Boyacioglu, 2007). Due to the complex nature of the physical, chemical, biological, and socio-economic processes that govern the water system, researchers are exploring ways to better understand the interrelationships and interactions of the components involved in these processes under various circumstances. Such understanding promises to further our capacity to preserve and manage our water systems. Several authors (Shyamala *et al*., 2008; Pradhan *et al*., 2009; Shirodkar *et al*., 2009) have used correlation as a tool to elucidate the interrelationship between and amongst water quality parameters as well as to trace the possible sources of contamination in a complex environment. Furthermore, conventional water quality assessments could involve as many as 20 parameters to adjudge a water system fit for use or otherwise. This could be very expensive especially for developing countries such as South Africa and could limit water quality evaluation in such countries. Correlation amongst other tools can also be used to identify parameters that are representative of others in order to cut down on the number of parameters that might be critical to adjudging the quality of a water system (Boyacioglu, 2007). In this section, we employ correlation as a tool to elucidate the interactions and interrelationships between water quality parameters and their usefulness in identifying possible sources of pollution.

The correlation matrix of the various physicochemical parameters is given in Table 3.2. There was significant positive correlation between and amongst pH, salinity, and DO ($P < 0.01$) while these parameters negatively correlated with EC, TDS and nitrate ($P < 0.01$) and with nitrite ($P < 0.05$). The positive correlation between pH and salinity is generally indicative of the higher pH concentration of the more saline receiving watershed compared to the less saline effluent (Table 3.1). The positive correlation between DO and salinity indicated that DO concentration

Table 3.2 Correlation matrix of physicochemical variables in treated final effluents and the receiving watershed

Variables	pH	Temperature	EC	Turbidity	Salinity	TDS	DO	COD	Nitrate	Nitrite	Phosphate
pH	1.0										
Temp	-.372	1.0									
EC	-.894**	.556*	1.0								
Turbidity	-.506*	-.523*	.300	1.0							
Salinity	.939**	-.553*	-.930**	-.523*	1.0						
TDS	-.908**	.534*	.999**	.322	-.935**	1.0					
DO	.732**	-.537*	-.905**	-.028	.796**	-.899**	1.0				
COD	.494	-.341	-.420	-.164	.418	-.425	.136	1.0			
Nitrate	-.704**	.417	.701**	.228	-.738**	.705**	-.767**	.082	1.0		
Nitrite	-.576*	-.120	.377	.781**	-.459	.401	-.015	-.507*	.034	1.0	
Phosphate	.172	-.293	-.233	.433	.086	-.236	.350	.136	-.295	.170	1.0

EC = electrical conductivity; TDS = total dissolved solid; *DO* = dissolved oxygen

**P* = 0.05, ***P* = 0.01; significant correlation (two tailed)

increased with increasing salinity, suggesting that the more saline receiving watershed is better oxygenated compared to the less saline effluent. The better oxygenation of the watershed must be sequel to the wind-induced turbulence and mixing of the marine water near the seashore where watershed samples (DP, UP, and DW) were collected (Shirodkar *et al*., 2009). The results revealed that the wastewater effluent was the main contributor of low dissolved oxygen to the watershed; however it is worthy of note that the receiving water quickly returned to DO levels similar to those observed upstream after flowing about 500 m downstream from the point of effluent discharge (Table 3.1); indicating its self-cleaning capacity.

There are several reports in the literature suggesting that EC and TDS were good and easy indicators of salinity (Oluyemi *et al*., 2006; Akan *et al*., 2008); results from this study however, reveals that this may not always be the case. While the near perfect correlation between EC and TDS suggests that these two parameters could very well represent one another in the determination of water quality irrespective of external and internal influences, their inverse relationship with salinity suggest that they may not always be good indicators of salinity. Our study showed that in the final effluent where chloride ions are dominant compared to sodium and other ions in the receiving waters (results not shown), EC and TDS values were significantly higher compared to salinity. Furthermore, if EC and TDS were very good indicators of salinity, it would be expected that the introduction of effluent high in EC and TDS levels into the saline receiving watershed would lead to an increase in EC and TDS levels with a concomitant increase in salinity, but the reverse was actually the case in this study (Table 3.1). This therefore implies that the type of dissolved ions present in a water system will to a large extent determine whether or not EC and/or TDS would be good surrogates of salinity.

The significant negative correlation of salinity with nitrate and nitrite also points to the

less saline municipal effluent as the source of these nutrients in the watershed. This could further be explained by the consistent higher concentrations of nitrate and nitrite observed in the effluent compared to other sampled points throughout this study (Table 3.1). The significant positive correlation between and amongst temperature, EC, and TDS ($P < 0.05$) and their (EC and TDS) negative correlation with salinity, pH and DO ($P < 0.01$) [Table 3.2] generally showed that the less saline effluent had higher temperatures compared to the more saline receiving watershed during this study (Table 3.1). The inverse relationship between turbidity and salinity suggests that the less saline effluent may be a source of turbidity in the watershed. However, the fact that turbidity did not correlate significantly with other prominent parameters in the effluent (e.g. EC and TDS), suggests that there may be other source(s) of turbidity in the receiving watershed apart from the effluent. This other source(s) may be responsible for the elevated levels of turbidity observed at DW compared to FE and other sampled sites (Table 3.1).

Chemical oxygen demand (COD) and orthophosphate did not correlate significantly with other parameters, suggesting diffuse origins of these parameters (COD and orthophosphate) in the watershed. The insignificant negative correlation of COD with EC and TDS and its insignificant positive correlation with salinity, pH, and DO however suggest that COD was introduced into the watershed by an unidentified source upstream of the effluent discharge. This observation is corroborated by the elevated levels of COD observed upstream compared to other sampling points especially FE and DW (Table 3.1). The slightly higher COD concentration observed in DP during summer and autumn is most likely a result of additional COD from the municipal effluent (FE) to the upstream water. A cursory look at Tables 3.1 and 3.2 suggest that orthophosphate followed a similar trend as COD and indicates a common source.

In general, for reasons mentioned earlier with respect to the complex nature of the

processes that govern water systems, it is difficult to compare the activities of one water system to another due to their uniqueness. For example, contrary to the observation of this study, Shirodkar *et al*. (2009) reported significant negative correlation between salinity and DO in the coastal waters of Mangalore in India. The authors explained that the incursion of the less saline riverine water compared to the more saline marine water was responsible for this observation. In a similar vein, Pradhan *et al*. (2009) reported positive correlation between pH and the nitrogenous nutrients (nitrate and nitrite) contrary to the observation of this study. Nutrient incursion was also cited as responsible for this observation. Consistent with our observation, Igbinosa and Okoh (2009) reported significant positive correlation amongst pH, DO and salinity ($P < 0.05$) and between EC and TDS ($P < 0.01$); while they reported significant negative correlation between pH and nitrate ($P < 0.01$) and between temperature and DO ($P < 0.01$). Contrary to our observation however, the authors (Igbinosa and Okoh, 2009) reported significant positive correlation for salinity with EC and TDS ($P < 0.01$) and for pH with TDS and EC ($P < 0.05$). The most stable relationship common to all the studies was seen between EC and TDS. This is an indication that external influence has little or no effect on these parameters and that they both represent each other very well, thus suggesting that either of the two parameters can be used to measure water quality in the stead of the other where limited resources is an issue.

4.4 Conclusion

The primary objective of this study was to evaluate the physicochemical qualities of the final effluent of an urban wastewater treatment facility in South Africa as a surrogate index of its capacity to remove selected pollutants from the wastewater influent prior to discharge into the

receiving environment. While the treated effluent met the recommended water quality standard for pH, TDS, NO_2^-, and NO_3^-, it fell short of stipulated standards for EC, turbidity, salinity, COD, DO, and PO_4^{3-}. The result generally showed a negative impact of the discharged effluent on the receiving watershed and calls for a regular and consistent monitoring programme by the relevant authorities to ensure best practices with regard to treatment and discharge of wastewater into the receiving aquatic milieu.

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CHAPTER 4

Prevalence and distribution of *Listeria* pathogens in the final effluents of a rural wastewater treatment facility in the Eastern Cape Province of South Africa

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CHAPTER 4

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Abstract

We assessed the prevalence of free-living and plankton-associated *Listeria* species in the final effluents of a South African (Alice) wastewater treatment facility and its receiving watershed between August 2007 and July 2008 as well as the antibiotic susceptibilities of effluent isolates. The physicochemical quality of raw sewage and treated effluent was also determined. Free-living *Listeria* were more prevalent (96%), compared to plankton-associated *Listeria* species (58-67%). *Listeria* pathogens were sensitive to 11 (55%) of the 20 tested antibiotics, and showed varying (7-71%) levels of resistance to 8 antibiotics. Turbidity, COD, NO_3, PO_4 and *Listeria* density fell short of recommended standards after treatment; while pH, temperature, TDS, DO and NO_2 were compliant with target quality after treatment. We conclude that final effluents of wastewater treatment plants are potential sources of *Listeria* pathogens in the aquatic milieu of South Africa.

Keywords Wastewater effluent; *Listeria*; free-living; plankton-associated; prevalence; antibiogram.

4.1 Introduction

Listeriosis is essentially a foodborne disease caused by *Listeria monocytogenes* and to some extent *L. ivanovii*. The disease conditions vary from severe invasive forms that affect immunocompromised patients to febrile gastroenteritis and perinatal infections associated with fetal loss or abortion in humans and animals (Siegman-Igra *et al*., 2002). Although rare, the disease is reported (Lyautey *et al*., 2007) to have very high mortality rate (20-50%), thus making it of serious public health concern. Despite the general consensus that food is the primary route of transmission of this disease, wastewater has long been reported to be a potential reservoir for these pathogens and possible route of transmission (Al-Ghazali and Al-Azawi, 1988; Arslan and Ozdemir, 2008; Czeszejko *et al*., 2003; Paillard *et al*., 2005; Watkins and Sleath, 1981). Watkins and Sleath (1981) reported the prevalence of *Listeria* species in sewage at numbers far higher than those of *Salmonella* species. And recent studies suggest that *Listeria* species readily survive conventional wastewater treatment processes even after tertiary treatment (Czeszejko *et al*., 2003; Paillard *et al*., 2005).

With reports of inadequate removal of *Listeria* pathogens from wastewater coming from the developed world (Czeszejko *et al*., 2003; Paillard *et al*., 2005), one can safely presume that wastewater treatment plants in developing countries such as South Africa are inefficient at removing these pathogens from wastewater influents prior to discharge of the final effluents into the receiving waters for obvious reasons. Most studies (Mackintosh and Colvin, 2003; Obi *et al*., 2007; Obi *et al*., 2008; Venkateswaran *et al*., 1989) in the area of water quality in South Africa had focused almost exclusively on drinking or potable water supply with scanty report in the literature on treated wastewater effluent as a source of pathogens for receiving waters. This may have serious public health implications as about 80 % of South Africans are reported to depend

on surface water bodies for drinking, domestic and agricultural purposes (Mackintosh and Colvin, 2003; Venter, 2001). It is little surprise therefore that about 43, 000 deaths (mostly children) are reported annually in South Africa due to diarrhea diseases (Mara, 2001). The situation is amongst the worst in the Eastern Cape Province due to high level of poverty, low level of sanitation, and lack of appropriate infrastructure (Mackintosh and Colvin, 2003). While reports in the media suggests that cholera may be responsible for majority of these infections, actual diagnosis suggests that these diseases could have been caused by any other waterborne pathogen apart from *Vibrio* species. A case in point was seen in the report of the Daily Dispatch of Thursday, 30th of January 2003, where out of 446 cases of water related diseases reported to the Eastern Cape health authorities, only 25 (5.6 %) were confirmed to be cholera and yet the disease was termed a 'cholera outbreak' without ascertaining the true identities of the pathogens responsible for over 84% of reported cases.

There is a general belief that the larger population of bacteria species grow as adherent to surfaces in all nutrient-sufficient aquatic ecosystems and that these sessile bacterial cells differ profoundly from their planktonic (free-living) counterpart (Costerton *et al*., 1978). It has also been reported that the existence of pathogens as free-living or plankton-associated cells, is critical to their survival in the environment as well as their transmission from one host to another (Donlan and Costerton, 2002). Several studies have revealed the preponderance of *Listeria* species to exist as biofilms attached to surfaces such as stainless steel, glass and propylene (Mafu *et al*., 1990), PVC (Djordjevic *et al*., 2002), and food and food processing environments (Lunden *et al*., 2000). There is however little or no report in the literature on *Listerio*-plankton association in the natural environment. Understanding the distribution of *Listeria* cells as free-living or plankton-associated niches may provide clues on how best to reduce the survival potentials of

these pathogens in the environment and during wastewater treatment, and consequently reduce their ability to interact with human and animal populations. In this study, we report the prevalence and distribution of *Listeria* pathogens in the treated effluents of a typical rural wastewater treatment facility in South Africa and its receiving watershed as well as the antibiotic susceptibility profiles of the *Listeria* pathogens isolated from treated effluent samples.

4.2 Materials and methods

4.2.1 Plant description

The wastewater treatment plant (Fig. 1) under study is located in Alice, a rural settlement in the Nkonkobe municipality of the Eastern Cape Province of South Africa and situated in the geographical coordinates 32^{o} 50" 36' S, 26^{o} 55" 00' E. The plant receives domestic sewage, some light industrial wastewater and run-off waters. It is an activated sludge treatment plant comprising a screen/grit channel, anaerobic and aeration basins, and clarifier. The final effluent is chlorinated and discharged into the Tyume River whilst water from the clarifier is channeled into a nearby pond for irrigation purpose. The design capacity of the plant is 2000 m^3/day and it currently operates at about 1100 m^3/day or 55% of its designed capacity.

4.2.2 Physicochemical analysis

All field meters and equipment were checked and appropriately calibrated according to the manufacturers' instructions. pH, temperature, total dissolve solid (TDS), and dissolved oxygen (DO), were all determined on site using the multi-parameter ion specific meter (Hanna-BDH laboratory supplies). Turbidity and the concentrations of free chlorine residual in the final

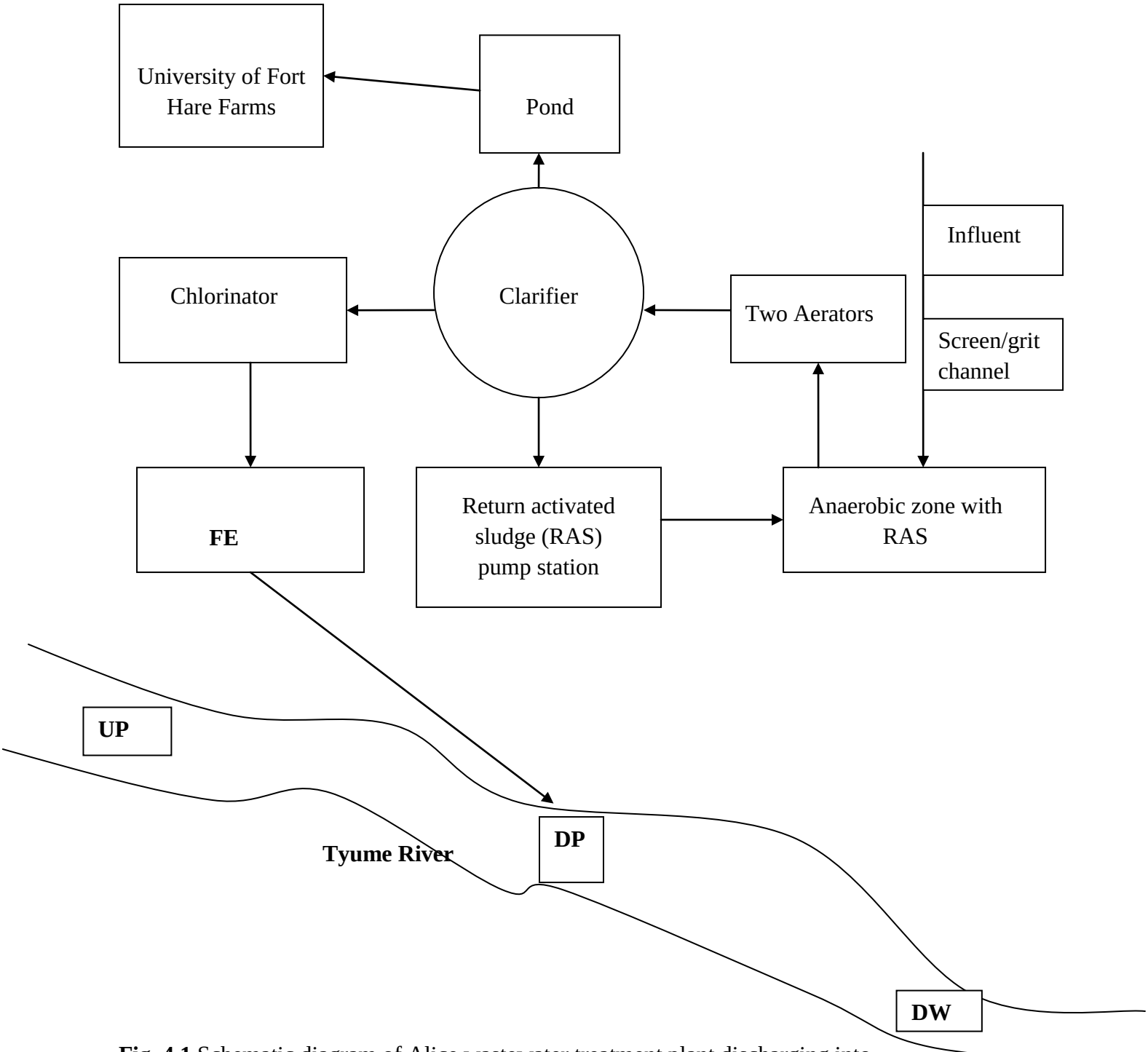

Fig. 4.1 Schematic diagram of Alice wastewater treatment plant discharging into the receiving Tyume river

Legend: *FE* = treated final effluent, *DP* = discharge point, *UP* = 500m upstream discharge point, *DW* = 500m downstream discharge point

effluent samples were also determined on site using a microprocessor turbidity meter (HACH Company, model 2100P) and an ion-specific meter (Hanna Instruments, HI 93711) respectively. The concentrations of orthophosphate as P (PO_4), Nitrate (NO_3), Nitrite (NO_2), and chemical oxygen demand (COD) were determined in the laboratory by the standard photometric method (DWAF, 1992) using the spectroquant NOVA 60 photometer (Merck Pty Ltd). Samples for COD analyses were digested with a thermoreactor model TR 300 (Merck Pty Ltd) prior to analysis using the spectroquant NOVA 60 photometer.

4.2.3 Sample collection

Wastewater samples were collected on a monthly basis from the treated effluent (FE), discharge point (DP), five hundred meters (500 m) upstream (UP) and five hundred meters (500 m) downstream (DW) of the discharge point between August 2007 and July 2008. Samples were collected from the surface of each site in duplicates in sterile one litre Nalgene bottles and transported in cooler boxes containing ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice campus for analyses. Sample bottles for the final effluents contained 0.1% sodium thiosulphate (3% solution) to neutralize the effect of the chlorine residual on the microflora. Processing of samples was done within 4 hours of sample collection.

4.2.4 Sample processing

Samples were processed according to the descriptions of Maugeri *et al*. (2004) with modifications. Briefly, samples (one litre in duplicates) were filtered in the laboratory through

180-, 60- and 20-µm pore size nylon nets (Millipore Corp., Ireland) respectively; the water that flowed through the 20-µm pore size nylon nets were collected in clean sterile containers for planktonic (free-living) *Listeria* cells analyses. To obtain a final volume corresponding to 40× of the original sample, trapped planktons on the nets and adhering bacteria were suspended in 25 ml of sterile phosphate-buffered saline (PBS). To detach adhering bacteria from the planktons, 12.5 g of sterile 0.1mm glass beads (Biospec Products Inc., Bartlesville, OK 74005, USA) was weighed into the bacteria-plankton suspension, vortexed at high speed for 30 s and centrifuged at 3000 × g for 10 min at ambient temperature using the Beckman Model TJ-6 centrifuge. The glass beads were allowed to settle to the bottom of the centrifuge tube and the supernatant was used for plankton-associated *Listeria* analyses. Henceforth in this study, plankton of sizes ≥ 180 µm, ≥ 60 µm ≤180 µm, and ≥ 20 µm ≤ 60 µm, shall simply be represented as 180 µm, 60 µm and 20 µm respectively.

4.2.5 Microbiological analysis

The cultural isolation of *Listeria* species were done according to the description of Hitchins (2001) with modifications. Briefly, aliquots of samples containing free-living and plankton-associated bacteria were directly inoculated onto *Listeria* chromogenic agar (LCA agar) (Pronadisa® Madrid, Spain) following standard spread plate technique and incubated for 24-48 h at 35 ^{o}C. Typical *Listeria* colonies appear blue-green on LCA agar plates while pathogenic *Listeria* species (*L. monocytogenes* and *L. ivanovii*) are surrounded by an opaque halo in addition to their blue-green color. Total *Listeria* counts were recorded and presumptive *Listeria* pathogens were isolated from the treated effluent samples, purified and stored on nutrient agar

slants at 4 ^{o}C for further analyses. The presumptive *Listeria* pathogens were further confirmed by standard cultural characteristics and biochemical reactions Hitchins (2001) and using the API *Listeria* kits (10 300, bioMerieux, South Africa). *Listeria monocytogenes* (ATCC 19115) and *Staphylococcus aureus* (ATCC 25923) were used as positive and negative controls respectively.

4.2.6 Antimicrobial agents

Twenty antibiotics commonly used as therapy in human and veterinary listeriosis were employed in the antibiogram test. The paper disks containing the antibiotics were obtained from Mast Diagnostics (Merseyside, United Kingdom) and includes: Amikacin (30 µg), Ciprofloxacin (5 µg), Aztreonam (30 µg), Linezolid (30 µg), Chloramphenicol (30 µg), Imipenem (10 µg), Ceftriaxone (30 µg), Meropenem (10 µg), Cephalothin (30 µg), Ertapenem (10 µg), Erythromycin (15 µg), Gatifloxacin (5 µg), Gentamycin (10 µg), Moxifloxacin (5 µg), Ampicillin (25 µg), Streptomycin (25 µg), Penicillin G (10 µg), Tetracyclin (30 µg), Trimethoprim (5 µg), and Sulphamethoxazole (25 µg).

4.2.7 Antibiotic susceptibility test

The antibiotic susceptibility test was performed and interpreted based on the disk agar diffusion method as described by the Clinical and Laboratory Standard Institute (CLSI, 2005), using Mueller Hinton agar plates (Biolab, Merck, South Africa). The inhibition zone diameters (IZD) were interpreted according to CLSI standards for staphylococci due to lack of specific standards for *Listeria* species (Conter *et al*., 2009). Interpretative standard for Linezolid was still under

investigation for staphylococci at the time of this study, thus standard for *Enterococcus* species was applied for this antimicrobial agent.

4.2.8 Statistical analysis

Calculation of means and standard deviations as well as scatter plot analysis for *Listeria* density and free residual chlorine concentrations were performed using Microsoft Excel office 2007 version. Correlations (paired T-test) and test of significance (independent t-test and one-way ANOVA) were performed using SPSS 15.0 version for Windows program (SPSS, Inc.). Independent t-test was used to compare differences in means between raw sewage and treated effluent parameters; while one-way ANOVA was used for all other tests of significance. All tests of significance and correlations were considered statistically significant at *P* values of <0.05 or <0.01.

4.3 Results

4.3.1 Physicochemical analysis

Table 4.1 shows the range and annual mean values of some wastewater quality parameters before and after treatment of the wastewater under study. Significant differences was observed between raw sewage and treated effluent for pH, TDS, and *Listeria* density ($P<0.05$) and for turbidity, DO, and nitrate ($P<0.01$). There was however no significant difference between treated and untreated sewage for temperature, COD, NO_2 and PO_4. Figure 4.2 shows the free chlorine residual (CR) concentration of the final effluents during the 12 month sampling period. Chlorine residual ranged between 0.097 mg/l (November 2007) and 1.4 mg/l (February 2008). There was

Table 4.1 Annual mean values of physicochemical and microbiological (*Listeria* density) parameters of raw and treated wastewater

Parameter	Raw sewage		Treated effluent		Recommended target limits
	Range	Mean±SD	Range	Mean±SD	
pH	7.1-9.6	7.5±0.6	5.53-9.38	6.7±0.97	6-9[a]
Temperature (^{o}C)	16-25	21±3	13-27	25±4	≤ 25 [a]
Turbidity (NTU)	10-388	143±89	1.59-25	6.68±5.7	0-1 [a]; ≤ 5[b]
TDS[h] (mg/l)	110-284	186±51	121-244	144±20	0-450 [a]
DO[i] (mg/l)	1.25-5.25	2.55±1	1.16-9.46	5.02±2	≥ 5[c]
COD[j] (mg/l)	10-700	250±193	10-975	129±235	30[d]; ≤ 1000[e]
NO_3 (mg/l)	0.3-4.4	1.96±1	4.4-18.8	10.04±3.8	6[a]; 1-5[d]
NO_2 (mg/l)	0.07-0.72	0.26±0.16	0.03-0.46	0.21±0.12	0-6[a]; <0.5[f]
PO_4 (mg/l)	0.06-7.4	2.26±1.83	0.12-4.3	2.02±1.41	0.005[f]
Total *Listeria* density (cfu/ml)	$7.0 \times 10^3 – 7.2 \times 10^5$	1.59×10^5	$0 – 6.25 \times 10^3$	1.38×10^2	0[g]

[a] Target limit for domestic water uses in South Africa (DWAF, 1996a); [b] Target limit for effluent to be discharged into surface waters (WHO, 2004); [c] Target limit for the support of aquatic life (Fatoki *et al*. 2003); [d] Target limit for effluent to be discharged into the environment (SA Government Gazette, 1984); [e] Target limit for effluent to be discharged into surface waters (Akan *et al*.2008); [f] Target limit that would reduce eutrophication in aquatic ecosystems (DWAF, 1996b); [g] Target limit (0 cfu/100ml of faecal coliform) for domestic water uses (DWAF, 1996a); [h] Total dissolved solids; [i] Dissolved oxygen; [j] Chemical oxygen demand

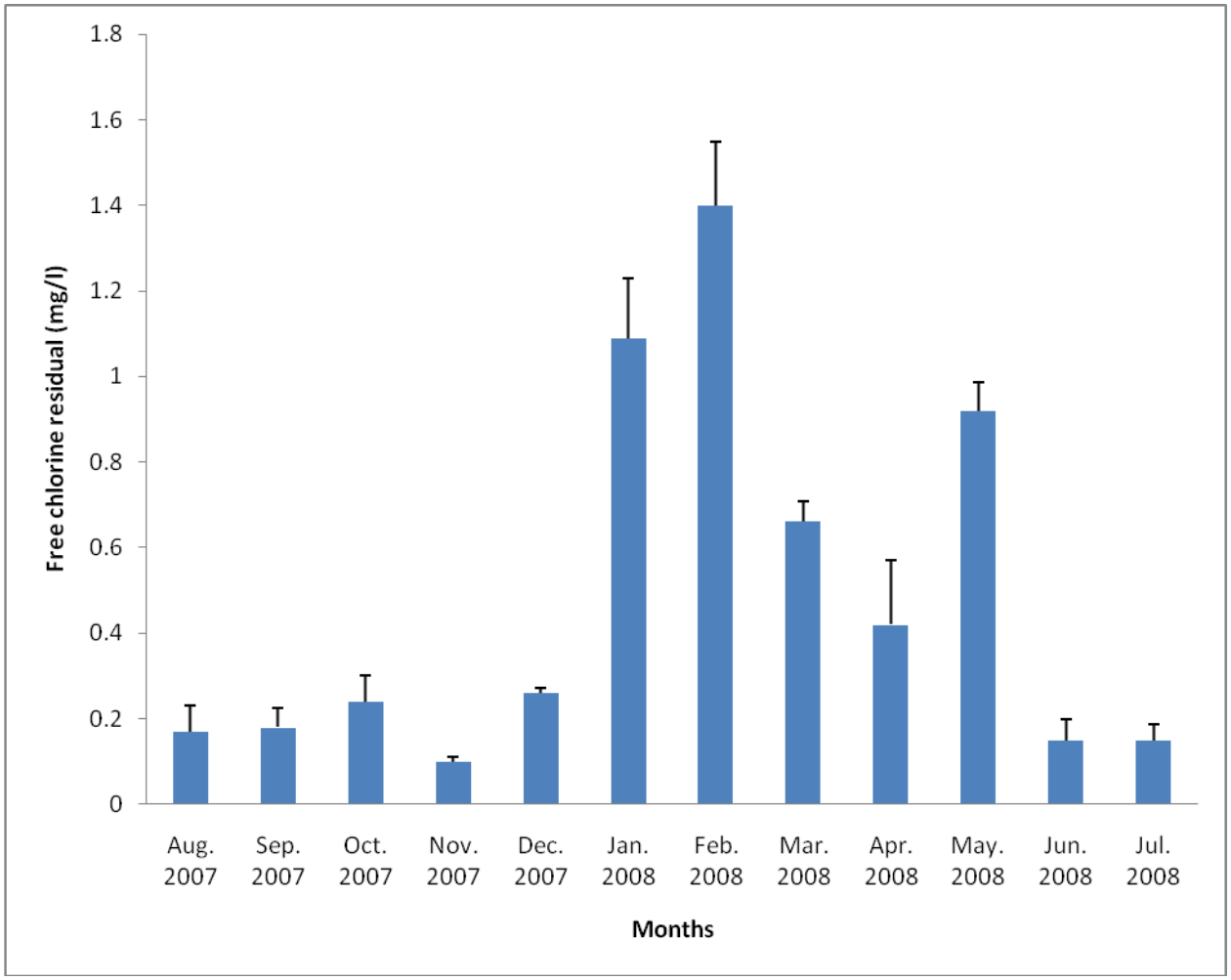

Fig. 4.2 Residual chlorine regime of the treated final effluents of the wastewater treatment plant

significant positive correlation ($P<0.05$) between free chlorine residual concentration and total *Listeria* count (Fig. 4.3).

