Carbapenem resistance profiling of selected bacterial species belonging to the *Enterobacteriaceae* family in various water matrices

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DECLARATION BY STUDENT

I hereby declare that this thesis entitled **"Carbapenem resistance profiling of selected bacterial species belonging to the *Enterobacteriaceae* family in various water matrices"**

1. Is my original work and has not been submitted for a degree at any other university.

2. I further declare that a detailed reference list has been provided on all cited literatures and resources.

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ABSTRACT

Carbapenems are broad-spectrum β-Lactams exhibiting bactericidal activity by binding to penicillin-binding proteins (PBPs). These antibiotics are the last resort drugs prescribed for severe infections when other administered ones have failed to produce enough responses in patients. Bacteria belonging to the *Enterobacteriaceae* family, including *Escherichia coli* and *Klebsiella pneumoniae* were identified as "priority pathogens" by the World Health Organization (WHO) in 2017 due to their resistance against carbapenems and have been ranked as "Priority 1: Critical" for research. Among the various types of carbapenem-resistant *Enterobacteriaceae* (CRE), carbapenemase-producing CRE (CP-CRE) have drawn the most attention since they can contribute to the overall challenge of antimicrobial resistance. Wastewater treatment plants (WWTPs) have been considered a potential hub for antibiotic resistance gene exchange and further release into the environment. Therefore, antimicrobial resistance surveillance using sewage isolates has been presented as a way to study the occurrence and spread of specific clonal groups or sequence types within a community or population. Thus, this study aimed to investigate the occurrence of carbapenem-resistant *K. pneumoniae* and *E. coli* and their virulence genes in selected WWTPs treating municipal wastewater in Durban, South Africa, and to assess the efficiency of these WWTPs in removing these microorganisms.

The samples were taken from two WWTPS (WWTP I and WWTP II) monthly from six sampling points (influent, aeration/biofilter, pre-chlorination, post-chlorination, upstream and downstream from the WWTP's discharge points) from January to September 2018. Primary isolation and enumeration were carried out on CHROMagar^{TM} ECC and Klebsiella *ChromoSelect* Selective Agar Base, for *E. coli* and *K. pneumoniae*, respectively. A total of 120 carbapenem-resistant *E. coli* (CR *E. coli*) and 100 carbapenem-resistant *K. pneumoniae* (CR *K. pneumoniae*) were

randomly selected, further identified using biochemical tests and confirmed using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), polymerase chain reaction (PCR) and 16S rRNA sequencing. The confirmed isolates were then subjected to antimicrobial susceptibility testing (AST) using eight antibiotics (third-generation cephalosporins and carbapenems) to determine their resistance profile. Extended-spectrum beta-lactamases (SHV, CTX-M, TEM, and OXA-1) and carbapenemase genes (NDM-1, OXA-48, IMP and VIM) and virulence genes such as *stx* 1 and 2, *rfbE*, *hly*, *eae,* and *fliC* were targeted using PCR.

While carbapenem-resistant *E. coli* was detected in both WWTPs, the carbapenem-resistant *K. pneumoniae* was only detected in WWTP II. The total *E. coli* count in the influent ranged from 6.8 to 7.1±0.06 $\log_{10}$ CFU/100 mL (WWTP I) and 6.9 to 7.2±0.08 $\log_{10}$ CFU/100 mL (WWTP II) with a carbapenem resistance percentage of 0.01% and 0.07%, respectively. A complete reduction of CR *E. coli* was observed in both WWTPs surveyed and was not detected from the receiving water bodies. The total *K. pneumoniae* in the influent of WWTP II ranged between 7.1 to 7.3±0.1 $\log_{10}$ CFU/100 mL. For carbapenem-resistant *K. pneumoniae*, the highest percentage was detected in the aeration tank (3.03%), followed by the pre-chlorinated effluent (1.33%), with the influent having the least (0.34%). However, carbapenem-resistant *K. pneumoniae* was not detected from the post-chlorinated effluent and the receiving water bodies, indicating a complete removal.

The third-generation cephalosporin resistance profile showed high resistance against cefixime in both *E. coli* and *K. pneumoniae* isolates. Among these, the influent of WWPT II showed the highest percentage of resistant *E. coli* against cefixime (92%) compared to WWTP I *E. coli* (62%) and WWTP II *K. pneumoniae* (81%). In addition, the resistance profile of carbapenems showed that most of the isolates were resistant to ertapenem in both WWTPs. Of the 120 *E. coli* isolates, 60 in

WWTP I and II showed 100% and 95% resistance against ertapenem, respectively. Additionally, *K. pneumoniae* showed 100% resistance against ertapenem.

The predominant ESBL genes detected in the *E. coli* isolates were TEM and SHV, showing a 100% carriage in both WWTPs. The least detected ESBL gene was OXA-1, at 98% and 52% carriage in WWTP I and II, respectively. The occurrence of the ESBL genes in *K. pneumoniae* isolates was slightly different between the sampling points. In the influent, the dominant genes were TEM and CTX-M at 75% and 62%, respectively. In the aeration and pre-chlorinated effluent, the dominant genes were TEM and SHV with 100% carriage. The least detected gene was the CTX-M at 13% in the influent, with no detection at the other sampling points. Both *E. coli* and *K. pneumoniae* isolates showed NDM-1 and OXA-48 to be the predominant genes identified of the carbapenemase-producing genes investigated. In the influent of WWTP I and II, the *E. coli* isolates that harboured NDM-1 were 100% and 82%, respectively. However, the modified Hodge test did not correlate with the detection of CRE since the test was positive for only 34 isolates (15%), while 168 isolates carried the carbapenemase genes based on PCR test.

Out of the six virulence genes tested in CR *E. coli*, three (*hly*, *rfbE*, and *eae*) were detected. The predominant gene in both WWTPs was *hly*, with the highest percentage in WWTP II (88%) compared to WWTP I (33%). The least detected gene was *eae*, only detected in WWTP II at 8%. For *K. pneumoniae*, out of the three virulence genes (*wabG*, *urea*, and *rmpA*) tested, *wabG* was the only gene detected. This gene was detected at all the sampling points, with the highest percentage being in the pre-chlorinated effluent (50%), followed by the influent (11%) and the least in the aeration (10%).

The investigated WWTPs showed carbapenem-resistant *E. coli* and *K. pneumoniae* isolates in their influent samples and the various stages of the treatment except in the post chlorinated effluent indicating their efficient removal during the disinfection process. Future research to determine the presence of viable but not culturable (VBNC) carbapenem-resistant Enterobacteriaceae in these treated effluent and recipient water bodies, and the use of advanced molecular methods capable of identifying these bacteria at lower concentrations is hereby recommended as these water bodies are routinely used for agricultural, industrial, and household purposes by the local communities.

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DEDICATION

This is dedicated to my family, especially my dad, who encouraged me to further my studies and my siblings, who provided support throughout my journey. To my mom (my angel), I hope you are proud of me.

CONFERENCES/PAPERS

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ABBREVIATIONS

ANOVA	Analysis of Variance
APHA	American Public Health Association
ARB	Antibiotic Resistant Bacteria
ARDs	Antimicrobial Resistance Determinants
ARGs	Antibiotic Resistance Genes
AST	Antibiotic Susceptibility Testing
ATTC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
C-KP	Classic non-virulent *K. pneumoniae*
CIM	Carbapenem inactivation method
CFU	Colony Forming Unit
CP-CRE	Carbapenemase producing carbapenem-resistant *Enterobacteriaceae*
CPE	Carbapenemase-producing *Enterobacteriaceae*
CRE	Carbapenem-resistant *Enterobacteriaceae*
CR	Carbapenem resistant
DAEC	Diffusely adherent *E. coli*
DEC	Diarrheagenic *E. coli*
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coli*
ECDC	European Centre for Disease Prevention and Con
EDTA	Ethylenediamine tetraacetic acid
EHEC/STEC	Enterohemorrhagic (Shiga toxin-producing) *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic E. *coli*
EU	European Union

ExPEC	Extra-intestinal pathogenic *E. coli*
FDA	Food and Drug Administration
FBI	Faecal Indicator Bacteria
GIM	German imipenemase
GES	Guiana extended-spectrum β-lactamase
HGT	Horizontal gene transfer
HV	Hypervirulent
Hv-KP	Hypervirulent strains of *Klebsiella pneumoniae*
IMP	Imipenemase/ imipenem-hydrolyzing β-lactamase
InPEC	Intestinal pathogenic *E. coli*
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	Lipopolysaccharide
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization-Time of Flight
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MBL	Metallo β-lactamase
MDR	Multi-drug Resistant
MGE	Mobile Genetic Elements
MHA	Muller-Hinton Agar
MHT	Modified Hodge test
NDM	New-Delhi metallo-β-lactamases
NMC-A	Non-metallo carbapenemase of class A
PBP	Penicillin Binding Proteins
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RE	Removal Efficiency
SA	South Africa
SFC	*Serratia fonticola* carbapenemase
SMI	Seoul imipenemase

SME	*Serratia marcescens* enzyme
UTI	Urinary Tract Infection
USEPA	United States Environmental Protection Agency
VIM	Verona integron-encoded metallo-β-lactamases
VGs	Virulence genes
WGS	Whole-genome sequencing
WHO	World Health Organization
WWTP	Wastewater Treatment Plant

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1 CHAPTER 1: INTRODUCTION

1.1 Background Study

The bacterial family, *Enterobacteriaceae,* has been recorded to have an increased prevalence of antibiotic resistance among their isolates (Hoelle *et al*., 2019). These are Gram-negative bacteria and are inhabitants of the intestinal flora of humans, where some of them are human pathogens and most harmless commensals. The pathogenic strains are involved in both hospital-acquired and community infections (Nordmann *et al*., 2011). Within the family, there are important species used as indicators of fecal contamination in water quality monitoring. The main fecal indicator organism in the *Enterobacteriaceae* family is *E. coli,* and some of the different coliforms include *Klebsiella* and *Enterobacter* (George *et al*., 2000). These organisms are part of the list of antibiotic resistant "priority pathogens" published by the World Health Organization (WHO) in 2017. They are ranked as "Priority 1: Critical" amongst other families and species of bacteria resistant to carbapenem antibiotics and pose a great threat to human health Rodríguez *et al.* (2020).

Carbapenems, including the four types viz., ertapenem, imipenem, meropenem, and doripenem, are β-lactam antibiotics with broad-spectrum bactericidal activity. They were first introduced in the 1980s and 1990s and are said to be antibiotics of last resort for treating infections caused by Gram-negative bacilli (Nordmann, 2014). Like all β-lactam antibacterial agents, they exhibit bactericidal activity by binding to penicillin-binding proteins (PBPs) (Zhanel *et al*., 2007). Because the β-lactam molecule binds to PBPs, bacteria are unable to complete transpeptidation (crosslinking) of peptidoglycan strands, preventing the formation of a complete bacterial cell wall (Zhanel *et al*., 2005). Carbapenems are stable to the majority of β-lactamases, including AmpC β-

lactamases and extended-spectrum β-lactamases (Zhanel *et al*., 2007) that cause resistance to third-generation cephalosporins (Zhanel *et al*., 2005).

Carbapenem resistance in *Enterobacteriaceae* is mainly caused by two different mechanisms. The first involves a reduction in antibiotic uptake caused by a lack of porin expression paired with overexpression of β-lactamases (ESBLs or AmpC) that have a low affinity for carbapenems (Wang *et al*., 2015a; Demir *et al*., 2015). Through this mechanism, carbapenem resistance was first detected in clinical isolates of enterobacteria, mainly among the *Enterobacter* species. This involved an increase in the expression of a chromosomal AmpC gene that encodes for intrinsic cephalosporinase, as well as modifications in the OmpC or OmpF porins (Lavigne *et al*., 2011). Ertapenem-resistant and ESBL-producing *K. pneumoniae* from clinical samples were first described in Italy (Garcia-Fernandez *et al*., 2010) and later reported from many other parts of the world (Indrajith *et al*., 2021; Tshitshi *et al*., 2020). These clinical isolates had a new OmpK36 porin variant (OmpK36 V), resulting in ertapenem resistance (Garcia-Fernandez *et al*., 2010). The second mechanism involves the expression of carbapenemase genes encoding for an enzyme that can break down carbapenems (Nordmann *et al*., 2012a). The first report on CRE was in 1993 where CR in *E. coli* and *E. cloacae* strains were reported in hospital isolates in Paris, France (Nordmann *et al*., 1993). Metalloenzymes such as Verona integron-encoded metallo-lactamase (VIM) and imipenemase (IMP) have been studied all over the world, with an increased prevalence in Europe, Asia, and Africa (Nordmann *et al*., 2011, Adam and Elhag, 2018, Kohler *et al*., 2020). A study in South Africa conducted by Lowman *et al*. (2011) reported the first case of the NDM-1 gene detected in clinical samples of *Enterobacteriaceae* isolates taken from patients who arrived from India. Since then, these ARGs have shown the ability to transfer genetic components and spread significantly among various bacterial species (El bomah and Okoh, 2020). Although carbapenem-

resistant bacteria have been primarily reported in hospital wastewater (Ferreira *et al*., 2011, Zhang *et al*., 2013), they were later isolated from environmental samples (Müller *et al*., 2018; Kohler *et al*., 2020).

However, only limited studies have earlier focused on understanding the role of WWTPs in the development and discharge of carbapenem-resistant microbes in the environment globally. This study, therefore, focused on investigating the occurrence of carbapenem-resistant *Enterobacteriaceae* in selected WWTPs and receiving surface water bodies in the city of Durban, South Africa. This study can form a basis for developing effective control measures against transmission of common waterborne pathogens, especially in developing countries like South Africa, where a significant population solely relies on surface water for all their needs. In addition, successful management of disease caused by such resistant strains requires an in-depth understanding of their diversity, resistance profile, virulence as well as molecular mechanisms underlying their expression and transmission.

1.2 Research Problem and Aims

Beta-lactam antibiotics are amongst the most widely used and diverse antimicrobial agents. Resistance genes for other antibiotics may also occur concurrently with those of carbapenem antibiotics. Therefore, carbapenem-resistant bacteria are a major and ongoing public health concern globally, especially to the communities exposed to contaminated water bodies. It would be more devastating in countries like South Africa due to high levels of immune-compromised individuals. Therefore, assessments followed by appropriate recommendation and planning is imperative to control their transmission.

AIM:

This study aimed to investigate the occurrence of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* and the virulence factors associated with these bacteria in water matrices in Durban, South Africa.

To achieve this aim, the following objectives were adopted

- To enumerate, isolate, characterize and identify carbapenem-resistant and *Escherichia coli* and *Klebsiella pneumoniae* using conventional bacteriological methods.
- To confirm the preliminary identification of the isolates using PCR and MALDI-TOF.
- To determine the antibiotic-resistant profile of the identified species using bioassay methods.
- To screen for the carbapenem resistance and virulence genes in the isolates using PCR.

2 CHAPTER 2: LITERATURE REVIEW

2.1 The *Enterobacteriaceae* family

Enterobacteriaceae is a family of Gram-negative bacteria with known characteristics such as being non-spore formers, motile with five polar flagella, or non-flagellated. They are facultative anaerobes that ferment glucose and other sugars, oxidase negative, reduce nitrate to nitrite, are catalase positive, and have DNA with a guanine-cytosine (GC) content of 39-59% (Farmer III *et al*., 2007; Murray *et al*., 2010). They are widely distributed in nature, appearing in soil, water, plants, and as part of the normal microbiota in the intestinal tract of both man and animals (Farmer III *et al*., 2007). Since they are found in the intestinal tract, the presence of some of these organisms in the environment indicates fecal contamination; thus, they are used as fecal indicator organisms in effluent/water quality monitoring (Bermudez and Hazen, 1988; van Elsas *et al*., 2011). The commonly used fecal indicator bacteria (FIB), in addition to *Escherichia coli* (*E. coli*), are the groups of fecal coliforms and enterococci (USEPA, 2006). Additionally, they are utilized to indicate the possible presence of pathogens in the environment (Stewart *et al*., 2008).

In addition to their importance in the environment, the *Enterobacteriaceae* family includes some common human pathogens that cause infections such as septicemia, meningitis, pneumoniae, cystitis, urinary tract infections, and intestinal infections (Farmer III *et al*., 2007; Flores-Mireles *et al*., 2015). They are involved in both community and nosocomial infections (Prabaker and Weinstein, 2011). The transmission routes of these infections include person-person transmission for example, by hands, or transmission through contaminated food and water (Pitout and Laupland,

2008). Some of the common members that cause these infections include *Enterobacter*, *Citrobacter*, *Escherichia*, *Providencia*, *Serratia*, *Shigella*, *Salmonella*, and *Klebsiella* (Horn *et al*., 2015).

2.2 Pathogenic attributes of *E. coli* and *K. pneumoniae*

2.2.1 *E. coli* pathogenic attributes

Pathogenic *E. coli* strains vary from those that dominate in the intestinal flora of healthy individuals since they are likely to display virulence factors, which are molecules directly involved in pathogenesis but are not required for normal metabolic activities (Donnenberg and Whittam, 2001). *E. coli* strains associated with diarrheal diseases are among the most important microbial pathogens of diarrhea. These strains have advanced by acquiring a specific set of features that have effectively persisted in the host, partially through horizontal gene transfer (HTG) (Croxen *et al*., 2013).

2.2.1.1 *E. coli* pathotypes

Generally, *E. coli* is a non-pathogenic bacterium that serves a crucial role in the gut of a healthy human. However, some pathogenic strains of *E. coli* possess virulence genes (VGs) that cause bacterial infections such as gastroenteritis, urinary tract infections (UTI), chronic diarrhea, sepsis, and respiratory illnesses (Sidhu *et al*., 2013). According to Gomi *et al*. (2015), *E. coli* is divided into two types depending on the site of the disease: intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* strains (ExPEC). In humans, the ExPEC strains are mainly associated with urinary tract infections (UTI) and neonatal meningitis, whereas InPEC is associated diarrheal diseases.

The InPEC strains consist of six known *E. coli* pathotypes collectively known as diarrheagenic *E. coli* (DEC) (Nataro and Kaper, 1998). DEC pathotypes differ in terms of preferred host colonization locations, virulence mechanisms, and clinical symptoms (Table 2.1). These are classified as enterotoxigenic *E. coli* (ETEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC) and enteroinvasive *E. coli* (EIEC) (Gomes *et al*., 2016).

2.2.1.2 *E. coli* virulence factors

2.2.1.2.1 Adhesion and colonization factors

Bacteria can directly interact with epithelial cells due to the presence of adhesins (Mainil, 2013). To prevent the removal by the peristaltic flow, organisms must adhere to the intestinal mucosa after passing through the intestine. *E. coli* pathogenic strains, such as EPEC and ETEC, have welldefined features that allow them to attach to the intestinal wall. Surface features such as outer membrane proteins (OMPs), fimbriae, and fimbrial adhesins mediate the adhesion (Mainil, 2005; Gebisa *et al*., 2019). One of the most common OMP is the intimin encoded by the *aea* gene found in the EPEC and EHEC pathotypes (Gebisa *et al*., 2019).

