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Assessment of *Listeria* Bacteria Abundance and Physicochemical Quality of the Effluents of a Typical Semi-urban Wastewater Treatment in South Africa

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The present study was undertaken to assess the efficiency of a typical semi-urban wastewater treatment plant in Eastern Cape Province of South Africa for the removal of *Listeria* pathogens in wastewater. The abundance of *Listeria* pathogens in the final effluent as well as their *in vitro* antibiogram characteristics was evaluated. Total *Listeria* counts ranged from 9.0×10^3 to 3.40×10^5 cfu/ml; 7.60×10^3 to 8.10×10^4 cfu/ml and 2.0×10^1 to 3.5×10^4 cfu/ml for mixed liquor, pre-chlorinated and final effluents respectively. The final effluents did not meet the standards limits for turbidity, EC, TDS, DO, COD, PO_4 and *Listeria* abundance, but fell within recommended limits for pH, temperature and salinity after treatment. *Listeria* strains showed resistance to at least one antibiotic, multiple antibiotic resistances ranging from 2 to 7 antibiotics. The study showed that treated final effluents in South Africa could be an important source of resistant *Listeria* pathogens in the environment.

Key words: Wastewater effluent, *Listeria* pathogens, Antibiogram, physicochemical qualities.

Wastewater effluents are major source of contamination to aquatic ecosystems causing severe disturbance in their ecological functioning (Tyagi, 2006). Wastewater treatment plants have been primarily designed to reduce pollution of natural waters by reducing suspended solids and organic matter in order to decrease public health risks associated with exposure (George *et al.,* 2002). Despite the fact that raw wastewater also carries large quantities and a wide variety of microorganisms including pathogens that causes humans infections (Tyagi, 2006) the reduction of bacteriological pollution in wastewater has not been a priority so far in developing countries (George *et al.,* 2002). In most communities of developed countries, liquid wastes are transformed by wastewater treatment plants to treated water which are discharged into the waterbodies (Gerba, 2000). Municipal wastewater contains substantial numbers of various microorganisms, including pathogens (Vilanova *et al.,* 2003). The numbers and types of pathogens in wastewater treatment plant effluents depend on the initial level of contamination of the influent and on the efficiency of subsequent treatment processes (Paillard *et al.,* 2005).

The current practice of using coliforms as indicator of water pollution has been proven to be unreliable, as coliforms are more susceptible to wastewater treatment process than some other wastewater pathogens including *Listeria* species

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(Tyagi, 2006). Furthermore, even where proper treatment was ensured, *Listeria* species have been reported to be resistant to disinfection by advanced treatment (Paillard *et al.,* 2005). Several cases of *Listeria* outbreaks associated with treated wastewater have been reported around the globe (Paillard *et al.,* 2005).

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 (Brugere-Picoux, 2008). However, there are reports of *L. seeligeri* and *L. ivanovii* causing illness in humans (Cocolin *et al.,* 2002), and *L. innocua* occasionally associated with encephalitis in ruminants (Walker *et al.,* 1994).

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 to regularly monitor the working efficiency of wastewater treatment plants. This is more so as the population and industrial growth across the Eastern Cape Province of South Africa over the years is posing a serious challenge to the capacities of existing wastewater treatment plants to adequately handle and treat current wastewater influents (Welgen, 2006; Okoh *et al.,* 2007). In this paper, we report the efficiency of a municipal wastewater treatment plant in the removal of *Listeria* pathogens as well as the antibiotics susceptibility profiles of the *Listeria* species isolated from the treated final effluents.

MATERIAL AND METHODS

Site description

The wastewater treatment plant under study is located in the geographical coordinates 32°47.071S within the Nkonkobe Municipality in the Eastern Cape Province of South Africa. It rests on the banks of the Kat River, surrounded by the majestic Katberg and Amatola mountain ranges. The plant receives domestic and light industrial sewage and the final effluent is discharged into the Kat River.

Sample collection

Wastewater samples were collected twice each month from June 2008 to August 2008. Samples were collected from three different points of treatment plants namely: the mixed liquor (ML), pre-chlorinated (PCH) and final effluents (FE). Samples were collected in duplicate in one litre clean sterile sample bottles. Sample bottles for the final effluents contained 0.1% sodium thiosulphate (3% solution) to neutralize the effect of the chlorine disinfectant on the microflora. Samples were then transported in cooler boxes to the laboratory for analyses. Samples were processed within six hours of sample collection.