4.3.2 Prevalence and distribution of *Listeria* species

Table 4.2 shows the prevalence and distribution of *Listeria* species during the 12 month sampling period. *Listeria* species were isolated throughout the year from all four sampling points. Forty-six (96 %) of all 48 samples (in duplicate) were positive for free-living *Listeria* species. Free-living *Listeria* species were isolated all year round except in the final effluent (FE) and discharge point (DP) where they were absent in the month of May 2008. Sixty seven percent of all samples were positive for *Listeria* species associated with large (180 µm) plankton. Of these *Listeria* was isolated from FE (10 samples), DP (8 samples from), DW (7 samples) and UP (7 samples). Twenty eight (58%) of all 48 samples were positive for *Listeria* species associated with medium-sized (60 µm) planktons; the bacteria was isolated from FE (8 samples), DP (6 samples), DW (7 samples) and UP (7 samples). *Listeria* species associated with small (20 µm) planktons were isolated in 31(65%) of the 48 samples. Final effluent (FE) samples were positive for this *Listeria* species in 8 samples, DP- 6 samples, DW- 8 samples and UP- 9 samples.

4.3.3 *Listeria* abundance

Total *Listeria* counts ranged from 1.0×10^1 to 1.1×10^4cfu/ml (Table 4.2). The lowest count was observed during winter in the month of July 2008 at DP while the highest count was observed in

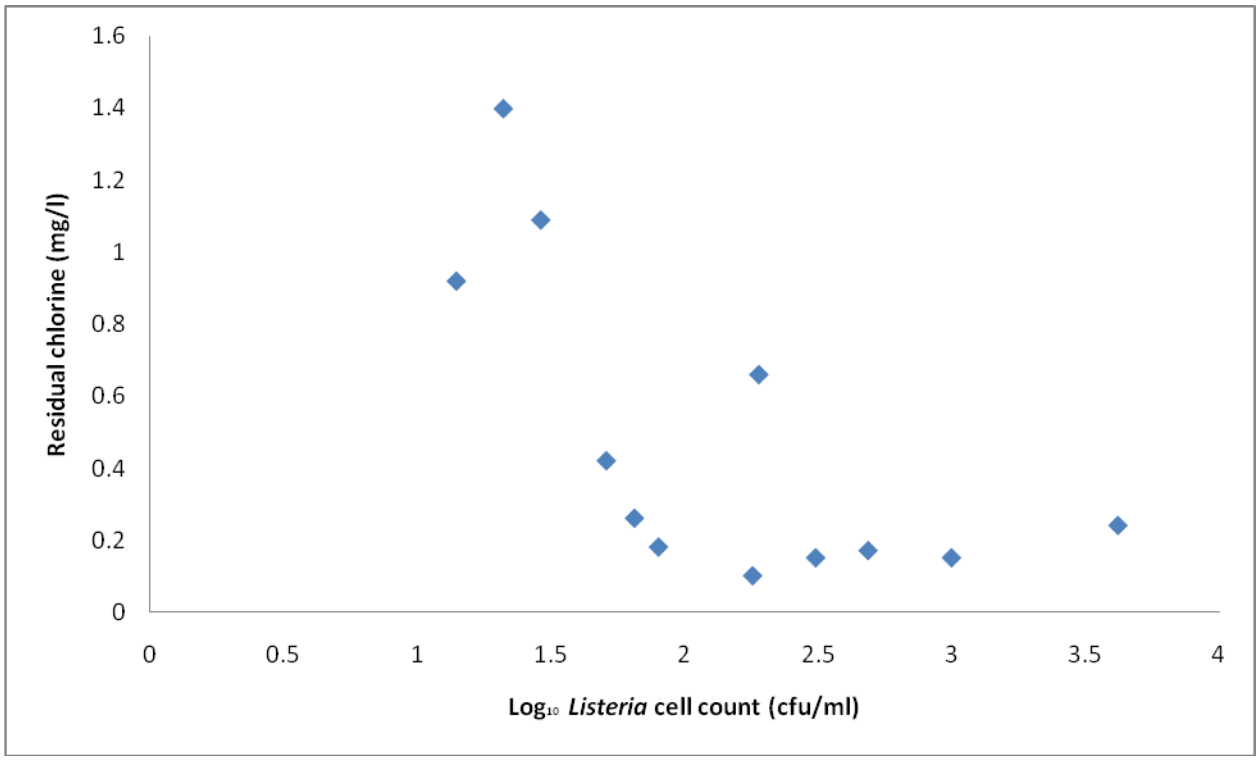

Fig. 4.3 Regression analysis of free chlorine residual with total *Listeria* counts from treated effluent samples

Table 4.2 Population density and distribution of the *Listeria* species in treated final effluents and the receiving watershed

Listeria density (cfu/ml)

Sampling sites	Net pore sizes	Spring			Summer			Autumn			Winter		
		Aug. 2007	Sep. 2007	Oct. 2007	Nov. 2007	Dec. 2007	Jan. 2008	Feb. 2008	Mar. 2008	Apr. 2008	May. 2008	Jun. 2008	Jul. 2008
FE	180µm	1×10^{0}	0.0	4.0×10^{3}	7.3×10^{0}	3.1×10^{0}	4.1×10^{0}	1.2×10^{1}	7.8×10^{0}	1.6×10^{1}	2.8×10^{0}	8.3×10^{1}	0.0
	60 µm	1.25×10^{0}	0.0	1.4×10^{1}	1.7×10^{1}	2.3×10^{0}	0.0	2.6×10^{0}	1.6×10^{1}	0.0	8.1×10^{0}	1.0×10^{0}	0.0
	20 µm	4.38×10^{2}	0.0	0.0	1.5×10^{1}	4.5×10^{0}	9.9×10^{0}	1.4×10^{0}	1.2×10^{1}	0.0	2.8×10^{0}	2.6×10^{1}	0.0
	Free	4.8×10^{2}	8.0×10^{1}	2.0×10^{2}	1.4×10^{2}	5.5×10^{1}	1.5×10^{1}	5.0×10^{0}	1.6×10^{2}	3.5×10^{1}	0.0	2.0×10^{2}	1.0×10^{3}
	Total	4.87×10^{2}	8.0×10^{1}	4.2×10^{3}	1.8×10^{2}	6.5×10^{1}	2.9×10^{1}	2.1×10^{1}	1.9×10^{2}	5.1×10^{1}	1.4×10^{1}	3.1×10^{2}	1.0×10^{3}
DP	180µm	0.0	0.0	0.0	ND	7.0×10^{0}	1.5×10^{0}	3.3×10^{0}	1.5×10^{1}	1.4×10^{0}	7.1×10^{0}	4.2×10^{1}	0.0
	60 µm	0.0	0.0	0.0	ND	2.1×10^{0}	6.0×10^{0}	0.0	6.8×10^{0}	0.0	5.4×10^{0}	3.1×10^{1}	0.0
	20 µm	0.0	0.0	2.9×10^{2}	ND	3.3×10^{0}	4.9×10^{0}	0.0	4.9×10^{0}	0.0	1.8×10^{1}	0.0	0.0
	Free	5.7×10^{2}	8.5×10^{1}	1.2×10^{3}	ND	6.0×10^{1}	2.5×10^{1}	4.5×10^{1}	1.4×10^{2}	2.0×10^{1}	0.0	2.0×10^{1}	1.0×10^{1}
	Total	5.7×10^{2}	8.5×10^{1}	1.5×10^{3}	ND	7.2×10^{1}	3.7×10^{1}	4.8×10^{1}	1.6×10^{2}	2.1×10^{1}	3.1×10^{1}	9.2×10^{1}	1.0×10^{1}
DW	180µm	0.0	0.0	2.2×10^{1}	1.1×10^{4}	6.3×10^{0}	6.0×10^{0}	0.0	3.5×10^{1}	0.0	2.8×10^{0}	0.0	1.6×10^{0}
	60 µm	0.0	0.0	1.1×10^{1}	5.0×10^{0}	5.5×10^{0}	3.5×10^{0}	1.3×10^{1}	9.3×10^{0}	0.0	1.1×10^{1}	0.0	0.0
	20 µm	0.0	0.0	1.3×10^{0}	1.0×10^{1}	8.5×10^{0}	7.6×10^{0}	1.3×10^{1}	8.8×10^{0}	0.0	1.2×10^{1}	1.8×10^{1}	0.0
	Free	9.0×10^{1}	7.0×10^{1}	9.5×10^{2}	1.1×10^{2}	1.2×10^{2}	6.5×10^{1}	3.0×10^{1}	7.7×10^{2}	7.0×10^{1}	2.2×10^{2}	1.5×10^{1}	1.5×10^{1}
	Total	9.0×10^{1}	7.0×10^{1}	9.8×10^{2}	1.1×10^{4}	1.4×10^{2}	8.2×10^{1}	5.7×10^{1}	8.2×10^{2}	7.0×10^{1}	2.5×10^{2}	3.3×10^{1}	1.7×10^{1}
UP	180µm	0.0	0.0	1.9×10^{2}	2.4×10^{0}	2.3×10^{0}	0.0	0.0	4.9×10^{1}	2.1×10^{1}	1.6×10^{0}	6.2×10^{1}	0.0
	60 µm	0.0	0.0	4.5×10^{1}	8.6×10^{0}	1.5×10^{0}	4.6×10^{0}	1.8×10^{0}	3.1×10^{1}	0.0	3.5×10^{0}	0.0	0.0
	20 µm	0.0	0.0	9.4×10^{0}	1.4×10^{1}	2.6×10^{1}	1.9×10^{0}	4.0×10^{0}	6.8×10^{0}	1.8×10^{1}	2.4×10^{0}	2.6×10^{1}	0.0
	Free	1.0×10^{2}	3.0×10^{1}	3.2×10^{3}	3.5×10^{1}	9.5×10^{1}	4.5×10^{1}	3.5×10^{1}	8.4×10^{2}	2.0×10^{2}	2.8×10^{2}	5.0×10^{0}	5.0×10^{1}
	Total	1.0×10^{2}	3.0×10^{1}	3.5×10^{3}	6.0×10^{1}	1.2×10^{2}	5.2×10^{1}	4.1×10^{1}	9.3×10^{2}	2.4×10^{2}	2.8×10^{2}	9.3×10^{1}	5.0×10^{1}

FE = treated final effluent, *DP* = discharge point, *DW* = 500m downstream discharge point, *UP* = 500m upstream discharge point

DW in the summer month of November 2007. Abundance of free-living *Listeria* species varied between 0 and 3.2×10^3 cfu/ml, with the highest count recorded at UP in October 2007.

Listeria species associated with plankton of sizes 180 µm, 60 µm, and 20 µm, were observed at densities of 0 to 1.1×10^4 cfu/ml, 0 to 4.5×10^1 cfu/ml and 0 to 4.38×10^3 cfu/ml respectively. The highest densities for the respective plankton-associated *Listeria* species were observed in DW (October 2007), UP (October 2007) and FE (August 2007). There was no significant correlation between *Listeria* abundance and season either as free-living or plankton-associated species. The population of free-living *Listeria* species in spring varied significantly with those of summer ($P<0.01$) but not with other seasons or treatments. Abundance of free-living *Listeria* isolates in the month of October 2007 also varied significantly ($P<0.05$) with those of other months except March 2008. With reference to Listerial association, free-living *Listeria* species negatively correlated with *Listeria* species attached to large (180 µm) plankton ($P<0.01$) and positively correlated with *Listeria* species attached to small (20 µm) planktons ($P<0.01$). There was no significant correlation however, between free-living *Listeria* species and other treatments. Significant negative correlations were also observed between *Listeria* species associated with small (20 µm) and large (180 µm) planktons ($P<0.05$), and between small (20 µm) and medium-sized (60 µm) planktons ($P<0.01$), while a positive correlation was observed between *Listeria* species attached to large (180 µm) and medium-sized (60 µm) planktons ($P<0.05$).

4.3.4 Antibiogram profile

Fifty-six presumptive *Listeria* pathogens were isolated from the final effluents by cultural and biochemical procedures as described earlier. Of these, 17 isolates (30 %) were confirmed to be

Listeria species by API out of which 11 (19.6%) were confirmed to be *L. ivanovii*; 1 (1.8%) was *L. monocytogenes*; 4 (7.14%) were *L. grayi*; and 1(1.8%) was *L. innocua*. The 12 pathogenic strains (*L. ivanovii* and *L. monocytogenes*) and 2 *L. grayi* strains were tested for antibiotic susceptibility. Results of the antibiotic susceptibility patterns are shown in Table 4.3. All 14 strains of *Listeria* species were sensitive to 11 (55%) of the 20 tested antibiotics including, amikacin, gentamycin, streptomycin (aminoglycosides); chloramphenicol (phenicol); tetracycline (tetracycline); ciprofloxacin, gatifloxacin, moxifloxacin (fluoroquinolones); and imipenem, meropenem, and ertapenem (carbapenems). Five (4 *L. ivanovii* and 1 *L. grayi*) of the 14 isolates were moderately susceptible to erythromycin, ceftriaxone and cephalothin; three *L. ivanovii* strains showed moderate sensitivity to erythromycin, while the other was moderately sensitive to ceftriaxone alone; and the *L. grayi* strain was moderately sensitive to both cephalothin and ceftriaxone. All 14 isolates were either moderately or completely sensitive to ceftriaxone. The test isolates were resistant to 8 (40%) of the 20 antibiotics tested. Resistance was expressed against ampicillin, penicillin G, linezolid, aztreonam, erythromycin, cephalothin, sulphametoxozole, and trimethoprim (Table 4.3). All strains showed resistance to at least one antibiotic; 3 (21.42%) showed resistance to only one antibiotic; while the other 11 (78.54%) strains displayed multiple antibiotic resistance ranging from 2 to 5 antibiotics (Table 4.4).

4.4 Discussion

The significant difference observed for most parameters between raw and treated sewage (Table 4.1), indicated that the wastewater treatment plant under study remarkably improved the quality

Table 4.3 *In vitro* antibiotic susceptibility profile of the *Listeria* strains isolated from treated final effluents of the wastewater treatment plant

Antibiotics	Number of isolates (%)		
	Susceptible	Intermediate	Resistant
Amikacin (30 µg)	14(100)	0(0)	0(0)
Gentamycin(10 µg)	14(100)	0(0)	0(0)
Streptomycin(25 µg)	14(100)	0(0)	0(0)
Chloramphenicol(30 µg)	14(100)	0(0)	0(0)
Tetracyclin(30 µg)	14(100)	0(0)	0(0)
Ciprofloxacin(5 µg)	14(100)	0(0)	0(0)
Gatifloxacin(5 µg)	14(100)	0(0)	0(0)
Moxifloxacin(5 µg)	14(100)	0(0)	0(0)
Imipenem(10 µg)	14(100)	0(0)	0(0)
Meropenem(10 µg)	14(100)	0(0)	0(0)
Ertapenem(10 µg)	14(100)	0(0)	0(0)
Ampicillin(30 µg)	4(29)	0(0)	10(71)
Penicillin G(10 µg)	5(36)	0(0)	9(64)
Linezolid(30 µg)	6(43)	0(0)	8(57)
Aztreonam(30 µg)	8(57)	0(0)	6(43)
Erythromycin(15 µg)	8(57)	0(0)	6(43)
Cephalothin(30 µg)	11(79)	1(7)	2(14)
Ceftriaxone(30 µg)	12(86)	2(14)	0(0)
Sulphamethoxazole (25 µg)	13(93)	0(0)	1(7)
Trimethoprim(5 µg)	13(93)	0(0)	1(7)

Table 4.4 Multiple antibiotic resistances of *Listeria* strains isolated from treated final effluents of the rural wastewater treatment plant

Antibiotics	n=14	Percentage (%)
ATM, E	1[c]	7.14
AP, ATM, E	1[b]	7.14
AP, LZD, PG	3[a,c]	21.42
AP, ATM, LZD, PG	1[c]	7.14
AP, E, LZD, PG	3[c]	21.42
AP, KF, E, LZD, PG	1[c]	7.14
AP, ATM, SMX, TM, PG	1[b]	7.14
Total	**11**	**78.54**

Legend: *ATM* = Aztreonam, *E* = Erythromycin, *AP* = Ampicillin, *LZD* = Linezolid, *PG* = Penicillin G, *KF* = Cephalothin, *SMX* = Sulphamethoxazole, *TM* = Trimethoprim,

[a]1 strain of *L. monocytogenes,* and 2 strains of *L. Ivanovii*

[b] Strains of *L. grayi*

[c] Strains of *L. ivanovii*

of the wastewater by the treatment process. Apart from COD and nitrate (SA Government Gazette, 1984), there are no South African standards for the evaluation of wastewater effluent quality meant for discharge into the environment. The quality of the treated effluent was therefore evaluated by other standards as shown on Table 4.1. The improvement on raw sewage quality notwithstanding, the final effluent did not measure up to desired target quality for turbidity, COD, NO_3, PO_4 and *Listeria* density (Table 4.1). This therefore disqualifies the effluent for use in domestic activities and indicates that discharge of the effluent into the receiving river could very well support eutrophication with all its negative consequences (DWAF, 1996a; DWAF, 1996b; Fatoki *et al*., 2003). The effluent quality however, fell within recommended limits for pH, temperature, TDS, DO, and NO_2; and for COD with reference to WHO standard (Akan *et al*., 2008).

Most South African wastewater treatment works disinfect wastewater by chlorination prior to discharge into receiving watersheds. The goal is to remove pathogens from wastewater. To achieve this goal, residual chlorine is maintained at sufficient levels and in contact with the microbial community in the chlorination tank. There is no recommended standard for residual chlorine concentration for wastewater effluent in South Africa, but the recommended limits of no risk at point of use vary from 0.3-0.6 mg/l (Obi *et al*., 2008). The residual chlorine concentration in this study ranged between 0.09 and 1.4 mg/l (Fig. 4.1). The concentration fell outside the recommended limit for most part of the year under review, except in April 2008 when the average concentration was 0.42 mg/l (Fig. 4.2). The chlorine residual concentration exceeded the maximum limit of 0.6 mg/l in January 2008, February 2008, March 2008 and May 2008 and fell below the minimum recommended concentration in the other months except April 2008. Similar ranges have been reported for chlorine residual concentration in South African water works (Momba *et al*., 2006; Obi *et al*., 2007; Obi *et al*., 2008) and indicate that some South African

water works do not comply with stipulated standards with reference to free chlorine residual concentration. There was significant correlation ($P<0.05$) between free chlorine residual concentration and *Listeria* density (Fig. 4.3), indicating that the chlorine residual concentration significantly influenced the abundance of *Listeria* species in this study. The effect of residual chlorine was however not enough to eliminate the pathogens (Tables 4.1 and 4.2). The attachment of *Listeria* species to planktons and/or other suspended particles could be responsible for the inability of chlorine to eliminate the pathogens even at relatively high concentrations (LeChevallier *et al*., 1988). This observation is supported by the fact that turbidity (which is a measure of suspended particles including planktons) fell short of recommended target limits throughout the sampling period even after sewage treatment (Table 4.1). However, the fact that free-living *Listeria* species were more abundant in the final treated effluent compared to plankton associated species, suggests that factors other than bacterial attachment may be responsible for the lack of decisive effect of chlorine on *Listeria* populations in the final effluent. Some other factors that may affect the efficiency of disinfectants such as chlorine include contact time, temperature, and pH (Obi *et al*., 2008).

Listeria species were isolated from all samples collected in this study. Although there are no recommended standards specific for *Listeria* species in water and wastewater samples in South Africa, the population density of the pathogen across all sampling sites and throughout the year exceeded the no risk limit of 0 cfu/100 ml of faecal coliform recommended for domestic water uses by the South African government (DWAF, 1996a). Consistent with our observation, high prevalence of *Listeria* species has been reported by other workers for treated wastewater effluent and its receiving watershed (Al-Ghazali and Al-Azawi, 1986; 1988; Paillard *et al*., 2005; Watkins and Sleath, 1981). Watkins and Sleath (1981) reported 100 % prevalence of *Listeria* species in sewage, river water, and trade effluent at densities (7.0×10^2 to $>1.8 \times 10^4$ MPN/ml)

slightly higher than those observed in this study. The sewage effluent reported by Watkins and colleague however, only underwent primary treatment unlike ours that was treated at the tertiary level by chlorination which could account for the differences. Al-Ghazali and Al-Azawi (1986; 1988) also reported 100 % prevalence in treated wastewater effluent in Iraq but at lower densities of < 3 to 28 MPN/ml. And Paillard *et al*. (2005) reported 84.4 % prevalence of *Listeria* species in treated wastewater in France at densities ranging from < 0.3 to 21 MPN/ml. Contrary to our observation, lower prevalence have been reported for *Listeria* species in a variety of surface waters. Frances *et al*. (1991) reported the isolation of *Listeria* species from 21 % of freshwater samples collected from sites in Cheshire and North Wales; while Lyautey *et al*. (2007) reported 64 % for surface waters of the South Nation River Watershed in Ontario, Canada. These observations were consistent with expectations for surface waters that are not impacted by wastewater effluent; Dijkstra (1982) reported the isolation of *L. monocytogenes* from 21 % of various surface waters, noting higher level of contamination (67%) in waters closer to sewage treatment plant effluents.

There is little or no report in the literature with regards to the prevalence and distribution of *Listeria* species as free-living or plankton-associated cells in the environment. Thus, to the best of our knowledge this is the first report that details the prevalence and distribution of *Listeria* species as free-living and/or plankton-associated cells in wastewater effluent and its receiving watershed. The preference for and identities of the specific planktons involved in this association are subjects of on going investigation in our group. In this study our discussion is restricted to *Listeria* species as free-living or plankton-associated cells.

Our study revealed that free-living *Listeria* species were most prevalent (96%) in both treated effluent and receiving surface water samples. This was followed by *Listeria* cells associated with planktons of sizes 180 µm (67%), 20 µm (65%), and 60 µm (58%), respectively.

This is consistent with the observation of Maugeri *et al*. (2004), who reported high prevalence for free-living and plankton-associated bacteria species including *Vibrio* spp. *E. coli, Aeromonas* spp. *Enterococcus* spp., *Campylobacter* spp. and *Arcobacter* species; and concluded that although prevalence varied from one bacteria species to another, free-living bacteria were generally more prevalent compared to the plankton-associated bacteria. Also consistent with our observation these workers (Maugeri *et al*., 2004) reported higher prevalence in bacterial cells associated with larger planktons (> 200 µm) than those associated with smaller planktons (> 64 µm). However, contrary to the observation of Maugeri and colleagues our study showed higher prevalence for *Listeria* species associated with a relatively smaller (20 µm) plankton compared to those attached to larger (60 µm) planktons, thus suggesting that the *Listeria* species have more affinity for very large or relatively small size planktons compared to planktons of medium sizes. In spite of the peak listerial density recorded by *Listeria* species associated with large (180 µm) planktons in the month of November 2007 in DW (Table 4.2), free-living *Listeria* species were generally more abundant during the sampling period and across all sampled sites. This was followed by *Listeria* species associated with planktons of sizes 20 µm, 180 µm, and 60 µm respectively. Consistent with our observation, Ilinsky and Gorshkov (2002) and Unanue *et al*. (1992) reported higher abundance for free-living bacteria compared to attached bacteria in costal waters. Conversely, Maugeri *et al*. (2004) reported higher abundance for plankton-associated bacteria compared to their free-living counterparts in coastal waters of Italy.

In general, *Listeria* species were more prevalent in the final effluent (FE) both as free-living and/or plankton-associated strains compared to other sites (Table 4.2), except in the 20 µm plankton category where *Listeria* species were isolated from UP in 9 out of 12 (75%) samples as against FE's 8 out of 12 (67%) samples. The observation could be as a result of higher nutrient levels in the wastewater effluents compared to receiving water bodies and is in agreement with

the report of other workers (Czeszejko *et al*., 2003; Dijkstra, 1982; Paillard *et al*., 2005). There was no significant correlation between *Listeria* abundance and season either as free-living or plankton-associated species in this study. This is consistent with the report of Murrel *et al*. (1999) but contrary to reports of other researchers (Maugeri *et al*., 2004; Unanue *et al*., 1992; Venkateswaran *et al*., 1989). The population density of free-living *Listeria* species for spring varied significantly with those of summer ($P<0.01$) but not with other seasons or treatments. Abundance of free-living *Listeria* isolates in the month of October 2007 also varied significantly ($P<0.05$) with those of other months except March 2008. The reasons for these observations were not clear.

With reference to Listerial association, counts of free-living *Listeria* species negatively correlated with counts of *Listeria* species attached to large (180 µm) planktons ($P<0.01$) and positively correlated with counts of *Listeria* species associated with small (20 µm) planktons ($P<0.01$). There was no significant correlation however, between free-living *Listeria* species and other treatments. Significant negative correlations were also observed between small (20 µm) plankton-associated *Listeria* species and their larger (180 µm) plankton-associated counterparts ($P<0.05$); and between small (20 µm) plankton-associated *Listeria* species and their medium-sized (60 µm) plankton counterparts ($P<0.01$); while a positive correlation was observed between large (180 µm) plankton-associated *Listeria* species and *Listeria* species attached to planktons in the size category of 60 µm ($P<0.05$). This generally indicates that the *Listeria* species associated with larger planktons (60 µm and 180 µm) occupy the same niches in the ecosystem separate from those occupied by free-living *Listeria* species and *Listeria* species attached to smaller planktons (20 µm). Contrary to our observation, Maugeri *et al*. (2004) reported no significant correlation between free-living bacteria and plankton associated bacterial populations in a marine coastal zone in Italy. Consistent with our observation however, Hsieh *et*

al. (2007) reported a negative correlation between planktonic *Vibrio* cells and attached populations. The authors explained that this trend could possibly mean that attachment provided refuge for cells under harsh conditions, thereby increasing the population of attached cells during such conditions while the abundance of planktonic cells decrease; on the other hand detachment of cells from plankton during favorable conditions would likely increase the planktonic population while reducing the abundance of attached cells.

Most study on the antimicrobial susceptibility profiles of *Listeria* species focus almost exclusively on clinical and/or food isolates with little information in the literature on antibiotic susceptibility profiles for *Listeria* strains isolated from treated municipal wastewater effluent. To our knowledge, this is the first study that specifically evaluated the antimicrobial susceptibility profile of *Listeria* strains isolated from treated municipal wastewater effluent in South Africa. All 14 strains of *Listeria* species were sensitive to 11 (55 %) of the 20 tested antibiotics (Table 4.3). Consistent with our observations, Hansen *et al*. (2005) reported that except for ciprofloxacin to which the test strains were moderately sensitive, meropenem, gentamycin, chloramphenicol, and tetracycline amongst other antibiotics were 'in the main sensitive' against the 106 strains of *L. monocytogenes* isolated from humans in Denmark between 1958 and 2001. Conter *et al*. (2009) also reported about hundredth percentile susceptibility for strains of *L. monocytogenes* isolated from food, to imipenem, gentamycin, ciprofloxacin and tetracycline; while Safdar and Armstrong (2003) reported a complete sensitivity (100%) of *Listeria* species to amikacin, ciprofloxacin and imipenem. Contrary to our observation however, other workers have reported *Listeria* resistance to these antibiotics. Srinivasan *et al*. (2005) reported *L. monocytogenes* resistance to streptomycin, tetracycline, chloramphenicol and gentamycin; while Li *et al*. (2007) reported *Listeria* resistance to ciprofloxacin, chloramphenicol and tetracycline, and moderate sensitivity to streptomycin and gentamycin.

Our *Listeria* strains were resistant to 8 of the 20 antibiotics tested at percentages ranging from 7 % - 71 % (Table 4.3). The penicillins are regarded as the drug of choice for the treatment of listeriosis as most report in the literature (Abuin *et al*., 1994; Conter *et al*., 2009; Hansen *et al*., 2005; Zhang *et al*., 2007) indicates a high sensitivity of *Listeria* species to these antibiotics. Conversely, results of this study show a high level of resistance to these antibiotics [ampicillin (71%), penicillin G (64%)] by the *Listeria* isolates. There are reports in the literature however, that support our observation of high resistance to the penicillins. Srinivasan *et al*. (2005) reported 92 % and 40 % resistance against ampicillin and penicillin G respectively, for strains of *L. monocytogenes* 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. The physicochemical character of the wastewater effluent may have influenced the level of resistance displayed by the *Listeria* strains isolated in this study. It has been widely reported in the literature that conventional wastewater treatment plants lack the capacity to effectively remove antibiotics and a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluent to develop resistance to common antibiotics due to selective pressure (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004). Although we did not attempt to assay for residual antibiotics in the treated effluents in the course of this study, lack of capacity to remove some chemicals from wastewater during the treatment process under review is evident in Table 4.1. The table shows that treated effluent fell short of recommended standard quality for critical parameters such as turbidity, COD, NO_3, and PO_4 and suggests a possible influence on the Listerial resistance.