2.2.1.2.2 Toxins

Many pathogenic bacteria produce toxins as virulence factors (Gebisa *et al*., 2019). These toxins are proteins that can target the cytoplasm, metabolism, or skeleton of a cell (Mainil, 2013). The common type of toxins produced by *E. coli* is the Shiga toxins (encoded by *stx1* and *stx2*). In individuals with hemolytic uremic syndrome and hemorrhagic colitis, they have been shown to cause vascular endothelial damage (Etcheverría and Padola, 2013).

Table 2.1: Classification and virulence factors of various *E. coli* pathotypes (Donnenberg and Whittam, 2001; Abbasi *et al*., 2017)

Pathotype	Clinical features	Epidemiological features	Virulence factors	Common virulence genes
Enteropathogenic (EPEC)	Watery diarrhea and vomiting	Infants in developing countries	Bundle-forming pilus, attaching and effacing	*bfpA* *eae*
Enterohemorrhagic (EHEC/STEC)	Watery diarrhea, hemorrhagic colitis, hemolyticuremic syndrome	Food-borne, waterborne outbreaks in developed countries	Shiga toxins, attaching and effacing	*stx* *eae*
Enterotoxigenic (ETEC)	Watery diarrhea	Childhood diarrhea in developing countries, traveler's diarrhea	Pili, heat-labile and heat-stable enterotoxins	*st* and *lt*
Enteroaggregative (EAEC)	Diarrhea with mucous	Childhood diarrhea	Pili, cytotoxins	*aggR*
Enteroinvasive (EIEC)	Dysentery, watery diarrhea	Food-borne outbreaks	Cellular invasion, intracellular motility	*ipaH* *ial*
Diffusely adherent (DAEC)	Watery or bloody diarrhea	Food-borne, waterborne outbreaks	Diffuse attachment	*daaD*

The commonly reported *E. coli* virulence genes in hospital settings include *bfpA*, *lt*, *st*, *eae* (Derakhshan *et al*., 2019; Salmani *et al*., 2016; Zhou *et al*., 2018). These virulence genes were detected in stools samples from patients with diarrhea and UTI samples. The identified isolates belonged to the EPEC, ETEC and EAEC pathotypes. The frequently reported virulence genes in the environment, particularly in surface and drinking water, are the *ipaH*, *eae*, *bfpA*, *lt* (Osińska *et al*., 2018; Titilawo *et al*., 2015). Pillay and Olaniran (2016) analysed river water samples in

Durban, South Africa, and found the most prevalent virulence gene to be the *hly* gene (32.35%) followed by *stx2* (30.39 %), *eae* (27.45 %), and *fliC* (17.65 %).

2.2.2 Virulence attributes of *K. pneumoniae*

K. pneumoniae, like many other opportunistic pathogens, possesses a variety of virulence factors that assist the bacterium's survival within the host (Podschun and Ullmann, 1998). Among these factors, the capsule has been the extensively studied protective structure in *K. pneumoniae* (March *et al*., 2013). Its function involves providing protection against antimicrobial peptides, phagocytosis, and complement-mediated lysis (Struve *et al*., 2015). To colonize the cell, *K. pneumoniae* utilizes type I pili to adhere to various surfaces, such as the mucosa (Rosen *et al*., 2015). The commonly reported *K. pneumoniae* virulence genes in hospital settings include *wcaG*, *rmpA*, *entB* and *fimH* (Bakr and Zaki, 2019; Remya *et al*., 2019; Albasha *et al*., 2020; Liu *et al*., 2019). These are frequently found in patients with UTIs from different specimens. The frequently reported virulence genes in the environment include *wabG*, which has been reported in estuarine water (85.4%) by Barati *et al*. (2016).

2.2.2.1 Protection by the capsule

The capsule of *K. pneumoniae* is a dense coating of polysaccharide fibers around 160nm thick that successfully protects the bacteria from harmful conditions (Amako *et al*., 1998). The virulence of *K. pneumoniae* is significantly reduced when the genes required for capsule formation are deleted in clinical strains, resulting in a bacterium that is basically non-pathogenic (Lawlor *et al*., 2005). Several studies have shown that the *K. pneumoniae* capsule acts as an effective barrier against bacterial death (Clements *et al*., 2007; Fang *et al*., 2004; Lin *et al*., 2012). The expression of two plasmid-borne transcriptional regulators, the regulator of mucoid phenotype A (*rmpA*) and *rmpA2*,

as well as the expression of *rmpA*'s chromosomal copy and the control of the capsule synthesis A and B genes (*rcsA* and *rcsB*), can all help to increase capsule production (Paczosa and Mecsas, 2016).

2.2.2.2 Lipopolysaccharide in membrane stability

Lipopolysaccharide (LPS), a significant component of Gram-negative cell walls, is vital for outer membrane stability and protection from the outside environment. LPS is essential for Gram-negative bacteria's survival yet changes in LPS are common and important to adapt to different environmental conditions (Needham and Trent, 2013). The genes involved in proper LPS production include *uge*, which encode a UDP galacturonate 4-epimerase, and *wabG*, which encode a *GalA* transferase (Regue *et al*., 2004). The majority of *K. pneumoniae* isolates, both disease-causing and commensal strains, have the *uge* gene. *K. pneumoniae* isolates lacking this gene produce rough LPS and are less capable of causing UTI, pneumonia, and sepsis than wild-type strains. (Lin *et al*., 2014). Meanwhile, *wabG* is likely present in most clinical isolates, with reports stating that *wabG* is present in 64.5, 88 and 88.5%, according to Jung *et al*. (2013), Candan and Aksöz (2015), and Hasani *et al.* (2020), respectively.

2.2.2.3 Outer membrane protein production

K. pneumoniae employs outer membrane proteins to avoid detection by the complement system in addition to modifying the capsular polysaccharides and LPS structures. (Hsieh *et al*., 2013). The complement system, which is part of the first line of immune defence, is made up of a network of plasma proteins that initiate a proteolytic cascade when microbial patterns are recognized (Ricklin *et al*., 2010; Walport, 2001). Upon activation of the complement system, complement proteins are deposited on the surface of bacteria, marking them for phagocytic uptake and eventual death.

2.3 Carbapenems and carbapenem resistance in *E. coli* and *K. pneumoniae*

2.3.1 Carbapenem antibiotics

For infections caused by bacteria belonging to the *Enterobacteriaceae* family, in this case, *E. coli* and *K. pneumoniae*, a range of antimicrobial agents are used to inhibit their growth, including the beta-lactams (Pitout, 2012). Beta-lactams are one of the commonly used antimicrobial agents (Bush and Bradford, 2016). Truter (2015) reported that β-lactams are one of the most prescribed antibiotics in South Africa. They exhibit a broad-spectrum activity with low toxicity to humans (Zango *et al*., 2019). Their mechanism of action includes inhibiting the growth of bacteria by binding to the penicillin-binding proteins (PBPs), thus preventing cell wall synthesis leading to cell lysis (Bush and Bradford, 2016).

Different generations of β-lactams have been introduced, with the first-generation being penicillins, followed by cephalosporins, then carbapenems and monocyclic β-lactams (Konaklieva, 2014). The development of β-lactam antibiotics has thus been a constant challenge of designing new compounds that can withstand inactivation by the increasing diversity of β-lactamases, which lead to antibiotic resistance (Essack, 2001).

Carbapenems are a subclass of β-lactam antibiotics, including imipenem, tebipenem, doripenem, ertapenem, panipenem, biapenem, and meropenem which are Food and Drug Administration (FDA) approved (Pei *et al*., 2016; Hazra *et al*., 2014). They have been proven to have a wider spectrum of activity against bacteria compared to other available β-lactam/β-lactamase inhibitor combinations (El-Gamal *et al*., 2017). Carbapenems are often only used in hospitals and other healthcare settings. They are last-resort drugs used to treat severe infections caused by multi-drug resistant bacteria (Papp-Wallace *et al*., 2011).

Carbapenems have three properties that contribute to their broad-spectrum activity (El-Herte *et al*., 2012; Parekh and Desai, 2009). Firstly, their charge characteristic that lets some of the porins in the outer membrane to enhance the access of the PBPs. Secondly, the chemical structure of the carbapenems which makes them resistant to cleavage by most β-lactamases, including carbapenemase. Thirdly, they display an affinity for a wide spectrum of PBPs from different bacterial strains. Because of these properties, carbapenems can access the periplasm and adhere to PBPs without being inactivated by β-lactamases (Parekh and Desai, 2009).

2.3.2 Mechanisms of carbapenem resistance in *Enterobacteriaceae*

2.3.2.1 Non-carbapenemase mediated resistance to carbapenem in *Enterobacteriaceae*

The following are the primary mechanisms of β-lactam resistance in *Enterobacteriaceae* (Figure 2.1): (i) enzymatic inactivation of the antibiotic by enzymes possessing hydrolytic activity against β-lactam molecules; (ii) decreased permeability of the outer membrane due to the production of modified porins, loss of porin expression, or a change in the types of porins present in the outer membrane; and (iii) through the production of an efflux pump, the antibiotic is effluxed to the exterior of the bacteria (Nordmann *et al*., 2012a).

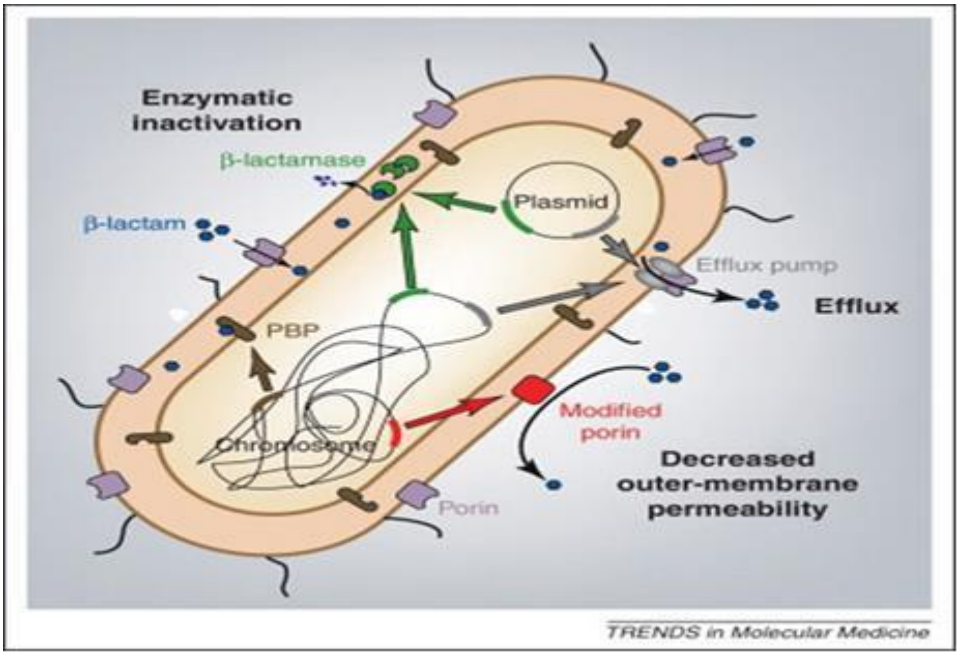


Figure 2.1: Mechanisms of β-lactam resistance in the *Enterobacteriaceae* family (Noordmann *et al*., 2012a).

2.3.2.1.1 ESBL production

Most β-lactams, including cephalosporins, have seen a rise in β-lactamases (ESBL) with an extended spectrum of activity (Bush and Fisher 2011). In *Enterobacteriaceae*, resistance to carbapenems relates to plasmid-borne genes such as CTX-M, SHV, and TEM, which code for ESBLs (Qin *et al.,* 2008). The first report on carbapenem resistance with an ESBL production was by a study by Lartigue *et al.* (2007). In this study, ertapenem resistance was detected in an *E. coli* isolate clinical isolate that produced CTX-M and, that was porin OmpC deficient. This was later confirmed by reports from around the world (Garcia-Fernandez *et al*., 2010; Eser *et al*., 2014; Yan *et al*., 2017; Tian *et al*., 2018).

2.3.2.1.2 AmpC production

Another form of non-carbapenemase-mediated resistance mechanism includes carbapenem-resistant isolates that display an expression of AmpC-type enzymes and porin loss (Qin *et al.,* 2008; Patel *et al.,* 2009). Extended-spectrum cephalosporin resistance is mediated by AmpC-type enzymes, which have limited carbapenem activity. However, carbapenem resistance is observed when the activity of this enzyme is paired with decreased carbapenem cellular penetration as a result of porin loss (Patel *et al.,* 2009). Imipenem resistance linked to porin loss and chromosomal AmpC β-lactamase hyperproduction was originally discovered in *Enterobacter* spp. (Livermore and Woodford, 2000). Another mechanism of imipenem resistance was also observed in *K. pneumoniae* due to porin loss and the presence of plasmid-mediated AmpC β-lactamase (Livermore and Woodford, 2000).

2.3.2.1.3 Outer Membrane impermeability and efflux

The outer membrane of Gram-negative bacteria is hydrophobic, thus inhibiting external substances from entering the cell. However, they also have hydrophilic proteins (porins) that constitute an entrance for required nutrients and compounds (Koebnik *et al.,* 2000; Pagès *et al.,* 2008). Porins responsible for antibiotic absorption in *Enterobacteriaceae* belong to the outer membrane protein F (OmpF) or outer-membrane protein C (OmpC) families. If the uptake efficiency of the porins is altered, antibiotic resistance may be affected (Nordmann *et al.,* 2012a). Antibiotic sensitivity may be reduced as a result of mutations in the porin proteins, a lack of porin expression, or a change in the types of porins present in the outer membrane (Patel *et al.,* 2009). Enterobacterial isolates, particularly *Enterobacter* spp., were the first to show carbapenem resistance by overexpressing a chromosomal AmpC gene, encoding an intrinsic cephalosporinase and modifications in the OmpC or OmpF porins (Lavigne *et al*., 2011). This carbapenem resistance mechanism has also been

discovered in enterobacterial species that lack an inherent cephalosporinase, such as *E. coli*, *K. pneumoniae* and *Salmonella* spp. Resistance in these cases is due to a combination of plasmid-encoded AmpC expression and a decreased cell membrane permeability caused by changes in OmpK35/36 for *K. pneumoniae*, OmpF and OmpC for *E. coli*, and OmpF for *Salmonella typhimurium* (Shin *et al*., 2011; Chia *et al*., 2009; Armand-Lefevre *et al*., 2003).

The efflux of antibiotics out of bacteria occurs due to an efflux pump, resulting in resistance to a variety of antimicrobials, including carbapenems (Nordmann *et al.*, 2012a). Imipenem selects bacteria that have active efflux pumps that expel quinolones, tetracycline, and chloramphenicol, among other antibiotics. In *Enterobacter aerogenes*, overexpression of the *AcrA* efflux pump component was found to cause imipenem resistance (Bornet *et al.*, 2003).

2.3.3.1 Carbapenemase-mediated resistance in *Enterobacteriaceae*

Among the different types of CRE, the carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE) have attracted the most attention because they have the greatest potential to contribute to the challenging antimicrobial resistance (Lutgring and Limbago, 2016). Carbapenemase production commonly confers resistance without the need for further chromosomal mutations or additional mechanisms (Lutgring and Limbago, 2016). Because carbapenemase genes are carried on mobile genetic elements, these can be spread horizontally, thus contributing to a reservoir of resistance in environmental and clinical *Enterobacteriaceae*. Moreover, plasmids in CP-CRE frequently incorporate additional resistance elements, increasing the potential of resistance to a range of drug classes (Lutgring and Limbago, 2016).

In *Enterobacteriaceae*, carbapenemases are divided into three main molecular classes of β-lactamases. These include Ambler class A, which comprises *Klebsiella pneumoniae*

carbapenemase (KPC); class B metallo-β-lactamases, which comprises Verona integron-encoded metallo-β-lactamases (VIM), imipenemase (IMP), and New-Delhi metallo-β-lactamases (NDM); and class D carbapenemase, which comprises Oxacillinase (OXA-48) (Livermore, 2012; Nordmann *et al.*, 2012b). The Ambler class C (AmpC) may also fall within these classes; however, it is a rare chromosome-encoded cephalosporinase (Nordmann *et al.*, 2012b).

2.3.3.1.1 Ambler Class A Carbapenemases

Serine-based carbapenemases are classified as Class A. These include the KPC (*Klebsiella pneumoniae* carbapenemase), NMC-A (non-metallo carbapenemase of class A), IMI (imipenem-hydrolyzing β-lactamase), SME (*Serratia marcescens* enzyme), SFC (*Serratia fonticola* carbapenemase), families, and some GES (Guiana extended-spectrum β-lactamase) (Table 2.2). They can hydrolyse a wide variety of beta-lactams, including carbapenems, cephalosporins, cephalosporins, aztreonam, and penicillins. However, they are inhibited by clavulanate and tazobactam (Walsh *et al*., 2005; Queenan and Bush, 2007). Because it is the most often detected enzyme in class A-lactamases, KPC is the most clinically significant enzyme (Arnold *et al.*, 2012; Patel *et al.*, 2009). KPC was first discovered in the United States (Woodford *et al*., 2004) and is now found all over the world (Zhu *et al*., 2020; Stoesser *et al*., 2017). The spread of the KPC may be partially due to its presence on the plasmid, plasmid-mediated fluoroquinolone resistance, and aminoglycoside resistance are all resistance mechanisms seen in KPC-encoding plasmids (Patel *et al.*, 2009).

Table 2.2: Ambler classification of clinically significant carbapenemases (Sawa *et al*., 2020, modified)

Ambler classification	Common enzymes	Hydrolytic activity against
Class A serine-based	KPC	carbapenems, cephalosporins, penicillins, aztreonam
Class B metallo-β-lactamases	VIM, IMP, NDM-1	carbapenems, cephalosporins, penicillins
Class D oxacillinase	OXA-48	cloxacillin, oxacillin, carbapenems

2.3.3.1.2 Ambler Class B Carbapenemases

This class of metallo-β-lactamases displays hydrolytic activity against penicillins, cephalosporins, and carbapenems, yet it is not inhibited by β-lactamase inhibitors (Nordmann *et al.,* 2012a). This action of hydrolysis relies on the interaction of β-lactams with Zn^{2+} ions as a co-factor in the active site and is therefore inhibited by EDTA (Patel *et al.,* 2009). The most frequent MBLs families include the Verona integron-encoded MBLs (VIM), "active on imipenem" (IMP), "German imipenemase" (GIM), and "Seoul imipenemase" (SIM) enzymes (Queenan and Bush, 2007). There has been a significant increase in the acquisition or transfer of MBL genes since the 1990s, which included NDM, IMP and VIM groups (Iaconis and Sanders, 1990; Yang and Bush, 1996).