Physicochemical parameters

All field meters and equipment were checked and appropriately calibrated according to the manufacturers' instructions. The 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.

Microbiological analysis

The cultural isolation of *Listeria* bacteria 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 °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 colour. 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 °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 (10300, bioMerieux, South Africa). *Listeria monocytogenes* (ATCC 19115) and *Staphylococcus aureus* (ATCC 25923) were used as positive and negative controls respectively

Test antibiotics

Eleven antibiotics were used for this bioassay. The paper disks containing the antibiotics were obtained from Mast Diagnostics (Merseyside, United Kingdom) and includes: Amikacin (30µg), Ampicillin (10µg), Cephalothin (30µg), Chloramphenicol (30µg), Ceftriaxone (30 µg), Erythromycin (15µg), Gentamycin (10µg), Nalidixic acid (30µg), Tetracycline (30µg), Sulphamethoxazole (25 µg) and Trimethoprim (5µg).

Antibiotic Susceptibility Profiling

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).

Statistical analysis

The obtained data were subjected to descriptive statistical analysis. Regression analysis for *Listeria* density and free residual chlorine concentrations, correlations (paired T-test) and test of significance (independent T-test and one-way ANOVA) were performed using SPSS 18.0 version for Windows program (SPSS, Inc.). Independent T-test was used to compare differences in means between mixed liquor, pre-chlorinated 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.

RESULTS AND DISCUSSION

Table 1 shows the range and total mean values of some wastewater quality parameters before and after treatment of the wastewater under study. Significant differences were observed between mixed liquor, pre-chlorinated and final effluents for pH, temperature, turbidity, salinity, DO, COD and PO_4, NO_3 ($P<0.01$) and *Listeria* densities ($P<0.05$). There was however no significant difference between mixed liquor, pre-chlorinated and final effluents for EC and TDS. There was significant correlation ($r = 0.554$; $P<0.05$) between free chlorine residual concentration and total *Listeria* count (Table 1). At the time of this study there was no guideline for regulating the range of the concentration of residual chlorine in treated wastewater final effluent in South Africa thus, we use regulations concerning domestic water supplies, which recommend ranges of 0.3 – 0.6 mg/l (Mooijiman *et al.,* 2001). The significant variation observed for most physicochemical parameters between mixed liquor, pre-chlorinated and final effluents of the wastewater treatment plant (Table 1), indicated that the wastewater treatment plant under study remarkably improved the quality of the wastewater by the treatment process. Despite improvement on mixed liquor and pre-chlorinated effluents qualities, the final treated effluent did not meet the desired target quality for turbidity, EC, TDS, DO, COD, PO_4 and *Listeria* density (Table 1). As a result it disqualifies the effluent for use in domestic and recreational activities and indicates that discharge of the effluent into the receiving waterbodies could 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 and salinity. Similar temperature values have been reported in the literature for similar environments (Igbinosa and Okoh, 2010a; Odjadjare and Okoh, 2009). The chlorine residual (Table 1) fell within acceptable target limits (0.3-0.6 mg/l) for domestic water at the point of use (Mooijiman *et al.,* 2001) indicates that the water is safe for domestic applications with reference to chlorine residual.

Table 1. Total mean values of physicochemical parameter and *Listeria* density from semi-urban wastewater treatment plant.