All 14 *Listeria* strains showed resistance to at least one antibiotic; 3 (21.42%) showed resistance to only one antibiotic (two strains to aztreonam and one to cephalothin); while the other 11 (78.54 %) strains gave multiple antibiotic resistance ranging from 2 to 5 antibiotics

(Table 4.4). Our result is consistent with that of Srinivasan *et al*. (2005), but contrary to those of other workers (Arslan and Ozdemir, 2008; Conter *et al*., 2009). While Srinivasan *et al*. (2005) reported all 38 strains (100%) of *L. monocytogenes* tested to be resistant to more than one antimicrobial agent, Conter *et al*. (2009) reported that 'resistance' to one antibiotic was more common than multiple resistance in 120 strains of *L. monocytogenes* tested against 19 antibiotics; and Arslan and Ozdemir (2008) reported more resistance to a single antibiotic with no record of multiple antibiotic resistance amongst 47 strains of *Listeria* species isolated from white cheese and tested against 13 antibiotics. Multiple drug resistance in *Listeria* species have been attributed to antimicrobial selective pressure and gene transfer mechanism between and amongst *Listeria* species and close relatives of the bacteria such as *Enterococcus*, *Streptococcus* and *Staphylococcus* species (Safdar and Armstrong, 2003). Donlan and Costerton (2002) also reported the acquisition of inherent resistance to antimicrobial agents by attached bacterial species; suggesting that attachment to plankton at one point or the other may have enhanced the multiple resistances of our isolates to several test antibiotics.

Listeria species were isolated from the treated final effluents of the rural wastewater plants as well as from the receiving watershed throughout the year. Free-living *Listeria* isolates were more prevalent and abundant compared to plankton-associated *Listeria* species, and the pathogens showed multiple resistance to common antibiotics used as therapy against human and veterinary listeriosis. Although annual mean values of wastewater quality parameters before and after treatment suggests a significant improvement in the sewage quality, the wastewater effluent still fell short of recommended standards for some critical parameters even after treatment. In light of the public health implication of the use of waters impacted by poor quality wastewater effluents, the intervention of relevant monitoring authorities becomes *sin quo non* pursuant to ensuring compliance of rural wastewater treatment facilities to regulatory standards.

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CHAPTER 5

Wastewater final effluent as a potential source of *Listeria* pathogens in the watershed: an urban community plant in South Africa as a case study

(Submitted to International Microbiology for publication)

CHAPTER 5

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Abstract

We assessed the abundance of free-living and plankton-associated *Listeria* pathogens in the final effluents of an urban wastewater treatment facility and its receiving watershed between August 2007 and July 2008, and elucidated the *in vitro* antibiotic susceptibilities and resistance genes profile of *Listeria* isolates from the chlorinated effluent. The physicochemical qualities of the raw sewage and treated effluents were also determined. Total listerial density varied between 2.9×10^{0} and 1.2×10^{5} cfu/ml; while free-living *Listeria* species were most prevalent (84%), compared to *Listeria* species attached to planktons (59-75%). The treated effluent quality fell short of recommended standards for turbidity, dissolved oxygen, chemical oxygen demand, nitrite, phosphate and *Listeria* density; while pH, temperature, total dissolved solids and nitrate contents were compliant with target quality limits after treatment. The *Listeria* isolates (23) were sensitive to 3 (15%) of the 20 test antibiotics, and showed varying (4.5-91%) levels of resistance to 17 antibiotics; *sulII* genes were also detected in five *Listeria* isolates. The study showed that treated municipal effluents in South Africa could be a significant source of antibiotic resistant *Listeria* pathogens in the receiving watershed.

Keywords *Listeria* abundance . Free-living . plankton-attached . Wastewater quality . Antibiogram

5.1 Introduction

Listeria is an emerging pathogen commonly associated with foodborne infections. The bacterium has been implicated in several foodborne outbreaks in the developed world (Rocourt *et al*., 2000; Siegman-Igra *et al*., 2002) with little information on the existence of the pathogen in developing countries (Rocourt *et al*., 2000). Although food is reported as the major route of transmission of the pathogen, wastewater may be significantly relevant in the epidemiology of the pathogen as *Listeria* is severally reported (Al-Ghazali and Al-Azawi, 1986, 1988; Czeszejko *et al*., 2003; Paillard *et al*., 2005; Watkins and Sleath, 1981) to survive conventional wastewater treatment processes even after disinfection. This has serious public health implications for developing countries such as South Africa where a larger percentage of the population depend on surface water bodies that are negatively impacted by untreated or inadequately treated wastewater (Mackintosh and Colvin, 2003; Okoh *et al*., 2007; Venter, 2001).

Listeria infections are reported to have the highest (up to 50%) (Rocourt *et al*., 2000) mortality rate amongst foodborne pathogens, making the South African public particularly vulnerable in the event of an outbreak due to the high HIV/AIDS prevalence level and rate of drug and alcohol abuse in the country (Obi *et al*., 2006). The existence of *Listeria* as free-living or attached cells was previously observed (Djordjevic *et al*., 2002; Lunden *et al*., 2000; Mafu *et al*., 1990) to influence the capacity of the bacteria to resist disinfection and enhance its resistance to antimicrobial therapy.

Listerial resistance to antimicrobial therapy was also reported (Davis and Jackson, 2009; Srinivasan *et al*., 2005) to be mediated by certain resistance genes which code for proteins that function in ways that inhibits or reduce the effects of antimicrobials on the pathogen. It has been documented (Environment Canada, 2001) that the quality of wastewater effluent and by extension its impact on the receiving environment vary considerably from place to place and is

influenced by the population and development patterns of each area. Thus in this study we report the prevalence and distribution of *Listeria* pathogens as free-living and plankton-associated cells in a typical urban wastewater treatment facility in South Africa as well as the antibiotic susceptibility characteristics of the *Listeria* species isolated from chlorinated final effluents.

5.2 Materials and methods

5.2.1 Plant description

The wastewater treatment plant (Fig. 1) is located in a large and highly populated urban community in the Eastern Cape Province of South Africa, with the geographical coordinates: 32.97^{o}S and 27.87^{o}E. The plant receives municipal domestic sewage and a heavy industrial effluent and comprise of four screens, a grit channel, two anaerobic and two anoxic tanks and two aerobic tanks (each equipped with three vertically mounted mechanical aerators). The plant has six sedimentation tanks with the return activated sludge (RAS) pumped from the bottom of the clarifiers via the screens with raw sewage to the aeration tanks. Chlorine contact is carried out by means of a water pressure operated, wall mounted, gas chlorinator in a baffled reinforced concrete contact tank and the final effluent is discharged into the Indian Ocean. The average daily inflow of raw sewage during the study period was 32 000 m^3/day, while the plant has a built in capacity of 40 000 m^3/day.

52.2 Sample collection

Wastewater samples were collected on a monthly basis from the final effluent (FE), discharge point (DP), five hundred meters (500 m) upstream (UP) and five hundred meters (500 m) downstream (DW) of the discharge point between August 2007 and July 2008. Aqueous effluent samples were collected in duplicates in sterile one litre Nalgene bottles and transported

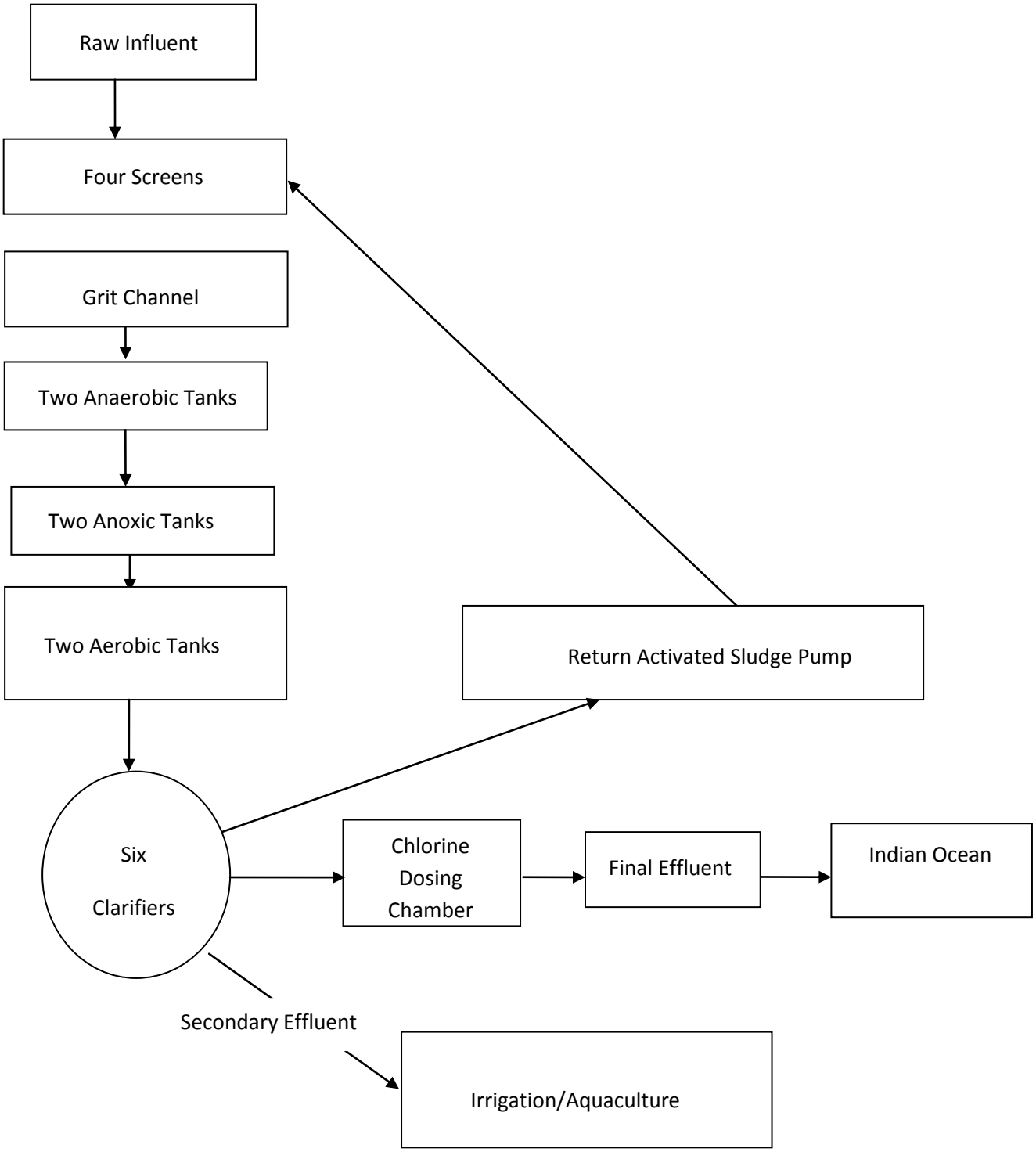

Fig. 5.1 Schematic representation of the study wastewater treatment plant

in cooler boxes containing ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, South Africa for analyses.Sample bottles for the final effluents contained 0.1% sodium thiosulphate (3% solution) to neutralize the effect of the chlorine residual on the microflora. Processing of samples was done within 6 hours of sample collection.

5.2.3 Sample processing

Samples were processed according to the descriptions of Maugeri *et al*. (2004) with modifications. Briefly, samples (one litre in duplicates) were filtered in the laboratory through 180-, 60- and 20-µm pore size nylon nets (Millipore Corp., Ireland) respectively; the water that flowed through the 20-µm pore size nylon nets were collected in clean sterile containers for planktonic (free-living) *Listeria* cells analyses. To obtain a final volume corresponding to 40× of the original sample, trapped planktons on the nets and adhering bacteria were resuspended in 25 ml of sterile phosphate-buffered saline (PBS). To detach adhering bacteria from the planktons, 12.5 g of sterile 0.1 mm glass beads (Biospec Products Inc., Bartlesville, OK 74005, USA) was weighed into the bacteria-plankton suspension, vortexed at high speed for 30 s and centrifuged at 3000 × g for 10 min at ambient temperature using the Beckman Model TJ-6 centrifuge. The glass beads were allowed to settle to the bottom of the centrifuge tube and the supernatant was used for plankton-associated *Listeria* analyses. Henceforth in this paper, plankton of sizes ≥ 180 µm, ≥ 60 µm ≤180 µm, and ≥ 20 µm ≤ 60 µm, shall simply be represented as 180 µm, 60 µm and 20 µm respectively.

5.2.4 Microbiological analysis

The isolation of *Listeria* species were done according to the description of Hitchins (2001) with modifications. Briefly, aliquots of samples containing free-living and plankton-associated bacteria were directly inoculated onto *Listeria* chromogenic agar (LCA agar) (Pronadisa® Madrid, Spain) following standard spread plate technique and incubated for 24-48 h at 35 ^{o}C. Typical *Listeria* colonies appear blue-green on LCA agar plates while pathogenic *Listeria* species (*Listeria monocytogenes* and *L. ivanovii*) are surrounded by an opaque halo in addition to their blue-green color. Total *Listeria* counts were recorded and presumptive *Listeria* pathogens were isolated from the treated (chlorinated) effluent samples, purified and stored on nutrient agar slants at 4^{o}C for further analyses. The presumptive *Listeria* pathogens were further confirmed by standard cultural characteristics and biochemical reactions (Hitchins, 2001) and using the API *Listeria* kits (10 300, bioMerieux, South Africa). *L. monocytogenes* (ATCC 19115) and *Staphylococcus aureus* (ATCC 25923) were used as positive and negative controls respectively.

5.2.5 Physicochemical analyses

All field meters and equipment were checked and appropriately calibrated according to the manufacturers' instructions. pH, temperature, total dissolve solid (TDS), and dissolved oxygen (DO), were all determined on site using the multi-parameter ion specific meter (Hanna-BDH laboratory supplies). Turbidity and the concentrations of free chlorine residual in the final effluent samples were also determined on site using a microprocessor turbidity meter (HACH Company, model 2100P) and an ion-specific meter (Hanna Instruments, HI 93711) respectively. The concentrations of orthophosphate as P (PO_4), Nitrate (NO_3), Nitrite (NO_2), and chemical oxygen demand (COD) were determined in the laboratory by the standard photometric method (DWAF, 1992) using the spectroquant NOVA 60 photometer (Merck Pty Ltd). Samples for COD

analyses were digested with a thermoreactor model TR 300 (Merck Pty Ltd) prior to analysis using the spectroquant NOVA 60 photometer.

5.2.6 Antimicrobial agents

Twenty antibiotics commonly used as therapy in human and veterinary listeriosis were employed in the antibiogram assay. The paper disks containing the antibiotics were obtained from Mast Diagnostics (Merseyside, United Kingdom) and includes: Amikacin (30 µg), Ciprofloxacin (5 µg), Aztreonam (30 µg), Linezolid (30 µg), Chloramphenicol (30 µg), Imipenem (10 µg), Ceftriaxone (30 µg), Meropenem (10 µg), Cephalothin (30 µg), Ertapenem (10 µg), Erythromycin (15 µg), Gatifloxacin (5 µg), Gentamycin (10 µg), Moxifloxacin (5 µg), Ampicillin (25 µg), Streptomycin (25 µg), Penicillin G (10 µg), Tetracyclin (30 µg), Trimethoprim (5 µg), and Sulphamethoxazole (25 µg).

5.2.7 Antibiotic susceptibility test

The antibiotic susceptibility test was performed and interpreted based on the disk agar diffusion method as described by the Clinical and Laboratory Standard Institute (CLSI, 2005), using Mueller Hinton agar plates (Biolab, Merck, South Africa). The inhibition zone diameters (IZD) were interpreted according to CLSI standards for staphylococci due to lack of specific standards for *Listeria* species (Conter *et al*., 2009). Interpretative standard for Linezolid was still under investigation for staphylococci at the time of this report, thus standard for *Enterococcus* species was applied for this antimicrobial agent.

5.2.8 Bacterial DNA extraction and amplification of antimicrobial resistance genes

DNA was isolated from pure cultures of the selected *Listeria* strains by the boiling method as described elsewhere (Naravaneni and Jamil, 2005). Based on the *in vitro* antimicrobial susceptibility profile of the *Listeria* isolates, seven antimicrobial resistance genes including those coding for penicillin binding protein (*penA*); dihydropteroate synthetase type I (*sulI*); dihydropteroate synthetase type II (*sulII*); adenine methylase (*ermA*); erythromycin resistance methylase (*ermB*); erythromycin esterase type II (*ereB*); and β-lactamase-ampicillin resistance gene (*ampC*); were selected for screening. Oligonucleotide sequences and predicted amplicon sizes for the different antimicrobial resistance genes are listed on Table 5.1. Presence of antimicrobial resistance genes in the *Listeria* species were all determined by PCR technique according to the description of Srinivasan *et al*. (2005).

5.2.9 Statistical analyses

Calculation of means and standard deviations were performed using Microsoft Excel Office 2007 version. Correlations (paired T-test) and test of significance (one-way ANOVA) were performed using SPSS 17.0 version for Windows program (SPSS, Inc.). All tests of significance and correlations were considered statistically significant at *P* values of < 0.05 or < 0.01.

5.3 Results

5.3.1 Abundance of *Listeria*

Total *Listeria* counts ranged from 2.9×10^0 to 1.2×10^5cfu/ml (Table 5.2). The lowest count was observed during summer in the month of November 2007 at DW while the highest count was observed at the DP, also in the summer month of December 2007. Abundance of free-living

Table 5.1 Primers used for resistance genes detection in the *Listeria* isolates from chlorinated wastewater effluents

Gene	Primer	Nucleotide sequence	Amplicon size	Reference
penA	PenA-F	ATCGAACAGGCGACGATGTC	500	Srinivasan *et al*. (2005)
	PenA-R	GATTAAGACGGTGTTTTACGG		
ampC	AmpC-F	TTCTATCAAMACTGGCARCC	550	,,
	AmpC-R	CCYTTTTATGTACCCAYGA		
ermB	ErmB-F	GAAAAGGTACTCAACCAAATA	639	,,
	ErmB-R	AGTAACGGTACTTAAATTGTTTAC		
ereA	EreA-F	AACACCCTGAACCCAAGGGACG	420	,,
	EreA-R	CTTCACATCCGGATTCGCTCGA		
ereB	EreB-F	AGAAATGGAGGTTCATACTTACCA	546	,,
	EreB-R	CATATAATCATCACCAATGGCA		
su1I	Su1I-F	GTGACGGTGTTCGGCATTCT	779	,,
	Su1I-R	TCCGAGAAGGTGATTGCGCT		
su1II	Su1II-F	CGGCATCGTCAACATAACCT	721	,,
	Su1II-R	TGTGCGGATGAAGTCAGCTC		

Listeria species varied between 0 and 2.4×10^3 cfu/ml, with the highest count recorded at FE and DW in April 2008. *Listeria* species associated with plankton of sizes 180 µm, 60 µm, and 20 µm, were observed at population densities of 0 to 1.95×10^3 cfu/ml, 0 to 1.8×10^2 cfu/ml and 0 to 1.15×10^5 cfu/ml respectively. The highest counts for the plankton-associated *Listeria* species were all observed at the DP in December 2007, June 2008 and December 2007 respectively for 180 µm, 60 µm, and 20 µm categories. Listerial abundance did not vary significantly with season either as free-living or plankton-associated entities. The population of free-living *Listeria* species in the FE samples varied significantly ($P < 0.05$) with those of large (180 µm) and medium sized (60 µm) planktons but not with small (20 µm) planktons. *Listeria* density did not vary significantly with the size of the planktons to which they attach at DP and DW. There was however significant difference ($P < 0.05$) in listerial density between free-living *Listeria* populations and plankton-attached species of all categories at the upstream sampling site; but significant variation was not observed for other treatments at this site. There was significant ($P < 0.01$) positive correlation between *Listeria* populations attached to large (180 µm) planktons and those attached to small (20 µm) planktons. Significant correlation was however not observed for other treatments with respect to listerio-plankton association.

Table 5.2 shows the prevalence of *Listeria* density during this study. *Listeria* species were isolated throughout the year from all four sampled sites. Thirty seven (84%) of all 44 samples (in duplicate) were positive for free-living *Listeria* species. Free-living *Listeria* species were isolated all year round except in downstream samples (DW) throughout summer and early winter (May 2008) and in upstream samples in December 2007 and during winter (May, June 2008). Seventy five percent of all samples were positive for *Listeria* species associated with large (180 µm) plankton. Of these, *Listeria* was isolated from FE (11 samples), DP (9 samples),

Table 5.2 Population density and distribution of the *Listeria* species in the treated final effluents and the receiving watershed

		***Listeria* density (cfu/ml)**											
		Spring			**Summer**			**Autumn**			**Winter**		
Sampling Sites	**Net pore sizes**	Aug. 2007	Sep. 2007	Oct. 2007	Nov. 2007	Dec. 2007	Jan. 2008	Feb. 2008	Mar. 2008	Apr. 2008	May 2008	Jun. 2008	Jul. 2008
FE	180µm	1.5×10^{0}	3.5×10^{0}	ND	4.0×10^{0}	8.6×10^{1}	2.5×10^{1}	7.6×10^{0}	3.5×10^{1}	1.1×10^{1}	2.7×10^{1}	4.3×10^{1}	1.8×0^{1}
	60 µm	2.9×10^{0}	2.4×10^{0}	ND	0.0	1.0×10^{1}	1.6×10^{1}	3.0×10^{0}	1.4×10^{1}	8.1×10^{0}	1.0×10^{1}	3.8×10^{1}	1.2×10^{1}
	20 µm	6.3×10^{2}	7.1×10^{0}	ND	0.0	3.0×10^{2}	1.2×10^{1}	9.3×10^{0}	3.9×10^{0}	9.4×10^{0}	1.2×10^{1}	9.3×10^{1}	1.1×10^{0}
	Free	2.6×10^{2}	$3.\times10^{2}$	ND	1.6×10^{2}	2.4×10^{2}	2.3×10^{2}	2.8×10^{2}	9.5×10^{2}	2.4×10^{3}	2.0×10^{1}	4.5×10^{2}	2.5×10^{1}
	Total	$\mathbf{8.8\times10^{2}}$	$\mathbf{3.3\times10^{2}}$	**ND**	$\mathbf{1.7\times10^{2}}$	$\mathbf{6.3\times10^{2}}$	$\mathbf{2.8\times10^{2}}$	$\mathbf{2.95\times10^{2}}$	$\mathbf{1.0\times10^{3}}$	$\mathbf{2.4\times10^{3}}$	$\mathbf{6.9\times10^{1}}$	$\mathbf{6.2\times10^{2}}$	$\mathbf{5.7\times10^{1}}$
DP	180µm	3.9×10^{0}	2.1×10^{0}	ND	3.0×10^{0}	1.95×10^{3}	9.9×10^{0}	1.5×10^{0}	2.1×10^{1}	0.0	1.0×10^{1}	1.8×10^{2}	0.0
	60 µm	3.5×10^{0}	0.0	ND	0.0	1.9×10^{1}	2.2×10^{1}	3.8×10^{0}	3.5×10^{0}	7.6×10^{0}	7.0×10^{0}	1.8×10^{2}	0.0
	20 µm	2.8×10^{0}	1.1×10^{0}	ND	0.0	1.2×10^{5}	6.3×10^{0}	6.1×10^{0}	4.7×10^{1}	6.7×10^{1}	1.6×10^{1}	6.9×10^{1}	0.0
	Free	5.7×10^{2}	2.1×10^{2}	ND	1.5×10^{1}	4.0×10^{2}	8.0×10^{1}	2.1×10^{2}	3.4×10^{2}	3.5×10^{1}	1.5×10^{2}	8.5×10^{1}	5.0×10^{0}
	Total	$\mathbf{5.8\times10^{2}}$	$\mathbf{2.1\times10^{2}}$	**ND**	$\mathbf{1.98\times10^{1}}$	$\mathbf{1.2\times10^{5}}$	$\mathbf{1.2\times10^{2}}$	$\mathbf{2.2\times10^{2}}$	$\mathbf{4.1\times10^{2}}$	$\mathbf{1.1\times10^{2}}$	$\mathbf{1.8\times10^{2}}$	$\mathbf{5.1\times10^{2}}$	$\mathbf{5.0\times10^{0}}$
DW	180µm	0.0	1.1×10^{0}	ND	2.9×10^{0}	0.0	2.1×10^{1}	1.1×10^{0}	2.9×10^{0}	0.0	4.3×10^{0}	2.6×10^{1}	0.0
	60 µm	0.0	0.0	ND	0.0	0.0	1.5×10^{1}	0.0	0.0	0.0	6.9×10^{0}	3.0×10^{1}	0.0
	20 µm	0.0	0.0	ND	0.0	0.0	1.2×10^{1}	1.6×10^{0}	9.6×10^{0}	0.0	1.96×10^{1}	1.8×10^{1}	0.0
	Free	3.5×10^{1}	3.5×10^{1}	ND	0.0	0.0	0.0	5.0×10^{1}	1.6×10^{2}	2.4×10^{3}	0.0	1.5×10^{1}	5.0×10^{0}
	Total	$\mathbf{3.5\times10^{1}}$	$\mathbf{3.6\times10^{1}}$	**ND**	$\mathbf{2.9\times10^{0}}$	**0.0**	$\mathbf{4.8\times10^{1}}$	$\mathbf{7.8\times10^{0}}$	$\mathbf{1.7\times10^{2}}$	$\mathbf{2.4\times10^{3}}$	$\mathbf{3.1\times10^{1}}$	$\mathbf{8.9\times10^{1}}$	$\mathbf{5.0\times10^{0}}$
UP	180µm	0.0	0.0	ND	3.5×10^{0}	0.0	2.5×10^{1}	1.0×10^{0}	4.4×10^{0}	0.0	4.3×10^{0}	9.9×10^{0}	0.0
	60 µm	0.0	0.0	ND	0.0	0.0	8.9×10^{0}	2.0×10^{0}	1.1×10^{0}	0.0	2.7×10^{1}	2.4×10^{1}	0.0
	20 µm	0.0	0.0	ND	3.6×10^{3}	0.0	7.6×10^{0}	1.5×10^{0}	2.4×10^{0}	0.0	1.7×10^{1}	3.1×10^{1}	0.0
	Free	1.5×10^{1}	5.0×10^{0}	ND	1.2×10^{2}	0.0	3.5×10^{1}	1.0×10^{1}	1.3×10^{2}	9.0×10^{1}	0.0	0.0	5.0×10^{0}
	Total	$\mathbf{1.5\times10^{1}}$	$\mathbf{5.0\times10^{0}}$	**ND**	$\mathbf{1.2\times10^{2}}$	**0.0**	$\mathbf{7.6\times10^{1}}$	$\mathbf{1.5\times10^{1}}$	$\mathbf{1.4\times10^{2}}$	$\mathbf{9.0\times10^{1}}$	$\mathbf{4.8\times10^{1}}$	$\mathbf{6.5\times10^{1}}$	$\mathbf{5.0\times10^{0}}$

FE = treated final effluent, *DP* = discharge point, *DW* = 500m downstream discharge point, *UP* = 500m upstream discharge point

DW (7 samples) and UP (6 samples). Twenty six (59%) of all 44 samples were positive for *Listeria* species associated with medium-sized (60 µm) planktons which were isolated from FE (10 samples), DP (8 samples), DW (3 samples) and UP (5 samples). *Listeria* species associated with small (20 µm) planktons were isolated in 30 (68%) of the 44 samples. FE samples were positive for this *Listeria* species in 10 samples, DP in 9 samples, DW in 5 samples and UP in 6 samples.

5.3.2 Physicochemical analyses

Table 5.3 shows the range and annual mean values of some wastewater quality parameters before and after treatment of the wastewater under study. Significant differences was observed between raw sewage and treated effluent for turbidity, DO, and PO_4 ($P < 0.01$) and for nitrate ($P < 0.05$). There was however no significant difference between treated and untreated sewage for pH, temperature, TDS, COD, and NO_2. Fig. 5.2 shows the free chlorine residual (CR) of the final effluents during the 12 month sampling period. Chlorine residual ranged between 0.197 mg/l (September 2007) and 0.71 mg/l (November 2007). The relationship between residual chlorine and total *Listeria* count did not follow any defined trend (Fig. 5.3).

5.3.3 Antibiogram and resistance gene detection

Fifty-one presumptive *Listeria* pathogens were isolated from the final effluents. Of these, 28 (55 %) were confirmed to be *Listeria* species by API out of which 27 (53%) were confirmed to be *L. ivanovii*; 1 (2%) was *L. innocua* and the identity of the remaining 23 (35%) isolates were indeterminate by the API test. Twenty-three isolates (22 *Listeria ivanovii* and 1 *L. innocua*) were tested for phenotypic antibiotic susceptibility and the result is shown on Table 5.4. All 23 *Listeria* species were sensitive to 3 (15%) of the 20 test antibiotics including, amikacin

Table 5.3 Some physicochemical qualities of the raw wastewater and treated final effluent

Parameter	Raw wastewater		Treated effluent		Recommended target limits
	Range	Mean±SD	Range	Mean±SD	
pH	4.97 - 7.75	7.1±0.44	6.7 – 7.7	7.1±0.28	6-9[a]
Temperature (^{o}C)	18 - 26	23±2.3	18 – 26	22±2.45	≤ 25 [a]
Turbidity (NTU)	86 - 1000	573±369	2.16 - 16	6.09±3.64	0-1 [a]; ≤ 5[b]
TDS (mg/l)	311 - 907	452±153	289 – 743	398±110	0-450 [a]
DO (mg/l)	0.14 - 7.32	1.76±1.78	2.38 – 6.78	4.46±0.94	≥ 5[c]
COD (mg/l)	40 - 2404	489±701	4 – 960	143±271	30[d]
NO_3 (mg/l)	0.026 - 5.1	3.17±1.32	0.25 – 6.95	4.56±2.53	6[a]; 1-5[d]
NO_2 (mg/l)	0.07 - 3.5	0.53±0.93	0.07 – 6.95	0.88±1.84	0-6[a]; <0.5[e]
PO_4 (mg/l)	1.33 - 5.91	3.78±1.26	0.05 – 0.73	0.34±0.16	0.005[e]

[a]Target limit for domestic water uses in South Africa (DWAF, 1996a); [b]Target limit for effluent to be discharged into surface waters (WHO, 2004);[c]Target limit for the support of aquatic life (Fatoki *et al*., 2003); [d]Target limit for effluent to be discharged into the environment [SA Government Gazette, 1984); [e]Target limit that would reduce eutrophication in aquatic ecosystems (DWAF, 1996b).