IMP was one of the earliest MBLs discovered in *Pseudomonas* spp., *Acinetobacter* spp., and *Enterobacteriaceae* (Patel *et al.,* 2009). The IMP-1 variant was the first of these genes to be detected in *S. marcescens*, and a single variant in *K. pneumoniae*, in Japan (1991) (Ito *et al.*, 1995). VIM is the most widely reported MBL in the world, with the highest prevalence in East and West

Africa, Central Europe, and the Middle East (Aruhomukama *et al*., 2019; Loqman *et al*., 2021; Gajdács *et al*., 2020).

NDM is a group from which NDM-1 positive *Enterobacteriaceae* variants emerged. The transmission of the NDM-1 enzyme differs from other carbapenemases. The plasmids that encode the NDM-1 gene have a broad range of hosts, which allows for easy dissemination among other *Enterobacteriaceae* and unrelated species (Coetzee and Brink, 2011). All NDM-1 producers possess the ability to express various unrelated resistance genes like those encoding carbapenemases (VIM-type, OXA-48-type), AmpC, cephalosporinases, ESBLs; and resistance to aminoglycosides (16S RNA methylases), macrolides (esterases), rifampicin (rifampicin-modifying enzymes), and sulfamethoxazole (Nordmann *et al.*, 2012a).

2.3.3.1.3 Ambler Class D Carbapenemases

Class D carbapenemases produced by *Enterobacteriaceae i*nclude the oxacillinase (OXA)-48-like β-lactamases. The organisms that produce OXA-48 and OXA-181 normally do not display high levels of resistance to the carbapenems. This may be problematic for detecting the OXA-48-like enzymes unless those organisms have mechanisms associated with resistance, such as ESBL production and/ or permeability defects (Nordmann *et al.*, 2012a; Poirel *et al.*, 2012; van Duin and Doi, 2017). OXA related enzymes now comprise the second largest family of beta-lactamases (Zhanel *et al*., 2007).

2.3.4 Emergence and spread of carbapenem-resistant *Enterobacteriaceae*

2.3.4.1 Reported cases of carbapenem-resistant *Enterobacteriaceae*

The first report on CRE was in 1993 (Nordmann *et al*., 1993), in carbapenem-resistant in *E. coli* and *Enterobacter cloacae* strains isolated from hospital samples in Paris, France. Studies on

metalloenzymes such as Verona integron-encoded metallo-β-lactamase (VIM), Imipenemase (IMP) have been reported globally, with an increased incidence in Europe and Asia (Nordmann *et al*., 2011). Carbapenemase-encoding genes of the oxacillinase-48-like type were previously reported in India, the Mediterranean, and some parts of Europe (Bakthavatchalam *et al*., 2016; Sharma *et al*., 2016).

A study by Mahon *et al*. (2017) in Ireland reported NDM-1-producing *Enterobacteriaceae* recovered from beach water samples. This study pointed out that there is a potential for CRE to exist in harsh environments, thereby contributing to a transition of CRE from largely healthcare-associated to organisms affecting the general population and the veterinary sector. NDM-1 has also been reported in Europe, China, South Africa, and Algeria (Gajdács *et al*., 2020; Xiang *et al*., 2020; Ramsamy *et al*., 2020; Abderrahim *et al*., 2017) and are presently one of the most important carbapenemases of worldwide concern (Ramsamy *et al*., 2020).

In Africa, NDM-1 was first identified in Kenya among seven clonally related *K. pneumoniae* isolates from urine or urethral pus of seven adult patients hospitalized in different wards (Poirel *et al*., 2011). Several reports indicated that OXA-48-producing *Enterobacteriaceae* are endemic in North African countries, such as Egypt, Algeria, and Tunisia (Khalifa *et al*., 2017; Yagoubat *et al*., 2016; Tanfous *et al*., 2017). These observations suggest the substantial dissemination of carbapenem resistance, mediated mostly by NDM-1 or OXA-48 carbapenemases in *K. pneumoniae, A. baumannii, E. cloacae, S. marcescens*, and other Gram-negative bacteria worldwide.

The number of carbapenem resistance reports has also increased in South Africa (Singh-Moodley and Perovic, 2016; Sekyere, 2016; Thomas and Duse, 2018; Nel *et al*., 2019). A study in South

Africa carried out by Lowman *et al.* (2011) was the first reported case of NDM-1 gene detected in *Enterobacteriaceae* isolated from clinical samples taken from patients that arrived from India. Another study focused on different provinces in South Africa reported that resistance to carbapenems was highest in the Gauteng and KwaZulu-Natal provinces, followed by the Eastern and Western Capes, respectively. The dominant carbapenemases in this study were NDM-1 (red ribbons) and OXA-48 (pink ribbons) (Figure 2.2) (Sekyere, 2016). Few resistance cases reported from other provinces, such as the Free state and Limpopo, while VIM, IMP, and GES were low. Most of these carbapenem resistance cases had no travel history outside South Africa, suggesting that these strains were selected from increased carbapenem use within the country (van Boeckel *et al*., 2014; Sekyere, 2016). The last reported case of carbapenem resistance from patients that travelled overseas was that of a study by Lowman *et al*. (2011).

2.3.4.2 Carbapenem-resistant *Enterobacteriaceae* in WWTPs

After intake by human beings or animals, either in clinical practices or in households, a major portion of the consumed antibiotics will be excreted to the sewerage system through urine and faeces either in their original form or metabolites (Devarajan *et al*., 2016). Depending on their water solubility and persistence within the environment, the compounds can either be degraded, or adsorb to the sewage sludge, or released to receiving freshwater bodies (Daughton and Ternes, 1999). Antibiotics for use in humans can also reach agricultural soils directly through irrigation with treated wastewaters and surface water (Kinney *et al*., 2006) or via sludge when used as fertilizers.

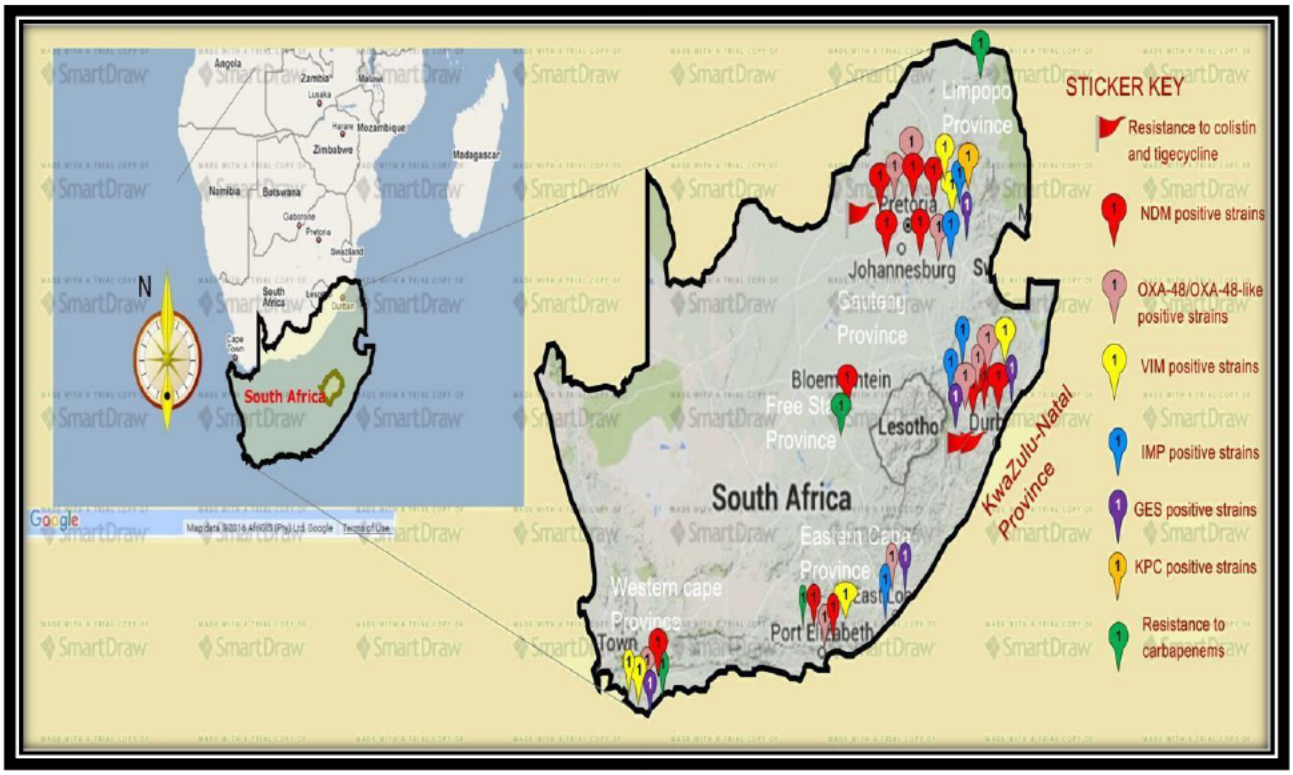


Figure 2.2: Occurrence and geographical distribution of carbapenemase-producing *Enterobacteriaceae* in South Africa based on clinical studies (Sekyere, 2016).

The WWTPs using activated sludge have been proposed as hotspots for the development of antibiotic resistance in bacteria (Berendonk *et al*., 2015; Rizzo *et al*., 2013b) and for HGT between bacteria due to the favourable conditions such as high-nutrient and high-bacterial and antibiotics load (Rizzo *et al*., 2013b). Furthermore, exposure of bacteria to the sub-inhibitory concentrations of antibiotics for a longer period, like those observed in WWTPs, have been shown to increase the frequency of transfer of resistance genes and facilitate resistance (Auerbach *et al*., 2007; Davies *et al*., 2006; Finley *et al*., 2013).

Most studies have focused on assessing the carbapenem-resistant bacteria in healthcare settings

(van Boeckel *et al*., 2014; Sekyere, 2016; Chandran *et al*., 2014, Arena *et al*., 2018, Perovic *et al*., 2020). However, due to their increased detection from various environmental matrices, there has been an increased interest in environmental samples in recent years (Bengtsson-Palme *et al*., 2016; Mills and Lee, 2019; Reinke *et al*., 2020). Additionally, there is limited information on the impact that the carbapenem-resistant bacteria have on the local rivers and public health (Mathys *et al*., 2019; Sivalingam *et al*., 2019). A study by Hrenovic *et al*. (2016) in Croatia in a WWTP reported an increase of carbapenem-resistant bacteria in activated sludge, compared to the effluent. It was assumed to be due to an attachment to the activated sludge flocs that was further removed by the secondary settling. In China, Zhang *et al.* (2020) detected carbapenemase genes in *Enterobacteriaceae*, *Acinetobacter* and *Aeromonas* isolate from the effluent and in the receiving rivers. Similarly, in the US, Mathys *et al.* (2019) detected carbapenemase-producing *Enterobacteriaceae* species in the effluent of a WWTP and the receiving surface waters.

2.4 Isolation and characterization of carbapenem-resistant *E. coli* and *K. pneumoniae* from the aquatic environment

2.4.1 Conventional isolation and enumeration of carbapenem-resistant *Enterobacteriaceae*

Although culture-independent approaches have been used, the determination of quantities and resistance patterns is more frequently based on culture-dependent methods (Jani *et al*., 2019; Nowrotek *et al*., 2019). One of the main advantages of cultivation is the possibility of determining phenotypic traits, many of which are crucial for understanding the ecology of a given bacterial group (Manaia *et al*., 2016). In addition, culture-based methods allow the enumeration of viable cells and the possibility for assessing antibiotic resistance profiles (i.e., minimal inhibitory concentration and antibiotic resistance spectrum) of isolates (Buthelezi *et al*., 2010; Garcha *et al*.,

2016); it is the most used method to determine multidrug resistance phenotypes. The membrane filtration method, commonly used for water microbiological analysis, is frequently adapted to isolate bacteria for further characterization of antibiotic resistance (APHA, 2005). With the presented advantages of culture-based methods, one of their drawbacks is that they provide a restricted view on microbial community and ARB (Nowrotek *et al*., 2019). Thus, methods such as targeted and sequence-based metagenomics provide an accurate assessment of the abundance of ARB (Nowrotek *et al*., 2019; Fitzpatrick and Walsh, 2016). Additionally, another method known as microbial culturomics was developed to address the shortcomings of metagenomics. It has been used to identify new species found in the human microbiome and used in clinical microbiology fields (Abdallah *et al*., 2017; Lagier *et al*., 2018).

Several researchers have used antibiotics in media to achieve selective inhibition of various groups of organisms to isolate specific drug-resistant strains. Previous reports favour the use of meropenem since it provides the best balance between sensitivity and specificity for the detection of carbapenemase activity (Giske *et al*., 2013; Nordmann *et al.,* 2012b), whereas ertapenem and imipenem have high sensitivity but lack specificity (Giske *et al*., 2013; Nordmann *et al.,* 2012b; Vading *et al*., 2011). It was further reported that the addition of cloxacillin might prevent the overexpression of AmpC and efflux pumps expressed by bacteria (Robert *et al*., 2017).

2.4.2. Identification of *E. coli* and *K. pneumoniae* isolates using PCR and MALDI-TOF

2.4.2.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) assays have been widely used on both pure cultures and mixed environmental samples for the detection of specific ARGs encoding resistance. Environmental target DNA or RNA at low concentrations can be amplified and detected by PCR-based methods.

For this reason, PCR is considered a sensitive assay (Garibyan and Avashia, 2013); however, a false-positive result is an often a challenge in the PCR assays (Zhang *et al*., 2009).

Malate dehydrogenase (*mdh*) is an essential enzyme in the tricarboxylic acid (TCA) cycle as well as the noncyclic anaplerotic pathway of *Escherichia coli* (Park *et al*., 1995). The *mdh* gene has been successfully used to detect *E. coli* isolates via PCR from different studies (Omar and Barnard, 2010; Pillay and Olaniran, 2016). For the detection of *K. pneumoniae*, the *its* (internal transcribed spacer) and *rcsA* genes were used. The *rcsA* gene was successfully used in a study by Liu *et al.* (2008) and Dong *et al.* (2015) for the detection of *K. pneumoniae* in infant formula and clinical samples, respectively.

2.4.2.2 Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF)

Phenotypic properties are unstable at times, and their expression is dependent upon changes in the environmental conditions, e.g., growth substrate, temperature, and pH levels (Rosselló-Mora and Amann, 2001). MALDI-TOF has been recommended as an efficient alternative technique for the detection and characterization of a wide range of microbial species (Rodrigues *et al*., 2017). It has attracted a lot of attention for its precise identification of several microorganisms at the species level (Rodrigues *et al*., 2017). The method is based on the accurate determination of their protein mass that is compared to available profiles stored in a software database identifying the species in a few minutes (Seng *et al*., 2010).

Several comparative studies have evaluated the reproducibility and accuracy of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). In 2011, a study demonstrated that MALDI-TOF MS is reliable (accuracy, 99.3%) in identifying various *Staphylococcus* species. It may be considered equivalent to the standard method of *rpoB* sequence-

based identification (Spanu *et al*., 2011). Another study suggested that MALDI-TOF MS is the method of choice for the identification of *Campylobacter* and related microorganisms compared with other commercial systems (Martiny *et al*., 2011). Few drawbacks of this method have also been reported in the literature. It has been reported that encapsulated microorganisms, such as *K. pneumoniae* and *H. influenza* can be misidentified by MALDI-TOF-MS (Hou *et al*., 2019). Although most of the bacterial species can be identified with the utilization of the reference spectra, a small number of the strains that have a very similar genetic or wide diversity interspecies cannot be distinguished by the mass spectrometry signal to obtain high confidence identification results (Hou *et al*., 2019).

2.5 Detection of carbapenem-resistant *E. coli* and *K. pneumoniae*

2.5.1 Phenotypic detection of Carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE)

Once carbapenem resistance is identified through standard susceptibility testing, additional phenotypic tests can help to identify CP-CRE. These include the modified Hodge test (MHT), the Carba NP test and its variants, and the carbapenem inactivation method (CIM). All target carbapenemase production but provide no guidance regarding the specific carbapenemase type (Lutgring and Limbago, 2016).

2.5.1.1 Modified Hodge test (MHT)

The MHT is simple and inexpensive to perform and is well established in many clinical microbiology laboratories based on its ability to detect KPC producers. The MHT also demonstrates good sensitivity for many other carbapenemases, including VIM, IMP, and OXA48-like enzymes. For the U.S. collections of *Enterobacteriaceae*, sensitivity has been documented to

be between 93 and 98% (Mathers *et al*., 2013; Vasoo *et al*., 2013). The test's performance in detection of NDM-1 is generally recognized as much lower; in one study, only 7 of 14 NDM-1 isolates were MHT-positive (Girlich *et al*., 2012).

2.5.1.2 Multidisc diffusion test

Multidisc diffusion tests involving numerous inhibitors of specific enzyme types, including PBA (inhibitor of KPC and AmpC), Ethylenediaminetetraacetic acid (EDTA) or dipicolinic acid (inhibitors of MBL), clavulanate (inhibitor of ESBL), and cloxacillin (inhibitor of AmpC), have been described for the differentiation of carbapenemases from each other (Miriagou *et al*., 2013; van Dijk *et al*., 2014; Tsakris *et al*., 2010). Such tests are relatively inexpensive and simple to perform, although interpretation, especially when more than one mechanism is present, can sometimes be complicated (Lutgring and Limbago, 2016).

2.5.2 Molecular detection of carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE)

Despite the availability of various phenotypic methods, molecular biology techniques (especially PCR and sequencing) serve as the gold standard for the detection, identification and differentiation of different β-lactamases (Bradford, 2001). Molecular assays for CP-CRE detection include PCR, microarrays, and whole-genome sequencing (WGS). These methods have the benefit of determining the exact mechanism conferring carbapenem resistance, which can be helpful during outbreak investigations and while performing epidemiological research (Lutgrind and Limbago, 2016). The primary limitation of PCR assays is that only known genes can be targeted; those encoding novel carbapenemases will be missed with molecular approaches (Lutgring and Limbago, 2016). The commonly targeted carbapenemase genes include NDM-1, OXA-48, KPC,

VIM, and IMP (Sheppard *et al*., 2016; Calero-Cáceres *et al*., 2017; Piedra-Carrasco *et al*., 2017; Singh-Moodley and Perovic, 2016; Thomas and Duse, 2018), in both clinical and environmental settings.