Parameter	Mixed Liquor		Pre-chlorinated		Final Effluent		F-value	P-value	Recommended target limits
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range			
pH	7.40 ± 0.2	7.04 – 7.91	7.36 ± 0.14	7.18 – 7.69	6.96 ± 0.22	6.70 – 7.57	163.882	0.007*	6-9 (DWAF, 1996a)
Temperature (°C)	14.50 ± 3.8	9.55 ± 21.63	15.98 ± 3.0	9.55 ± 18.63	16.97 ± 3.09	12.96 – 23.16	580.415	0.001*	≤25°C (DWAF, 1996a)
EC (µS/cm)	718.61 ± 80.5	613 – 879	845.94 ± 135.6	632 – 1087	863.94 ± 93.79	784 - 1077	451.963	0.892^{ns}	70mS/m or 700µS/cm (DWAF, 1996b)
Turbidity (NTU)	373.24 ± 210	92.4 – 788	138.51 ± 62.0	70.2 – 272	42.60 ± 19.7	15.2 – 70.8	182.635	0.008*	0-1 NTU; ≤ 5 NTU (DWAF, 1996a,b)
Salinity (PSU)	0.39 ± 0.04	0.34 – 0.49	0.46 ± 0.08	0.32 – 0.61	0.47 ± 0.05	0.4 – 0.6	381.407	0.001*	33-35 psu (SANCOR, 1984; Whitefield and Bate, 2007)
DO (mg/l)	5.96 ± 2.8	2.25 – 8.88	5.07 ± 2.6	2.38 ± 8.92	2.46 ± 1.2	0.36 – 4.65	154.323	0.000*	≤ 5 mg/l (Fatoki *et al.,* 2003)
TDS (mg/l)	359.5 ± 39.9	307 – 439	421.27 ± 69.2	316 – 543	431.38 ± 48.90	376 - 538	468.476	0.497^{ns}	0-450 mg/l (DWAF, 1996a)
COD (mg/l)	473.66 ± 226.0	182 – 896	313.22 ± 190.0	36 – 712	164.16 ± 65.46	70 -260	175.602	0.009*	30 mg/l (South Africa Government Gazette, 1984)
NO_3 (mg/l)	0.64 ± 0.3	0.19 -1.12	0.36 ± 0.3	0.12 – 1.1	0.54 ± 0.01	0.15 – 1.1	28.505	0.004*	6 mg/l; 1-5 mg/l DWAF, 1996a and WHO, 2004)
PO_4 (mg/l)	0.56 ± 0.31	0.16 -1.06	0.34 ± 0.2	0.21 – 1.04	0.33 ± 0.01	0.11 – 1.05	15.341	0.005*	0.005 mg/l (DWAF, 1996b)
RC (mg/l)					0.40 ± 0.1	0.013 – 1.57	3.837	0.041**	0.3 – 0.6 mg/l (Mooijiman *et al.,* 2001).
Total *Listeria* density (cfu/ml)	6.55×10^4 ± 2.8	9.0×10^3– 3.40×10^5	4.43×10^4± 2.78	7.60×10^3– 8.10×10^4	1.05×10^4 ± 1.12	2.0×10^1– 3.5×10^4	168.764	0.021**	0 cfu/ml (DWAF, 1996a)

Legend: EC-Electrical conductivity; DO-Dissolved oxygen; TDS-Total dissolved solid; NO_3-Nitrite; PO_4-Orthophosphate; RC-Residual Chlorine Values are means of duplicates ± Standard deviations (SD) Significant value at $P < 0.01$*; $P < 0.05$** ns= Not significant.

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.

Total *Listeria* counts ranged significantly ($P<0.05$) from 9.0×10^3 to 3.40×10^5 cfu/ml; 7.60×10^3 to 8.10×10^4 cfu/ml and 2.0×10^1 to 3.5×10^4 cfu/ml for mixed liquor, pre-chlorinated and final effluents respectively (Table 1). Also, the identities of a total of 30 *Listeria* strains isolated from the final effluent were confirmed. Eighteen of the isolates (60%) were identified as *L. ivanovii*, while seven (23%) were identified *L. grayi*; four (13%) as *L. welshimerii*; and one as *L. seeligeri* (3%). Although there are no recommended standards specific for *Listeria* species in wastewater samples in South Africa, the population density of the pathogen across all sampling stations exceeded the no risk limit of 0 cfu/100 ml for faecal coliform recommended for domestic water uses by the South African government (DWAF, 1996a). In line with our observation, high prevalence of *Listeria* species has been reported by other workers for treated wastewater effluents. For example, Al-Ghazali *et al.* (1986; 1988) reported 100 % prevalence in treated wastewater effluent in Iraq but at lower densities of <3 to 2.8×10^1 MPN/ml. Also, Paillard *et al.* (2005) reported 84.4 % prevalence of *Listeria* species in treated wastewater in France at densities ranging from <0.3 to 2.1×10^1 MPN/ml. The significant reduction in listerial density regardless of the treatment process did not adequately eliminate the bacteria from the wastewater. This is consistent with previous reports (Czeszejko *et al.,* 2003; Odjadjare and Okoh, 2010b), and confirm the resilience of the bacteria to conventional wastewater treatment processes including disinfection (Czeszejko *et al.,* 2003; Paillard *et al.,* 2005; Odjadjare and Okoh, 2010b).