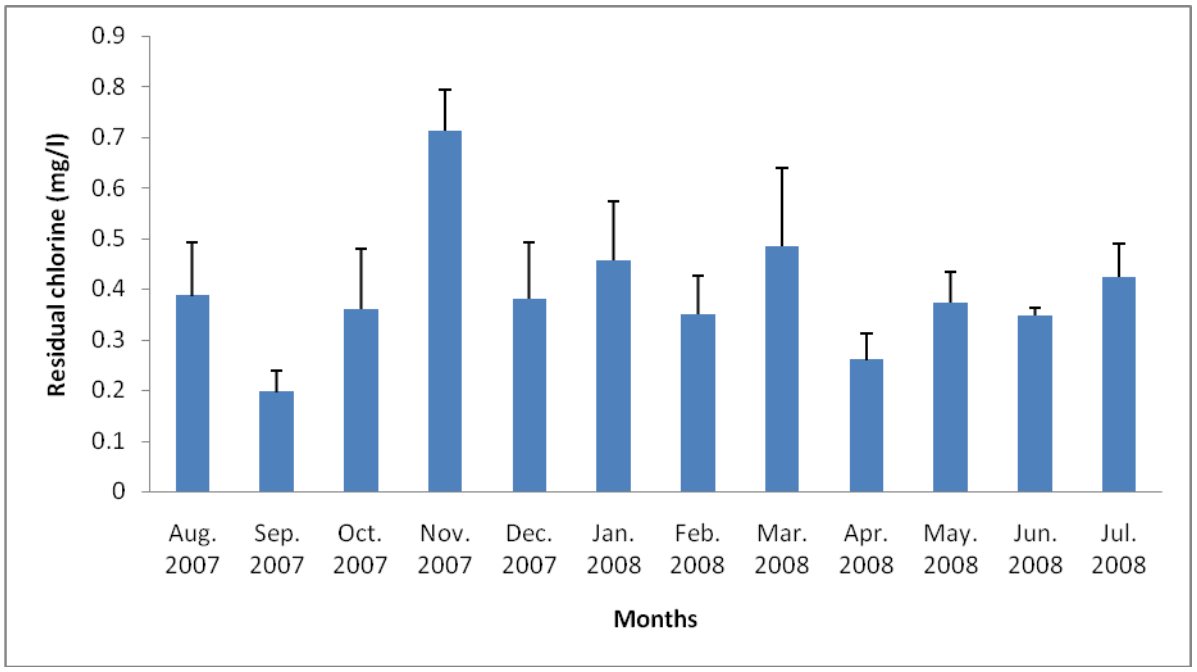

Fig. 5.2 Chlorine residual regime of the treated final effluents

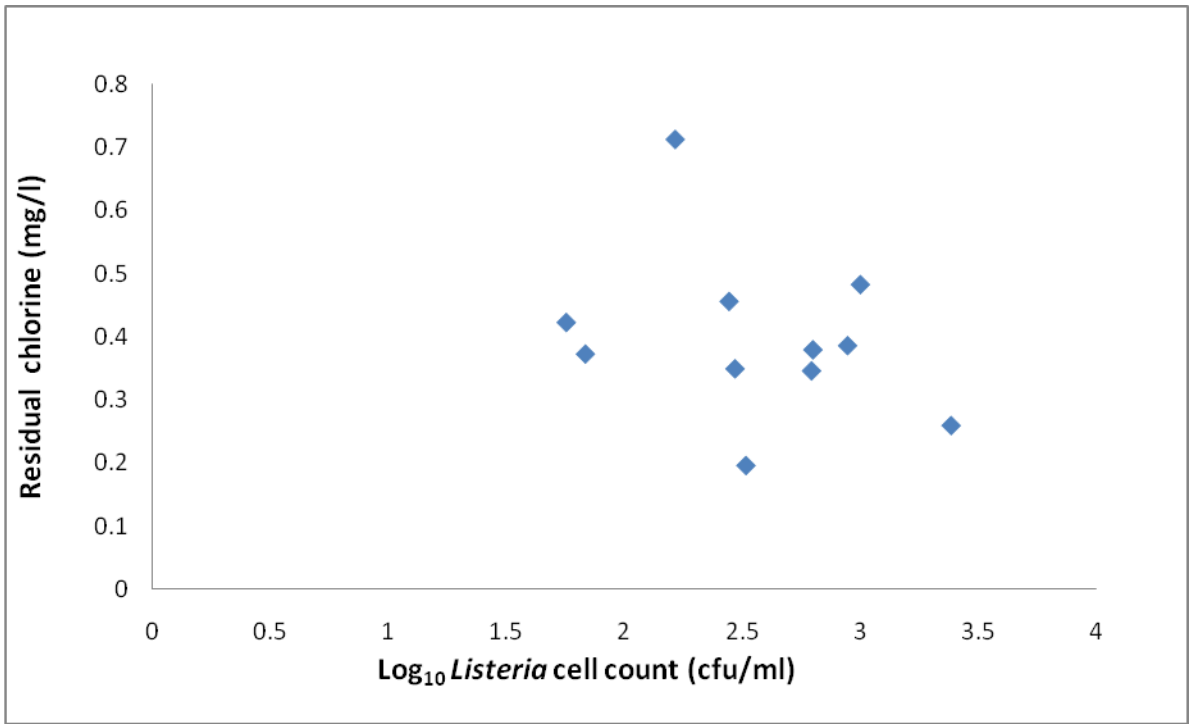

Fig. 5.3 Scatter plot of listerial density with chlorine residual. Total listerial density was not determined for the final effluent in the month of October, hence the missing data for that month

Table 5.4 *In vitro* antibiotic susceptibility profile of the *Listeria* strains isolated from the chlorinated effluents (n=23)

Antibiotics	Number of isolates (%)		
	Susceptible	**Intermediate**	**Resistant**
Amikacin (30 µg)	23(100)	0(0)	0(0)
Gentamycin(10 µg)	19(83)	0(0)	4(17)
Streptomycin(25 µg)	(15)65	0(0)	8(35)
Chloramphenicol(30 µg)	20(87)	0(0)	3(13)
Tetracyclin(30 µg)	19(83)	0(0)	4(17)
Ciprofloxacin(5 µg)	21(91)	1(4.5)	1(4.5)
Gatifloxacin(5 µg)	19(83)	2(8.5)	2(8.5)
Moxifloxacin(5 µg)	17(74)	3(13)	3(13)
Imipenem(10 µg)	19(83)	0(0)	4(17)
Meropenem(10 µg)	23(100)	0(0)	0(0)
Ertapenem(10 µg)	23(100)	0(0)	0(0)
Ampicillin(30 µg)	3(13)	0(0)	20(87)
Penicillin G(10 µg)	1(4.5)	1(4.5)	21(91)
Linezolid(30 µg)	18(78)	0(0)	5(22)
Aztreonam(30 µg)	21(91)	0(0)	2(9)
Erythromycin(15 µg)	4(17)	0(0)	19(83)
Cephalothin(30 µg)	17(74)	1(4)	5(22)
Ceftriaxone(30 µg)	21(91)	1(4.5)	1(4.5)
Sulphamethoxazole (25 µg)	8(35)	0(0)	15(65)
Trimethoprim(5 µg)	17(74)	0(0)	6(26)

n, number of isolates tested

(aminoglycosides), meropenem, and ertapenem (carbapenems). Eight (35%) of the 23 *Listeria* isolates were moderately sensitive to moxifloxacin, cephalothin, gatifloxacin, ciprofloxacin and ceftriaxone; three strains showed moderate sensitivity to moxifloxacin, 2 to gatifloxacin, while the other three were each moderately sensitive to cephalothin, ciprofloxacin, and ceftriaxone. The test isolates showed resistance to 17 (85%) of the 20 antibiotics at percentages ranging from 4.5% - 91% (Table 5.4). Only one *L. ivanovii* isolate showed resistance to a single antibiotic (aztreonam); while multiple antibiotic resistances was observed in the other 22 (95.7%) isolates in combinations ranging from four to ten antibiotics (Table 5.5). Of the seven antimicrobial genes assayed in this study, only *sulII genes* were detected in 5 (22%) strains of *Listeria ivanovii* (Table 5.6).

5.4 Discussion

Although the peak listerial density was observed for cells attached to small planktons, free-living *Listeria* species were generally more abundant in comparison to plankton associated cells during this study and across all sampled sites. The observation was corroborated by the significant difference observed in listerial abundance between free-living *Listeria* species and plankton attached cells in the FE and UP and consistent with reports elsewhere (Venkateswaran *et al*., 1989). There are no recommended standards specific for *Listeria* pathogens in water and wastewater samples in South Africa; hence the faecal coliforms standard (0 cfu/100 ml) for domestic water uses (DWAF, 1996a) was applied in this report. Based on this standard the water quality across all sampled sites and throughout the year (Table 5.2) fell short of acceptable target limits for domestic applications and thus disqualifies the waters for such (domestic) uses. *Listeria* abundance did not vary significantly with season either as free-living or plankton-

Table 5.5 Multiple antibiotic resistances of *Listeria* strains isolated from the chlorinated effluents

Antibiotics	**Number of isolates involved**	**Percentage (%)**
E, SMX, LZD, PG, AP	7[a]	31
E, LZD, PG, AP	2[b]	8.7
KF, E, SMX, LZD, PG, AP	2[b]	8.7
E, TM, LZD, MFX, PG, AP	1[b]	4.3
E, LZD, MFX, PG, AP	1[b]	4.3
C, KF, E, S, T, SMX, LZD, GAT, PG, AP	1[b]	4.3
E, S, T, SMX, LZD, MFX, PG, AP	1[b]	4.3
KF, E, S, SMX, TM, LZD, PG, AP	1[b]	4.3
CRO, KF, E, S, SMX, LZD, PG, AP,	1[b]	4.3
E, S, SMX, LZD, PG	1[b]	4.3
C, E, GM, S, SMX, TM, IMI, PG	1[b]	4.3
GM, TM, IMI, AP	1[b]	4.3
ATM, C, GM, S, T, TM, CIP, IMI, PG, AP	1[b]	4.3
GM, S, T, TM, LZD, IMI, PG, AP	1[b]	4.3
Total	**22**	**95.7**

Legend. ATM = Aztreonam; E = Erythromycin; AP = Ampicillin; LZD = Linezolid; PG = Penicillin G; KF = Cephalothin; SMX = Sulphamethoxazole; TM = Trimethoprim; MFX = Moxifloxacin; C = Chloramphenicol; S = Streptomycin; GAT = Gatifloxacin; CRO = Ceftriaxone; IMI = Imipenem; GM = Gentamycin; T = Tetracycline; CIP = Ciprofloxacin;

[a]1 strain of *L. innocua* and 6 strains of *L. ivanovii*; [b] Strains of *L. ivanovii*

Table 5.6 Occurrence of antimicrobial resistance genes in *Listeria* strains isolated from the final effluents

***Listeria* isolate codes**	**Antibiotic resistance genes**						
	penA	*ampC*	*ermB*	*ereA*	*ereB*	*su1I*	*su1II*
LEL 1[a]	–	–	–	–	–	–	–
LEL 2[b]	–	–	–	–	–	–	–
LEL 3[b]	–	–	–	–	–	–	–
LEL 4[b]	–	–	–	–	–	–	–
LEL 5[b]	–	–	–	–	–	–	+
LEL 6[b]	–	–	–	–	–	–	–
LEL 7[b]	–	–	–	–	–	–	+
LEL 8[b]	–	–	–	–	–	–	–
LEL 9[b]	–	–	–	–	–	–	–
LEL 10[b]	–	–	–	–	–	–	+
LEL 11[b]	–	–	–	–	–	–	+
LEL 12[b]	–	–	–	–	–	–	+
LEL 13[b]	–	–	–	–	–	–	–
LEL 14[b]	–	–	–	–	–	–	–
LEL 15[b]	–	–	–	–	–	–	–
LEL 16[b]	–	–	–	–	–	–	–
LEL 17[b]	–	–	–	–	–	–	–
LEL 18[b]	–	–	–	–	–	–	–
LEL 19[b]	–	–	–	–	–	–	–
LEL 20[b]	–	–	–	–	–	–	–
LEL 21[b]	–	–	–	–	–	–	–
LEL 22[b]	–	–	–	–	–	–	–
LEL 23[b]	–	–	–	–	–	–	–

[a]*Listeria innocua*; [b]Strains of *L. ivanovii;* + = Genes detected; - = Genes not detected

associated species consistent with the observation of Murrel *et al*. (1999). The significant positive correlation observed between *Listeria* species attached to large (180 µm) planktons and those attached to small (20 µm) planktons suggests that the two groups of *Listeria* species may occupy the same niche in the ecosystem. The lack of significant correlations between and amongst other treatments in this study, suggests that free-living *Listeria* species and *Listeria* species attached to medium-sized (60 µm) planktons occupy separate niches in the ecosystem, different from those occupied by *Listeria* species attached to large (180 µm) and small (20 µm) planktons. The observation is consistent with the report of Maugeri *et al*. (2004) who observed lack of significant correlation between free-living bacteria and plankton associated bacterial populations in a marine coastal zone in Italy. However, another study (Hsieh *et al*., 2007) reported a negative correlation between planktonic *Vibrio* cells and sessile populations.

Listeria species were isolated from all sampled sites and throughout the year in this study, suggesting a hundred percent prevalence of the pathogen in the water system. Free-living *Listeria* species were most prevalent (84%) both in treated effluent and the receiving watershed; followed by *Listeria* cells associated with planktons of sizes 180 µm (75%), 20 µm (68%), and 60 µm (59%), respectively. Corroborating this observation Maugeri *et al*. (2004) reported higher prevalence for free-living bacteria compared to their plankton-associated counterparts. *Listeria* species were generally more prevalent in the treated effluents (FE) both as free-living and/or plankton-associated cells compared to other sampled points (Table 5.2). The observation could be as a result of higher nutrient levels in the wastewater effluents compared to the receiving watershed in agreement with previous reports (Czeszejko *et al*., 2003; Dijkstra, 1982; Paillard *et al*., 2005). Consistent with the observation of this study, high prevalence of *Listeria* species has been reported by other workers for treated wastewater effluent and its receiving watershed (Al-

Ghazali and Al-Azawi, 1986, 1988; Paillard *et al*., 2005; Watkins and Sleath, 1981). Watkins and Sleath (1981) reported 100% prevalence of *Listeria* species in sewage, river water, and trade effluent at densities (7.0×10^2 to $>1.8\times10^4$ MPN/ml) slightly higher than those observed in this study. The sewage effluent reported by Watkins and colleague however, only underwent primary treatment unlike ours that was disinfected by chlorination which could account for the differences. Al-Ghazali and Al-Azawi (1986, 1988) also reported 100% prevalence in treated wastewater effluent in Iraq but at lower densities of < 3 to 28 MPN/ml, and Paillard *et al*. (2005) reported 84.4% prevalence of *Listeria* species in treated wastewater in France at densities ranging from < 0.3 to 21 MPN/ml. Contrary to our observation, lower prevalence have been reported for *Listeria* species in a variety of surface waters. Frances *et al*. (1991) reported the isolation of *Listeria* species from 21% of freshwater samples collected from sites in Cheshire and North Wales; while Lyautey *et al*. (2007) reported 64% for surface waters of the South Nation River Watershed in Ontario, Canada. These observations were consistent with expectations for surface waters that are not impacted by wastewater effluent in agreement with a report elsewhere (Dijkstra, 1982).

The significant variation observed between raw and treated sewage for most physicochemical parameters (Table 5.3), is an indication that the wastewater treatment process remarkably improved the quality of the raw wastewater. The improvement on raw sewage quality notwithstanding, the treated effluent did not measure up to desired target quality for turbidity, DO, COD, NO_2 (DWAF, 1996a) and PO_4; however it was of acceptable quality for pH, temperature, TDS, and NO_3 (Table 5.3). The observation generally implies that the final effluent is not safe for use in domestic activities and may support eutrophication in the receiving watershed (DWAF, 1996a,b; Fatoki *et al*., 2003).

The chlorine residual (Fig. 5.2) generally fell within acceptable target limits (0.3-0.6 mg/l) for domestic water at the point of use (Obi *et al*., 2008) except in September and November 2007 and indicates that the water is safe for domestic applications with reference to chlorine residual. The scatter plot (Fig. 5.3) indicates that the relationship between chlorine residual and listerial density did not follow any particular trend. This observation suggests that factors other than chlorine residual affected the abundance of *Listeria* species during this study; some of these factors may also be responsible for the inability of chlorine to adequately eliminate the pathogens from the wastewater even at relatively high doses. LeChevallier *et al*. (1988) observed attachment of bacteria to planktons and/or other suspended particles as a factor which enhanced resistance of bacteria to chlorine disinfection while Obi *et al*. (2008) reported other factors to include contact time, temperature, and pH. This suggests that turbidity (which is a measure of suspended particles including planktons) could be a factor in the ineffectiveness of chlorine disinfection during this study; the parameter fell short of recommended target limits throughout the study (Table 5.3). Attachment of *Listeria* species to plankton may however, not be a significant factor in the bacterial survival of chlorine disinfection in this study as free-living *Listeria* species were more abundant compared to their plankton attached counterparts even after chlorine disinfection.

Most study on the antimicrobial susceptibility profiles of *Listeria* species focus almost exclusively on clinical and/or food isolates with little information in the literature on antibiotic susceptibility profiles for *Listeria* strains isolated from treated municipal wastewater effluent. All 23 *Listeria* species tested in this study were completely sensitive to 3 (15%) of the 20 test antibiotics including, amikacin (aminoglycosides), meropenem, and ertapenem (carbapenems) (Table 5.4). Consistent with our observation, Hansen *et al*. (2005) reported complete sensitivity

of 106 *Listeria* species isolated from humans to meropenem, while Safdar and Armstrong (2003) observed 100% sensitivity to amikacin, and we reported complete sensitivity to the three antibiotics by all 14 *Listeria* species isolated from chlorinated wastewater effluent in a previous study (Odjadjare and Okoh, 2009).

Listeria strains in this study showed resistance to at least one of 17 antibiotics at percentages ranging from 4.5% - 91% (Table 5.4), and particularly high levels for penicillin G (91%), ampicillin (87%), erythromycin (83%), and sulphamethoxazole (65%). Contrary to the observation of this study, *Listeria* species were generally reported to be susceptible to penicillin G (Abuin *et al*., 1994), ampicillin (Zhang *et al*., 2007), erythromycin (Conter *et al*., 2009; Safdar and Armstrong, 2003), and sulphamethoxazole (Hansen *et al*., 2005). Conversely, considerable resistance has been reported in the literature for *Listeria* species against the penicillis (penicillin G and ampicillin) (Srinivasan *et al*., 2005), erythromycin (Aureli *et al*., 2008), and sulphamethoxazole (Zhang *et al*., 2007). The high resistance observed for penicillin G, ampicillin and sulphamethoxazole could be of serious public health concern as penicillin G and ampicillin are reported to be the antibiotics of choice in listeriosis therapy (Conter *et al*., 2009; Hansen *et al*., 2005) while sulphamethoxazole usually in combination with trimethoprim is considered second choice especially for patients who are allergic to the penicillins (Zhang *et al*., 2007). The physicochemical quality of the wastewater effluent may be a factor in the level of resistance observed in this study as it is widely reported (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004) in the literature that conventional wastewater treatment plants lack the capacity to effectively remove antibiotics and a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluent to develop resistance to common antibiotics due to selective pressure. Although we did not attempt

to assay for residual antibiotics in the treated final effluents in the course of this study, lack of capacity to remove some chemicals from the wastewater during the treatment process is evident in Table 5.3. The table shows that the treated effluent fell short of recommended standard quality for critical parameters such as turbidity, DO, COD, NO_2, and PO_4 and suggests a possible influence on the listerial resistance.

Twenty-two (95.7%) of the 23 test isolates in this study showed multiple antibiotic resistance in combinations ranging from four to ten antibiotics (Table 5.5). Similar observation has been reported elsewhere (Srinivasan *et al*., 2005). On the contrary Conter *et al*. (2009) reported that 'resistance to one antibiotic was more common than multiple resistance' amongst their *Listeria* isolates, while Arslan and Ozdemir (2008) reported resistance to single antibiotics with no record of multiple antibiotic resistance amongst 47 strains of *Listeria* species isolated from white cheese and tested against 13 antibiotics. Multiple drug resistance in *Listeria* species have been attributed to antimicrobial selective pressure and gene transfer mechanism between and amongst *Listeria* species and close relatives of the bacteria such as *Enterococcus, Streptococcus* and *Staphylococcus* species Safdar and Armstrong (2003). Donlan and Costerton (2002) also reported the acquisition of inherent resistance to antimicrobial agents by attached bacterial species; suggesting that attachment to plankton at one point or the other may have enhanced the multiple resistances of our isolates to several test antibiotics.

Although the penicillins (penicillin G and ampicillin) and erythromycin showed the highest phenotypic resistance during this study, the genes responsible for resistance to these antibiotics were not detected in our *Listeria* isolates (Table 5.6). In a similar report, Srinivasan *et al*. (2005) observed high level (92%) of phenotypic resistance to ampicillin but failed to detect the genes responsible for ampicillin resistance in all of their 38 *Listeria* isolates. Consistent with

the observation of this study, Davis and Jackson (2009) could not detect *penA* genes (responsible for penicillin resistance) in *Listeria* from various sources and Srinivasan *et al*. (2005) reported their inability to detect genes responsible for erythromycin and ampicillin resistance in 38 *Listeria* isolates from dairy farms. Conversely Srinivasan *et al*. (2005) reported the detection of *penA* genes in 37% of their *Listeria* isolates while Roberts *et al*. (1996) reported the detection of erythromycin resistance genes in *Listeria* species isolated from food samples. To the best of our knowledge, this is the first report on the detection of dihydropteroate synthetase type II (*sulII*) genes in *Listeria* species (Table 5.6). Previous attempt by other workers (Davis and Jackson, 2009; Srinivasan *et al*., 2005) did not detect the genes in *Listeria* species. The percentage of *Listeria* isolates that harbored this gene was however relatively small (22%) compared to the high (65%) level of phenotypic resistance observed for the antibiotic (sulphametoxazole) in this study. The observations generally suggests that the presence of antimicrobial resistance genes in bacterial isolates do not always correlate with phenotypic antibiotic resistance and indicates that other mechanisms such as decreased outer membrane permeability, activation of efflux pump, or mutation in a ribosomal protein may have contributed to antimicrobial resistance phenotypes observed in this study (Srinivasan *et al*., 2005).

This study demonstrated that *Listeria* pathogens very well survives the activated sludge treatment process as free-living and plankton attached entities and that the wastewater treatment plant studied could be a significant source of the pathogen in the receiving aquatic ecosystem. There is need therefore for the relevant monitoring agencies to take proactive steps aimed at curtailing an impending listeriosis outbreak in the interest of the public health.

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CHAPTER 6

Listeria abundance and physicochemical quality of a reclaimed wastewater used for irrigation and aquaculture in South Africa

(Submitted to Journal of The American Water Resources Association for publication)

CHAPTER 6

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Abstract

We evaluated *Listeria* pathogen abundance and the physicochemical quality of raw sewage influent (INF) and secondary treated effluent (SE) of an urban (East London) wastewater treatment facility in South Africa, and its suitability for agriculture and aquaculture. Listerial density ranged between 1.3×10^5 to 1.4×10^7 cfu/100 ml in INF and 9.6×10^3 to 2.8×10^5 cfu/100 ml in SE. Secondary treatment reduced listerial density by 77.8 - 99.5 %. The pH of the INF ranged between 6.8 and 7.4, while those of the SE vary between 6.8 and 7.6. Temperature at both sampled sites varied from 19^o to 25°C while turbidity ranged between 258 NTU - 678 NTU (INF) and 2.75 NTU - 10.37 NTU (SE). Also, total dissolved solids varied between 365 - 642 mg/l (INF) and 321 - 528 mg/l (SE); and dissolved oxygen was in the range of 0.18 - 4.04 mg/l (INF) and 1.77 - 6.2 mg/l (SE). Chemical oxygen demand varied between 43 - 1116 mg/l in the INF and 36 - 109 mg/l in the SE. Other physicochemical parameters were as follows: Nitrate [1.65 - 4.05 mg NO_3-N/l (INF) & 2.23 - 6.35 mg NO_3-N/l (SE)]; Nitrite [0.14 - 1.73 mg NO_2-N/l (INF) & 0.25 - 3.21 mg NO_2-N/l (SE)]; and Phosphate [2.42 - 5.31 mg PO_4-P/l (INF) & 0.23 - 0.36 mg PO_4-P/l (SE)]. Although the microbial quality of the wastewater fell short of recommended standard after secondary treatment, its physicochemical quality reaffirms its potential as a cheaper water resource in agriculture and aquaculture in South Africa.

(Keywords: irrigation; environmental impact; public health; reuse wastewater; *Listeria;* physicochemical; aquaculture.)

6.1 INTRODUCTION

Growing economic and physical scarcity of water, made worse by global climatic changes and increasing demands for freshwater, calls for innovative ways of water use and development (Inocencio *et al.*, 2003). The Southern African region is predicted to experience more and longer droughts over the next 70 years (Palitza, 2009); according to the report the impending water-shortage will result in more strain on available freshwater resources and in turn lead to increased crop failures, less pasture for livestock and ultimately less food for the growing population. The United Nations Environment Program (UNEP, 2009) also predicted that the situation may get so bad in the coming years that wastewater may account for 25-75% of the total available irrigation water in the region, especially in the very dry zones. The bleak future of freshwater availability is thus forcing planners and stakeholders to consider any sources of water which might be useful economically to effectively promote food security and further development (FAO, 1992). Hence reuse of wastewater may be an inevitable option for most farmers in South Africa and neighboring States in the near future for obvious reasons.

Innovative approaches to agricultural water use have been reported to have the capacity not only to raise agricultural productivity and food security in sub-Saharan Africa, but also lead to the general improvement of living standard of the poor (Inocencio *et al.*, 2003). It is little wonder therefore that wastewater reuse for agriculture is increasingly becoming an attractive option to many stakeholders in the Southern Africa region due to its potential to efficiently conserve water resources, recycle nutrients, and minimize pollution of surface water bodies (Al-Sa'ed, 2007). UNEP (2009) reported the use of sewage in the cultivation of fishes in Malawi, South Africa and Zimbabwe with fish yields in Malawi reaching 4-5 tons/ha/growth period as against yields of 0.8-1.2 tons/ha/year in South Africa. The report also indicated that South Africa

recycles about 8% of her total sewage output as against up to 50% in Namibia, and about 65% in Botswana. While it is necessary to encourage the reuse of wastewater especially in the very dry zones of the Southern African region, conscious steps must be taken to ensure acceptable reuse wastewater quality in order to preserve the public health and protect the environment. Unfortunately, there is a dearth of information on the quality of treated wastewater effluent used for agricultural purposes in South Africa. Information on the quality of wastewater for reuse in agriculture will enable farmers and other stakeholders to make adequate plans with regards to the type of crops or fish(es) that will best suit the available water quality; in other words, such information will elucidate the effluent utilization potential. Effluent quality information will also enable planners to determine the best measure to take at improving the quality of the said irrigation water for intended purposes.

This study therefore reports the *Listeria* abundance and physicochemical quality of a secondary treated wastewater effluent from a typical urban wastewater treatment facility in South Africa used for irrigation and fish farming and its suitability for these purposes. The investigation of *Listeria* pathogens as against the popular coliforms in this study was deliberate based on relevant information in the literature. A report by the Food and Agricultural Organization (FAO, 1992) suggests that the coliforms were unsuitable for the monitoring of wastewater reuse systems due to the fact that several species in this group are able to grow outside the gut. In a similar vein, the organization disqualifies the use of faecal streptococci as an indicator of wastewater reuse potential on the basis that "the possible presence of the non-faecal biotypes as part of the natural microflora on crops may detract from their utility in assessing the bacterial quality of wastewater irrigated crops; and the poorer survival of faecal streptococci at higher than at low temperatures." In contrast, FAO (1992) recommended *Salmonella* as one of

the pathogens to be monitored in wastewater meant for agricultural uses, due to the fact that this pathogen is typically present in good numbers in urban sewage. Reports in the literature (Watkins and Sleath, 1981; Paillard *et al.*, 2005; Odjadjare and Okoh, 2009) however, suggest that *Listeria* species are very resilient and survive conventional wastewater treatment processes better than *Salmonella* species. In addition, the bacteria is reported to be capable of saprophytic existence on plant and in soil for years (Al-Ghazali and Al-Azawi, 1986; Beuchat, 1996); this coupled with the high mortality rate of listeriosis (30-51%) (Rocourt *et al.*, 2000), makes the pathogen a preferred candidate for investigation in reuse wastewater meant for agriculture and aquaculture as done in this study.