3 CHAPTER 3: METHODOLOGY

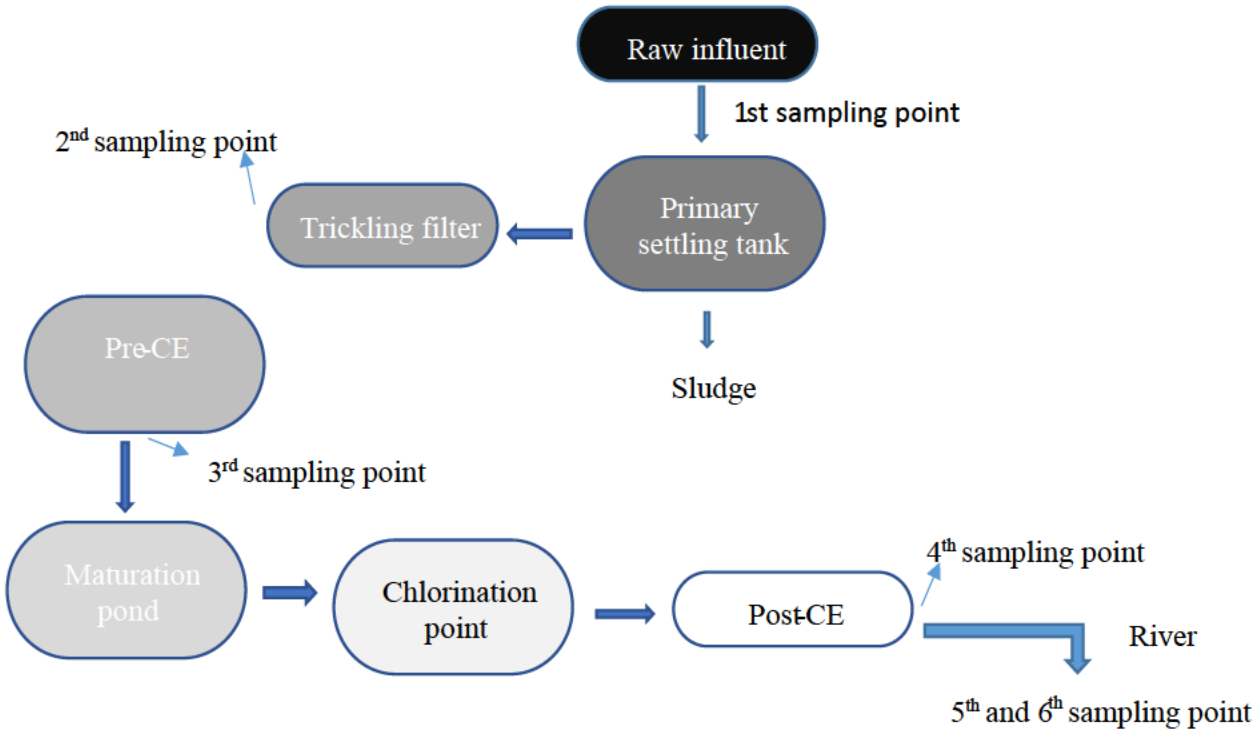
3.1 Description of sampling site and collection

Two WWTPs (WWTP I and WWTP II) treating municipal wastewater in the eThekwini Municipality (City of Durban) and their recipient rivers were selected for this study. The rivers flow through various informal settlements where water may be used for irrigation and other purposes. WWTP I has a designed capacity of 15 ML/D and working capacity of 10.9 ML/D. The biological treatment is based on a trickling filter configuration with four primary settling tanks, six trickling filters, six settling tanks, and three (2000 m^3) anaerobic digesters (unheated and unmixed). WWTP II is larger with the biological treatment based on activated sludge and with a design and working capacity of 25 ML/D and 22.5 ML/D, respectively. It has two primary settling tanks, the activated sludge reactors, three clarifiers, and three (2600 m^3) anaerobic digesters (heated and mixed).

The sampling points for WWTP I are specified in Fig 3.1 and included the influent after mechanical screening (raw sewage) (point 1), after the biofilter (point 2), after pre-chlorinated effluent (point 3), and after the discharge point, post-chlorinated effluent (point 4). In addition, sampling was done one km upstream (U.S - point 5) as well as one km downstream (D.S - point 6) from the discharge point. For WWTP II, the sampling points (Fig 3.2) were raw influent after mechanical screening (point 1), at the aeration tanks (point 2), pre-chlorinated effluent (point 3) and post-chlorinated effluent (point 4). Receiving surface water sampling was done one km upstream (U.S – point 5) and 1 km downstream (D.S - point 6) from the discharge point.

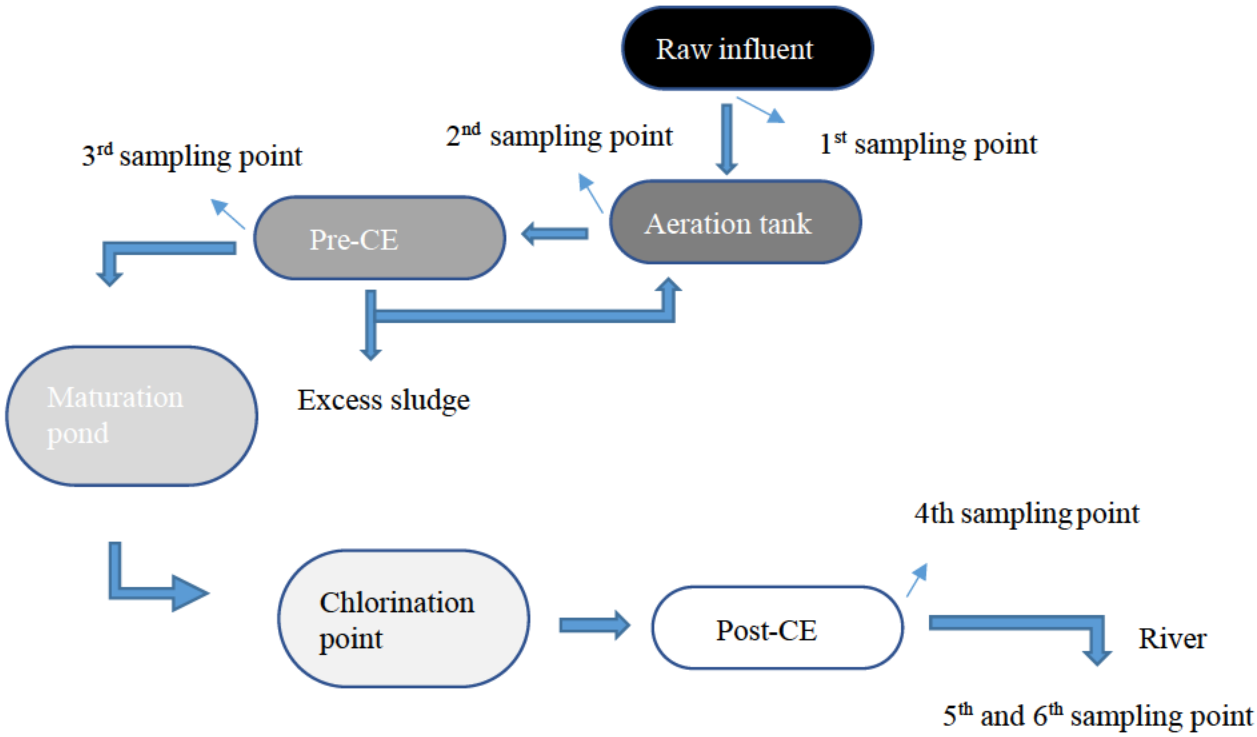
Samples were collected from each sampling point as a pooled composite sample, composed of sub-sample (taken every minute for 30-minutes). Samples were collected in 1 L plastic containers,

which were disinfected 24 h prior to collection by rinsing with deionized water and soaking in 70% ethanol. During the collection of samples, the containers were rinsed with the sampled water before filling to three-quarter of the container, leaving space to allow for proper mixing/aeration. The samples collected were transported in ice in the dark to the laboratory and analysed within 24 hours in line with existing guidelines (US EPA, 2006). Sampling was done monthly from January 2018 to September 2018.



Pre-CE: pre-chlorinated effluent; post-CE: post-chlorinated effluent

Figure 3.1: Schematic of WWTP I and its sampling points



Pre-CE: pre-chlorinated effluent; post-CE: post-chlorinated effluent

Figure 3.2: Schematic of WWTP II and the sampling points

3.2 Isolation of *E. coli* and *K. pneumoniae*

Serial dilutions (10^{-1} to 10^{-3}) of the samples (influent, aeration/biofilter, and pre-chlorinated effluent) were prepared in phosphate buffered saline (PBS), and 0.1 mL aliquots were used to inoculate the selective media in triplicates using the spread plate method. The CHROMagar ECC™ (MediaMage, South Africa) and Klebsiella *ChromoSelect* Selective Agar Base (Sigma Aldrich, South Africa) media were used for selective isolation of *E. coli* and *K. pneumoniae,* respectively. For the post-chlorinated effluent and river samples, a volume of 100 mL of undiluted samples was filtered using 0.45 µm membrane filters (Millipore). The membrane filters were then placed aseptically on the selective media plates for isolation of bacteria and incubated at 37°C for 24 h.

The selective media contained 0.25 µg/mL meropenem and 200 µg/mL cloxacillin for the selection of carbapenemase producers (Yamamoto *et a*l., 2017). A volume of 0.1 mL of undiluted samples (from the sampling points mentioned before) was inoculated on the prepared plates. The plates were incubated at 37^{o}C for 24 h. Enumeration of each of the selected species was done and the colonies that grew on the media supplemented with antibiotics were selected (following consistent attributes), purified, and stored with 50% glycerol at -80^{o}C for further characterization. The prevalence of carbapenem-resistant isolates was estimated according to Watkinson *et al*. (2007) as the percentage of resistant colonies:

$$\text{Percentage resistance} = \frac{\text{bacterial count on plate with carbapenem}}{\text{bacterial count on plate without carbapenem}} \times 100$$

3.3 Enumeration and purification of the isolates

Representative colonies were selected from the primary culture plates based on colonial size, shape, and the colour given (blue/turquoise coloured *E. coli* colonies, purple-magenta coloured *K. pneumoniae* colonies). Pure colonies were picked from each plate and purified by the streak plate method using Tryptone Soy agar (TSA). The obtained pure colonies were then stored in Tryptone Soy broth (TSB) with 50% glycerol at -80^{o}C for further studies.

3.4 Confirmation of the presumptive *E. coli* and *K. pneumoniae* isolates

3.4.1 Biochemical tests

The purified isolates were initially characterized using Methyl-Red Voges-Proskauer (MR-VP) test and Triple Iron Sugar agar (TSIA) test as described below.

3.4.1.1 Methyl Red-Voges-Proskauer test

A sterilized Methyl Red-Voges-Proskauer (MR-VP) broth was inoculated with overnight bacterial cultures and was incubated for 24 h at 37^{o}C. After incubation, the test tube was vortexed, and three drops of Barritt's 1/A reagent (5% alpha-napthol solution) and a drop of Barritt's 2/B reagents (40% potassium hydroxide solution) were added. The tube was vortexed and kept at room temperature (±22^{o}C) for 1 hour while observing a colour change in the broth. A red colour indicates a positive result for acid fermentation; a pink or red interface is a positive result for acetoin (McDevitt, 2009).

3.4.1.2 Triple Iron Sugar agar test

A sterilized Triple Iron Sugar agar (TSIA) slant of 5 mL in a test tube was inoculated with overnight bacterial cultures. The cultures were inoculated by streaking the surface of the slants and stabbing the bottom of the test tube, these were incubated at 37°C for 24 h. The colour change was observed on the slant and bottom of the test tube, as well as H_2S production. Glucose, lactose, and sucrose utilization results in a yellow/yellow colour in the slant and butt. Glucose only and peptone utilization give a red colour in the butt and yellow colour in the slant. If no sugar, but only peptone is utilized, the colour remains red (Karki, 2018). The blackening of the media indicates H_2S gas production, and gas production is indicated by bubbles in the butt (Karki, 2018).

3.4.2 Confirmation of the isolates through MALDI-TOF

Identification of the isolated colonies using MALDI-TOF was done at the Centre for Antibiotic Resistance Research (CARe), University of Gothenburg, Sweden. From fresh overnight cultures on blood agar, tiny amounts of biomass were transferred with a 1 µL plastic loop to individual spots on a VITEK® MS-DS slide (BioMérieux). Immediately after depositing the biomass, 1 µL of matrix α-cyano-4-hydroxycinnamic acid (***CHCA***) ***matrix*** solution (BioMérieux) was added to

the spots. The slides were left at room temperature (±22°C) to dry before being loaded into a VITEK® mass spectrometry system (BioMérieux). In order to determine the species of each tested isolate, the resulting mass spectra were analysed using the VITEK® MS databases IVD version 3.0 and SARAMIS version 4.15.

3.4.3 Molecular confirmation of the presumptive *E. coli* and *K. pneumoniae* isolates by PCR

3.4.3.1 DNA extraction

DNA extraction was carried out using the modified boiling method described by Paniagua-Contreras *et al*., 2017. The glycerol stock was streaked on Tryptone Soy Agar and incubated at 37°C for 24 h. After incubation, a loopful of pure colonies was transferred to a Tryptone Soy Broth, vortexed, and incubated at 37°C for 24 h. This broth was then transferred into a 2 mL Eppendorf tube, centrifuged at 14,000 rpm for 5 min, and the supernatant discarded. The pellets were rinsed with sterile distilled water two times. The pellets were resuspended in 500 µL of sterile distilled water and placed in a heat block to boil for 10 min and centrifuged at 13 500 rpm for 15 min. A volume of 450 µL of the supernatant containing the DNA was removed and stored at -20°C. This was used as a DNA template for all PCR-based experiments.

3.4.3.2 PCR confirmation of *E. coli* and *K. pneumoniae*

Molecular confirmation of the *E. coli* isolates was carried out via PCR amplification of the conserved malate dehydrogenase (*mdh*) gene (Pillay and Olaniran, 2016), and for *K. pneumoniae*, internal transcribed spacer gene (*its*) (Liu *et al.* 2008), and capsular polysaccharide synthesis regulating gene (*rcsA*) (Dong *et al*., 2015 (Table 3.1).

The 25 µL PCR mixture assay contained 5 µL DNA template, 12.5 µL hot-start green master mix

(Thermo Fisher), 6.5 µl RNAse free water, and 0.5 µL each of 10 µM primer (Table 3.1). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as a positive control, and a negative control consisted of the reaction mixture without a DNA template Amplification of the *mdh E. coli* target genes was performed in a T100 Thermal Cycler (Bio-rad). under the following conditions: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. For *K. pneumoniae*, the amplification conditions for the *its* gene were as follows; initial denaturation at 95°C for 15 min, followed by 35 cycles consisting of 30 s denaturation at 94°C, primer annealing at 58°C for 90 s, extension at 72°C for 90 s, and a final extension at 72°C for 10 min. For the *rcsA* gene, the amplification conditions were as follows; denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s and final extension at 72°C for 10min. The amplicons (5 µL aliquots) were resolved by electrophoresis (80 V for 45 min) on a 1.5% agarose gel (Merck, SA) stained with ethidium bromide and visualized under the UV trans-illuminator (Biorad, USA). A 100-bp DNA ladder (Promega, USA) was included on each gel as a molecular size standard. The amplification products of *its* gene were sequenced, and the sequences were analysed in the BioEdit Sequence Alignment Editor. They were subsequently compared with sequences available in GenBank, using the BLAST (Basic Local Alignment Search Tool) program of the National Center for Biotechnology Information of the United States (NCBI) (www.ncbi.nlm.nih.gov/BLAST).

Table 3.1: Primers and their product size for the identification of *E. coli* and *K. pneumoniae*

Bacterial species	Gene	Primer sequence (5'-3')	Expected product size (bp)
E. coli	*mdh*	GGTATGGATCGTTCCGACCT GGCAGAATGGTAACACCAGAGT	300
K. pneumoniae	*its*	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130
K. pneumoniae	*rcsA*	GGATATCTGACCAGTCGG GGGTTTTGCGTAATGATCTG	176

3.5 Antimicrobial susceptibility test

3.5.1 Antimicrobial agents

The isolates were screened against a predetermined and commercially available panel of 7 antibiotics (Shalom laboratory). These included carbapenem; imipenem (10 µg), meropenem (10 µg), doripenem (10 µg) and ertapenem (10 µg). Others were the third-generation cephalosporins; cefotaxime (30 µg), ceftazidime (30 µg) and cefixime (5 µg).

3.5.2 Disk diffusion test

Standardization of inoculum for antibiotic susceptibility testing was done following Komolafe and Adegoke (2008) method. Pure colonies of each isolate from a 24 h plate culture were inoculated into 2 mL sterile saline water and incubated at 37ºC for six hours. The turbidity was adjusted following the MacFarland standard. A volume of 100 µL of the adjusted inoculum was used to inoculate the Nutrient agar plates for antibiotic sensitivity testing. Antibiotic discs were aseptically placed at equidistance, on the surface of the agar, using sterile forceps for confluence growth, and

incubated for 24 h at incubation at 37ºC. The diameter of the zone of inhibition was measured to the nearest millimetre and interpreted using charts recommended by the Clinical and Laboratory Standard Institute (CLSI, 2017; CLSI, 2020).

3.6 Molecular analysis of antibiotic resistance genes in *E. coli* and *K. pneumoniae* isolates

3.6.1 Detection of ESBL genes

The detection of the resistance and virulence genes was done on isolates confirmed as described in section 3.4.1. For the detection of the ESBL genes (TEM, CTX-M, and SHV) in both *E. coli* and *K. pneumoniae* isolates, PCR conditions and primers published by Cai *et al.* (2012) were employed (Table 3.2). Positive controls were isolates (from this study) confirmed through sequencing, and negative controls were reaction mixtures for the respective genes, without a DNA template. Amplification of the target genes was performed in a T100 Thermal Cycler (Bio-Rad) under the following optimized PCR conditions; initialization at 95^{O}C for 5 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 56ºC for 40 s, and extension at 72ºC for 50 s and a final extension at 72ºC 10 min. For the detection of ARGs of OXA-1, the conditions and primer adopted by Braun *et al.* (2014) were used. The PCR conditions were as follows; initialization at 95ºC for 4 min, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 52ºC for 30 s, and extension at 72ºC for 60 s and a final extension at 72ºC 10 min. The amplicons (5 µL aliquots) were resolved by electrophoresis (80 V for 45 min) on a 1.5% agarose gel (Merck, SA) stained with ethidium bromide and visualized under the UV trans-illuminator (Biorad, USA). A 100-bp DNA ladder (Promega, USA) was included on each gel as a molecular size standard.