Table 2. Antibiotics susceptibility profile of *Listeria* species isolated from semi-urban wastewater final effluent treatment plant

Antibiotics	Number of isolates (%)		
	Susceptible	Intermediate	Resistant
Amikacin (30µg)	15(100)	0(0)	0(0)
Ampicillin (10µg)	6(40)	0(0)	9(60)
Cephalothin (30µg)	12(80)	3(20)	0(0)
Chloramphenicol (30µg)	0(0)	0(0)	15(100)
Ceftriaxone (30µg)	11(73)	3(20)	1(7)
Erythromycin (15µg)	7(47)	2(13)	6(40)
Gentamycin (10µg)	15(100)	0(0)	0(0)
Nalidixic acid (30µg)	10(67)	0(0)	5(33)
Tetracycline (30µg)	14(93)	1(7)	0(0)
Sulphamethoxazole (25µg)	10(67)	0(0)	5(33)
Trimethoprim (5µg)	13(87)	0(0)	2(13)

Table 3. Antibiogram of *Listeria* species isolated from final effluent of semi-urban wastewater treatment plant

Antiobiotypes[r]	n=15	Percentage (%)
AMP, CHL, SMX, TM	1[a]	6.66
AMP, CHL, ERY	1[b]	6.66
CHL, NA	6[c]	40.0
AMP, CHL, CRO, ERY, NA, SMX, TM	7[d]	46.66
Total	15	99.98

Legend: AMP, amplicillin; CHL, chloramphenicol; SMX, sulphamethoxazole, TM, trimethoprim; ERY, erythromycin; NA, nalidixic acid; CRO, ceftriaxone
[a]*Listeria seeligeri*; [b]*Listeria welshimerii*; [c]*Listeria grayi;* [d]*Listeria ivanovii*; [r]resistance

Of the 30 identified isolates, 15 were tested for antibiotic susceptibilities and the results are as shown in Table 2. All 15 isolates were 100% susceptible to amikacin and gentamycin, and 93% were susceptible to tetracycline. Also ceftriaxone, cephalothin and trimethoprim were active against 73%, 80% and 87% of the isolates respectively, while 67% of the isolates were susceptible to

nalidixic acid and sulphamethoxazole, and 100 % were resistant to chloramphenicol. All the isolates displayed multiple antibiotic resistances against 2 to 7 antibiotics (Table 3). Resistance to the antibiotics most commonly used to treat human listeriosis was not observed in all other test *Listeria* species except for *L. seeligeri* where resistance was observed to all the antibiotics used for the treatment of listeriosis. This observation is at variance with the report of Chee-Sanford *et al.* (2001), who reported that tetracycline resistance is the most frequent resistance trait in *Listeria* species. It is noteworthy that most isolates from clinical as well as environmental sources are generally uniformly susceptible to these antibiotics (Jones and MacGowan, 1995). Although most *Listeria* strains have been found to be highly susceptible to most of the antibiotics tested, significant differences in susceptibilities among the species have been seen with quinolones and trimethoprim-sulfamethoxazole (Charpentier and Courvalin, 1999). Resistance to other antibiotics such as erythromycin and ampicillin in *L. ivanovii* and chloramphenicol has been observed in our study. 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.

All 15 *Listeria* strains showed resistance to at least one antibiotic; 6 (40.0%) showed resistance to only two antibiotics (chloramphenicol and nalidixic acid); while the others exhibited multiple antibiotic resistances ranging from 3 to 7 antibiotics (Table 3). Our finding is in agreement with that of Srinivasan *et al.* (2005) who reported all 38 strains (100%) of *L. monocytogenes* tested to be resistant to more than one antimicrobial agent. 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).

CONCLUSION

Listeria pathogens were isolated from the treated final effluents of the study wastewater treatment plant. *Listeria* strains showed multiple resistance to common antibiotics used as therapy against human and veterinary listeriosis. While total mean values of wastewater quality parameters before and after treatment suggests a considerable improvement in the effluents quality, the wastewater effluent still fell short of recommended standards for some critical parameters even after treatment. There is a need for improvement of wastewater treatment systems, as well as more efficient monitoring, regulation, and enforcement procedures for wastewater disposal into waterbodies pursuant to ensuring a safer and healthier environment.

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