6.2 MATERIALS AND METHODS

6.2.1 Description of Study Site

The wastewater treatment plant (FIGURE 6.1) is located in a large and highly populated urban community in the Eastern Cape Province of South Africa, with the geographical coordinates: 32.97^{o}S and 27.87^{o}E. The plant receives municipal domestic sewage and a heavy industrial effluent and comprise of four screens, a grit channel, two anaerobic tanks, two anoxic tanks and two aerobic tanks (each equipped with three vertically mounted mechanical aerators). The plant has six sedimentation tanks (clarifiers) with the return activated sludge (RAS) pumped from the bottom of the clarifiers via the screens with raw sewage to the aeration tanks. Supernatant liquor from the sedimentation tanks (secondary effluent) is used as a water resource for irrigation of a nearby golf course and a fish pond located within the treatment plant premises. The average daily inflow of raw sewage during the period of study was 32 000 m^3/day, while the plant has a built in capacity of 40 000 m^3/day.

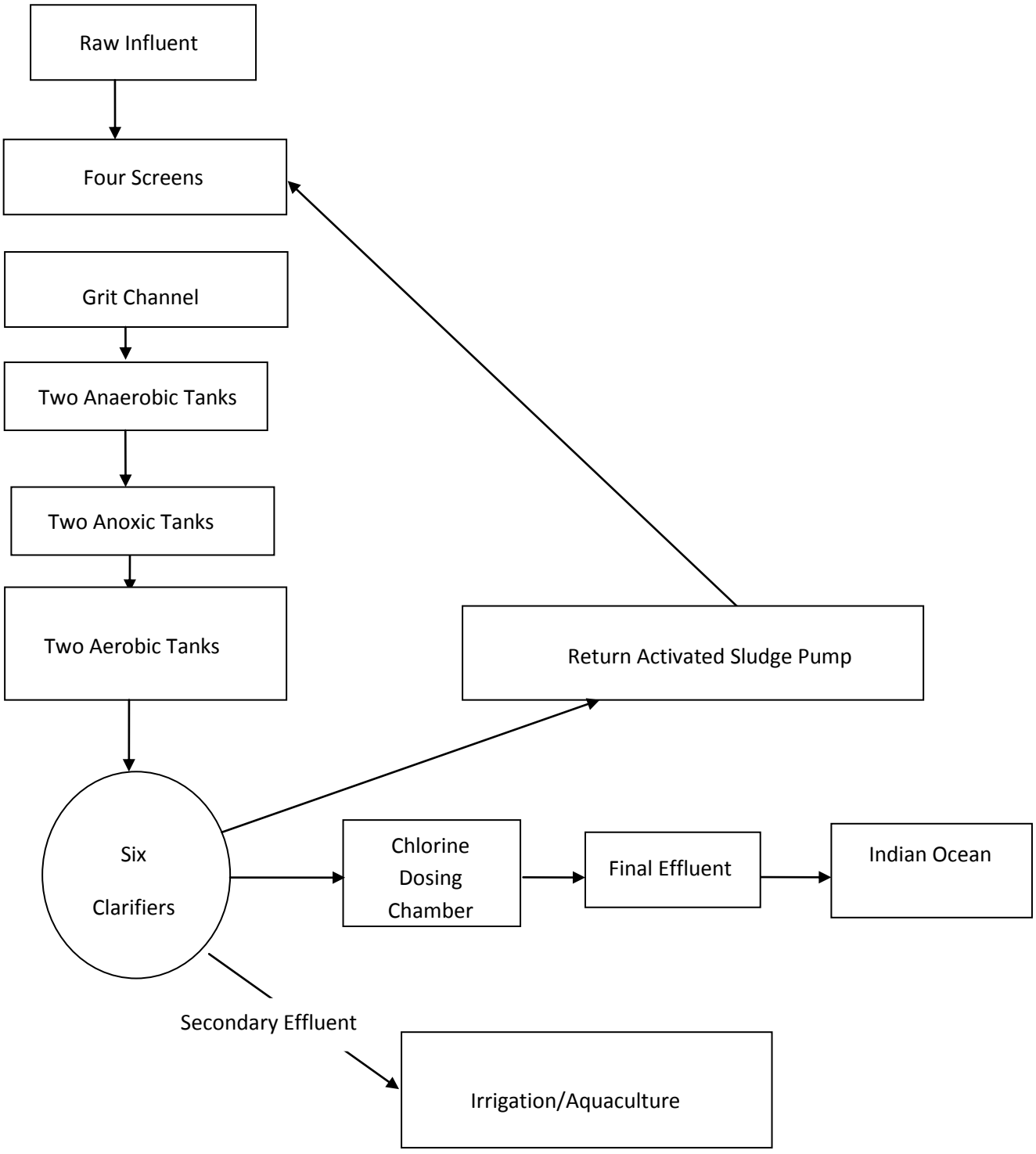

FIGURE 6.1. Schematic Representation of the East Bank Reclamation Works.

6.2.2 Sample Collection

Wastewater samples were collected on a monthly basis from the raw sewage influent (INF) and the secondary effluent (SE) between August, 2007 and July, 2008. Samples were collected in duplicates from the surface of each site in clean sterile one litre Nalgene bottles and transported in cooler boxes containing ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice campus for analyses. Processing of samples was done within 6 hours of sample collection.

6.2.3 Estimation of Listeria Abundance

The isolation of *Listeria* species were done according to the description of Hitchins (2001) with modifications. Briefly, aliquots of samples were directly inoculated onto *Listeria* chromogenic agar (LCA agar) (Pronadisa® Madrid, Spain) following standard spread plate technique and incubated for 24-48 h at 35^{o}C. Typical *Listeria* colonies appeared blue-green on LCA agar plates while pathogenic strains (*L. monocytogenes* and *L. ivanovii*) are surrounded by an opaque halo in addition to their blue-green color. Total *Listeria* counts were recorded and the isolates purified and stored on nutrient agar slants at 4^{o}C for further analyses.

6.2.4 Physicochemical Analyses

All field meters and equipment were checked and appropriately calibrated according to the manufacturers' instructions. pH, temperature, total dissolve solid (TDS), and dissolved oxygen (DO), were all determined on site using the multi-parameter ion specific meter (Hanna-BDH laboratory supplies). Turbidity was also determined on site using a microprocessor turbidity meter (HACH Company, model 2100P) while concentrations of orthophosphate (PO_4) as P,

Nitrate (NO_3), Nitrite (NO_2), and chemical oxygen demand (COD) were determined in the laboratory by the standard photometric method (DWAF, 1992) using the spectroquant NOVA 60 photometer (Merck Pty Ltd). Samples for COD analyses were digested with a thermoreactor model TR 300 (Merck Pty Ltd) prior to analysis using the spectroquant NOVA 60 photometer.

6.2.5 Statistical Analysis

Calculation of means and standard deviations were performed using Microsoft Excel office 2007 version. Correlations (paired T-test) and test of significance (ANOVA) were performed using SPSS 17.0 version for Windows program (SPSS, Inc.). All tests of significance and correlations were considered statistically significant at *P* values <0.05 or <0.01.

6.3 RESULTS

Tables 6.1, 6.2, and 6.3 show results of *Listeria* abundance and physicochemical quality of the raw sewage (INF) and secondary effluent (SE) as well as the correlation matrix of the parameters evaluated.

6.3.1 Listeria Abundance

Table 6.1 shows the average listerial densities of the wastewater before and after treatment. Listerial density ranged between 1.3×10^5 to 1.4×10^7 cfu/100 ml in INF and 9.6×10^3 to 2.8×10^5 cfu/100 ml in SE. The highest listerial density was recorded in the summer month of

TABLE 6.1. *Listeria* Density in Raw and Treated Sewage.

Seasons	Months	*Listeria* density (cfu/100 ml)		
		Raw sewage	**Secondary effluent**	**Reduction (%)**
Spring	August 2007	3.5×10^{6}	6.4×10^{4}	98.2
	September 2007	1.2×10^{6}	1.6×10^{4}	98.6
	October 2007	ND	ND	ND
Summer	November 2007	1.9×10^{6}	9.6×10^{3}	99.5
	December 2007	5.0×10^{6}	2.3×10^{4}	99.5
	January 2008	1.3×10^{5}	2.9×10^{4}	77.8
Autumn	February 2008	3.1×10^{6}	4.0×10^{4}	98.7
	March 2008	4.9×10^{6}	9.7×10^{4}	98.0
	April 2008	1.4×10^{7}	2.8×10^{5}	98.0
Winter	May 2008	6.1×10^{6}	4.1×10^{4}	99.3
	June 2008	1.6×10^{6}	6.2×10^{4}	96.1
	July 2008	2.1×10^{6}	1.4×10^{4}	99.3
Annual Average		3.9×10^{6}	6.1×10^{4}	96.6
Range		1.3×10^{5} - 1.4×10^{7}	9.6×10^{3} - 2.8×10^{5}	77.8 -99.5

Legend: ND = Not Determined

TABLE 6.2. Physicochemical Quality of the Wastewater before and after Secondary Treatment.

PARAMETER	Spring		Summer		Autumn		Winter	
	INF[a]	SE[b]	INF	SE	INF	SE	INF	SE
pH	6.8±0.91	7.1±0.31	7.1±0.04	7.6±0.78	7.4±0.35	7.5±0.14	6.9±0.13	6.8±0.11
Temperature	22±1.93	21±0.93	24±0.84	24±0.97	25±1.51	25±2	19±1.61	19±1.43
Turbidity	258±183	6.86±3.5	550±430	10.37±10	620±375	3.5±1.36	678±363	2.75±0.28
TDS[c]	377±8	401±113	365±48	321±36	642±290	528±206	414±14	391±13
DO[d]	4.04±2	6.2±1.4	1.57±0.85	3.34±0.19	0.18±0.04	1.77±0.74	2.28±1.71	3.12±1.3
COD[e]	74±1.4	81±2	43±4	52±5	880±650	109±89	1116±1037	36±26
Nitrate	1.65±1.8	3.26±3	3.35±0.21	6.35±0.21	2.8±0.73	2.23±1	4.05±0.21	4.8±0.42
Nitrite	1.73±1.9	3.21±3.5	0.14±0.08	0.33±0.07	0.27±0.09	0.25±0.05	0.47±0.22	1.01±0.03
Phosphate	4.17±0.45	0.36±0.06	2.81±1	0.23±0.13	5.31±05	0.34±0.31	2.41±1.29	0.25±0.15

[a] Raw sewage influent [b] Secondary effluent [c] Total dissolved solids [d]Dissolved oxygen [e]Chemical oxygen demand

TABLE 6.3. Correlation Matrix of the Wastewater Quality Parameters.

	pH	Temperature	Turbidity	TDS	DO	COD	Nitrate	Nitrite	Phosphate	*Listeria* spcies
pH	1	.562**	-.060	.506**	-.272	.047	-.288	.112	.157	.376*
Temp		1	.169	.061	-.311*	.075	.146	-.355*	.194	.144
Turbidity			1	.014	-.615**	.411*	-.198	-.144	.646**	.303
TDS				1	-.434**	.073	-.260	-.149	.305*	.670**
DO					1	-.339*	.324*	.183	-.473**	-.461**
COD						1	-.072	-.050	.090	.148
Nitrate							1	-.602**	-.334*	-.389*
Nitrite								1	-.091	-.115
Phosphate									1	.652**
Listeria species										1

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

April, 2008 in INF while the lowest density was observed in the SE in the autumn month of November, 2007. The annual mean listerial density was 3.9×10^6 cfu/100 ml for INF and 6.1×10^4 cfu/100 ml for the SE. The percentage reduction achieved by the secondary treatment ranged from 77.8 to 99.5% with the highest percentage reduction observed in the summer months of November and December, 2007 and the lowest recorded in January, 2008 (late summer). Listerial density varied significantly with sampling site ($P < 0.05$) but not with season. *Listeria* abundance showed significant positive correlation with TDS and PO_4 ($P < 0.01$) and pH ($P < 0.05$) and negatively correlated with DO ($P < 0.01$) and NO_3 ($P < 0.05$).

6.3.2 pH

pH in the raw sewage varied from 6.8 to 7.4 while that of the secondary effluent ranged from 6.8 to 7.6 (Table 6.2). Values of pH for spring varied significantly ($P < 0.05$) with those of autumn and winter but not with summer. pH values for winter also varied significantly with those of summer ($P < 0.05$) and autumn ($P < 0.01$) but did not vary significantly with sampling site. pH significantly (positive) correlated with temperature and TDS ($P < 0.01$) and with listerial density ($P < 0.05$) but not with other parameters.

6.3.3 Temperature

Temperature ranged between 19^oC (winter) and 25^oC (autumn) across the sampled sites during the study; and in comparison with the spring temperature, those of summer and autumn were significantly different ($P < 0.01$). Similarly, temperature values for winter varied significantly

with those of summer and autumn ($P < 0.01$). Temperature did not vary significantly with sampling site, and it showed significant negative correlations with DO and nitrite ($P < 0.05$) but not with other parameters except as previously cited for pH.

6.3.4 Turbidity

Turbidity was in the range of 258 NTU - 678 NTU (INF) and 2.75 NTU - 10.37 NTU (SE) during the study. The values varied significantly with sampling site ($P < 0.01$) but not with season. Turbidity negatively correlated with DO ($P < 0.01$) and positively correlated with COD ($P < 0.05$) and PO_4 ($P < 0.01$). It however, did not significantly correlate with other parameters.

6.3.5 Total Dissolved Solids

TDS varied between 365 - 642 mg/l (INF) and 321 - 528 mg/l (SE); concentrations in autumn were significantly different ($P < 0.05$) from those of spring and summer, but not with winter. TDS did not vary significantly with sampling site; it is positively correlated with PO_4 ($P < 0.05$) and negatively correlated with DO ($P < 0.01$). There was no significant correlation between TDS and other parameters except as previously cited for *Listeria* abundance and pH.

6.3.6 Dissolved Oxygen

DO was in the range of 0.18 - 4.04 mg/l (INF) and 1.77 - 6.2 mg/l (SE). There were significant differences in DO values for spring with those of summer and winter ($P < 0.05$) and autumn ($P <$

0.01). DO also vary significantly with sampling site ($P < 0.05$). It showed significant negative correlation with COD ($P < 0.05$), and PO_4 ($P < 0.01$); and positively correlated with nitrate ($P < 0.05$). There was however, no significant correlation between DO and other parameters except as previously cited for *Listeria* abundance, temperature and turbidity.

6.3.7 Chemical Oxygen Demand

COD varied between 43 - 1116 mg/l in the INF and 36 - 109 mg/l in the SE. COD did not show significant difference with regards to season and sampling site. There was also no significant correlation between COD and other parameters except as cited previously for turbidity and DO.

6.3.8 Nitrate

Concentration of nitrate ranged between 1.65 - 4.05 mg NO_3-N/l (INF) and 2.23 - 6.35 mg NO_3-N/l (SE) and varied significantly with sampling site ($P < 0.05$) but not with season. Nitrate showed significant negative correlations with PO_4 ($P <0.05$) and nitrite ($P <0.01$) but did not significantly correlate with other parameters except as was earlier cited above for listerial density and DO.

6.3.9 Nitrite

Nitrite concentration varied from 0.14 -1.73 mg NO_2-N/l (INF) and 0.25-3.21 mg NO_2-N/l (SE) and showed significant difference with regard to sampling site ($P < 0.05$). Nitrite concentration

in spring varied significantly with those of summer, autumn and winter ($P < 0.05$). There was however no significant correlation between NO_2 and other parameters except as earlier cited above for temperature and NO_3.

6.3.10 Phosphate

Orthophosphate (PO_4) concentration during the study ranged between 2.42 - 5.31 mg PO_4-P/l (INF) and 0.23 - 0.36 mg PO_4-P/l (SE) and varied significantly with sampling site ($P < 0.05$) but not with season. It did not significantly correlate with other parameters except as earlier cited above for *Listeria* abundance, TDS, turbidity, DO, and NO_3.

6.4 DISCUSSION

Quality parameters for wastewater reuse in agriculture are usually evaluated based on their relevance to yield and quality of agricultural products, maintenance of soil productivity, and protection of the environment and public health (FAO, 1992). The optimal quality of irrigation water required to achieve the above-mentioned goals depends on (but are not exclusive to) a number of factors including the physical and chemical qualities of the receiving environment, and the type of agricultural product (crop or fish) to be cultivated (WHO, 2006a,b). The determination of the fitness of reclaimed wastewater for agriculture and aquaculture purposes involves physicochemical and microbiological quality assay, some of which were evaluated in this study and discussed below.

The listerial densities reported in this study was similar to those reported by Watkins and

Sleath (1981), but remarkably higher than those reported in other studies (Al-Ghazali and Al-Azawi, 1986, 1988; Paillard *et al.,* 2005; Odjadjare and Okoh, 2009). Similar reduction rates as observed in this study have been reported elsewhere for *Listeria* species (Al-Ghazali and Al-Azawi, 1988) and faecal coliforms (Saleem *et al.*, 2000; Al-Sa'ed, 2007). The high reduction rate observed in this study reflects the effects of settling and aeration as part of the secondary treatment (Al-Ghazali and Al-Azawi, 1988). The significant reduction in listerial density notwithstanding, the treatment did not adequately eliminate the bacteria from the wastewater. This is consistent with previous reports (Czeszejko *et al.,* 2003; Odjadjare and Okoh, 2009), and reaffirms the resilience of the bacteria to conventional wastewater treatment processes including disinfection (Czeszejko *et al.,* 2003; Paillard *et al.,* 2005; Odjadjare and Okoh, 2009). The negative correlation observed between DO and *Listeria* species points to the higher density of the bacteria in the (low oxygen-containing) raw sewage compared to the (higher oxygen-containing) secondary effluent in line with previous documentations (Watkins and Sleath, 1981; Paillard *et al.,* 2005).

The WHO guidelines for unrestricted irrigation (irrigation of crops likely to be eaten uncooked), requires that no detectable faecal coliform bacteria be allowed in 100 ml of irrigation water (Blumenthal *et al.,* 2000). For irrigation of commercially processed and fodder crops the guideline limit is $\leq$ 200 faecal coliform bacteria/100 ml of irrigation water. To the best of our knowledge, there is no bacterial guideline for restricted irrigation in the WHO guidelines as at the time of compiling this report. In order to prevent pathogen invasion of fish muscle FAO (1992) recommended guideline limits of wastewater fed aquaculture of $\leq 10^3$ coliform bacteria/100 ml if the water will not be further diluted and $\leq 10^4$ coliform bacteria/100 ml if the water will be further diluted in the pond. Based on these guidelines, the quality of the wastewater

effluent under study fell short of recommended standards for unrestricted irrigation and aquaculture and must therefore be improved upon in the interest of public health. As an alternative, food products grown by reclaimed wastewater of this bacterial quality should be properly cooked prior to consumption where necessary in order to mitigate possible health hazards (WHO, 2006a,b).

Listeria species are reported to survive and multiply in soil and plant surfaces for as long as 10-12 years (Beuchat, 1996), making them of epidemiological significance in reuse wastewater applied in agriculture. Although listeriosis is rare, the mortality rate of the disease could be as high as 51% (Rocourt *et al.* 2000) making it of serious public health concern. *Listeria* pathogens have been implicated in several foodborne outbreaks around the globe (Beuchat, 1996; Rocourt *et al.,* 2000; Paillard *et al.,* 2005); and the significance of wastewater in the epidemiology of these pathogens have long been recognized (Watkins and Sleath, 1981; Al-Ghazali and Al-Azawi, 1986, 1988) but relatively understudied.

The range of pH observed in this study fell within the recommended target limits (6.5-8.5) for agriculture and aquaculture (FAO, 1992; WHO, 2006a,b) and indicates that the wastewater is of good quality for agriculture with reference to pH. Similar pH values as observed in this study have been previously reported in the literature (Al-Ghazali and Al-Azawi, 1986; El-Shafai *et al.,* 2004). Conversely, Ogunfowokan *et al.* (2005) reported lower pH values (5.23-6.32) while Akan *et al.* (2008) reported higher pH (8.94 - 10.34). Temperature also fell within acceptable limits ($\leq 25^{o}C$) for maintaining the stability of the receiving ecosystem as stipulated by the South African government (DWAF, 1996). This observation implies that the secondary effluent was of standard quality with reference to temperature and may not significantly offset the homeostatic balance of the receiving ecosystems vis-à-vis its application in agriculture and

aquaculture. Similar temperature values have been reported in the literature for similar environments (Igbinosa and Okoh, 2009; Odjadjare and Okoh, 2009).

The effluent quality fell short of target turbidity limit (<1 - <5 NTU) for reclaimed wastewater for irrigation (Lazarova *et al.*, 2008) in spring and summer, but was compliant in autumn and winter. This implies that the organic matter load during spring and summer were higher than those of autumn and winter and indicates a higher chance of soil clogging and oxygen depletion in the former seasons than in the later (FAO, 1992). However, this may not be a problem if the organic matter is readily degradable in the soil (FAO, 1992). In a similar vein, high organic matter content may lead to depletion of available oxygen in water systems, and result in death of fishes grown in wastewater fed aquaculture (WHO, 2006b). Based on the USEPA (2004) recommended standard (<20 - 90 mg/l) for COD levels in reclaimed wastewater, the secondary effluent quality during this study could be adjudged fit for application in agriculture except for values recorded during autumn (Table 6.1).

Total dissolved solids (TDS) target limit in reclaimed wastewater for agriculture range from <500-2000 mg/l (FAO, 1992; Abu-Zeid, 1998; WHO, 2006a) depending on the sensitivity (Table 6.4) of the crop to salinity. Abu-Zeid (1998) reported that at TDS concentration of <500 mg/l, no noticeable effect has been reported for soil or crops, indicating that the quality of the wastewater under study was generally good for agriculture with reference to TDS after secondary treatment. Although there are no recommended limits for TDS concentration in aquaculture, Morrison *et al.* (2001) reported that high salt concentration in wastewater can result in adverse ecological effects on aquatic biota. TDS concentration did not vary significantly with sampling site in this study and suggests that the secondary treatment did not significantly remove dissolved salts from the raw sewage (Table 6.2). The strong positive correlation between TDS

TABLE 6.4. Salt Tolerance of Selected Crops.

[a]Sensitive	[b]Moderately sensitive	[c]Moderately tolerant	[d]Tolerant
Bean	Broad bean	Cowpea	Barley
Paddy rice	Corn	Kenaf	Cotton
Sesame	Flax	Oats	Guar
Carrot	Millet	Safflower	Rye
Onion	Peanut	Sorghum	Sugar beet
Okra	Sugarcane	Soybean	Triticale
Pea	Sunflower	Wheat	Semi-dwarf wheat
Parsnip	Alfalfa	Barley (forage)	Durum wheat
Strawberry	Bentgrass	Grass canary	Alkali grass
Almond	Angleton bluestem	Hubam clover	Nuttail alkali
Apple	Smooth brome	Sweet clover	Bermuda grass
Apricot	Buffelgrass	Tall fescue	Kallar grass
Avocado	Burnet	Meadow fescue	Desert salt grass
Blackberry	Alsike clover	Harding grass	Wheat grass
Boysenberry	Strawberry clover	Broadleaf trefoil	Fairway wheat
Cherimoya	White Dutch clover	Wheat (forage)	Crested wheat
Sweet cherry	Corn (forage)	Artichoke	Tall wheat grass
Sand cherry	Cowpea (forage)	Red beet	Altai wild rye
Currant	Grass dallis	Zuchinni squash	Russian wild rye
Gooseberry	Meadow foxtail	Fig	Asparagus
Grapefruit	Blue grama	Jujube	Guayule
Lemon	Love grass	Papaya	Jojoba
Lime	Oats (forage)	Pomegranate	
Loquat	Cabbage	Rhodes grass	

Source: Abu-Zeid, 1998

Note: [a]Toletates total dissolved salt (TDS) concentration at ≤ 500 mg/l; [b]tolerates TDS concentration between 500 and 1,000 mg/l; [c]tolerates TDS concentration between 1,000 and 2,000 mg/l; and [d]tolerates TDS concentration ≥ 2,000 mg/l.

and listerial density is consistent with previous reports (Al-Ghazali and Al-Azawi, 1986; Czeszejko *et al.,* 2003) on the capacity of the bacteria to survive high salt concentrations.

Dissolved oxygen (DO) levels in this study fell short of the acceptable limit (≥ 5 mg/l) of no risk for the support of aquatic life (Fatoki *et al.,* 2003) except in spring when the secondary effluent (6.2 mg/l) was compliant with the stipulated standard (Table 6.2). This is an indication that the wastewater may not be fit for aquaculture except in the growth of oxygen tolerant fish species (WHO, 2006b). Dissolved oxygen is essential in maintaining the oxygen balance in the environment. Low dissolved oxygen levels in irrigation and aquaculture water may adversely affect plant and aquatic life (FAO, 1992; Abu-Zeid, 1998; WHO, 2006a,b). The nitrate concentration observed during this study fell within recommended limits(< 30 mg NO_3-N/l) that may increase productivity in agriculture (WHO, 2006a). Although there are no recommended standards for nitrate in aquaculture, high nitrate levels in water systems is reported to result in eutrophication leading to loss of diversity in the aquatic biota and overall ecosystem degradation through algal blooms, excessive plant growth, oxygen depletion, reduced sunlight penetration and ultimately, death of aquatic life (CCME, 2006).

Nitrites like nitrates enhance plant productivity at appropriate concentrations (WHO, 2006a). However, high nitrite concentration may encourage the infection of fish organ and increase mortality rate in fishes (El-Shafai *et al.,* 2004). Nitrites concentration during this study fell within acceptable limits for agriculture (< 30 mg NO_2-N/l) (WHO, 2006a) but not for the preservation of the aquatic ecosystem (< 0.5 mg NO_2-N/l) as recommended by the South African government (DWAF, 1996). This therefore implies that whilst the wastewater may be suitable for agriculture it may not be beneficial for aquaculture. Phosphate levels similar to those observed in this study had been previously reported (Igbinosa and Okoh, 2009).

Conversely,Fatoki *et al.* (2003) reported lower PO_4 levels and Ogunfowokan *et al.* (2005) reported higher PO_4 levels in their studies. The phosphate concentration observed during this study complied with recommended limits for agriculture (< 20 mg PO_4-P/l) but fell short of aquaculture target limits (5 µg/l or 0.005 mg PO_4-P/l) in lieu of risk of eutrophication (DWAF, 1996; WHO, 2006a) suggesting that the treated wastewater is suitable for agriculture but not for aquaculture. The strong positive correlation between phosphate and listerial density indicates that phosphate enhanced the growth of *Listeria* species in agreement with the report of Prescott *et al.* (1996) which states that phosphate is required for bacterial growth.

6.5 CONCLUSION

The suitability of secondary treated municipal wastewater of a typical urban community of South Africa as a cheap resource in agriculture and aquaculture was demonstrated in this study.

Although the water quality fell short of recommended target limit for microbial standard, its physicochemical qualities were generally acceptable for application in agriculture and aquaculture. While we call on relevant stakeholders to continually take steps at improving reclaimed wastewater quality in South Africa, we submit that an outright disuse of this water resource may be costlier than its reuse in agriculture and aquaculture with reference to the consequent food shortage, environmental pollution, and high cost of sourcing for the 'perfect' irrigation water in a developing and water scarce nation like South Africa.

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CHAPTER 7

Prevalence and antibiogram of *Listeria* pathogens in the final effluent of a peri-urban wastewater treatment facility and its receiving watershed

CHAPTER 7

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Abstract

We assessed the abundance of free-living and plankton-associated *Listeria* pathogens in the final effluents of a wastewater treatment facility and its receiving watershed in a typical peri-urban (Dimbaza) community in South Africa between August 2007 and July 2008, and elucidated the *in vitro* antibiogram of the listerial isolates as well as the physicochemical qualities of the raw sewage and treated effluents. Total *Listeria* counts ranged between 4.0×10^2 and 3.52×10^5cfu/ml. *Listeria* species associated with small (20 µm) planktons were most prevalent (100%), compared to the free-living cells and those attached to larger (60 and 180 µm) plankton sizes (90-95%). The treated effluent quality fell short of recommended standards for turbidity, chemical oxygen demand, phosphate and *Listeria* density while complying with target limits for pH, temperature, total dissolved solids, dissolved oxygen, nitrate, and nitrite after treatment. The *Listeria* isolates were sensitive to 6 (30%) of the 20 test antibiotics, and showed varying (6-94%) levels of resistance to 12 antibiotics. The study demonstrated that municipal wastewater effluents could be a source of multiple resistant *Listeria* pathogens in the South African aquatic milieu.

Keywords: Wastewater effluent; *Listeria*; free-living; plankton-associated; abundance; antibiogram.

7.1 Introduction

Listeriosis is an infectious disease caused by the bacterium *Listeria*. The disease is mainly caused by *Listeria monocytogenes* (in humans) and *L. ivanovii* (in animals) (Schuchat *et al*., 1991) and commonly affects the pregnant, newborns, the elderly and immunocompromised subjects (Srinivasan *et al*., 2005). Despite the low global incidence of the infection (2-15 cases per million people per year), the pathogen is under close surveillance due to its relative resilience to adverse conditions and high mortality rate (20-51%) (Siegman-Igra *et al*., 2002; Rocourt *et al*., 2000; Lyautey *et al*., 2007). Although food and food products were widely reported to be the route of transmission of *Listeria* pathogens, recent reports (Czeszejko *et al*., 2003; Paillard *et al*., 2005; Watkins and Sleath, 1981) indicate that *Listeria* species very easily survive conventional wastewater treatment processes and suggests that wastewater effluent could play a significant role in the epidemiology of the pathogen in the population. The discharge of inadequately treated wastewater into the receiving watershed could pose serious health hazards to developing nations such as South Africa where majority of her populace depend on these surface water bodies for their daily subsistence as a consequence of poor infrastructure (Mackintosh and Colvin, 2003; Okoh *et al*., 2007; Venter, 2001).