Table 3.2: Primers for the detection of ESBL producers

Gene(s)	Sequence (5'-3')	Expected product Size (bp)	References
bla*TEM*	GTCGCCGCATACACTATTCTCA CGCTCGTCGTTTGGTATGG	258	Cai *et al.,* 2012
bla*SHV*	GCCTTGACCGCTGGGAAAC GGCGTATCCCGCAGATAAAT	319	Cai *et al*., 2012
bla$_{CTX-M}$	CGGGAGGCAGACTGGGTGT TCGGCTCGGTACGGTCGA	381	Cai *et al.,* 2012
bla*OXA-1*	TTCTGTTGTTTGGGTTTCGC ACGCAGGAATTGAATTTGTTC	190	Braun *et al.,* 2014

3.6.2 Detection of the carbapenemase genes

Carbapenemase genes targeted in this study are listed in Table 3.3. The positive controls were isolates (from this study) confirmed through sequencing, and negative controls were reaction mixtures for the respective genes, without a DNA template. Amplification was performed in a T100 Thermal Cycler (Bio-Rad) using the following conditions The PCR conditions followed for the amplification of the VIM gene was as follow; 94°C for 10 min, followed by 36 cycles of denaturation at 94°C for 30 secs, annealing at 52°C for 40 secs, and extension at 72°C for 50 secs, followed by a final extension at 72°C for 5 min (Ellington *et al*., 2007). For the detection of OXA48, the PCR conditions were as follows; follows pre-denaturation at 95^{o}C for 4 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 52^{o}C for 30 s, and extension at 72^{o}C for 60 s and a final extension at 72^{o}C 10 min. For IMP and NDM-1, the following PCR conditions were used; initialization at 95^{o}C for 4 min, followed by 35 cycles of denaturation at 94^{o}C for 30 s, annealing at 52^{o}C for 30 s, and extension at 72^{o}C for 60 s and a final extension at 72^{o}C 10 min.

The amplicons (5 µL aliquots) were resolved by electrophoresis (80 V for 45 min) on a 1.5% agarose gel (Merck, SA) stained with ethidium bromide and visualized under the UV transilluminator (Biorad, USA). A 100-bp DNA ladder (Promega, USA) was included on each gel as a molecular size standard.

Table 3.3: Primers used for the detection of carbapenemase genes

Gene(s)	Sequence (5'-3')	Expected product Size (bp)	References
bla*VIM*	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	Ellington *et al*., 2007
bla*IMP*	GAGTGGCTTAATTCTCRATC AACTAYCCAATAYRTAAC	120	Monteiro *et al*., 2012
bla*OXA-48*	GCTTCCCACTGTGCAGCTCATTC CGCCCAACTCCTTCAGCAACAAATTG	450	Monteiro *et al*., 2012
bla*NDM-1*	ATGCGGCCTTGGGAACG GGTGCATCCCGGTGAAAT	660	Bonnin *et al*., 2012

3.7 Phenotypic detection of carbapenemase producers - modified Hodge test

An overnight grown culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland standard was inoculated onto Petri plates containing Mueller-Hinton agar (MHA). A paper disk impregnated with meropenem (10 µg, Shalom laboratory supplies) was placed on the Petri plate. An overnight culture of the isolates was streaked from the edge of the paper disk to the periphery of the plate in four different directions, inoculated with four different isolates. Inoculated plates were dried for 15 min at room temperature and incubated at 35 ±2^{o}C for 16 - 24 h. The test was considered positive when a clover leaf-like shape was observed as a result of the presence of

growing isolates within the meropenem antibacterial circle due to carbapenemase production by the isolate (Saban and Gopal, 2017).

3.8 Detection of virulence genes

3.8.1 Detection of virulence genes in carbapenem-resistant *E. coli*

A multiplex PCR protocol was employed to determine the presence of six *E. coli* virulence genes (Table 3.4) as described by Pillay and Olaniran (2016). Briefly, genomic DNA was extracted using the boiling method as previously described in section 3.4.3 and used as the template for the PCR analysis. The multiplex PCR mixture (25µl) was prepared as follows; 10 µM of each primer (total of 6 primers), 0.5 U Taq DNA Polymerase, 1 mM dNTP, 25 mM $MgCl_2$, 10× reaction buffer, and RNAse free water. *E. coli* O157:H7 (ATCC 35150) was used as a positive control, and a negative control was a reaction mixture without a DNA template. Amplification was performed in a T100 Thermal Cycler (Bio-Rad) using the following conditions: initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and elongation at 68°C for 75 s. A final elongation step was done at 68°C for 7 min. The amplicons (5 µL aliquots) were resolved by electrophoresis (80 V for 45 min) on a 1.5% agarose gel (Merck, SA) stained with ethidium bromide and visualized under the UV trans-illuminator (Biorad, USA). A 100-bp DNA ladder (Promega, USA) was included on each gel as a molecular size standard.

3.8.2. Detection of virulence genes in carbapenem-resistant *K. pneumoniae*

For *K. pneumoniae*, a single-plex PCR protocol was used to determine the presence of three virulence genes (Table 3.5). The positive controls were isolates (from this study) confirmed through sequencing, and negative controls were reaction mixtures for the respective genes, without a DNA template. The PCR conditions and primers used were adapted from Brisse *et al.* (2009).

Table 3.4: Primers used for the detection of virulent genes in *E. coli* (Pillay and Olaniran, 2016)

Genes	Sequence (5'-3')	Expected product (bp)
hly	GCGAGCTAAGCAGCTTGAAT	199
rfbE	CAGGTGAAGGTGGAATGGTTGTC TTAGAATTGAGACCATCCAATAAG	296
eae	CATTATGGAACGGCAGAGGT ACGGATATCGAAGCCATTTG	375
stx1	TGTCGCATAGTGGAACCTCA TGCGCACTGAGAAGAAGAGA	655
stx2	CCATGACAACGGACAGCAGTT TGTCGCCAGTTATCTGACATTC	477
fliC	AGCTGCAACGGTAAGTGATTT GGCAGCAAGCGGGTTGGTC	949

Each 25 µL PCR mixture contained 10 µM of each primer, 0.5 U Taq DNA Polymerase, 1 mM dNTP, 25 mM $MgCl_2$, 10× reaction buffer and RNAse free water. PCR conditions were the same for all genes except the annealing temperature; denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at; 55°C for *urea*, 53°C for *wabG* and 46°C *rmpA*) for 30s, elongation at 72°C for 1 min and a final elongation step at 72°C for 1 min. The amplicons (5 µL aliquots) were resolved by electrophoresis (80 V for 45 min) on a 1.5% agarose gel (Merck, SA) stained with ethidium bromide and visualized under the UV trans-illuminator (Biorad, USA). A 100-bp DNA ladder (Promega, USA) was included on each gel as a molecular size standard.

Table 3.5: Primers used for the detection of virulent genes in *K. pneumoniae* (Brisse *et al*., 2009)

Genes	Sequence (5'-3')	Expected product (bp)
urea	GATCATGGCGCTACCT(C/T)A GCTGACTTAAGAGAACGTTATG	377
wabG	CGGACTGGCAGATCCATATC ACCATCGGCCATTTGATAGA	683
rmpA	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	535

3.9 Statistical Analysis

The results were analysed and presented using relevant statistical models, which included descriptive statistics and ANOVA to determine statistical differences, as appropriate. The mean values were compared using ANOVA. The SPSS statistics software was used for the analysis. A p-value < 0.05 was considered statistically significant.

4 CHAPTER 4: PREVALENCE OF CARBAPENEM-RESISTANT *E. COLI* AND *K. PNEUMONIAE* IN WASTEWATER TREATMENT PLANTS

4.1 Introduction

Carbapenem antibiotics have been effective against multidrug-resistant Gram-negative bacilli and are a mainstay of therapy for infections due to such organisms (Hoelle *et al*., 2019). The therapeutic use of these antibiotics has been followed by the emergence and dissemination of clinically important carbapenemase-producing *Enterobacteriaceae* (CPE). These are rare, multi drug-resistant organisms commonly associated with hospitalized patients (Mathys *et al*., 2019). The dissemination of clinically relevant antibiotic-resistant bacteria such as CPE into the environment poses a direct threat to public health as a potential reservoir for community-acquired infections (Mollenkopf *et al*., 2017). There have been reports of CPE in wastewater effluent and surface water (Woodford *et al*., 2014; Mathys *et al*., 2019).

The WWTPs have been identified as reservoirs of antibiotic resistance genes (ARG) (Rizzo *et al.,* 2013b; Berendonk *et al*., 2015). Due to the presence of these genes and the proximity between the microbes, susceptible bacteria may be transformed into antibiotic-resistant bacteria (ARB) as well as become reservoirs of resistance genes (Dolejska *et al*., 2011). Although the treatment processes in WWTP can help in removing or reducing the ARB load, it has limited impact on ARGs since they are not degradable (Fouz et al., 2020). Therefore, effluents from WWTPs may represent a vehicle for the dissemination of antibiotic-resistant organisms and a route through which ARGs are introduced to the natural ecosystem (Fouz *et al*., 2020).

This chapter focus on understanding the prevalence and fate of CR *E. coli* and *K. pneumoniae* in different WWTPs within the city of Durban, South Africa. To achieve the aim, this chapter had the following specific objectives:

1. Isolation and quantification of carbapenem-resistant *E. coli* and *K. pneumoniae* from two WWTPs in Durban, South Africa

2. Determination of the efficiency of the selected WWTPs in the removal of carbapenem-resistant *E. coli* and *K. pneumoniae* at different stages of wastewater treatment.

3. Determination of the effect of the selected WWTPs on the prevalence and concentration of the selected bacteria in the receiving water bodies

4. *In vitro* analysis of isolates on the susceptibility against third-generation cephalosporin and carbapenem antibiotics.

4.2 Methodology

The two WWTPs treating municipal wastewater in the Durban metropolis (WWTP I and WWTP II) selected for this study are further summarised and illustrated in Chapter 3.1. The recipient rivers flow past various settlements where the water is used for agricultural purposes. Enumeration and isolation were done using the spread plate method on CHROMagar ECC (MediaMage, South Africa) and Klebsiella *ChromoSelect* Selective Agar Base (Sigma Aldrich, South Africa) for total *E. coli* and *K. pneumoniae,* respectively. For the isolation of carbapenemase producers, the media was modified with the addition of 0.25 µg/mL meropenem and 200 µg/mL cloxacillin (see section 3.2). The isolates were then purified as described in section 3.3 and characterized (section 3.4.1). Identification of the presumptive isolated colonies was done using the MALDI-TOF (as described in section 3.4.2). In parallel, molecular confirmation of the *E. coli* isolates was carried out via PCR

amplification of the conserved malate dehydrogenase (*mdh*) gene, internal transcribed spacer gene (*its*) and capsular polysaccharide synthesis regulating gene (*rcsA*) for *K. pneumoniae* (section 3.4.3). Antimicrobial susceptibility was determined against carbapenems and third-generation cephalosporin antibiotics (see section 3.5.1) using the disk diffusion method described in section 3.5.2. The results were analysed and presented using descriptive statistics and ANOVA. A p-value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 Isolation, identification, and confirmation of carbapenem-resistant *E. coli* and *K. pneumoniae*

The average total counts of *E. coli* in the influent were 6.9±0.15 $\log_{10}$ CFU/100 mL and 7.1±0.11 $\log_{10}$ CFU/100 mL, in WWTP I and II, respectively. The average CRE counts were found to be 3.0±0.31 $\log_{10}$ CFU/100 mL and 3.3±0.16 $\log_{10}$ CFU/100 mL in WWTP I and WWTP II, respectively. From the CRE counts, 120 isolates (60 from each WWTP) were obtained from the influent samples. All of the 120 isolates were positive for biochemical tests and also positive for further confirmation through MALDI-TOF and PCR (Figure 4.1).

CR *K. pneumoniae* was only detected in WWTP II. With an average total *K. pneumoniae* count of 7.3±0.1 $\log_{10}$ CFU/100 mL in the influent of WWTP II, the average CR *K. pneumoniae* was found to be 4.9±0.11 $\log_{10}$ CFU/100 mL. From the CR *K. pneumoniae*, a total of 60 isolates were isolated from the influent, 20 isolates from the aeration, and 20 isolates from the pre-chlorinated effluent samples. This resulted in a total of 100 randomly selected CR *K. pneumoniae* for further confirmation and identification. The isolates were further verified through biochemical tests, PCR

and MALDI-TOF. All isolates were positive for biochemical tests. Out of the 100 isolates, 60 randomly picked ones were analysed by MALDI-TOF. Only 12 isolates were identified as *Klebsiella* species, subdivided into eight *K. pneumoniae* (13%) and four as *K. oxytoca*. Of the remaining, 48 isolates were identified as *Enterobacter* (20), *Aeromonas* (15), unidentified (8) and others (5). MALDI-TOF was run simultaneously with PCR using the *rcsA* and *its* gene, which confirmed the isolates as 57% and 100% positive, respectively. Further confirmation of the 60 isolates was performed through sequencing, and 53 isolates were identified as *K. pneumoniae* and the remaining seven as *Enterobacter* species. Of the remaining 43% of the isolates detected as negative for the *rcsA* gene, all were negative for MALDI-TOF, and 16% were negative for sequencing. The remaining 40 isolates (of the initial 100) were identified as *K. pneumoniae* by *its* gene through PCR (Figure 4.1).

4.3.2. Total and carbapenem-resistant *E. coli* counts in the influent

The water samples were collected for 10 months, and no seasonal variation in the overall number of isolates recovered between the two WWTPs was observed. However, certain sampling events showed incidences of slightly higher concentrations. The total counts in the influent of WWTP I was slightly lower when compared to that of WWTP II. It ranged from 6.8 to 7.1±0.06 $\log_{10}$ CFU/100 mL for total *E. coli*. For CR *E. coli*, the counts ranged from 2.9 to 3.1±0.31 $\log_{10}$ CFU/100 mL (Table 4.1), constituting a total of 0.01% of CR *E. coli*.

In WWTP II, the total count in the influent ranged from 6.9 to 7.2±0.08 $\log_{10}$ CFU/100 mL. The difference in monthly concentrations of total *E coli* for both WWTPs was not significant ($p \geq 0.05$) (Table 4.1). For CR *E. coli*, WWTP II showed slightly higher counts as compared to WWTP I, with a range from 3.1 to 3.5±0.16 $\log_{10}$ CFU/100 mL (0.07%); however, the difference was not significant between the WWTPs ($p \geq 0.05$) (Table 4.1).

Table 4.1: Total and carbapenem-resistant (CR) *E. coli* and *K. pneumoniae* counts for WWTP I and II in the influent (log_{10}CFU/100 mL)

	WWTP I		WWTP II			
	Total *E. coli*	CR *E. coli*	Total *E. coli*	CR *E. coli*	Total *K. pneumoniae*	CR K. *pneumoniae*
Jan	7,0	2,9	7,2	3,3	7,3	4,8
Feb	6,8	2,9	6,9	3,1	7,3	4,8
Mar	7,1	3,1	7,1	3,1	7,2	4,8
Apr	6,9	2,8	6,9	3,2	7,2	4,8
May	7,1	2,9	6,9	3,4	7,2	4,8
Jun	6,8	2,8	7,0	3,3	7,1	4,8
Jul	6,7	2,9	7,1	3,4	7,3	4,7
Aug	7,0	2,9	7,1	3,5	7,4	4,7
Sept	7,1	3,0	7,2	3,4	7,3	4,9
Oct	7,1	2,0	7,1	3,5	7,2	4,9
Mean	**6.9**	**2,9**	**7,0**	**3,3**	**7,2**	**4,8**
SD	0,2	0,3	0,1	0,1	0,1	0,1

WWTP: wastewater treatment plants; CR; carbapenem-resistant; SD: standard deviation

4.3.3 Log reduction and removal efficiency of *E. coli* in the WWTPs and the impact of the effluent on the receiving rivers

The total *E. coli* counts in the biofilter samples of WWTP I ranged from 5.8 and 6.0±0.04 log_{10} CFU/100 mL, 4.1 to 4.5±0.13 log_{10} CFU/100 mL in the pre-chlorinated effluent, and 1.9 to 2.3±0.14 log_{10} CFU/100 mL in the post-chlorinated effluent (Figure 4.2 and Table 4.2). The difference between the total *E. coli* count in the influent and biofilter, biofilter and pre-chlorinated effluent was not significant ($p \geq 0.05$). However, the difference was significant when comparing the pre-chlorinated effluent and the post-chlorinated effluent ($p \geq 0.0001$) and between the raw influent and the post-chlorinated effluent ($p \geq 0.0001$).

In WWTP II, the *E. coli* total counts in the aeration tank ranged from 5.7 to 6.4±0.32 log_{10} CFU/100 mL, 4.2 to 4.6±0.10 log_{10} CFU/100 mL in the pre-chlorinated, 2.3 to 2.7±0.12 log_{10} CFU/100 mL in the post-chlorinated effluent (Figure 4.2 and Table 4.2). WWTP II showed similar trends as WWTP I when comparing between influent and aeration (no significant difference $p \geq 0.05$) as well as the aeration and pre-chlorinated effluent (no significant difference $p \geq 0.05$). However, the difference between the total count of *E. coli* in the pre and post-chlorinated effluent was statistically significant ($p \geq 0.0004$). In addition, a higher overall removal rate in WWTP II as compared to WWTP I was observed.

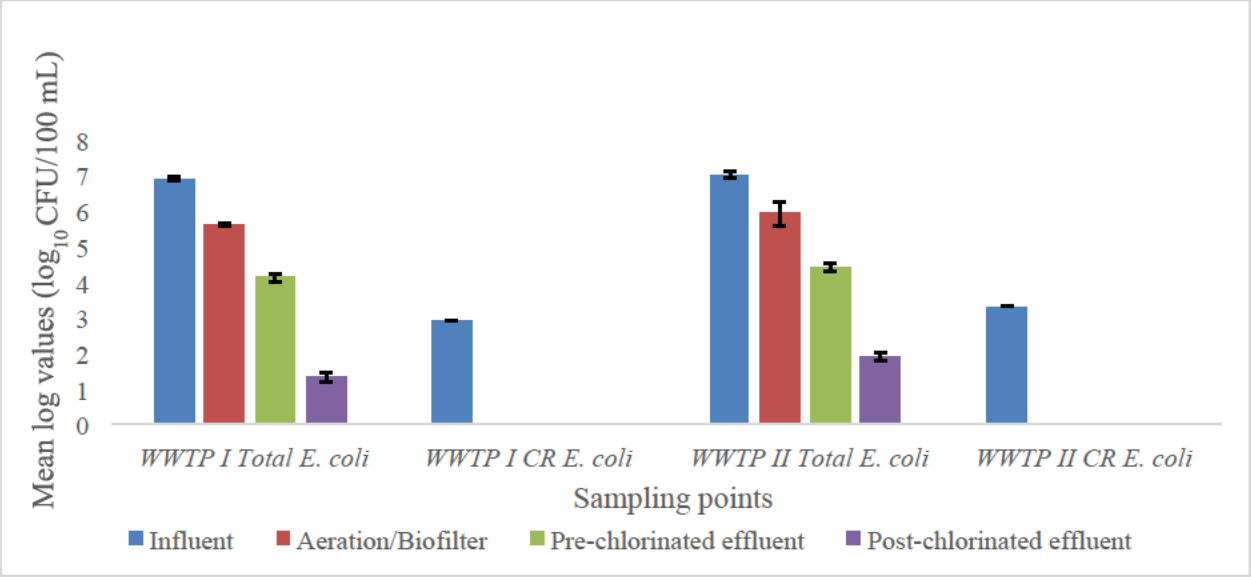
Table 4.2: Mean log values of total *E. coli* and *K. pneumoniae* from different sampling points of WWTP I and II (log_{10} CFU/100mL)

Sampling point(s)	WWTP I	WWTP II	
	Total *E. coli*	Total *E. coli*	Total *K. pneumomiae*
Bio/Aera	5,6	5,9	6,1
Pre-CRE	4,1	4,4	4,9
PCE	2,1	2,5	3,4

WWTP: wastewater treatment plants; Infl: influent; Bio: biofilter; Aera: aeration; PreCE: pre-chlorinated effluent; PCE: post-chlorinated effluent

The overall log_{10} removal of total E. coli was higher in WWTP II compared to WWTP I. In WWTP I, the log_{10} removal of total E. coli in the influent to the biofilter ranged from 1.05 to 1.16log_{10} CFU/100 mL, 1015 to 1.8 log_{10} CFU/100 mL from the biofilter to the pre-chlorinated effluent, and 1.94 to 2.38 log_{10} CFU/100 mL from pre-chlorinated effluent to post-chlorinated effluent.