Listeriosis is mainly reported in industrialized nations with few or no reports from Africa, Asia, and South America (Rocourt *et al*., 2000). It is not clear whether the infection was restricted to the geographical boundaries of industrialized nations or the observation was borne out of little or no studies on the pathogens in developing nations. The existence of *Listeria* as free-living or attached cells was previously observed (Djordjevic *et al*., 2002; Lunden *et al*., 2000; Mafu *et al*., 1990) to influence the capacity of the bacteria to resist disinfection and enhance its resistance to antimicrobial therapy. Although *Listeria* species were reported to be

susceptible to common antimicrobials (Abuin *et al*., 1994; Arslan and Ozdemir, 2008; Aureli *et al*., 2003), recent reports (Conter *et al*., 2009; Roberts *et al*., 1996; Safdar and Armstrong, 2003) suggests a growing antibiotic resistance level in *Listeria* isolates from various sources. Most studies on the antimicrobial susceptibilities of *Listeria* species focused mostly on clinical and/or food isolates with little information in the literature on those from municipal wastewater.

It has been reported (Fatoki *et al*., 2003) that wastewater treatment facilities in South Africa find it difficult to adequately treat sewage prior to discharge into the receiving environment. It would be safe to assume therefore that given the resilience of *Listeria* species to conventional wastewater treatment in advanced countries (Czeszejko *et al*., 2003; Paillard *et al*., 2005) municipal wastewater effluents in South Africa will readily harbor the pathogen even after treatment. In this study we report the prevalence and distribution of *Listeria* pathogens as free-living and plankton-associated cells in a typical peri-urban wastewater treatment facility in South Africa and its receiving watershed, as well as the antibiotic susceptibility characteristics of the *Listeria* species isolated from the chlorinated final effluents.

7.2 Materials and Methods

7.2.1 Plant Description

The wastewater treatment plant (Figure 7.1) is located in a peri-urban (Dimbaza) community in the Eastern Cape Province of South Africa, with the geographical coordinates: 32° 50' 0" South, 27° 14' 0" East. The plant receives municipal domestic sewage and a heavy industrial effluent and comprise of two screens, three grit channels, two anaerobic, two aerobic tanks (each equipped with three vertically mounted mechanical aerators), and two sedimentation tanks with the return activated sludge (RAS) pumped from the bottom of the clarifiers via the screens

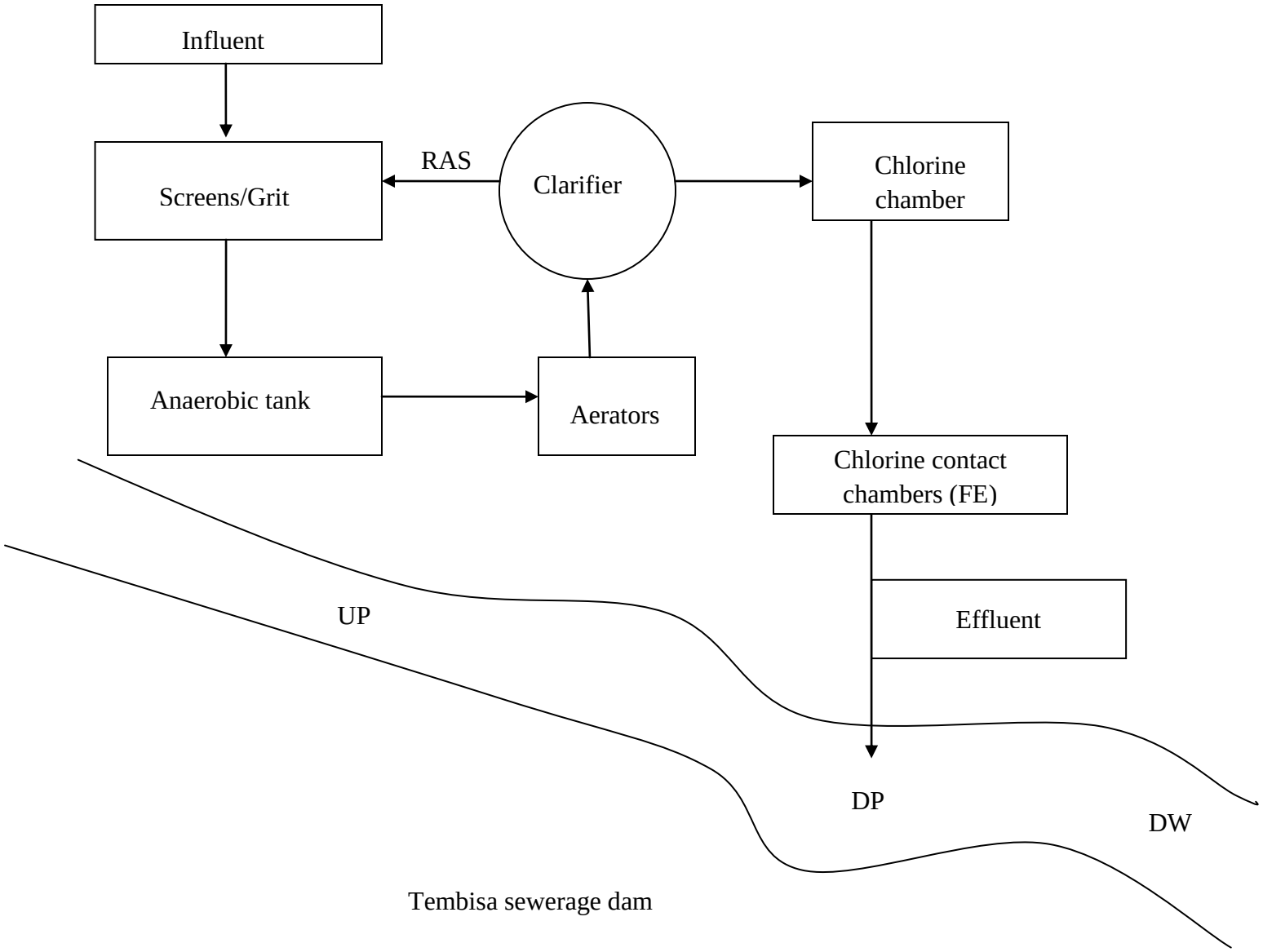

Figure 7.1. Schematic diagram of the wastewater treatment plant discharging into the receiving Tembisa River.

Legend: *FE* = treated final effluent, *DP* = discharge point, *UP* = 500 m upstream discharge point, *DW* = 500m downstream discharge point

with raw sewage to the aeration tanks. Chlorine contact is carried out by means of a water pressure operated, wall mounted, gas chlorinator in a baffled reinforced concrete contact tank and the final effluent is discharged into the Tembisa sewerage dams.

7.2.2 Sample Collection

Wastewater samples were collected on a monthly basis from the final treated effluent (FE), discharge point (DP), five hundred meters (500 m) upstream (UP) and five hundred meters (500 m) downstream (DW) of the discharge point between August 2007 and July 2008. Aqueous effluent samples were collected in duplicates in sterile one litre Nalgene bottles and transported in cooler boxes containing ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, South Africa for analyses. Sample bottles for the final effluents contained 0.1% sodium thiosulphate (3% solution) to neutralize the effect of the chlorine residual on the microflora. Processing of samples was done within 4 hours of sample collection.

7.2.3 Sample Processing

Samples were processed according to the descriptions of Maugeri *et al.* (2004) with modifications. Briefly, samples (one litre in duplicates) were filtered in the laboratory through 180-, 60- and 20-µm pore size nylon nets (Millipore Corp., Ireland) respectively; the water that flowed through the 20-µm pore size nylon nets were collected in clean sterile containers for planktonic (free-living) *Listeria* cells analyses. To obtain a final volume corresponding to 40× of the original sample, trapped planktons on the nets and adhering bacteria were resuspended in 25

ml of sterile phosphate-buffered saline (PBS). To detach adhering bacteria from the planktons, 12.5 g of sterile 0.1 mm glass beads (Biospec Products Inc., Bartlesville, OK 74005, USA) was weighed into the bacteria-plankton suspension, vortexed at high speed for 30 s and centrifuged at 3000 × g for 10 min at ambient temperature using the Beckman Model TJ-6 centrifuge. The glass beads were allowed to settle to the bottom of the centrifuge tube and the supernatant was used for plankton-associated *Listeria* analyses. Henceforth in this study, plankton of sizes ≥ 180 μm, ≥ 60 μm ≤180 μm, and ≥ 20 μm ≤ 60 μm, shall simply be represented as 180 μm, 60 μm and 20 μm respectively.

7.2.4 Microbiological Analyses

The isolation of *Listeria* species were done according to the description of Hitchins (2001) with modifications. Briefly, aliquots of samples containing free-living and plankton-associated bacteria were directly inoculated onto *Listeria* chromogenic agar (LCA agar) (Pronadisa® Madrid, Spain) following standard spread plate technique and incubated for 24-48 h at 35 ^{o}C. Typical *Listeria* colonies appear blue-green on LCA agar plates while pathogenic *Listeria* species (*L. monocytogenes* and *L. ivanovii*) are surrounded by an opaque halo in addition to their blue-green color. Total *Listeria* counts were recorded and presumptive *Listeria* pathogens were isolated from the treated (chlorinated) effluent samples, purified and stored on nutrient agar slants at 4^{o}C for further analyses. The presumptive *Listeria* pathogens were further confirmed by standard cultural characteristics and biochemical reactions (Hitchins, 2001) and using the API *Listeria* kits (10 300, bioMerieux, South Africa). *Listeria monocytogenes* (ATCC 19115) and *Staphylococcus aureus* (ATCC 25923) were used as positive and negative controls respectively.

7.2.5 Physicochemical Analyses

All field meters and equipment were checked and appropriately calibrated according to the manufacturers' instructions. pH, temperature, total dissolve solid (TDS), and dissolved oxygen (DO), were all determined on site using the multi-parameter ion specific meter (Hanna-BDH laboratory supplies). Turbidity and the concentrations of free chlorine residual in the final effluent samples were also determined on site using a microprocessor turbidity meter (HACH Company, model 2100P) and an ion-specific meter (Hanna Instruments, HI 93711) respectively. The concentrations of orthophosphate as P (PO_4), nitrate (NO_3), nitrite (NO_2), and chemical oxygen demand (COD) were determined in the laboratory by the standard photometric method (DWAF, 1992) using the spectroquant NOVA 60 photometer (Merck Pty Ltd). Samples for COD analyses were digested with a thermoreactor model TR 300 (Merck Pty Ltd) prior to analysis using the spectroquant NOVA 60 photometer.

7.2.6 Antimicrobial Agents

Twenty antibiotics commonly used as therapy in human and veterinary listeriosis were employed in the antibiogram assay. The paper disks containing the antibiotics were obtained from Mast Diagnostics (Merseyside, United Kingdom) and includes: Amikacin (30 µg), Ciprofloxacin (5 µg), Aztreonam (30 µg), Linezolid (30 µg), Chloramphenicol (30 µg), Imipenem (10 µg), Ceftriaxone (30 µg), Meropenem (10 µg), Cephalothin (30 µg), Ertapenem (10 µg), Erythromycin (15 µg), Gatifloxacin (5 µg), Gentamycin (10 µg), Moxifloxacin (5 µg), Ampicillin (25 µg), Streptomycin (25 µg), Penicillin G (10 µg), Tetracyclin (30 µg), Trimethoprim (5 µg), and Sulphamethoxazole (25 µg).

7.2.7 Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed and interpreted based on the disk agar diffusion method as described by the Clinical and Laboratory Standard Institute (CLSI, 2005), using Mueller Hinton agar plates (Biolab, Merck, South Africa). The inhibition zone diameters (IZD) were interpreted according to CLSI standards for staphylococci due to lack of specific standards for *Listeria* species (Conter *et al*., 2009). Interpretative standard for Linezolid was still under investigation for staphylococci at the time of this report, thus standard for *Enterococcus* species was applied for this antimicrobial agent.

7.2.8 Statistical Analysis

Calculation of means and standard deviations were performed using Microsoft Excel Office 2007 version. Correlations (paired T-test) and test of significance (two-way ANOVA) were performed using SPSS 17.0 version for Windows program (SPSS, Inc.). All tests of significance and correlations were considered statistically significant at *P* values of < 0.05 or < 0.01.

7.3 Results

7.3.1 Estimation of *Listeria* Abundance

Listeria abundance ranged between 4.0×10^2 and 3.52×10^5cfu/ml (Table 7.1). The lowest count was observed during summer in the month of November 2007 at DP while the highest count was observed at DW in the winter month of June 2008. Abundance of free-living *Listeria* species ranged between 0 and 1.48×10^3 cfu/ml, with the highest count recorded at FE in January 2008 and the lowest in FE (December 2007) and DP (November 2007). *Listeria* cells attached to

plankton of sizes 180 µm, 60 µm, and 20 µm, were observed at densities of 0 to 1.58×10^5 cfu/ml, 0 to 1.32×10^5 cfu/ml and 0 to 2.82×10^5 cfu/ml respectively. The highest counts for the plankton-associated *Listeria* species were observed in June 2008 (DP), June 2008 (DW), and January 2008(DP) respectively for 180 µm, 60 µm, and 20 µm categories. Listerial abundance varied significantly with plankton affiliation ($P < 0.01$), sampling point ($P < 0.05$) and season ($P < 0.05$); while the interaction effects between and amongst plankton size, sampling point, and season was significant ($P < 0.05$) on listeria density. The population of free-living *Listeria* species across the sampling points varied significantly with those of cells attached to small (20 µm) plankton ($P < 0.01$) and larger (60 µm, 180 µm) plankton associated species ($P < 0.05$); while *Listeria* density for the small (20 µm) plankton category showed significant ($P < 0.05$) variance with those of larger (60 µm, 180 µm) plankton associated *Listeria* species. Listerial density in summer varied significantly ($P < 0.05$) with those of winter but not with other seasons. There was no significant correlation between listerial abundance and plankton sizes, sampling point or season.

Table 7.1 also shows the prevalence of *Listeria* species during the study. *Listeria* species were isolated throughout the year from all four sampled points. Thirty-eight (95%) of all 40 samples (in duplicate) were positive for free-living *Listeria* species. Free-living *Listeria* species were isolated all year round except in FE (December 2007) and DP (November 2007). Ninety percent of all samples were positive for *Listeria* species associated with large (180 µm) plankton. Of these, *Listeria* was isolated from FE (10 samples), DP (9 samples), DW (8 samples) and UP (9 samples).Thirty seven (92.5%) of all 40 samples were positive for *Listeria* species attached to medium-sized (60 µm) planktons which were isolated from FE (9 samples), DP (9 samples), DW (10 samples) and UP (9 samples). *Listeria* species associated with small (20 µm) planktons were

Table 7.1. Population density and distribution of the *Listeria* species in the treated final effluents and the receiving watershed.

		***Listeria* density (cfu/ml)**											
		Spring			**Summer**			**Autumn**			**Winter**		
Sample Points	**Net sizes**	Aug. 2007	Sep 2007	Oct. 2007	Nov. 2007	Dec. 2007	Jan. 2008	Feb. 2008	Mar. 2008	Apr. 2008	May. 2008	Jun. 2008	Jul. 2008
FE	180µm	1.04×10^{4}	ND	ND	4.0×10^{2}	4.6×10^{3}	1.82×10^{4}	1.34×10^{4}	2.14×10^{4}	3.4×10^{3}	8.8×10^{3}	4.08×10^{4}	2.0×10^{2}
	60 µm	2.46×10^{4}	ND	ND	0.0	2.2×10^{3}	9.8×10^{3}	4.2×10^{3}	2.1×10^{4}	5.6×10^{3}	1.64×10^{4}	4.6×10^{4}	1.2×10^{3}
	20 µm	1.94×10^{5}	ND	ND	8.0×10^{2}	8.6×10^{3}	1.04×10^{5}	3.52×10^{4}	3.52×10^{4}	3.20×10^{4}	1.44×10^{4}	5.4×10^{4}	3.2×10^{3}
	Free	3.55×10^{2}	ND	ND	1.0×10^{1}	0.0	1.48×10^{3}	2.3×10^{2}	3.7×10^{2}	4.6×10^{2}	3.0×10^{2}	2.0×10^{1}	6.0×10^{1}
	Total	$\mathbf{2.29\times10^{5}}$	**ND**	**ND**	$\mathbf{1.21\times10^{3}}$	$\mathbf{1.54\times10^{4}}$	$\mathbf{1.33\times10^{5}}$	$\mathbf{5.30\times10^{4}}$	$\mathbf{7.8\times10^{4}}$	$\mathbf{4.15\times10^{4}}$	$\mathbf{3.99\times10^{4}}$	$\mathbf{1.41\times10^{5}}$	$\mathbf{4.66\times10^{3}}$
DP	180µm	2.0×10^{2}	ND	ND	0.0	2.0×10^{2}	5.4×10^{3}	4.80×10^{3}	1.94×10^{4}	4.8×10^{3}	9.4×10^{3}	1.58×10^{5}	4.0×10^{2}
	60 µm	2.0×10^{2}	ND	ND	0.0	6.6×10^{3}	1.26×10^{4}	2.6×10^{3}	9.8×10^{3}	1.04×10^{4}	1.33×10^{4}	7.34×10^{4}	6.0×10^{2}
	20 µm	1.8×10^{3}	ND	ND	4.0×10^{2}	1.92×10^{4}	2.82×10^{5}	2.14×10^{4}	5.08×10^{4}	7.36×10^{4}	1.0×10^{4}	1.34×10^{4}	1.2×10^{3}
	Free	6.0×10^{1}	ND	ND	0.0	1.5×10^{1}	9.65×10^{2}	9.4×10^{2}	6.45×10^{2}	6.9×10^{2}	3.5×10^{1}	2.05×10^{2}	2.0×10^{1}
	Total	$\mathbf{2.26\times10^{3}}$	**ND**	**ND**	$\mathbf{4.0\times10^{2}}$	$\mathbf{2.6\times10^{4}}$	$\mathbf{3.01\times10^{5}}$	$\mathbf{2.97\times10^{4}}$	$\mathbf{8.06\times10^{4}}$	$\mathbf{8.95\times10^{4}}$	$\mathbf{3.27\times10^{4}}$	$\mathbf{2.45\times10^{5}}$	$\mathbf{2.22\times10^{3}}$
DW	180µm	2.8×10^{3}	ND	ND	0.0	4.6×10^{3}	0.0	5.2×10^{3}	3.6×10^{3}	2.56×10^{4}	1.8×10^{3}	1.28×10^{5}	6.0×10^{2}
	60 µm	1.4×10^{3}	ND	ND	2.0×10^{2}	3.6×10^{3}	6.0×10^{2}	1.8×10^{3}	1.58×10^{4}	3.0×10^{3}	8.0×10^{3}	1.32×10^{5}	4.0×10^{2}
	20 µm	2.74×10^{4}	ND	ND	6.0×10^{2}	1.2×10^{4}	5.5×10^{3}	9.6×10^{3}	2.74×10^{4}	2.52×10^{4}	5.8×10^{3}	9.24×10^{4}	1.8×10^{3}
	Free	2.05×10^{2}	ND	ND	5.0	1.0×10^{1}	6.0×10^{1}	3.9×10^{2}	4.2×10^{2}	5.35×10^{2}	2.05×10^{2}	9.0×10^{1}	3.5×10^{1}
	Total	$\mathbf{3.18\times10^{4}}$	**ND**	**ND**	$\mathbf{8.05\times10^{2}}$	$\mathbf{2.02\times10^{4}}$	$\mathbf{6.16\times10^{3}}$	$\mathbf{1.7\times10^{4}}$	$\mathbf{4.72\times10^{4}}$	$\mathbf{5.43\times10^{4}}$	$\mathbf{1.58\times10^{4}}$	$\mathbf{3.52\times10^{5}}$	$\mathbf{2.84\times10^{3}}$
UP	180µm	2.0×10^{2}	ND	ND	0.0	2.8×10^{3}	6.0×10^{2}	2.8×10^{3}	1.44×10^{4}	1.6×10^{3}	1.2×10^{3}	9.36×10^{4}	1.8×10^{3}
	60 µm	2.0×10^{2}	ND	ND	0.0	1.4×10^{3}	6.0×10^{2}	5.6×10^{3}	1.8×10^{4}	1.6×10^{3}	2.0×10^{3}	7.92×10^{4}	2.0×10^{2}
	20 µm	1.4×10^{3}	ND	ND	7.0×10^{3}	5.2×10^{3}	8.2×10^{3}	5.6×10^{3}	1.8×10^{4}	1.14×10^{4}	1.2×10^{3}	7.76×10^{4}	2.0×10^{3}
	Free	3.5×10^{1}	ND	ND	1.5×10^{1}	2.1×10^{2}	1.35×10^{2}	1.9×10^{2}	2.15×10^{2}	2.25×10^{2}	7.0×10^{1}	6.5×10^{1}	4.5×10^{1}
	Total	$\mathbf{1.84\times10^{3}}$	**ND**	**ND**	$\mathbf{7.02\times10^{3}}$	$\mathbf{9.61\times10^{3}}$	$\mathbf{9.54\times10^{3}}$	$\mathbf{1.42\times10^{4}}$	$\mathbf{5.06\times10^{4}}$	$\mathbf{1.48\times10^{4}}$	$\mathbf{4.47\times10^{3}}$	$\mathbf{2.5\times10^{5}}$	$\mathbf{4.05\times10^{3}}$

Legend: *FE* =treated final effluent *DP* =discharge point *DW* =500m downstream discharge point *UP* =500m upstream discharge point

isolated in all (100%) of the 40 samples.

7.3.2 Physicochemical Analyses

Table 7.2 shows the range and annual mean values of the raw sewage and treated wastewater during this study. The parameters did not vary significantly with sampling points. Figure 7.2 shows the free chlorine residual (CR) of the final effluents during the study. CR ranged between 0.07 mg/l (September 2007) and 3.85 mg/l (October 2007). There was a significant (r^2=0.99) inverse relationship between residual chlorine and total *Listeria* count (Figure 7.3).

7.3.3 Antibiogram

Fifty-three presumptive *Listeria* pathogens were isolated from the final effluents. Of these, 21 (40%) were identified as *Listeria* species by API, all of which were confirmed to be *L. ivanovii;* while the identity of the remaining 32 (60%) isolates were indeterminate by the API test. Seventeen of the *Listeria* isolates were all identified to be *L. ivanovii* and these were tested for antibiotic susceptibility and the result is shown on Table 7.3. All 17 *Listeria* strains were completely sensitive to 6 (30%) of the 20 test antibiotics including, amikacin, ciprofloxacin, gatifloxacin, moxifloxacin, meropenem, and ertapenem. Five (25%) of the 17 *Listeria* isolates were moderately sensitive to gentamycin (1 strain), linezolid (1 strain), cephalothin (1 strain), and ceftriaxone (2 strains). The test isolates showed resistance to 12 (60%) of the 20 antibiotics at percentages ranging from 6% - 94% (Table 7.3). All the *Listeria* isolates exhibited multiple antibiotic resistances in combinations ranging from four to eight antibiotics (Table 7.4).

Table 7.2. . Some physicochemical qualities of the raw wastewater and treated final effluents.

Parameter	Raw sewage		Treated effluent		Recommended target limits
	Range	Mean±SD	Range	Mean±SD	
pH	6.63 - 7.69	7.2±0.3	6.63 - 7.74	7.06±0.29	6-9[a]
Temperature (^{o}C)	14 – 24	19±3	14 – 23	20±2	≤ 25 [a]
Turbidity (NTU)	9.99 – 137	76±27	5.19 - 37.5	11±2	0-1 [a]; ≤ 5[b]
Total dissolved solids (mg/l)	111 – 212	156±22	108 – 160	128±16	0-450 [a]
Dissolved oxygen (mg/l)	0.82 - 5.33	2.38±1.43	4.17 - 6.33	5±0.43	≥ 5[c]
Chemical oxygen demand (mg/l)	10 – 315	105±91	12 – 945	136±27	30[d]
Nitrate (mg/l)	0.5 - 3.4	1.82±0.83	0.6 – 9	4.24±2	6[a]; 1-5[d]
Nitrite (mg/l)	0.053 - 0.42	0.23±0.1	0.063 - 0.68	0.29±0.18	0-6[a]; <0.5[e]
Phosphate (mg/l)	0.03 - 9.9	2.4±2.5	0.02 - 4.36	2.07±1.49	0.005[e]

[a] Target limit for domestic water uses in South Africa (DWAF, 1996a); [b] Target limit for effluent to be discharged into surface waters (Watkins and Sleath, 1981); [c] Target limit for the support of aquatic life (Fatoki *et al*., 2003); [d] Target limit for effluent to be discharged into the environment (SA Government Gazette, 1984); [e] Target limit that would reduce eutrophication in aquatic ecosystems (DWAF, 1996b).

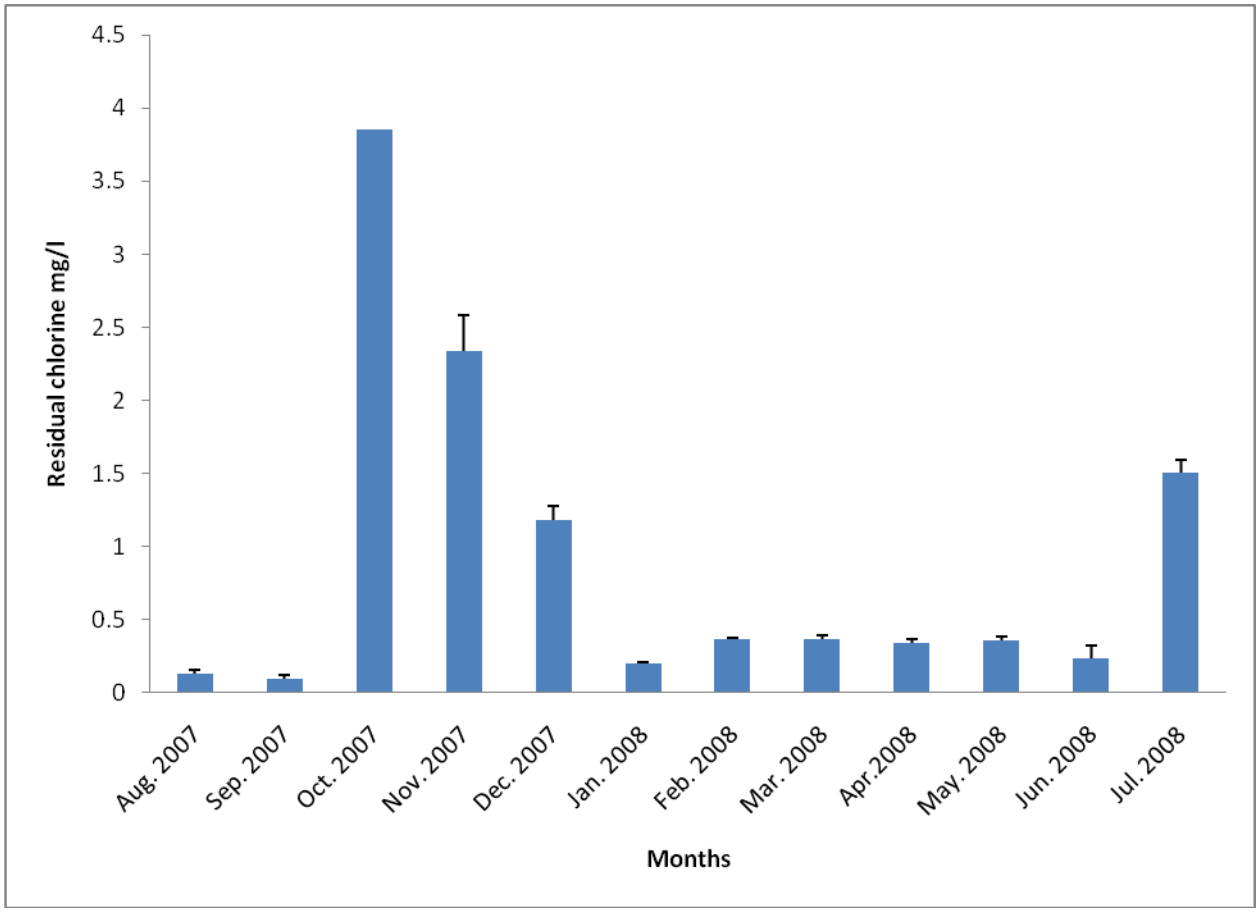

Figure 7.2. Chlorine residual regime of the treated effluents.

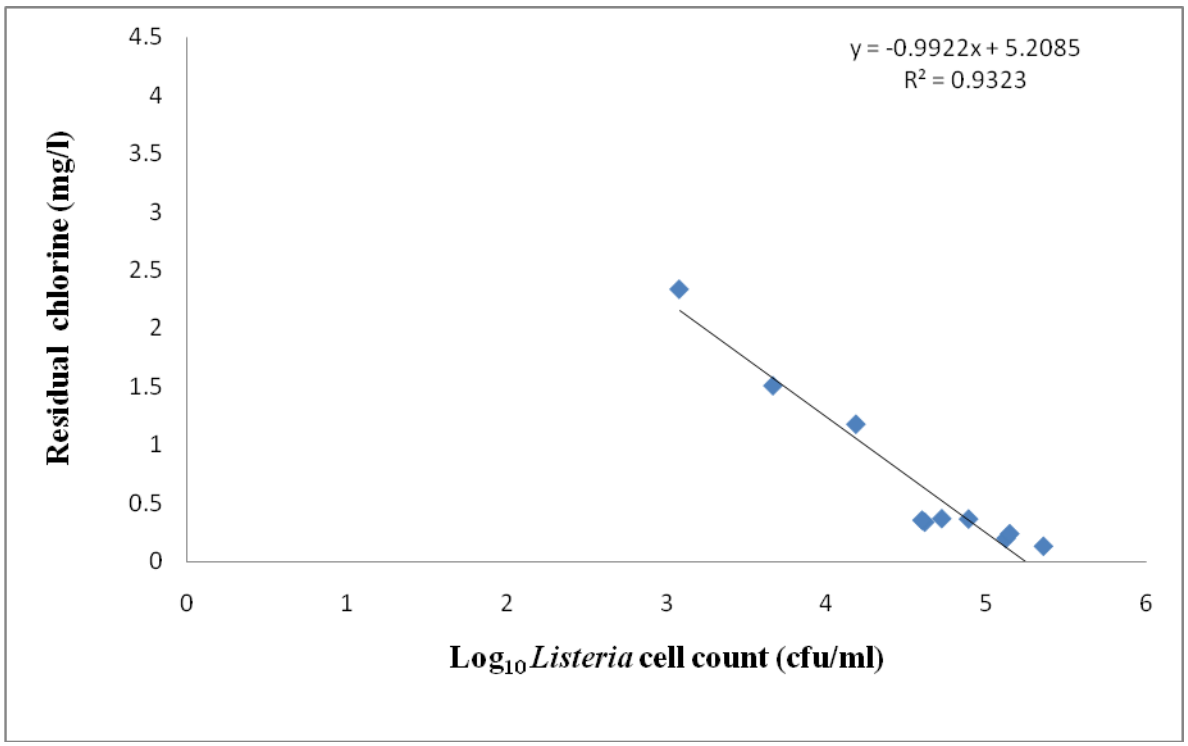

Figure 7.3. Scatter plot of listerial density with chlorine residual.

N.B: Total listerial density was not determined for final effluent in the months of September and October, hence the missing data for those months.

7.4 Discussion

Listeria spp were isolated from all samples collected in this study at densities ranging from 4.0×10^2 to 3.5×10^5 cfu/ml (Table 7.1). There are no recommended standards specific for *Listeria* pathogens in water and wastewater samples in South Africa; hence the faecal coliforms standard (0 cfu/100 ml) for domestic water uses (DWAF, 1996a) was applied in this report. Based on this standard the water quality across all sampled points and throughout the year (Table 7.2) fell short of acceptable target limits for domestic applications and thus disqualifies the waters for such uses. The high density of *Listeria* species in the final treated effluent during this study is an indication of the inefficiency of the wastewater treatment plant at removing the pathogen from sewage influent prior to discharge into the receiving watershed. Listerial density in this study were similar to those (7.0×10^2 to $>1.8\times10^4$ MPN/ml) reported by Watkins and Sleath (Watkins and Sleath, 1981) for sewage effluent that only underwent primary treatment. The lack of significant variation in listerial density with sampling points however, suggests that the wastewater effluent contributes *Listeria* pathogens to the receiving aquatic milieu as much as other unidentified sources. These unidentified sources may include birds and cattle (Schuchat *et al*., 1991) seen in and around the water stream during this study.

The significant interaction effects between and amongst mesh net sizes (indicative of plankton sizes), sampling points, and season on listerial density, suggests that the significant difference observed in *Listeria* density with respect to sampling point and season were mainly dependent on variation in listerial density with regards to plankton affiliation. This observation was corroborated by the lack of significant variance in *Listeria* density with sampling point independent of plankton size (data not shown); and only a weak significant variation in *Listeria* density based on season, occasioned by the significant ($P < 0.05$) variation observed in listerial

Table 7.3. *In vitro* antibiotic susceptibility profile of the *Listeria* strains isolated from the chlorinated effluents (n=17).

Antibiotics	Number of isolates (%) Susceptible	Intermediate	Resistant
Amikacin (30 µg)	17(100)	0(0)	0(0)
Gentamycin(10 µg)	16(94)	1(6)	0(0)
Streptomycin(25 µg)	14(82)	0(0)	3(18)
Chloramphenicol(30 µg)	15(88)	0(0)	2(12)
Tetracyclin(30 µg)	14(82)	0(0)	3(18)
Ciprofloxacin(5 µg)	17(100)	0(0)	0(0)
Gatifloxacin(5 µg)	17(100)	0(0)	0(0)
Moxifloxacin(5 µg)	17(100)	0(0)	0(0)
Imipenem(10 µg)	16(94)	0(0)	1(6)
Meropenem(10 µg)	17(100)	0(0)	0(0)
Ertapenem(10 µg)	17(100)	0(0)	0(0)
Ampicillin(30 µg)	2(12)	0(0)	(15)88
Penicillin G(10 µg)	2(12)	0(0)	(15)88
Linezolid(30 µg)	1(6)	1(6)	15(88)
Aztreonam(30 µg)	15(88)	0(0)	2(12)
Erythromycin(15 µg)	1(6)	0(0)	16(94)
Cephalothin(30 µg)	11(65)	1(6)	5(29)
Ceftriaxone(30 µg)	15(88)	2(12)	0(0)
Sulphamethoxazole (25 µg)	1(6)	0(0)	16(94)
Trimethoprim(5 µg)	13(76)	0(0)	4(24)

Table 7.4. Multiple antibiotic resistances of *Listeria* strains isolated from the chlorinated effluents.

Antibiotics	**Number of isolates involved**	**Percentage (%)**
E, SMX, LZD, PG	2	11.8
AP, E, SMX, LZD	1	5.9
AP, T, SMX, TM, PG	1	5.9
AP, ATM, E, LZD, PG	1	5.9
AP, E, SMX, LZD, PG	2	11.8
AP, E, S, SMX, LZD, PG	2	11.8
AP, KF, E, SMX, LZD, PG	3	17.4
AP, E, SMX, TM, LZD, PG	1	5.9
AP, C, E, T, SMX, TM, IMI	1	5.9
AP, KF, E, S, SMX, LZD, PG	1	5.9
AP, KF, E, T, SMX, LZD, PG	1	5.9
AP, ATM, KF, E, SMX, TM, LZD, PG	1	5.9
Total	**17**	**100**

ATM = Aztreonam, *E* = Erythromycin, *AP* = Ampicillin, *LZD* = Linezolid, *PG* = Penicillin G, *KF* = Cephalothin, *SMX* = Sulphamethoxazole, *TM* = Trimethoprim, *C* = Chloramphenicol, *S* = Streptomycin, *IMI* = Imipenem, *T* = Tetracycline

density between summer and winter samples.This therefore suggests that *Listeria* association with planktons was the major determinant of variance in *Listeria* density in this study. The observed variation in listerial density with cell affiliation to plankton of different sizes was mainly due to the significant ($P < 0.05$; $P < 0.01$) lower density (0 to 1.48×10^3 cfu/ml) of free-living *Listeria* species compared to the plankton associated categories (0 to 2.82×10^5 cfu/ml); and the higher density (4.0×10^2 to 2.82×10^5 cfu/ml) observed for small (20 µm) plankton attached cells in comparison (0 to 1.58×10^5 cfu/ml) with cells attached to larger (60 µm and 180 µm) planktons ($P < 0.05$). Consistent with the observation of this study Maugeri *et al*. (2004) reported higher abundance for plankton-associated bacteria compared to free-living species in coastal waters of Italy. Conversely, Ilinsky and Gorshkov (2002) observed a higher density for free-living bacteria species compared to their plankton-associated counterparts. The lack of significant correlation between listerial abundance and plankton sizes, sampling point and season suggests that all four categories of *Listeria* species with respect to plankton affiliation occupy separate niches in the ecosystem independent of one another and in agreement with another report (Maugeri *et al*., 2004). However, another study (Hsieh *et al*., 2007) observed a negative correlation between planktonic *Vibrio* cells and sessile populations.

In this study we observed *Listeria* species all through the study period. However, contrary to our previous report (Odjadjare and Okoh, 2009) *Listeria* species attached to small (20 µm) planktons were most prevalent (100%) both in treated effluent and the receiving watershed; followed by free-living *Listeria* species (95%), 60 µm (92.5%), and 180 µm (90%) categories respectively. Consistent with the observation of this study high prevalence of *Listeria* species has been reported by other workers (Al-Ghazali and Al-Azawi, 1986, 1988; Paillard *et al*., 2005; Watkins and Sleath, 1981) for treated wastewater effluent and their receiving watershed. Al-

Ghazali and Al-Azawi (1986, 1988) reported 100% prevalence of *Listeria* species in treated wastewater effluent in Iraq but at lower densities of < 3 to 28 MPN/ml, and Paillard *et al*. (2005) reported 84.4% prevalence of *Listeria* species in treated wastewater in France at densities ranging from < 0.3 to 21 MPN/ml. Contrary to our observation, lower prevalence have been reported for *Listeria* species in a variety of surface waters. Frances *et al*. (1991) reported the isolation of *Listeria* species from 21% of freshwater samples collected from sites in Cheshire and North Wales; while Lyautey *et al*. (2007) reported 64% for surface waters of the South Nation River Watershed in Ontario, Canada. These observations were consistent with expectations for surface waters that are not impacted by wastewater effluent as suggested by Dijkstra (1982).

The lack of significant variation between raw and treated sewage for most physicochemical parameters in this study (Table 7.3), indicates the inefficiency of the wastewater treatment facility under investigation to adequately treat the raw sewage prior to discharge into the receiving environment. The wastewater effluent fell short of recommended quality standard for turbidity, COD (SA Government Gazette, 1984), and phosphate (Table 7.3). The elevated turbidity levels observed in the final effluents indicates that the effluent was high in organic matter and may not be safe for domestic application due to increased chances of infection (Obi *et al.*, 2008). The relatively high phosphate concentration of the effluent after treatment also suggests that the receiving river is at risk of eutrophication (Fatoki *et al*., 2003). The treated effluent was however, compliant with set standards for pH, temperature, TDS, DO, nitrate, and nitrite (Table 7.3).

The chlorine residual (Figure 7.2) generally fell short of acceptable target limits (0.3-0.6 mg/l) for domestic water at the point of use (Obi *et al*., 2008) except in January, February, March and April, 2008 and indicates that the water may not be safe for domestic applications with

reference to chlorine residual. There are increased chances of trihalomethane (THM) precursor formation in the effluent (Fatoki *et al*., 2003) with the level of turbidity observed at FE during this study. THM is a carcinogenic compound formed as a by-product of chlorine and organic matter reaction in water systems and has serious health implications for aquatic life and humans exposed to it (Environment Canada, 2001). Scatter plot analysis (Figure 7.3) indicates a significant (r^2=0.93) inverse relationship between chlorine residual and listerial density and suggests that the chlorine disinfectant reduced the density of the pathogen in the water system, however the concentration was not enough to eliminate the pathogen completely (Table 7.2). The observation suggests that factors other than chlorine residual affected the abundance of *Listeria* species in the wastewater effluent. LeChevallier *et al*. (1988) identified attachment of bacteria to planktons and/or other suspended particles as a factor which enhanced resistance of bacteria to chlorine disinfection, suggesting that the relatively high turbidities (as a measure of suspended particles) observed at FE throughout this study might be a considerable factor in the ineffectiveness of chlorine disinfection (Table 7.3); Obi *et al*. (2008) also reported other factors to include contact time, temperature, and pH.

All 17 *Listeria* strains in this study were completely sensitive to 6 (30%) of the 20 tested antibiotics including, amikacin, ciprofloxacin, gatifloxacin, moxifloxacin, meropenem, and ertapenem (Table 7.4). Consistent with our observation, Hansen *et al*. (2005) reported complete sensitivity of 106 *Listeria* species isolated from humans to meropenem, while Safdar and Armstrong (2003) observed 100% sensitivity to amikacin and ciprofloxacin, and we reported complete sensitivity to the six antibiotics by all 14 *Listeria* species isolated from chlorinated wastewater effluent in a previous study (Odjadjare and Okoh, 2009).

Listeria strains in this study showed resistance to 12 antibiotics at percentages ranging from 6%-94% (Table 7.4), and particularly high levels for erythromycin (94%), sulphamethoxazole (94%), penicillin G (88%), ampicillin (88%), and linezolid (88%). Contrary to the observation of this study, *Listeria* species were generally reported to be susceptible to erythromycin (Conter *et al*., 2009; Safdar and Armstrong, 2003), sulphamethoxazole (Hansen *et al*., 2005), penicillin G (Abuin *et al*., 1994), ampicillin (Zhang *et al*., 2007) and linezolid (Conter *et al*., 2009). Conversely, considerable resistance has been reported in the literature for *Listeria* species against erythromycin (Aureli *et al*., 2003), sulphamethoxazole (Zhang *et al*., 2007), the penicillis (penicillin G and ampicillin) (Srinivasan *et al*., 2005), and linezolid (Odjadjare and Okoh, 2009). The high resistance observed for penicillin G, ampicillin and sulphamethoxazole could be of serious public health implication as penicillin and ampicillin are reported to be the antibiotics of choice in listeriosis therapy (Conter *et al*., 2009; Hansen *et al*., 2005) while sulphamethoxazole usually in combination with trimethoprim is considered second choice especially for patients who are allergic to the penicillins (Zhang *et al*., 2007). The physicochemical quality of the wastewater effluent may be a factor in the level of resistance observed in this study as it is widely reported (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004) in the literature that conventional wastewater treatment plants lack the capacity to effectively remove antibiotics and a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluent to develop resistance to common antibiotics due to selective pressure. Although we did not attempt to assay for residual antibiotics in the treated effluents in the course of this study, lack of capacity to remove some chemicals from the wastewater during the treatment process may be evident in Table 7.3. The table shows that the treated effluent fell short of recommended standard quality

for critical parameters such as turbidity, COD, and PO_4 and suggests a possible influence on the listerial resistance.

All seventeen *Listeria* isolates in this study showed multiple antibiotic resistances in combinations ranging from four to eight antibiotics (Table 7.5). Similar observation has been reported elsewhere (Srinivasan *et al*., 2005). On the contrary Conter *et al*. (2009) reported that 'resistance to one antibiotic was more common than multiple resistance' amongst their *Listeria* isolates, while Arslan and Ozdemir (2008) reported resistance to single antibiotics with no record of multiple antibiotic resistance amongst 47 strains of *Listeria* species isolated from white cheese and tested against 13 antibiotics. Multiple drug resistance in *Listeria* species have been attributed to antimicrobial selective pressure and gene transfer mechanism between and amongst *Listeria* species and close relatives of the bacteria such as *Enterococcus*, *Streptococcus* and *Staphylococcus* species (Safdar and Armstrong, 2003). Donlan and Costerton (2002) also reported the acquisition of inherent resistance to antimicrobial agents by attached bacterial species; suggesting that attachment to plankton at one point or the other may have enhanced the multiple resistances of our isolates to several test antibiotics.

7.5 Conclusion

The study demonstrated that *Listeria* pathogens existed in high densities as free-living and plankton-associated entities in the treated wastewater effluents and its receiving watershed; and the pathogens showed elevated levels of multiple antibiotic resistances to first choice antibiotics administered in human and veterinary listeriosis. The wastewater effluent was thus a significant source of resistant *Listeria* pathogens in the South African aquatic environment; an observation

that calls for more attention to be given to the role of wastewater effluents in the epidemiology of this pathogen in the population.

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CHAPTER 8

GENERAL DISCUSSION

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GENERAL DISCUSSION

Sanitary practices for the disposal of sewage, source water protection, and the filtration and chlorination of drinking water have dramatically decreased the risk of waterborne diseases since the 1900s such that the treatment of drinking water was acclaimed the number one public health achievement in the 20th century (NRC 1999; 2004). Nevertheless, waterborne outbreaks still occur around the globe even in advanced counties like the United States (Craun *et al*., 2006). In South Africa, Mara (2001) observed that about 43, 000 deaths were recorded annually due to diarrhea diseases especially amongst children while Mackintosh and Colvin (2003) reported diarrhea as responsible for 20% of all deaths in South Africa within the age bracket of 1-5 years.

Ironically, a substantial fraction of waterborne illness may not be reported as they are mostly self-limiting (Bennett *et al*., 1987); and in cases where they are reported the causative agents are mostly unknown (Mead *et al*., 1999) due to the erroneous practice of depending on indicator organisms for the determination of microbial quality of water. Several studies (FAO, 1992; Ashbolt *et al*., 2001; Bitton, 2005) have shown that indicator organisms may be quite unreliable in the evaluation of water quality as some of these organisms die off faster than the waterborne pathogens they were supposed to indicate (Bitton, 2005). The implication of this therefore is that while a water system may be adjudged clean and safe for consumption due to the absence of indicator organisms, the water may in reality contain deadly pathogens.

One pathogen of considerable ecological tolerance is *Listeria* species. The bacteria can survive wide ranges of temperature (3-43^{o}C) and pH (4.3-9.6), high salt concentrations (10-30%), decreased oxygen concentrations and CO_2 in the environment (Czeszejko *et al*., 2003). Despite the low global incidence of listeriosis (2-15 cases per million people per year), the pathogen is under close surveillance due to its high mortality rate (up to 51%) and common

source epidemic potential (Rocourt *et al*., 2000; de Valk *et al*., 2001). Although food and food products were widely reported to be the route of transmission of *Listeria* pathogens, recent reports (Watkins and Sleath, 1981; Al-Ghazali and Al-Azawi; 1986, 1988; Czeszejko *et al*. 2003; Paillard *et al*. 2005) indicate that *Listeria* species very easily survive conventional wastewater treatment processes and suggests that wastewater effluent could play a significant role in the epidemiology of the pathogen in the population.

With reports of inadequate removal of *Listeria* pathogens in wastewater coming from the developed world (Czeszejko *et al*. 2003; Paillard *et al*. 2005), one can safely presume that most wastewater treatment plants in developing countries such as South Africa are inefficient at removing these pathogens from wastewater influents prior to discharge of the final effluents into the receiving waters for obvious reasons. Failure of existing wastewater treatment plants in South Africa to adequately treat wastewater effluent prior to discharge into the receiving environment has been reported in the literature (Morrisson *et al*., 2001; Fatoki *et al*., 2003). This has led to several waterborne outbreaks in South Africa resulting in loss of lives (Coovadia *et al*., 1992; Pelgrum *et al*., 1998; DPLG, 2001).

While other pathogens (*Shigella*, *Salmonella* and *Vibrio* spp.) were implicated in these outbreaks, there is a dearth of information on waterborne listeriosis in South Africa. Of considerable interest however, is the fact that in most cases the identities of the pathogens responsible for these outbreaks were unknown. A case in point was seen in the report of the Daily Dispatch of Thursday, 30th of January 2003, where out of 446 cases of water related diseases reported to the Eastern Cape health authorities, only 25 (5.6 %) were confirmed to be cholera and yet the disease was termed a 'cholera outbreak' without ascertaining the true identities of the pathogens responsible for over 84% of reported cases.

Given the resilience of *Listeria* species in relation to other common waterborne pathogens like *Salmonella* and *E. coli,* (Watkins and Sleath, 1981; Czeszejko *et al*., 2003) it would not be out of place to assume that some of these unidentified waterborne pathogens were indeed *Listeria* species. According to Rocourt *et al*. (2000), listeriosis is mainly reported in industrialized nations with few or no reports from Africa, Asia, and South America. It was not clear however whether the infection was restricted to the geographical boundaries of industrialized nations or the observation was borne out of little or no surveillance studies on the pathogens in developing nations. Hence there was a dire need to carry out a surveillance study on *Listeria* pathogens in the water systems in South Africa in order to prove or disprove the hypothesis that *Listeria* species are not only present in the water system but could very easily survive the wastewater treatment processes in South Africa.

Results from the current study confirmed that *Listeria* species very easily survived the activated sludge treatment process even after disinfection. The listerial density in the final effluents and the receiving watershed throughout the study and at all locations exceeded the recommended target limit of 0 cfu/100 ml for waters to be applied in domestic concerns in (DWAF, 1996a), and suggests that domestic applications of the water system under study could pose serious threat to the public health. The observation also suggests that the dearth of information on the prevalence of *Listeria* pathogen in developing countries such as South Africa was not as a result of restriction of the pathogen to certain geographical enclaves, but most likely due to lack of adequate surveillance studies. There is need however for more surveillance studies especially in developing countries to ascertain the spread of *Listeria* strains of epidemic importance in these regions as a basis of comparison with those of the developed world which are considerably well characterized (Rocourt *et al*., 2000; Siegman-Igra *et al*., 2002).

The high listerial abundance across the sampling locations was related to turbidity of the final effluents across the wastewater treatment plants and suggests that effluents from the peri-urban treatment plant were the poorest of all three sampled plants. Corroborating this observation, Mackintosh and Colvin (2003) reported high turbidity levels in water systems in the Eastern Cape Province of South Africa and noted a sharp difference in the water quality of the more rural and less populated Eastern Cape Province in comparison with the densely populated urban and mountainous catchment streams of the Western Cape Province. Also supporting the variation of water quality with location, a report elsewhere (Environment Canada, 2001) asserted that the quality of wastewater effluent and by extension its impact on the receiving environment vary from place to place and with population and development patterns of each area. The lack of significant variance in listerial density with sampling location however suggests that listerial abundance was virtually the same across the locations and indicates that population density and lifestyles at the various locations had little or no effect on the abundance of the pathogen (Environment Canada, 2001).

The high listerial abundance observed in the treated effluents across the three wastewater treatment facilities during the current study suggests that the wastewater effluents were significant sources of the pathogen in the South African aquatic milieu. The observation is consistent with previous reports (Watkins and Sleath, 1981; Czeszejko *et al*., 2003; Paillard *et al*., 2005) and indicates increased chance of listeriosis outbreak in South Africa as about 80 % of the population is reported to depend on surface water bodies that serve as receptacles for wastewater effluents for drinking, domestic and agricultural purposes (Venter 2001; Momba *et al*., 2006).

Listeriosis was reported to commonly affect the pregnant, newborns, the elderly and immunocompromised subjects (Siegman-Igra *et al*., 2002); thus making the South African public particularly vulnerable in the event of an outbreak due to the high HIV/AIDS prevalence level and rate of drug and alcohol abuse in the country (Obi *et al*., 2006). The situation in the Eastern Cape Province is further worsened by high level of poverty, low level of sanitation, and lack of appropriate infrastructure (Mackintosh and Colvin 2003).

Ironically, there is little or no report of listeriosis outbreak in South Africa in spite of the high listerial abundance observed in the water systems. The reason for this observation can only be determined by a properly designed epidemiologic study aimed at understanding the intricate pathogen-host-environment relationship. However, some probable explanations for this observation include the possibility of the pathogen conferring immunity on the resident population over time thereby making them asymptomatic carriers of the pathogen (Craun *et al*., 2006); another explanation could be that the *Listeria* species in this part of the world are not pathogenic; and lastly, because the bacteria is not normally classified as a waterborne pathogen by health officials, chances are that it may have been recorded severally as one of the unidentified pathogens involved in waterborne outbreaks as it is not normally investigated in relevant specimens.

The existence of pathogens as free-living or plankton-associated cells was reported to be critical to their survival in the environment as well as their transmission from one host to another (Donlan and Costerton, 2002). Several studies have revealed the preponderance of *Listeria* species to exist as biofilms attached to surfaces such as stainless steel, glass and propylene (Mafu *et al*., 1990), PVC (Djordjevic *et al*., 2002), and food and food processing environments (Lunden *et al*., 2000). There is however little or no report in the literature on *Listerio*-plankton association

in the natural environment. Understanding the distribution of *Listeria* cells as free-living or plankton-associated niches may provide clues on how best to reduce the survival potentials of these pathogens in the environment and during wastewater treatment, and consequently reduce their ability to interact with human and animal populations.

To the best of my knowledge this is the first report that details the prevalence and distribution of *Listeria* species as free-living and/or plankton-associated cells in wastewater effluent and their receiving watersheds. Contrary to the general belief that the larger population of bacteria species grow as adherent to surfaces in all nutrient-sufficient aquatic ecosystems (Costerton *et al*., 1978), results from this study suggests otherwise. Free-living *Listeria* species were generally more abundant in comparison to plankton associated cells except in the peri-urban location. The high turbidity observed at the peri-urban location in relation to other locations may be responsible for the observed difference in agreement with the observation of Bidle and Fletcher (1995), who reported a proportional increase in particle-associated bacteria with greater particle loads. Corroborating the observations of this study Ilinsky and Gorshkov (2002) reported higher abundance for free-living bacteria compared to plankton associated bacteria while Maugeri *et al*. (2004) observed a higher density for plankton attached bacterial cells in comparison with their free-living counterparts in support of our observation at the peri-urban location.

Although reports (Venkateswaran *et al*., 1989; Unanue *et al*., 1992) in the literature suggested variance of bacterial density with season, observations from the current study indicated otherwise in agreement with another report (Murrel *et al*., 1999). The observation suggests that season did not significantly affect listerial abundance, affirming the capacity of the bacteria to survive a wide range of temperature fluctuations (Czeszejko *et al*., 2003). The high

prevalence of *Listeria* pathogens observed in this study (58-100%) corroborates the findings of other studies (Watkins and Sleath, 1981; Al-Ghazali and Al-Azawi, 1986, 1988). However, low prevalence was reported by other workers (Frances *et al.*, 1991; Lyautey *et al*., 2007) in water systems that were not impacted by wastewater effluents.

Quality parameters for wastewater reuse in agriculture are usually evaluated based on their relevance to yield and quality of agricultural products, maintenance of soil productivity, and protection of the environment and public health (FAO, 1992). The suitability of the urban wastewater effluent for application in agriculture and aquaculture was also evaluated as a case study of the state of reclaimed wastewater in South Africa. It is worthy of note however, that the results of this study are by no means a representation of secondary wastewater effluent quality across South Africa.

Listerial density in the secondary treated effluent at the urban community during this study exceeded the target limits for agriculture and aquaculture, except for restricted agriculture where crops are expected to be properly cooked before eating (Blumenthal *et al*., 2000; WHO, 2006 a,b). The observation suggests that application of the secondary effluent in agriculture and aquaculture may compromise the public health especially as the pathogen is reported to survive and multiply in soil and plant surfaces for as long as 10-12 years (Beuchat, 1996). Reports elsewhere (Farber, 1991; Ben-Embarek, 1994; Rocourt *et al*., 2000) has also implicated fish and fish products in a number of listeriosis outbreaks, suggesting a very high epidemiologic potential of reuse wastewater in aquaculture. Consistent with previous studies (Al-Ghazali and Al-Azawi, 1986, 1988) secondary treatment did not adequately eliminate the pathogen from the wastewater in spite of the significant reduction in listerial density, thus reaffirming the resilience of the

bacteria to conventional wastewater treatment processes (Czeszejko *et al.*, 2003; Paillard *et al.*, 2005; Odjadjare and Okoh, 2009).

The physicochemical quality of the secondary treated wastewater effluent generally indicated a high nutrient quality and demonstrates the promising potential of the reclaimed water as a cheap alternative water resource for agriculture and aquaculture. This may be good news for stakeholders in the agriculture and agro-allied sector in South Africa following predictions of serious droughts in the Southern African region over the next 70 years (Palitza, 2009). There is need however, to address the health hazard that high *Listeria* abundance may pose to the consumers of wastewater grown food products. According to WHO (2006a) one way of addressing the potential health concerns of the pathogen is by properly cooking food products where necessary before eating.

Acceptability of reuse wastewater for agriculture and aquaculture vary from country to country and from culture to culture; the age long debate on the merit and demerit of applying reclaimed wastewater for 'beneficial' uses of man is still on till date (Higgins *et al*., 2002). The objection of opponents of reuse wastewater is mainly based on a combination of prejudiced beliefs, fear, attitudes, lack of knowledge and general distrust, which, on the whole, is often not unjustified, judging by the frequent (and highly publicized) failures of wastewater treatment facilities worldwide (Friedler *et al*., 2006). On the other hand, most proponents of reuse wastewater believe that where an alternative water resource is available and readily affordable reuse wastewater may not be considered; but where the otherwise is the case, it may be more economical to apply reuse water than to abstain from it, especially with the availability of several guidelines for the reuse of wastewater (Innocencio *et al*., 2003; Raschid-Sally and Jayakody, 2008). In line with the stance of proponents of wastewater reuse it is the opinion of this writer

that an outright disuse of reclaimed water may be costlier than its reuse in agriculture and aquaculture with reference to the consequent food shortage, environmental pollution, and high cost of sourcing for the 'perfect' irrigation water in a developing and water scarce nation like South Africa.

The presence of carcinogenic substances (e.g. heavy metals, trihalomethanes, etc), and/or chemicals in wastewater effluents may cause adverse environmental impacts such as changes in aquatic habitats and species composition, decrease in biodiversity, impaired use of recreational waters and shellfish harvesting areas, and contaminated drinking water (Environment Canada, 2001; CCME, 2006). All of these impact leads to a less valuable environment, poor health, a less prosperous economy, and ultimately, a diminished quality of life (Environment Canada, 2001). Physicochemical parameters such as temperature, pH, DO, salinity, and nutrient loads have been reported to influence biochemical reactions within water systems. Such changes in the concentration of these parameters are indicative of changes in the condition of the water system (Hacioglu and Dulger, 2009); the consequence of such is the compromise of the water quality for beneficial uses.

The physicochemical quality of the final effluents across the sampling locations during the current study indicated a high polluting power and nutrient loading and suggests that the wastewater effluents were significant sources of pollution to the respective receiving watersheds (DWAF, 1996a, 1996b; Fatoki *et al*., 2003). The variance in level of compliance of effluents quality to recommended target limits with location is consistent with a previous observation (Environment Canada, 2003) that the efficiency of wastewater treatment vary from plant to plant and with design of the treatment plant. According to Okoh *et al*. (2007) some of the challenges that currently affects the effective treatment of wastewater in South Africa include: old and

worn-out collection facilities; complex character and quantity of contemporary contaminants in relation to the original design of the treatment plants; industrial and population growth; and lack of competent personnel to man the treatment plants; to mention a few.