In WWTP II, the log_{10} removal of total *E. coli* in the influent to the aeration ranged from 0.97 to 1.19 log_{10} CFU/100 mL, 1.36 to 2.37 log_{10} CFU/100 mL in the aeration to the pre-chlorinated effluent, and 2.03 to 2.47 log_{10} CFU/100 mL in the pre-chlorinated effluent to post-chlorination. The overall removal efficiency for both WWTPs was 100 % for CR *E. coli*, whereas for total *E. coli* for both plants it was 99.99% (Figure 4.3).



WWTP: wastewater treatment plants; CR: carbapenem-resistant

Figure 4.2: Mean log values and log reduction of total and CR *E. coli* for WWTP I and II (log_{10} CFU/100 mL)

There was no significant variation in the overall number of isolates recovered from the receiving water bodies between the months. WWTP II showed higher *E. coli* concentrations in the upstream and downstream samples compared to WWTP I. The upstream values in WWTP I and II ranged from 2.3 to 2.80±0.14 log10 CFU/100 mL and 2.7 to 3.0±0.10 log10 CFU/100 mL, respectively. The downstream values in WWTP I and II ranged from 2.3 to 2.60±0.09 log10 CFU/100 mL, and 2.7 to 2.9±0.10 log10 CFU/100 mL, respectively (Table 4.3). The post-chlorinated effluent from both WWTPs did not have an effect on the downstream samples as their concentrations were lower than that of those of upstream samples (Figure 4.4).

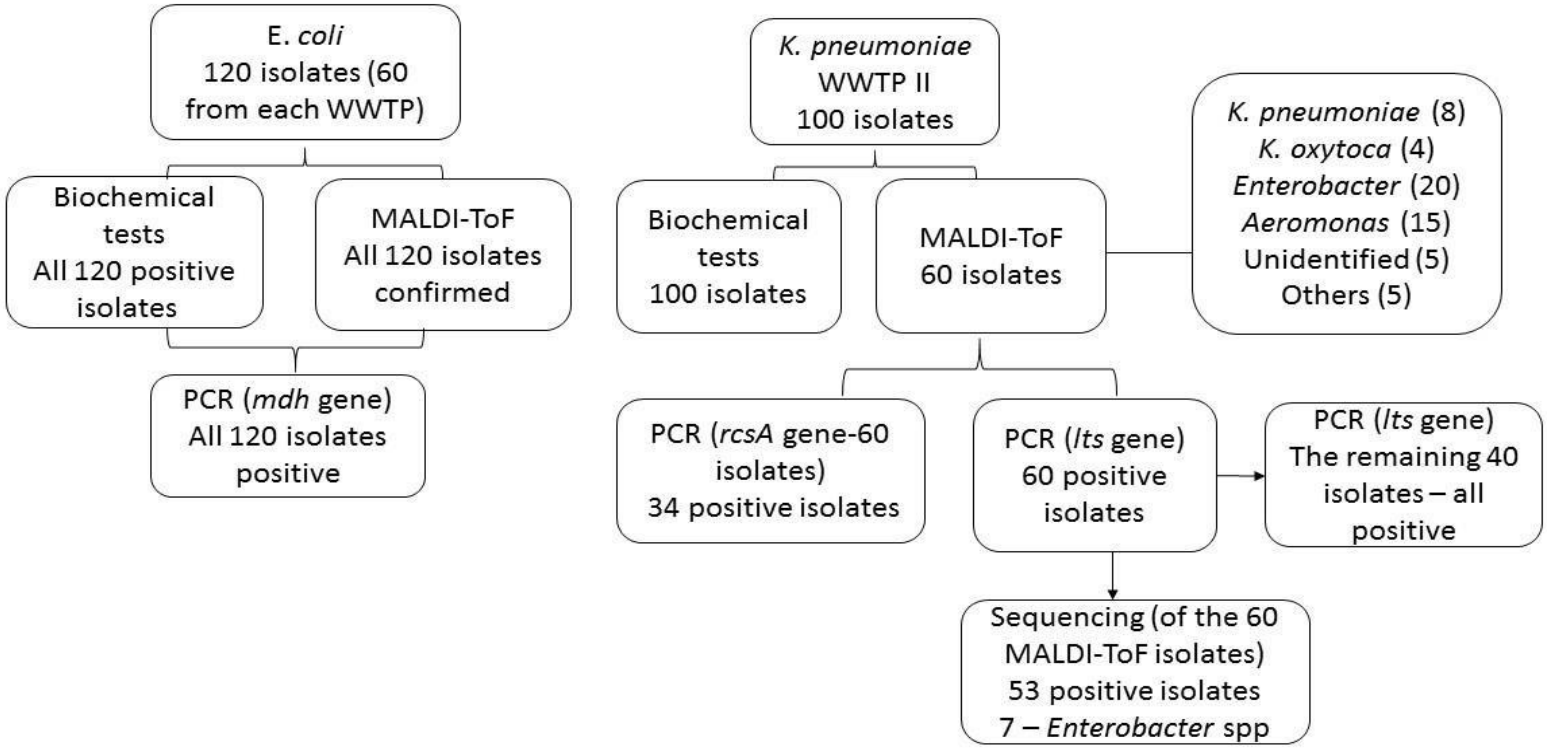
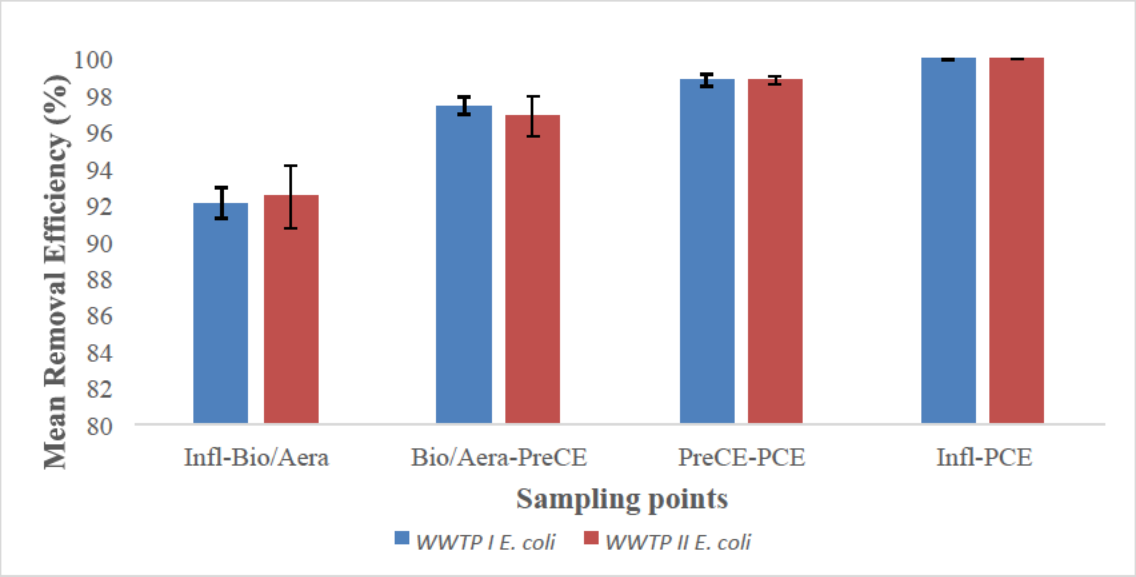


Figure 4.1: Overview of the results for the identification of isolates using biochemical test, MALDI-TOF and PCR



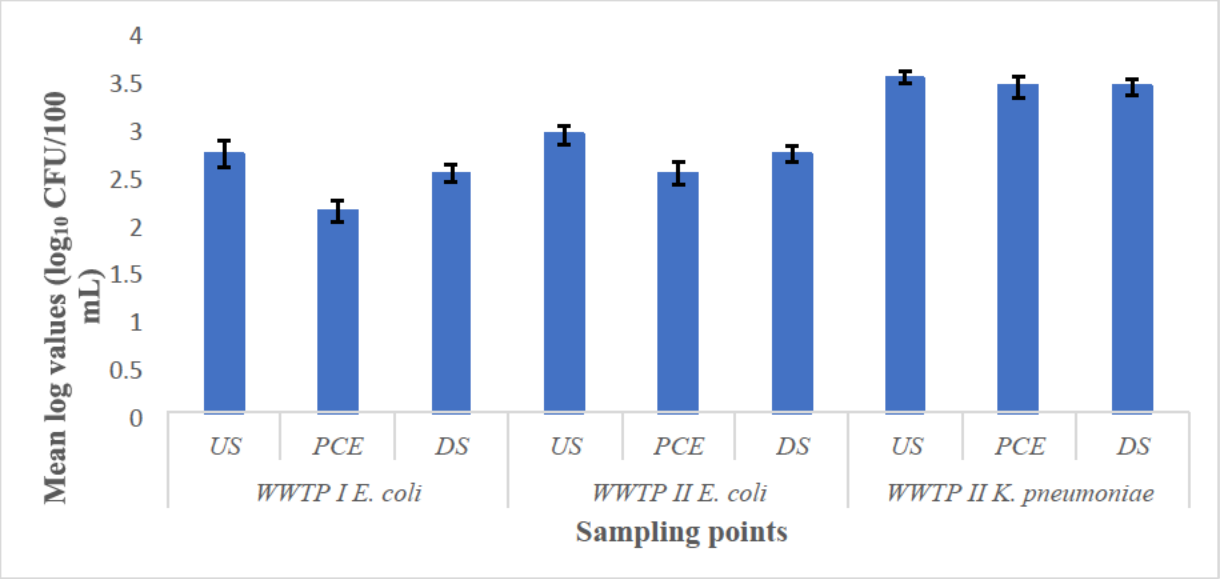
WWTP: wastewater treatment plants; Infl: influent; Bio: biofilter; Aera: aeration; PreCE: pre-chlorinated effluent; PCE: post-chlorinated effluent

Figure 4.3: Comparison of the removal efficiency of total *E. coli* in WWTP I and II between the treatment processes

Table 4.3: Mean log values of *E. coli* and *K. pneumoniae* and the effect of the post-chlorinated effluent on the receiving waters (log_{10} CFU/100 mL)

	WWTP I *E. coli*			WWTP II *E. coli*			WWTP II *K. pneumoniae*		
	US	PCE	DS	US	PCE	DS	US	PCE	DS
Mean log values	2.7	2.1	2.5	2.9	2.5	2.7	3.5	3.4	3.4
SD	0.14	0.11	0.09	0.1	0.12	0.09	0.06	0.11	0.08

WWTP: wastewater treatment plant; SD: standard deviation; US: upstream; PCE: post-chlorinated effluent; DS: downstream



WWTP: wastewater treatment plant; US: upstream; PCE: post-chlorinated effluent; DS: downstream

Figure 4.4: Effect of the total *E. coli* and *K. pneumoniae* in the receiving water bodies (log_{10} CFU/100 mL)

4.3.4 Total count and carbapenem-resistant *K. pneumoniae* concentration in WWTP II

The total *K. pneumoniae* was only determined in WWTP II as this was the only plant where carbapenem-resistant *K. pneumoniae* was detected. The total *K. pneumoniae* counts in the influent wastewater ranged from 7.1 to 7.4±0.1 log_{10} CFU/100 mL, 5.9 to 6.3±0.12 log_{10} CFU/100 mL (Table 4.1) in the aeration tank, 4.6 to 5.2±0.14 log_{10} CFU/100 mL in the pre-chlorinated effluent and 2.5 to 3.6±0.15 log_{10} CFU/100 mL in the post-chlorinated effluent. The difference between the total count of *K. pneumoniae* in the influent and the aeration, aeration and pre-chlorinated effluent was not statistically significant ($p \geq 0.05$). However, the difference was significant between the pre-chlorinated effluent and post-chlorinated effluent ($p \geq 0.0001$) and between the influent and post-chlorinated effluent ($p \geq 0.0001$).

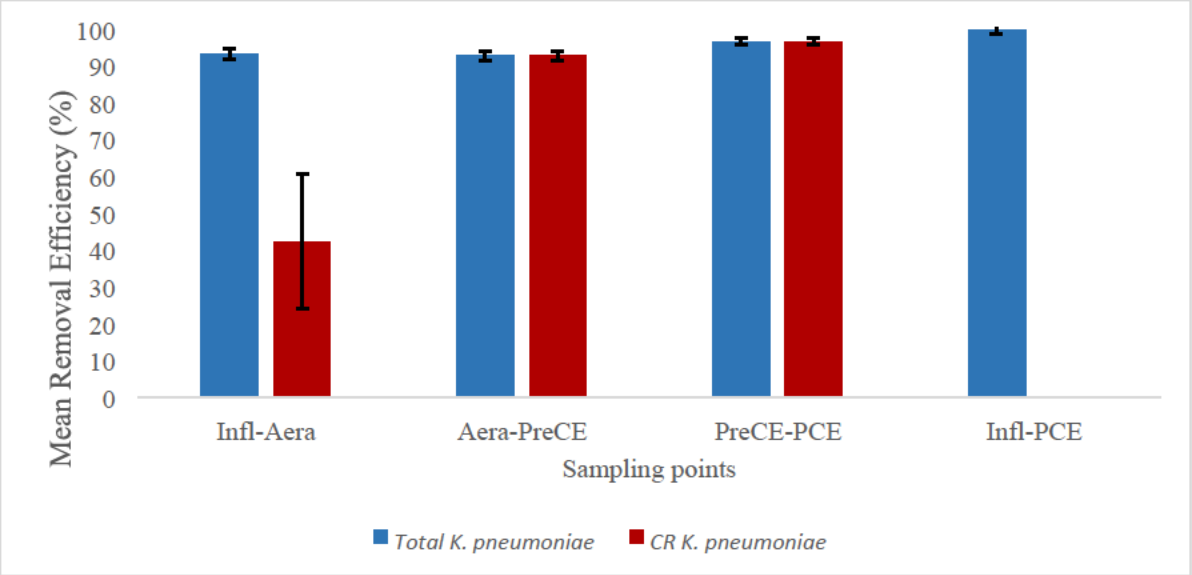
Carbapenem-resistant *K. pneumoniae* were isolated from the influent to the pre-chlorinated effluent. No isolates were recovered on the post-chlorinated effluent. CR *K. pneumoniae* counts in

the influent ranged from 4.7 to 4.9±0.11 $\log_{10}$ CFU/100 mL, 4.1 to 4.8±0.22 $\log_{10}$ CFU/100 mL in the aeration tank, and 2.5 to 3.5 ±0.26 $\log_{10}$ CFU/100 mL in the clarifier. Between the sampling points, the aeration tanks had a high prevalence of CR *K. pneumoniae* at 3.03%, followed by the pre-chlorinated effluent a 0.33% and the least being the influent at 0.34%. The difference in CR *K. pneumoniae* between the influent and the aeration tanks, between the aeration and pre-chlorinated effluent, and between the influent and pre-chlorinated effluent was statistically significant ($p \geq 0.0006$, $p \geq 0.0008$, and $p \geq 0.0004$, respectively). There was no major difference in the overall counts of CR *K. pneumoniae* isolates recovered.

4.3.5 Log reduction and removal efficiency of *K. pneumoniae* in WWTP II and the impact of the effluent on the receiving river

The *K. pneumoniae* total counts in the aeration samples of WWTP II ranged between 5.9 to 6.2±0.12 $\log_{10}$ CFU/100 mL, in the pre-chlorinated effluent, it ranged between 4.7 to 5.1±0.13 $\log_{10}$ CFU/100 mL and in the post-chlorinated effluent between 3.3 to 3.6±0.12 $\log_{10}$ CFU/100 mL (Figure 4.2 and Table 4.2). The difference between the total count of *K. pneumoniae* in the influent and the aeration, aeration and pre-chlorinated effluent was not statistically significant ($p \geq 0.05$). However, the difference was significant between the pre-chlorinated effluent and post-chlorinated effluent ($p \geq 0.0001$) and between the influent and post-chlorinated effluent ($p \geq 0.0001$).

In WWTP II, the $\log_{10}$ removal of total *K. pneumoniae* in the influent to the aeration ranged from 1.07 to 1.33 $\log_{10}$ CFU/100 mL. In the aeration to the post-chlorinated effluent, it ranged from 1.04 to 1.28 $\log_{10}$ CFU/100 mL, and in the pre-chlorinated effluent to post-chlorination, it was 1.38 to 1.65 $\log_{10}$ CFU/100 mL. The overall removal efficiency of total *K. pneumoniae* from the influent to the post-chlorinated effluent was 99.98% (Figure 4.5).



WWTP: wastewater treatment plant; CR: carbapenem-resistant; Infl: influent; Aera: aeration; PreCE: pre-chlorinated effluent; PCE: post-chlorinated effluent

Figure 4.5: Removal efficiency between the total and carbapenem-resistant (CR) *K. pnuemoniae* in WWTP II in the treatment processes

The $\log_{10}$ removal of CR *K. pneumoniae* in the influent to the aeration ranged from 0.09 to 0.59 $\log_{10}$ CFU/100 mL, aeration to the pre-chlorinated effluent ranged from 1.29 to 1.65 $\log_{10}$ CFU/100 mL, and complete removal was observed after chlorination. The overall removal of CR *K. pneumoniae* in WWTP II was 100%.