Most South African wastewater treatment works disinfect wastewater by chlorination prior to discharge into receiving watersheds. The goal is to remove pathogens from wastewater. To achieve this goal, chlorine residual is maintained at sufficient levels and in contact with the microbial community in the chlorination tank. Chlorination across all sampled location could not eliminate the pathogens from the raw sewage prior to discharge into the receiving watershed. The observation is consistent with reports elsewhere (Tree *et al*., 2003; Gomez *et al*., 2006) and suggests that factors other than chlorine residual affected the abundance of *Listeria* species in the wastewater effluents across the sampled locations. LeChevallier *et al*. (1988) identified attachment of bacteria to planktons and/or other suspended particles as one of such factors while Obi *et al*. (2008) reported other factors to include contact time, temperature, and pH.

Large quantities of antibiotics are administered to humans and animals to treat diseases and infections every year. They are also used at subtherapeutic levels to prevent diseases and promote growth in livestock. These antibiotics are likely to be released into the aquatic environment via wastewater effluent as a result of incomplete metabolism, ineffective treatment removal or improper disposal (Huang *et al*., 2001). Recent studies (Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004) indicate the presence of antibiotics in municipal wastewater effluents at considerable concentrations, thus raising concerns of antibiotic contaminants perturbing the microbial ecology; increasing proliferation of antibiotic resistant pathogens; and posing threats to human health as well as create challenges for the water industry on issues of water reuse and water resource planning (Daughton and Ternes, 1999).

Although reports on antibiotic susceptibility profile of bacteria species isolated from treated final municipal effluents are available in the literature (Goni-Urriza *et al*., 2000; da Silva *et al*., 2006, 2007), to the best of my knowledge this is the first study that specifically evaluated the antibiogram profile of *Listeria* strains isolated from chlorinated municipal wastewater effluents in South Africa. Based on previous reports (Goni-Urriza *et al*., 2000; da Silva *et al*., 2006, 2007) on the influence of municipal wastewater effluents on antibiotic susceptibility profile of resident bacterial flora, it was generally projected that listerial isolates from municipal effluents in South Africa may manifest a different antibiogram profile compared to their counterparts from other sources.

The current study reveals that *Listeria* strains (54) isolated from the treated effluents were completely sensitive to amikacin, meropenem and ertapenem irrespective of their location of origin. In addition, strains isolated from the peri-urban location showed complete sensitivity to 3 (ciprofloxacin, gatifloxacin, and moxifloxacin) other test antibiotics, while isolates from the rural location displayed phenotypic sensitivity to another 8 antibiotics including gentamycin, streptomycin, chloramphenicol, tetracycline, ciprofloxacin, gatifloxacin, moxifloxacin and imipenem. The observations suggest that while antibiotic susceptibilities varied from location to location, amikacin, meropenem, and ertapenem, appeared to be the best antibiotics for listeriosis therapy in South Africa.

Consistent with the observation of this study Hansen *et al.* (2005) reported sensitivity of 106 strains of *L. monocytogenes* isolated from humans in Denmark to meropenem, gentamycin, chloramphenicol, and tetracycline, while Conter *et al.* (2009) also reported susceptibility of *L. monocytogenes* strains isolated from food against imipenem, gentamycin, ciprofloxacin and tetracycline. And Safdar and Armstrong (2003) reported complete sensitivity of *Listeria* species

to amikacin, ciprofloxacin and imipenem. Other workers have however reported *Listeria* resistance to some of these antibiotics. Srinivasan *et al*. (2005) reported *L. monocytogenes* resistance to streptomycin, tetracycline, chloramphenicol and gentamycin; while Li and colleagues (Li *et al*. 2007) reported *Listeria* resistance to ciprofloxacin, chloramphenicol and tetracycline.

Pencillin G and ampicillin resistance were particularly high (64-91%) amongst isolates from all the three study communities. Whereas linezolid resistance was relatively low (22%) at the urban location, the antibiotic displayed high resistance at the rural (57%) and peri-urban (88%) locations; in a similar manner *Listeria* species showed relatively low (43%) resistance to erythromycin at the rural location compared to the urban (83%) and peri-urban (94%) locations. Sulphamethoxazole followed a similar trend as observed for erythromycin as the antibiotic showed a very low resistance (7%) for *Listeria* pathogens isolated from the rural location in comparison to 65% for the urban location and 94% for the peri-urban. Antibiotic resistance was relatively highest in the peri-urban location, followed by the rural and urban locations respectively and suggests that antimicrobial resistance was a reflection of the effluent quality.

Contrary to the observation of this study, *Listeria* species were generally reported to be susceptible to erythromycin (Conter *et al*., 2009, Safdar and Armstrong, 2003), sulphamethoxazole (Arslan and Ozdemir, 2008), penicillin G (Abuin *et al*., 1994), ampicillin (Zhang *et al*., 2007) and linezolid (Li *et al*., 2007). Conversely, considerable resistance was reported in the literature for *Listeria* species against erythromycin (Aureli *et al*., 2003), sulphamethoxazole (Zhang *et al*., 2007), and the penicillins (penicillin G and ampicillin) (Srinivasan *et al*., 2005); while low resistance (3.2%) was reported for linezolid (Conter *et al*., 2009).

The high resistance observed for penicillin G, ampicillin and sulphamethoxazole could be of serious public health concern as penicillin and ampicillin were reported to be the antibiotics of choice in listeriosis therapy (Conter *et al*., 2009; Hansen *et al*., 2005) while sulphamethoxazole usually in combination with trimethoprim is considered second choice therapy especially for patients who are allergic to the penicillins (Zhang *et al*., 2007). Equally alarming is the remarkably high resistance (22-88%) exhibited by listerial isolates to linezolid in this study compared to those (1.2-3.2%) of other studies (Li *et al*., 2007; Conter *et al*., 2009). Bacteria resistance to linezolid is very rare in the literature as the antibiotic is reported to be effective against most Gram-positive pathogens including notorious strains like methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (Mayers, 2009); suggesting that municipal wastewater effluents modifies bacterial antibiotic resistance.

The physicochemical quality of the wastewater effluent may be a factor in the level of resistance observed in this study as it is widely reported (Huang *et al*., 2001; Giger *et al*., 2003; Kummerer, 2003; Volkmann *et al*., 2004) in the literature that conventional wastewater treatment plants lack the capacity to effectively remove antibiotics and a number of other chemicals from wastewater, thereby increasing the chances of bacterial pathogens resident in such wastewater effluents to develop resistance to common antibiotics due to selective pressure. Although we did not attempt to assay for residual antibiotics in the treated effluents in the course of this study, lack of capacity to remove some chemicals from the wastewater during the treatment process was indicated by the non-compliance of the treated wastewater effluents to critical parameters (such as turbidity, COD, and PO_4) across the three studied plants and suggests a possible influence on the listerial resistance. The observation was further corroborated by a report of the common presence of sulphamethoxazole in municipal wastewater effluent (Huang *et al*., 2001)

thereby reaffirming that the effluent environment might have influenced the high resistance exhibited by municipal effluents isolates in comparison with isolates from other sources (Safdar and Armstrong, 2003; Zhang *et al*., 2007; Arslan and Ozdemir, 2008).

A high level of multiple antibiotic resistance similar to that observed in this study was reported elsewhere (Srinivasan *et al*., 2005). On the contrary Conter *et al*. (2009) reported that 'resistance to one antibiotic was more common than multiple resistance' amongst their *Listeria* isolates, while Arslan and Ozdemir (2008) reported resistance to single antibiotics with no record of multiple antibiotic resistance amongst 47 strains of *Listeria* species isolated from white cheese. Multiple drug resistance in *Listeria* species was attributed to antimicrobial selective pressure and gene transfer mechanism between and amongst *Listeria* species and close relatives of the bacteria such as *Enterococcus*, *Streptococcus* and *Staphylococcus* species (Safdar and Armstrong, 2003). Donlan and Costerton (2002) also reported the acquisition of inherent resistance to antimicrobial agents by attached bacterial species; suggesting that attachment to plankton at one point or the other may have enhanced the multiple resistances of isolates in this study to several test antibiotics.

In spite of the high levels of phenotypic antibiotic resistance exhibited by the *Listeria* isolates against penicillin G, and ampicillin across the sampled locations during this study, the genes responsible for resistance to these antibiotics were not detected. In a similar report, Srinivasan *et al*. (2005) observed high level (92%) of phenotypic resistance to ampicillin but failed to detect the genes responsible for ampicillin resistance, while Davis and Jackson (2009) could not detect *penA* genes in *Listeria* isolates from various sources. Conversely Srinivasan *et al*. (2005) reported the detection of *penA* genes in 37% of their *Listeria* isolates.

To the best of my knowledge, this is the first report on the detection of *sulII* and *ereA* genes (Appendix A1) in *Listeria* species. Previous attempt by other workers (Srinivasan *et al*., 2005; Davis and Jackson, 2009) did not detect the genes in *Listeria* species. The levels of occurrence of the genes were however low compared to the observed phenotypic resistances for sulphamethoxazole (22% *vs* 65%) and erythromycin (14% *vs* 43%). Interestingly, while only one strain of *Listeria* species showed phenotypic resistance to sulphamethoxazole at the rural location, two strains of the pathogen indicated presence of *sul1* genes in that location. The observation suggests that the presence of *sul1* genes may not necessarily confer phenotypic resistance on the bacteria in agreement with a report elsewhere (Enne *et al*., 2006). Srinivasan *et al*. (2005) also reported *sul1*genes in listerial isolates albeit at lower levels compared to the observation of this study. The observations put together, generally suggests that the presence of antimicrobial resistance genes in bacterial isolates do not always correlate with phenotypic antibiotic resistance and indicates that other mechanisms such as decreased outer membrane permeability, activation of efflux pump, or mutation in a ribosomal protein may have contributed to the antimicrobial resistance phenotypes observed in this study (Srinivasan *et al*., 2005).

Conclusions

The aim of this study was to evaluate the effluent quality of three wastewater treatment plants in the Eastern Cape Province of South Africa, set across rural, peri-urban and urban communities and their impact on the receiving watershed as well as the suitability of the secondary effluent of the urban treatment plant as reuse water in agriculture and aquaculture.

Although the secondary treated effluent quality of the urban treatment plant fell short of

recommended target limit for microbial standard for application in agriculture and aquaculture, its physicochemical qualities were generally acceptable; suggesting a plausible potential of the secondary treated wastewater as a cheap alternative water resource in agriculture and aquaculture. Thus, while advocating that steps be taken by the relevant authorities to improve reclaimed wastewater quality in South Africa, there is a feeling that an outright disuse of this water resource may be costlier than its reuse in agriculture and aquaculture with reference to the consequent food shortage, environmental pollution, and high cost of sourcing for the 'perfect' irrigation water in a developing and water scarce nation like South Africa.

Listeria species were isolated from the treated final effluents of all three wastewater treatment plants as well as from the receiving watershed throughout the year. Free-living *Listeria* isolates were more prevalent and abundant compared to plankton-associated *Listeria* species except at the peri-urban location, and the pathogens showed multiple antibiotic resistance to common antibiotics used as therapy against human and veterinary listeriosis. While a few antibiotic resistance genes (*sulII*- urban location; *ereA* and *sulI*- rural location) were detected during this study, resistance gene detection did not correlate with phenotypic antibiotic susceptibilities. Although annual mean values of physicochemical quality parameters before and after treatment suggests a significant improvement in the sewage quality (except in the peri-urban location), the wastewater effluents across all three locations still fell short of recommended standards for some critical water quality parameters (e.g. turbidity, COD, and phosphate) after treatment. In light of the public health implication of the use of waters impacted by poor quality wastewater effluents, the intervention of relevant monitoring authorities becomes *sin quo non* pursuant to ensuring compliance of wastewater treatment facilities to regulatory standards.

Further Prospect

Although the potential role of wastewater in the epidemiology of *Listeria* pathogens is long established, there is limited information in the literature on the risk assessment of this pathogen in the population vis-à-vis the extent to which wastewater contributes to the epidemiology of the pathogen. Hence the need for future studies to focus on this area. Whereas this study demonstrated that *Listeria* pathogens existed as free-living and plankton-associated entities, the preference for and identities of the specific planktons involved in this association were not investigated. Information from such investigation may build on our current knowledge of the pathogen-host-environment relationship and help in the general control and management of listeriosis. Furthermore, it may be necessary to investigate to what extent plankton association influences the resilience of the pathogen to wastewater treatment and antibiotic resistance. This is pertinent as results from the current study indicated that free-living *Listeria* species were generally more abundant even after chlorine disinfection against the expectation that chlorination would easily clear or drastically reduce the free-living cells leaving the supposedly 'resilient' attached cells.

Recommendations

- There is need for a continuous evaluation and reevaluation of the working efficiency of wastewater treatment plants in South Africa vis-à-vis their compliance to set standards in the interest of the public heath and the environment.

- The need for a well organized pathogen surveillance system in South Africa to determine the morbidity and mortality levels due to *Listeria* infection and to ascertain the incidence rate of the disease in the population.

- To determine the extent to which water systems affect other forms of listeriosis including food and clinical cases.

- The need for a systematic study of *Listeria* pathogens isolated from different regions of the world in order to determine the prevalence and geographical spread of virulent and resistant strains of the pathogen across the globe.

- The need to establish large reuse schemes to serve protection of receiving water bodies, public health, ecosystems and landscape, besides its benefits in agriculture.

- There is need also to develop policies and locally viable practices for safer wastewater use to maintain its benefits for food supply and livelihoods while reducing health and environmental risks.

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APPENDICES

Appendix A1

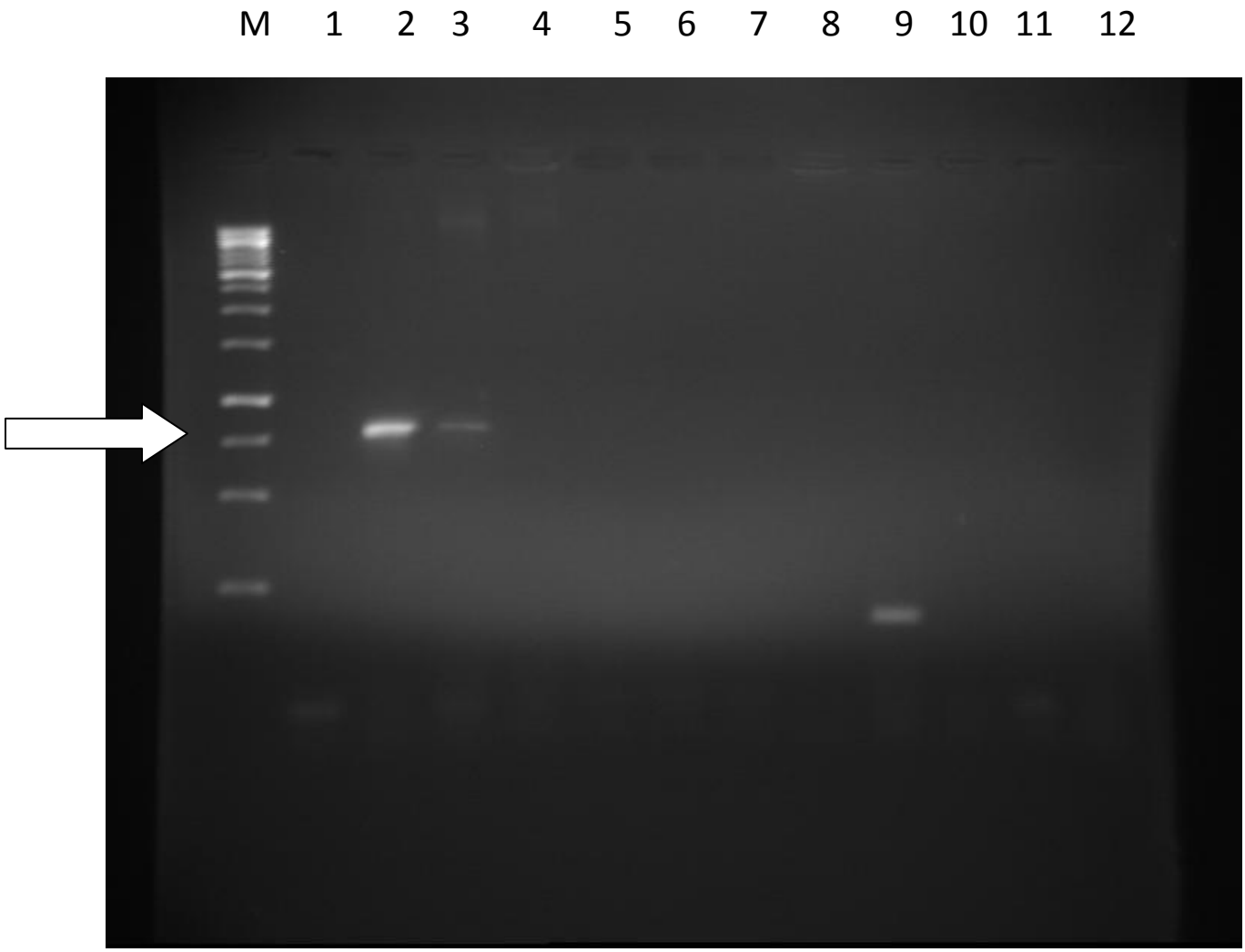

Fig. A1.1: The amplified *suII* genes detected in *Listeria* strains isolated from effluent of the rural community. Lane M: 1kb DNA ladder marker (Fermentas), Lane 1: Negative control; Lanes 2-12: strains of *Listeria* species.

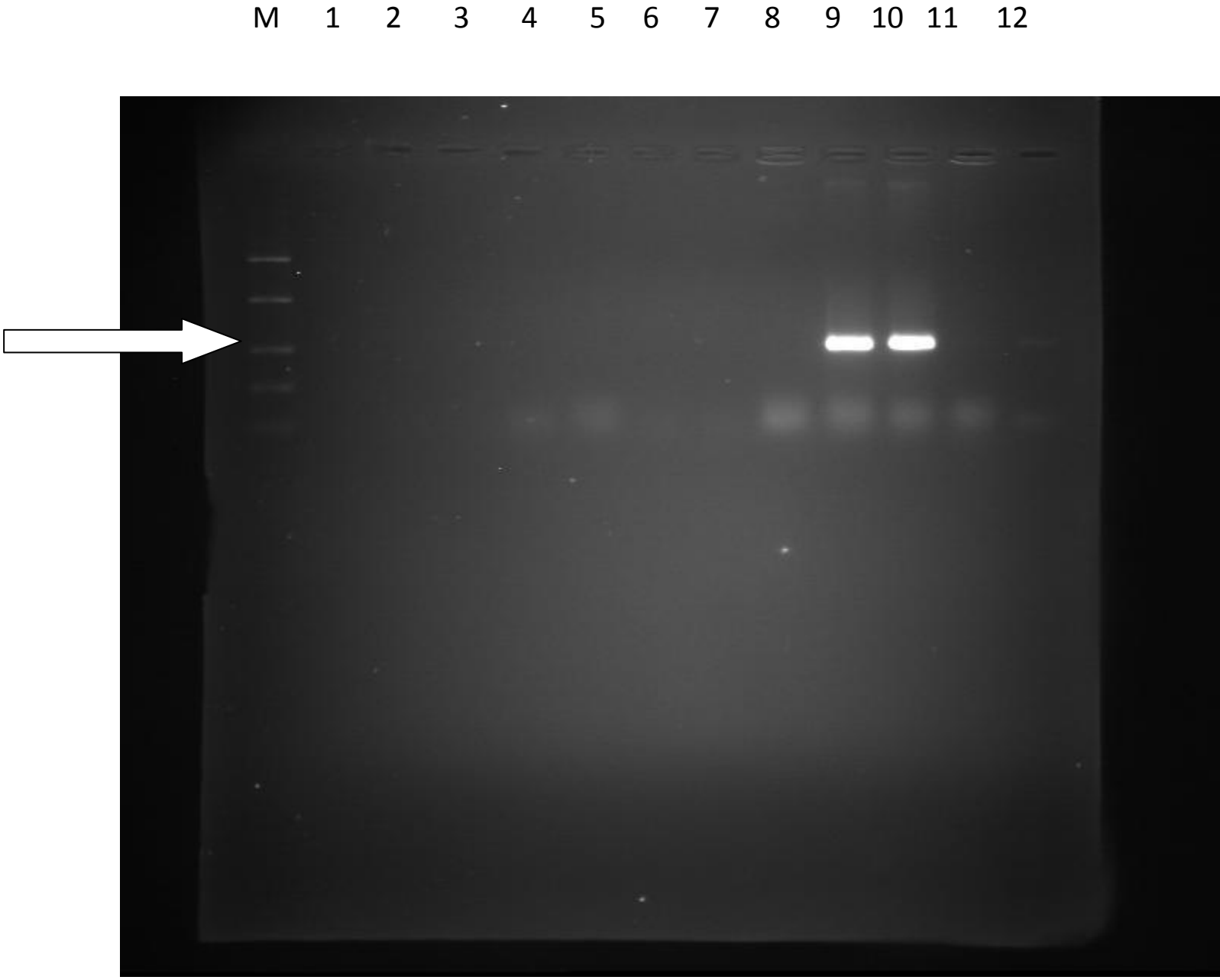

Fig. A1.2: The amplified *ereA* genes detected in *Listeria* strains isolated from effluent of the rural community. Lane M: Low range DNA ladder (Fermentas), Lane 1: Negative control; Lanes 2-12: strains of *Listeria* species.

Appendix A2

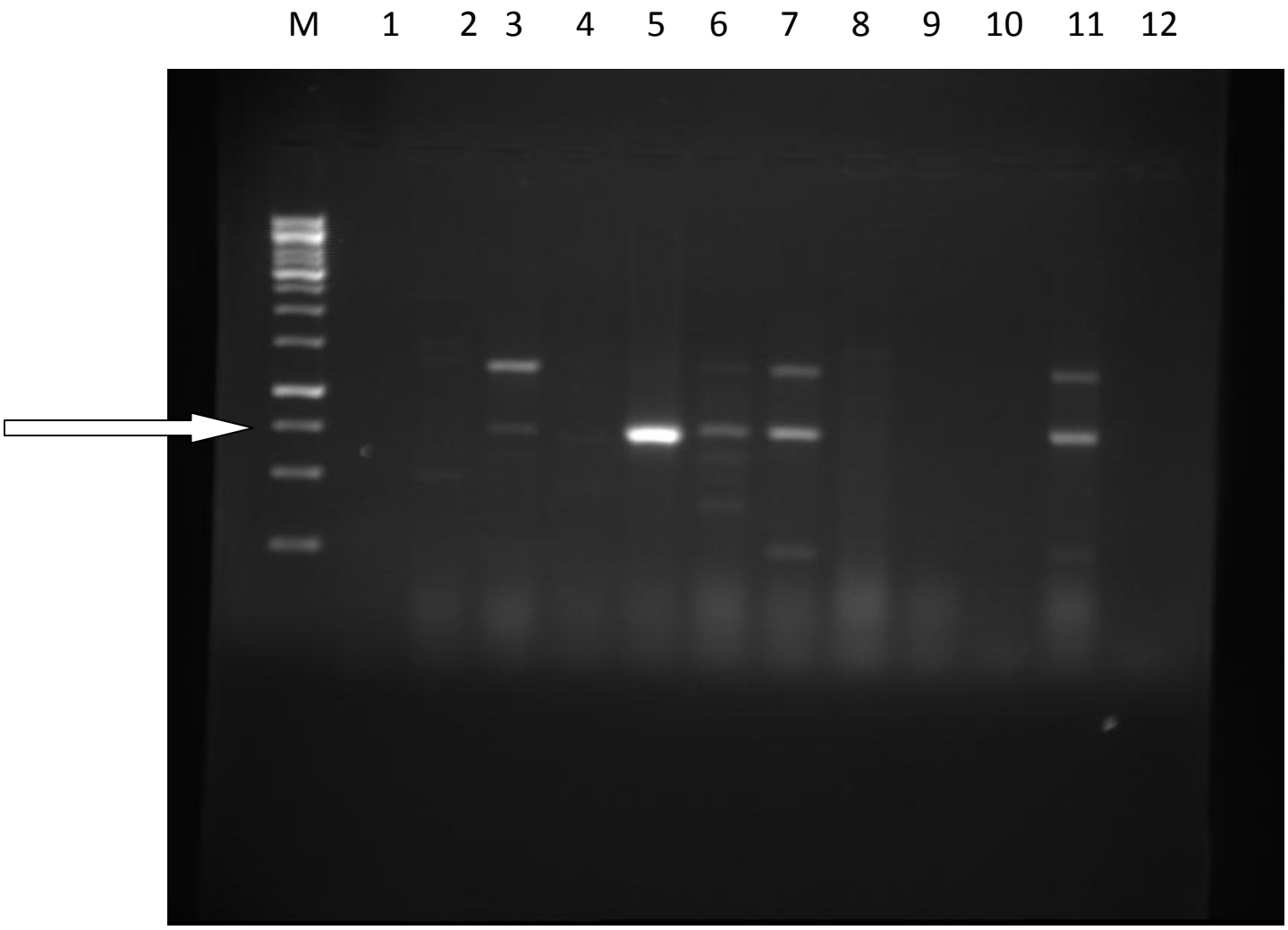

Fig A2.1: The amplified *sulII* genes detected in Listeria strains isolated from effluent of the urban community. Lane M: 1kb DNA ladder marker (Fermentas), Lane 1: Negative control; Lanes 2-12: strains of *Listeria* species.

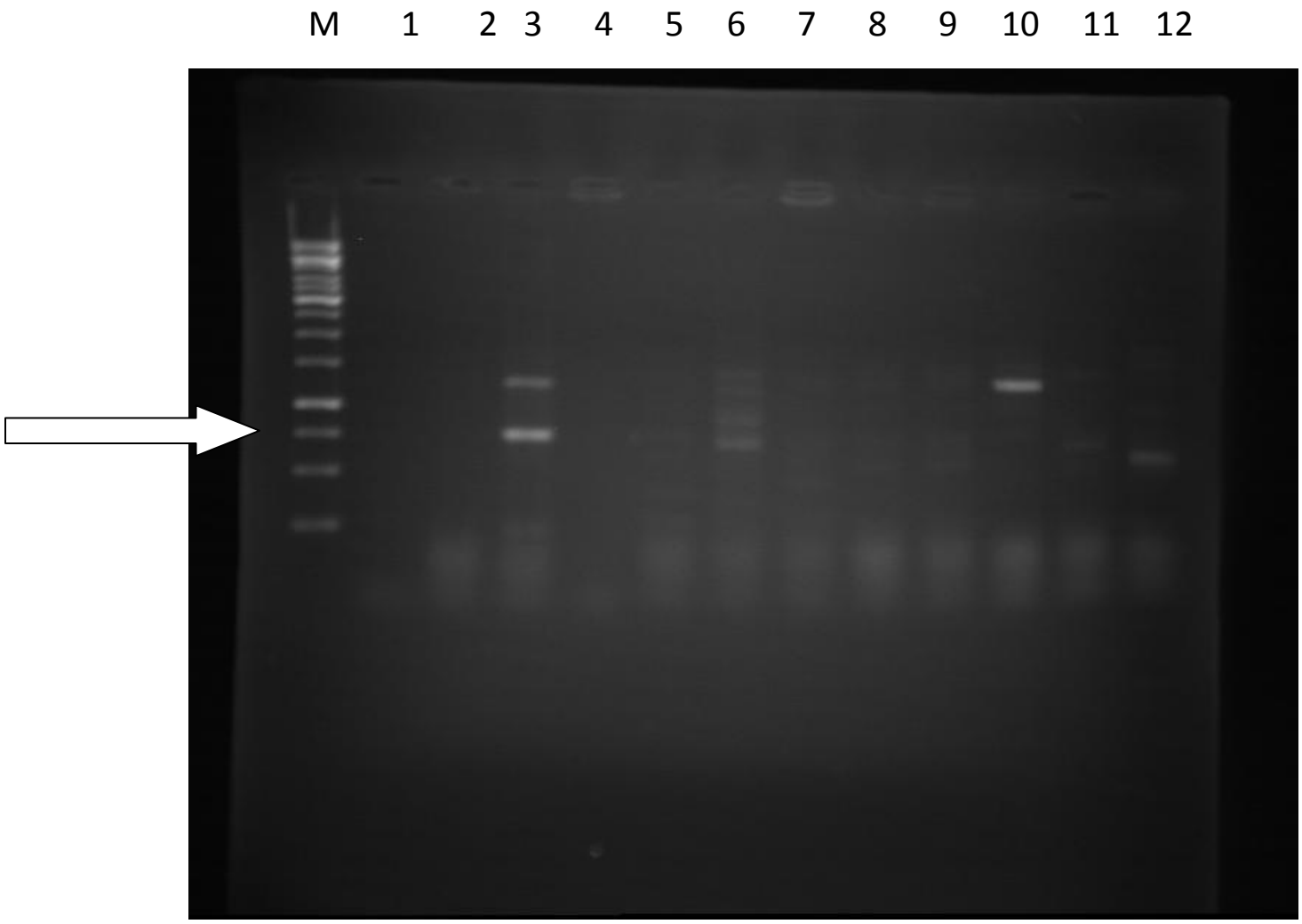

Fig. A2.2: The amplified *sulII* genes detected in *Listeria* strains isolated from effluent of the urban community. Lane M: 1kb DNA ladder marker (Fermentas), Lane 1: Negative control; Lanes 2-12: strains of *Listeria* species.