Similarly, the upstream values for *K. pneumoniae* in the recipient river of WWTP II were higher as compared to downstream and post-chlorinated effluent. They ranged between 3.4 to 3.60±0.06 $\log_{10}$ CFU/100 mL for upstream, 3.3 to 3.5 $\log_{10}$ CFU/100 mL for both post-chlorinated effluent and downstream samples (Table 4.3). CR *K. pneumoniae* was not detected in the river samples.

4.3.6 Antimicrobial susceptibility test – disk diffusion

4.3.6.1 Susceptibility against third-generation cephalosporins

The prevalence of resistance in *E. coli* against the third-generation cephalosporins did not differ between the two WWTPs samples. The isolates had high resistance against cefixime followed by cefotaxime, and lastly, ceftazidime with WWTP II displaying higher percentages of resistance than WWTP I. Similarly, *K. pneumoniae* isolates from WWTP II also showed high resistance against cefixime (81%), followed by cefotaxime (65%) and ceftazidime (48%) (Table 4.4).

Table 4.4: Susceptibility against third-generation cephalosporins using disk diffusion test (n=220)

WWTP		**Ceftazidime**			**Cefixime**			**Cefotaxime**		
		Percentage (%)								
		S	I	R	S	I	R	S	I	R
WWTP I	*E. coli*	57	-	43	-	32	68	52	-	48
WWTP II	*E. coli*	-	17	83	-	8	92	-	15	85
	K. pneumoniae	-	52	48	-	19	81	-	35	65

S: susceptible; I: intermediate; R: resistant, (-): none

4.3.6.2 Susceptibility against carbapenems

High resistance against ertapenem (100% - 95%) was seen in CR *E. coli* in both WWTPs, whereas CR *K. pneumoniae* from WWTP II showed 100% resistance to ertapenem (based on the CLSI guidelines). In this study, the lowest resistance was displayed against meropenem by both the bacteria (Table 4.5). However, all isolates were resistant to more than one carbapenem antibiotic, exhibiting a potential multidrug-resistant phenotype.

Table 4.5: Susceptibility against carbapenem antibiotics using disk diffusion test (n=220)

WWTP		Ertapenem		Imipenem		Meropenem		Doripenem	
		Percentage (%)							
		I	R	I	R	I	R	I	R
WWTP I	*E. coli*	-	100	49	51	42	58	39	61
WWTP II	*E. coli*	5	95	14	86	22	78	15	85
	K. pneumoniae	-	100	35	65	14	86	19	81

I: intermediate; R: resistance, (-): none

4.4 Discussion

E. coli is expected in the raw wastewater since it is a normal part of the gut microflora (van Elsas *et al*., 2011; Wu *et al*., 2020). The total *E. coli* counts in raw wastewater have been found to vary (Reinthaler *et al*., 2003; Adegoke *et al*., 2020). The WWTPs investigated in this study yielded a total *E. coli* count range from 6.8 to 7.1 $\log_{10}$ CFU/100 mL in WWTP I and 6.9 to 7.2 and $\log_{10}$ CFU/100 mL in WWTP II. The difference in the concentration between the WWTPs may be attributed to many factors, including the total capacity/volume of the WWTP, the technology used for treatment, and the composition of wastewater (Michałkiewicz, 2018). In this study, however, there was no significant difference in the total *E coli*, even though the two WWTPs varied in terms of total population served and volume of the wastewater treated.

Wastewater treatment plants receive sewage from various sources, including hospitals, which are important sources of pathogens, antibiotics and ARBs (Devarajan *et al*., 2016). In addition, most of the antibiotics used, whether in clinical practices or in households, are excreted in urine and faeces. This is later released to the municipal wastewater treatment plants. Because of this, WWTPs have been projected to influence the development and dissemination of ARB (Devarajan *et al*., 2016). This study used differential media (ECC CHROMagar™) supplemented with

meropenem and cloxacillin, thus making it selective to carbapenem-resistant bacteria. This was motivated by a study done by Yamamoto *et al.* (2017), where they evaluated different media with more sensitivity in recovering carbapenemase-producing *Enterobacteriaceae*. It has also been reported that meropenem provides the best balance between sensitivity and specificity for the detection of carbapenemase activity (Giske *et al*., 2013; Nordmann *et al*., 2012b). The carbapenem-resistant *E. coli* (CR *E. coli*) percentage in the raw wastewater of WWTP I was lower (0.01%) than that of WWTP II (0.07%). However, there was no significant difference between these concentrations ($p \geq 0.05$). Compared to clinical isolates, Weiner *et al.* (2016) found the percentage of CR *E. coli* to range from 0.7 to 1.9% in a survey of clinical infections, depending on the site of infection. Clinical settings will naturally concentrate antibiotic-resistant isolates compared to wastewater treatment plants, leading to higher concentrations (Hoelle *et al*., 2019). It was further noted that both WWTPs configurations investigated in this study were effective in removing these carbapenem-resistant bacteria completely from wastewater due to their absence in the post-chlorinated effluent samples.

There was a high overall removal efficiency of *E. coli* in both WWTPs (99.99%). The difference in the total *E. coli* between the raw wastewater and effluent of both WWTPs was significant ($p \geq 0.0001$). This could be attributed to the different treatment processes employed since WWTP II uses aeration tanks whilst WWTP I uses biofilter systems. When comparing these two systems, the aeration had a slightly higher removal rate (influent to biofilter/aeration) compared to the biofilter.

The total *K. pneumoniae* counts from WWTP II in the raw wastewater ranged from 7.1 to 7.3 $\log_{10}$ CFU/100 mL, which was much higher compared to a study done by King *et al*. (2020) from South Africa, where they reported viable counts for *Klebsiella* spp., including *K. pneumoniae* ranging

between 3.2 to 3.7 $\log_{10}$ CFU/100 mL. CR *K. pneumoniae* was more persistent in WWTP II since its detection was observed from the raw wastewater up to the pre-chlorinated effluent samples. The occurrence of resistant *K. pneumoniae* isolates in raw wastewater, aeration tanks and in the pre-chlorinated effluent was in the range of 0.34%, 3.03% and 1.33%, respectively. However, chlorination was found effective in removing the CR *K. pneumoniae* as no isolates were detected from the post-chlorinated effluent in any of the sampling events.

There was a progressive reduction in the load of total *K. pneumoniae* from the aeration to the post-chlorinated effluent. However, a considerable concentration of total *K. pneumoniae* was still detected in the final effluent, with counts ranging between 2.5 to 3.6 $\log_{10}$ CFU/100 mL. A study that investigated chlorine demand has shown that different organisms have unique chlorine demands, even at the same bacterial concentration (Shang and Blatchley, 2001). Thus, a higher concentration of chlorine may have been efficient in removing total *K. pneumoniae*.

Duong *et al.* (2008) reported that during the wastewater disinfection process in different WWTPs, the number of selected resistant bacteria was reduced up to 99%; this corresponded with this study where the overall reduction was 99.98%. Moreover, the concentrations of the tested bacteria in the post-chlorinated effluent did not affect the receiving water bodies judging from higher concentrations in the upstream samples compared to the downstream samples. Furthermore, the risk reduction is supported by the fact that the microbial load was within the guidelines for irrigation use, which is 10^3 CFU/100 mL (WHO, 2011; Ansa *et al*., 2017). In South Africa, the standards for reclaimed water use in agriculture had been set from the minimum counts of 5 to 300 CFU/100 mL for various vegetables (Kgopa *et al*., 2021). Therefore, the counts observed in this study were higher than recommended.

The biochemical test used in this study confirmed all the presumptive isolates as *E. coli* and *K. pneumoniae,* suggesting the specificity of the methods used, thus making them appropriate for preliminary detection of these organisms. In addition, a 100% confirmation of *E. coli* by MALDITOF was obtained. Similar sensitivity was also reported by Verburg *et al*. (2019), where 97-100% of *E. coli* isolates were identified by MALDI-TOF. However, MALDI-TOF did not identify all presumed *Klebsiella* isolates as *K. pneumoniae*. The other isolates were identified as *K. oxytoca, Enterobacter,* and *Aeromonas* species. This was also reported in a study by Rodrigues *et al.* (2017), where a *K. pneumoniae* isolate was misidentified at the genus level by MALDI-TOF. It was identified as *K. oxytoca* and *Enterobacter cloacae*. Although belonging to a different genus, it is included in the same tribe – Klebsielleae, along with *Hafnia, Serratia,* and *Pantoea* genera (Rodrigues *et al*., 2017). The discrepancy in the results could be due to the difficulty in MALDITOF to distinguish between some closely genetically related organisms (Randell, 2014) or the lack of the quality of the reference database (Hou *et al*., 2019). Also, it is reported that encapsulated organisms, such as *K. pneumoniae* can be misidentified by MALDI-TOF (Hou *et al*., 2019).

Molecular identification via PCR amplification was done using the rRNA genes as they are ideal for identification because of the highly conserved regions within the species (Fuks *et al*., 2018). The identification of presumptive *E. coli* isolates was confirmed through the detection of the *mdh* gene. These findings are similar to as reported by Pillay and Olaniran (2016), as well as Omar and Barnard (2010). Confirmation of *K. pneumoniae* was done using the *RcsA* and *its* gene, yielding 56% and 100%, respectively, for positive isolates. Due to the differences in PCR results, the 60 isolates were further analysed via sequencing. Using the *its* gene, 88% of the isolates were identified as *K. pneumoniae* and the remaining 12% as *Enterobacter* species. The NCBI Blast

revealed that the *its* reverse primer sequence used in this study (5'TTCACTCTGAAGTTTTCTTGTGTTC-3') had complementary sequences in *K. pneumoniae*, as well as *Enterobacter* species. This may have led to false-positive results on PCR confirmation (Zhang *et al*., 2009).

The objective behind performing a susceptibility test against third-generation cephalosporin antibiotics is that carbapenemase-producing *Enterobacteriaceae* (CPE) are, in most cases, resistant to extended-spectrum (oxyimino) cephalosporins (i.e., cefotaxime, ceftriaxone, ceftazidime and/or cefepime) (Nordmann *et al*., 2012a). Additionally, the antibiotics were selected based on the 2015 Centers for Disease Control and Prevention's definition for carbapenem-resistant *Enterobacteriaceae*: *in vitro* resistance to any carbapenem antimicrobial (CDC, 2015). The third-generation cephalosporin susceptibility test results showed that all the isolates (CR *E. coli* and CR *K. pneumoniae*) were highly resistant to cefixime, followed by cefotaxime and ceftazidime. This was also seen in a study done by Hoelle *et al*. (2019) in samples isolated from WWTPs, where out of the two third-generation cephalosporins investigated, cefotaxime had a higher number of resistant isolates than ceftazidime. Moreover, in a clinical study by Feizabadi *et al.* (2010), isolates were reported to be resistant to cefixime. Thus, this shows that isolates, either environmental or clinical, are showing resistance to the cefixime antibiotic.

The findings on carbapenem resistance showed that all the isolates were resistant to ertapenem. Potron *et al*. (2013) showed the same trend of carbapenem resistance (excluding doripenem), where isolates were more resistant to ertapenem and less resistant to imipenem. A carbapenem-resistant *Enterobacteriaceae* is usually suspected when resistance to ertapenem is detected. This

is because an ertapenem disk is used to screen for carbapenem resistance in the tested isolates (El Herte *et al*., 2012).

4.5 Conclusions

Two WWTPs were investigated in this study and found to harbour carbapenem-resistant *E. coli* and *K. pneumoniae* at varying concentrations. The PCR primers used to confirm the *E. coli* isolates had a high specificity as all the isolates were identified as positive. For the confirmation of *K. pneumoniae*, the *its* gene had a high specificity compared to the *rcsA* gene. Other confirmation methods should be used in parallel with MALDI-TOF as a significant number of *K. pneumoniae* isolates were not identified by this method. It was further noted that the MALDI-TOF and the *its* sequencing did not produce similar results.

The method of screening for resistant bacteria using selective media supplemented with carbapenem, made it possible to understand the prevalence of carbapenem-resistant bacteria in both WWTPs. For *E. coli*, carbapenem-resistant isolates were obtained in the influent from both WWTPs. For *K. pneumoniae*, carbapenem-resistant isolates were observed from the influent up to the pre-chlorinated effluent. This indicates that *K. pneumoniae* may have survival mechanisms that allowed its detection in the different sampling points as compared to *E. coli*. Most of the isolates, both *E. coli* and *K. pneumoniae*, showed higher resistance against cefixime among the third-generation cephalosporins antibiotics used. WWTP II showed a high prevalence of carbapenem-resistant *E. coli* compared to WWTP I. Most isolates, both *E. coli* and *K. pneumoniae*, showed resistance against ertapenem. The resistance to these antibiotics may reflect the consumption of antibiotics in the community. Though carbapenemase resistance could be deduced from the

resistance profile of the isolate, further phenotypic and genotypic tests should be done for confirmation.

5 CHAPTER 5: CARBAPENEMASE-ENCODING GENES, EXTENDED SPECTRUM β-LACTAMASES AND VIRULENCE GENES IN THE *E. COLI* AND *K. PNEUMONIAE* ISOLATES FROM WASTEWATER TREATMENT PLANTS

5.1 Introduction

Bacteria may become resistant to carbapenem through overproduction of ESBL or AmpC enzymes combined with porin loss or upregulated efflux pumps and production of carbapenem-hydrolysing enzymes, the carbapenemases (Wang *et al*., 2015a; Kocsis and Szabó, 2013). The latter is of clinical importance since bacteria producing these enzymes can result in resistance to several other antibiotics, including fluoroquinolones, trimethoprim-sulfamethoxazole, and aminoglycosides, thus limiting treatment options (Lutgring and Limbago, 2016; Bae *et al*., 2015). Moreover, with the presence of carbapenem resistance genes, they can confer resistance without the need for additional mechanisms or chromosomal mutations (Lutgring and Limbago, 2016).

Resistance to carbapenems through carbapenemase production is mediated through different classes of carbapenemases (see section 2.4.2.2) (Bush and Fisher, 2011). Initially, carbapenemase genes were chromosomally mediated, which led to resistance in a few species. Based on reports, the majority of these genes are plasmid-mediated, enabling transfer from one organism to another species than those within the *Enterobacteriaceae* family (Rodriguez-Martinez *et al*., 2009). Thus, the clinically important CRE are those with a confirmed carbapenemase gene, called the carbapenemase-producing *Enterobacteriaceae* (CPE) (Livermore, 2012). Studies on metalloenzymes such as Verona integron-encoded Metallo-β-lactamase (VIM) and Imipenemase

(IMP) have been reported globally (Nordmann *et al*., 2011; Lutgrind and Limbago, 2016). South Africa also reported its first NDM-1 gene detected in *Enterobacteriaceae* isolated from clinical samples taken from patients that arrived from India in 2011 (Lowman *et al*. 2011).

The methods used to detect carbapenemase production in CRE include phenotypic and genotypic screening tests (Hrabák *et al*., 2014). The available phenotypic tests include the modified Hodge test (MHT), among other methods. Even though the method is sensitive for detecting carbapenemase-mediated mechanisms of resistance to carbapenems, it does not specify the type of carbapenemases involved (Hrabák *et al*., 2014). Therefore, the genotypic approach based on a specific carbapenemase gene is recommended for more specific identification.

The presence of virulent factors leading to clinical infections caused by antibiotic-resistant strains enhances the potential for dissemination and increased morbidity and mortality (Hoelle *et al*., 2019). Therefore, the health challenges associated with virulent resistant bacteria call for the determination of virulence in resistant strains from environmental samples. The *E. coli* pathotypes investigated in this study are those related to diarrhea, collectively known as diarrheagenic *E. coli* (DEC) (see section 2.5.1) (Nataro and Kaper, 1998). The virulence factors related to DEC pathotypes may include genes encoding toxin production (*stx1* and *stx2*), α haemolysin production (*hlyA*), and adherence factor such as intimin (*eae*) (Mainil, 2013). The virulence factors in *K. pneumoniae* include capsule polysaccharide (CPS), lipopolysaccharide (LPS), fimbriae, and iron, encoded by genes such as *rmpA*, *urea*, and *wabG*. Among the virulence factors, CPS is an important one as it protects the bacteria from being engulfed by phagocytes or to be affected by serum bactericidal factors (Su *et al*., 2018).

The major objectives of this chapter, therefore are:

1. Determine the prevalence and distribution of carbapenem resistance genes in *E. coli* and *K. pneumoniae* using molecular methods,

2. Ascertain expression of the carbapenemase genes through carbapenemase production using the modified Hodge test method,

3. Investigate the prevalence of virulence genes and the relationship between antimicrobial resistance and virulence.

5.2 Methodology

The previously isolated and confirmed carbapenem-resistant *E. coli and K. pneumoniae* were subjected to PCR for the detection of ESBL and carbapenemase-producing genes. For the detection of the ESBL genes (TEM, CTX-M, SHV, and OXA-1), PCR conditions and primers adopted by Cai *et al.* (2012) were used (as described in section 3.6.1). For the detection of carbapenemase-producing genes (OXA-48, VIM, IMP, and NDM-1), the PCR conditions and primers used are described in section 3.6.2. To determine the production of carbapenemases by the isolates, the modified Hodge test was performed as adopted by Saban and Gopal (2017) (section 3.6). In addition, a multiplex PCR protocol was used to determine the presence of six *E. coli* virulence genes with PCR conditions and primers adopted from Pillay and Olaniran (2016) (section 3.7.1). For *K. pneumoniae*, a singleplex PCR protocol was used to determine the presence of three virulence genes (*wabG*, *urea* and *rmpA*). The PCR conditions and primers used were adopted from Brisse *et al.* (2009) (section 3.7.2). For data analysis, descriptive statistics were used to determine

and compare the prevalence of ESBL genes, carbapenemase-producing genes, and virulence genes between the WWTPs.

5.3 Results

5.3.1 Detection of ESBL genes

5.3.1.1 *E. coli* isolates

The detection of the resistance and virulence genes was done on isolates confirmed in accordance with the description in chapter 3.4. The antibiotic resistance genes were detected in all the *E. coli* isolates from both WWTPs in the influent, but the total prevalence varied between different genes. The ESBL genes such as TEM and SHV were found in all isolates from both WWTPs, but of the total isolates, CTX-M and OXA-1 were only present in 92% and 69% of the isolates, respectively (Table 5.1). A difference in the prevalence of the ESBL genes was observed in the isolates from WWTP I and WWTP II. All *E. coli* isolates from WWTP I were found to contain TEM, CTX-M and SHV; only 98% had the OXA-1 gene. Similarly, TEM and SHV were detected in all isolates from WWTP II; however, only 83% and 52% of isolates had the CTX-M and OXA-1, respectively. Thus, a higher prevalence of the ESBL genes in *E. coli* occurred in WWTP I compared to WWTP II.

Table 5.1: Distribution of selected ESBL genes in *E. coli* (n=120) from the influent of the WWTPs

	WWTP I *E. coli* (n=60)		**WWTP II *E. coli* (n=60)**		
Genes	n	%	n	%	Total n (%)
TEM	60	100	60	100	120 (100)
SHV	60	100	60	100	120 (100)
CTX-M	60	100	50	83	110 (92)
OXA-1	59	98	24	52	83 (69)

n: total number of isolates; WWTP: wastewater treatment plants

5.3.1.2 *K. pneumoniae* isolates

The ESBL genes in *K. pneumoniae* isolates from WWTP II were detected in three treatment steps (influent, aeration, and pre-chlorinated effluent). Among these, pre-chlorinated effluent showed the highest number of isolates with ESBL genes, followed by the aeration point and the lowest frequency in the influent. All the isolates obtained from the aeration point as well as pre-chlorinated effluent carried TEM and SHV genes (100%). However, CTX-M was only detected in two isolates from the aeration tank; however, it was more prevalent in the pre-chlorinated effluent (100%) (Table 5.2). The OXA-1 also occurred in some of the influent isolates (13%) but was not detected in the aeration and pre-chlorinated effluent (see Table 5.2).

Table 5.2: Distribution of selected ESBL genes in *K. pneumoniae* (n=100) in different sampling points from WWTP II

	Influent (n=60)		Aeration (n=20)		Pre-CE (n=20)		Total
Genes	n	%	n	%	n	%	(%)
TEM	45	75	20	100	20	100	85
SHV	32	53	20	100	20	100	72
CTX-M	37	62	2	10	8	40	47
OXA-1	8	13	0	0	0	0	8

n: total number of isolates; pre-CE: pre-chlorinated effluent

5.3.2 Detection of carbapenemase-producing genes

5.3.2.1 Carbapenemase-producing genes in CR *E. coli*

The frequency of the occurrence of the carbapenemase genes differed between the isolates of the two WWTPs. The predominant carbapenemase type for WWTP I was NDM-1 (100%), followed by OXA-48 (82%), while the other genes investigated (VIM and IMP) were not detected in WWTP I. In addition, a total of 17 (28%) isolates were found to harbour both NDM-1 and OXA-48 genes. The detection of carbapenemase genes for WWTP II showed the highest occurrence for

OXA-48 (52%), followed by NDM-1 (28%), VIM (7%), while IMP was not detected. In two isolates, the combination of VIM and OXA-48 genes was detected for WWTP II (3%), while a single isolate showed a combination of NDM-1 + OXA-48 + VIM (2%) (see Table 5.3).

Table 5.3: Distribution of selected carbapenemase genes in *E. coli* (n=120) between the WWTP I and II

	WWTP I *E. coli* (n=60)		**WWTP II *E. coli* (n=60)**	
Genes	n	%	n	%
NDM-1	60	100	17	28
OXA-48	49	82	31	52
VIM	0	0	4	7
IMP	0	0	0	0
NDM-1+OXA-48	17	28	0	0
VIM+OXA-48	0	0	2	3
NDM-1 + OXA48 + VIM	0	0	1	2

n = total number of isolates; WWTP: wastewater treatments plants

5.3.2.2 Carbapenemase-producing genes in CR *K. pneumoniae*

The occurrence of the carbapenemase genes detected in *K. pneumoniae* differed between the isolates obtained from different stages of the treatment. The influent samples showed more diverse gene occurrence (NDM-1 and OXA-48) and the highest number of isolates harbouring both these carbapenemase genes (Table 5.4). However, this was not the case in the subsequent treatment steps, where only the NDM-1 gene was detected in some of the isolates from the aeration samples (35%). Additionally, VIM was the only gene detected in the pre-chlorinated effluent among the 20 isolates screened (Table 5.4).

Table 5.4: Distribution of selected carbapenemase genes in *K. pneumoniae* (n=100) in different sampling points from WWTP II

	Influent (n=60)		Aeration (n=20)		Pre-CE (n=20)		Total
Genes	n	%	n	%	n	%	(%)
NDM-1	50	83	7	35	0	0	57
OXA-48	14	23	0	0	0	0	14
IMP	0	0	0	0	20	100	20
VIM	0	0	0	0	0	0	0
NDM-1 + OXA-48	7	12	0	0	0	0	7

n: total number of isolates; pre-CE: pre-chlorinated effluent

5.3.3 Modified Hodge test

5.3.3.1 *E. coli* carbapenemase production

Among the isolates screened for carbapenemase production using the modified Hodge test in the influent, only 8% of isolates from WWTP I and 32% from WWTP II were positive. The isolates that produced the carbapenemase enzyme in WWTP I had a combination of the two carbapenemase genes, NDM-1, and OXA-48, while WWTP II only had OXA-48 (Table 5.5).

5.3.3.2 *K. pneumoniae* carbapenemase production

For *K. pneumoniae* isolates between the three treatment stages, the pre-chlorinated effluent had the highest number of isolates producing this enzyme, followed by aeration and the influent points. In the influent, 8% isolates produced the enzyme. The corresponding percentage in the aeration was 30% and 100% in the pre-chlorinated effluent. The isolates in the influent and aeration harboured the same carbapenemase gene, NDM-1, but in the pre-chlorinated effluent, VIM was the only gene detected (Table 5.5). It was further noted that a total of 13% isolates from the influent did not harbour carbapenemase-encoding genes but grew on the plate supplemented with the carbapenem antibiotic. These isolates only had the ESBL genes in their genome.

Table 5.5: MHT results and carbapenemase genes associated with the expression of the carbapenemase

	WWTP I *E. coli*	**WWTP II *E. coli***	**WWTP II *K. pneumoniae***		
Sampling points	Influent	Influent	Influent	Aeration	Pre-CE
% of positive isolates	8%	32%	8%	30%	100%
Common gene (s)	NDM-1 and OXA-48	OXA-48	NDM-1	NDM-1	VIM

WWTP: wastewater treatment plant; Pre-CE

5.3.4 Detection of virulence genes

5.3.4.1 Virulence genes in CR *E. coli*

A total of six genes (*hly, rfbE, eae, stx1, stx2,* and *fliC*) were used to determine the virulence characteristics of CR *E. coli* isolates. The WWTP II had a higher number of isolates harbouring these genes than WWTP I and with an additional gene only detected in WWTP II. Among those screened, *hly* was the most prevalent virulence gene in WWTP I (42%) and WWTP II (88%), followed by *rfbE* at 18% in WWTP I and 33% in WWTP II. The additional gene only detected in WWTP II was *eae,* with 8% of the isolates positive (Table 5.6). The genes, *Stx1, stx2,* and *fliC* were not detected in any of the isolates.

Table 5.6: Detected virulence genes in CR *E. coli* from the influents of both plants

	WWTP I (n=60)		**WWTP II (n=60)**	
Genes	n	%	n	%
hly	25	42	53	88
rfbE	11	18	20	33
eae	0	0	5	8
stx1	0	0	0	0
stx2	0	0	0	0
fliC	0	0	0	0

n: total number of isolates

5.3.4.2 Virulence genes in CR *K. pneumoniae*

Three genes (*wabG*, *rmpA*, and *urea*) were assessed to determine the virulence profile of the CR *K. pneumoniae* isolates from WWTP II. The only virulent gene found was *wabG*, while *urea and rmpA* were not detected in any of the isolates screened. The *wabG* gene was detected from the influent (18%, aeration (10%), and pre-chlorinated effluent (50%) (Table 5.7).

Table 5.7: Distribution of the virulence genes in CR *K. pneumoniae* from WWTP II

	Influent (n=60)		**Aeration** (n=20)		**Pre-CE** (n=20)		**Total**
Genes	n	%	n	%	n	%	%
wabG	11	18	2	10	10	50	23
rmpA	0	0	0	0	0	0	0
urea	0	0	0	0	0	0	0

n: total number of isolates, pre-CE: pre-chlorinated effluent

The *E. coli* and *K. pneumoniae* isolates harboured a number of ESBL, carbapenem resistance and virulence genes. With the resistance genes, most isolates harboured two or more. The number of isolates, both *E. coli* and *K. pneumoniae*, harbouring the virulence genes investigated in the study was low compared to the resistance genes.

5.4 Discussion

A concept called One Health has been created to recognise that human health is inextricably linked to the health of animals and the shared environment (Mackenzie and Jeggo, 2019). In this concept, the topics of interest include antibiotic resistance, amongst others (Mackenzie and Jeggo, 2019). In relation to this concept, this study focuses on carbapenem resistance in the environment. It is important to note that although most antibiotic resistance research focuses on clinical samples, it also occurs in environmental organisms inhabiting terrestrial and aquatic habitats (Lösch *et al*., 2008; Moges *et al*., 2014). The concern in the occurrence of antibiotic resistance genes in

environmental bacteria is the potential transfer of these genes to pathogens, thus reducing the therapeutic potential against human and animal pathogens (Rizzo *et al*., 2013b; Bengtsson-Palme *et al*., 2017). The limited information regarding antibiotic resistance in the environment has sparked an increase in investigations on the presence of resistant organisms and their key genes in different environments and their possible pathogenicity (Cornejova *et al*., 2015; Hoelle *et al*., 2019).

An increase in the transmission of carbapenemase-producing *Enterobacteriaceae* (CPE) has been reported in areas of Africa, Asia, and Europe (Thomas and Duse, 2018; Xiang *et al*., 2020; Adam and Elhag, 2018; Gajdács *et al*., 2020). It has been reported that the overproduction of ESBL, combined with porin loss, as well as alteration or upregulated efflux pumps, may lead to carbapenem resistance in microorganisms (Wang *et al*., 2015a). A clinical study by Garcia-Fernandez *et al.* (2010) reported on ertapenem-resistant and ESBL-producing *K. pneumoniae* in Italy. These isolates carried a novel OmpK36 porin variant (OmpK36 V) that contributed to ertapenem resistance. The most prevalent ESBL genes found in *E. coli* isolates from the WWTP I and II were TEM and SHV (100%), the most reported ESBL genes in *E. coli* and *K. pneumoniae* (Bradford, 2001). A recent study by Zieliński *et al.* (2019) had also shown a high prevalence of the TEM gene in wastewater samples. Despite SHV being commonly identified in *K. pneumoniae* (Ojdana *et al*., 2014), *E. coli* isolates in this study showed a significant number harbouring this ESBL-type gene compared to *K. pneumoniae*.

A significant variation in the prevalence of OXA-1 and CTX-M genes was observed in the *E. coli* isolates obtained from WWP I and II. It has been reported that CTX-M hydrolyse cefotaxime, and OXA-1 hydrolyse ceftazidime (Ghafourian *et al*., 2015; Mandal *et al*., 2017). About 48% of CR

E. coli isolates from WWTP I showed resistance against cefotaxime, while 100% of the isolates harboured the CTX-M gene. This was similar to a study reported by Chandran *et al*. (2014), where *E. coli* isolates from hospital wastewater displayed a 48% resistance to cefotaxime while 88% of the isolates harboured the CTX-M gene. Similarly, 43% of the isolates were resistant to ceftazidime, while 98% harboured the OXA-1 gene. In WWTP II, majority of the CR *E. coli* isolates that were resistant to cefotaxime (85%) harboured the CTX-M gene which codes for resistance against cefotaxime. This indicated that a possible resistance against cefotaxime might be attributed to the presence of the CTX-M gene in their genome.

All the *K. pneumoniae* isolates obtained harboured the TEM gene. This correlated with a study done by Ojdana *et al.* (2014), which showed that TEM is the most prevalent ESBL gene in Gram-negative bacteria. Even though only 20 isolates were selected from aeration and pre-chlorinated effluent, both SHV and TEM genes were present in all the isolates. Ghafourin *et al.* (2015) reported that SHV was initially found in *K. pneumoniae* and has now spread to other microorganisms. This may have been attributed to their dominant detection in all the *K. pneumoniae* isolates from the aeration and pre-chlorinated effluent. A variation in the distribution of the ESBL genes was detected between the three treatment stages. The isolates obtained from the influent showed a higher prevalence of TEM and CTX-M genes, whereas, in the isolates from aeration and pre-chlorinated effluent, TEM and SHV genes were prevalent. Wagner *et al*. (2002) reported that a considerable change occurs in the distribution of the bacterial population during the treatment processes, which may have resulted in gene transfer (Fouz *et al*., 2020). The low resistance against ceftazidime (48%) may have contributed to the low detection of OXA-1 (13%) since this gene hydrolyses ceftazidime (Bradford, 2001). A considerable number of *K. pneumoniae* isolates were resistant to the cefotaxime antibiotics. This may have been influenced by the presence of the

CTXM gene since it hydrolyses this class of antibiotics. Several other studies have reported the prevalence of the CTX-M ESBL type gene in the clinical setting, making it the most prevalent ESBL in several countries, including Spain, France, India and the UK, and Argentina (El bouamria *et al*., 2015; D'Andrea *et al*., 2013).

All the ESBL genes detected in the isolates (both *E. coli* and *K. pneumoniae*) were reported from the clinical settings. This is not surprising because WWTP I sewage source included hospital effluent, and in WWTP II, the sewage was mainly from domestic wastewater. Although WWTP II mainly treated domestic waste, it also received some clinical waste from small community health settings.

The importance of these organisms includes their ability to transfer the resistant genes horizontally, from one organism to another since they are located on plasmids; they also carried additional resistance elements that may increase resistance to other antibiotic classes (Taggar *et al*., 2020; Lutgring and Limbago, 2016). Carbapenemase-producing *Enterobacteriaceae* are a threat to patients' safety due to their resistance to multiple antimicrobials, minimizing the therapeutic options to treat infected patients (ECDC, 2011). CPE infections are also associated with high mortality, where 26% - 44% was reported by Falagas *et al*. (2014). Currently, the three carbapenemases categorized as being most important are *Klebsiella pneumoniae* carbapenemase (KPC; Ambler class A), certain metalloenzymes (VIM, NDM; Ambler class B), and OXA-type enzymes (e.g., OXA-48; Ambler class D) (Nordmann *et al*., 2012a). These carbapenemases (NDM-1, OXA-48, and VIM) were also dominant in this study, both in *E. coli* and *K. pneumoniae*. The associated carbapenemase genes are frequently found on mobile genetic elements and have the potential to spread to other Gram-negative bacteria (Nordmann *et al*., 2012a).

Though the three classes of carbapenemases stated above were detected in both WWTPs, their distribution differed. The most prevalent carbapenemase genes detected in *E. coli* in WWTP I and II were NDM-1 (100%) and OXA-48 (52%), respectively. These results were similar to those reported by Singh-Moodley and Perovic (2016), where isolates from 46 laboratories (for diagnostic purposes) from seven provinces in South Africa displayed NDM-1 and OXA-48 as the dominant carbapenemase genes. Similarly, in a hospital setting, Thomas and Duse (2018) reported NDM-1 and OXA-48 genes as the dominant carbapenemase-encoding genes identified. The *K. pneumoniae* isolates also displayed the NDM-1 as the most prevalent gene. The NDM-1 gene has been reported in Europe, China, South Africa, and Algeria (Gajdács *et al*., 2020; Xiang *et al*., 2020; Ramsamy *et al*., 2020; Abderrahim *et al*., 2017). It has been proposed that it is suitable to use ertapenem antibiotics to screen for NDM-1 producers (Thomson, 2010). This correlates with the findings in this study, where most ertapenem-resistant isolates harboured the NDM-1 gene. The OXA-related enzymes have been reported by Dahiya *et al.* (2015) to be the second-largest family of β-lactamases (Dahiya *et al*., 2015). Its occurrence was also evident in our study, where the OXA-48 carbapenemase gene was the second most frequent detected gene. The high detection of OXA-48 (both *E. coli* and K. *pneumoniae*) may have contributed to the finding of resistance against imipenem. This is because the OXA-48 hydrolyses imipenem (Papp-Wallace *et al*., 2011). VIM carbapenemase gene was the least detected gene in this study and was only found in isolates from WWTP II. This correlates with a study reported by Zurfluh *et al.* (2017), where the VIM gene was also sporadically detected in *K. pneumoniae* isolates from wastewater. A specific pattern has been demonstrated by *K. pneumoniae*, where the distribution of genes differed from the influent to the pre-chlorinated effluent. This shows the dynamic of gene distribution that occurs within the WWTPs. This study showed several isolates that harboured two metallo-β-lactamase genes

(OXA48 and NDM-1, OXA-48, and VIM), which had also been reported by Bakthavatchalam *et al* (2016).

Limitations of the genotypic method employed should be noted, which may have resulted in false-negative results. Several carbapenemase genes were not included in the screening assay, for example, IMI, SPM, SIM, GES, KPC, which are reported in other studies (Yang *et al*., 2016; Zurfluh *et al*., 2017; Rizek *et al*., 2014; Zhou *et al*., 2020). Therefore, some carbapenemase genes may have been missed, and the actual proportion of CPE in this study could have been higher than that reported (Singh-Moodley and Perovic, 2016). Additionally, the difference in ESBL and carbapenemase gene prevalence may also be due to the difference in the methodology used for the isolation and cultivation of *E. coli* and *K. pneumoniae* isolates (Akiba *et al*., 2016).

To detect whether carbapenem-resistance is mediated by the carbapenemase enzyme production, the modified Hodge test was employed (Sharma *et al*., 2016). Based on the results obtained, WWTP II had a high prevalence of the OXA-48 (n=19) carbapenemase gene compared to WWTP I (n=5), thus displaying more positive results for carbapenemase expression. However, many other studies have reported that this method has some pitfalls which may lead to false-negative results though recommended by the CLSI (Hrabák *et al*., 2014; Yamada *et al*., 2016; Kumudunie *et al*., 2021). Several investigations, however, have shown that this phenotypic-based method often yielded low sensitivity and specificity for detection of carbapenemases, especially for class B carbapenemases, which includes NDM-1 and which mainly was present in almost all the isolates (Carvalhaes *et al*., 2010; Girlich *et al*., 2012; Netikul and Kiratisin, 2015; Pasteran *et al*., 2009). This was the case for *E. coli* isolates obtained from WWTP I and *K. pneumoniae* isolates from WWTP II, which showed a high prevalence of the NDM-1 gene. Additionally, for isolates that harboured the NDM-1 gene, resistance may also have been attributed to mechanisms such as efflux

pumps and porin loss channels. The efflux porins are the main resistance mechanism mediating resistance to carbapenems. Not all carbapenems interact in the same way with outer membrane proteins (OMPs); some OMPs are affected by certain carbapenems more than others (PappWallace *et al*., 2011). The mechanisms usually observed for loss of porin channels are plasmid-encoded AmpC enzymes combined with the loss of porin channels OmpK35/36, OmpF or OmpC for *E. coli* and *K. pneumoniae* (Fernández and Hancock, 2012; Hamzaoui *et al*., 2018). Another mechanism responsible for carbapenem resistance is the overproduction of the ESBL genes. This could have been the cause of carbapenem resistance in this study, as most isolates harboured a combination of more than one ESBL gene.

The assessment of virulence in *E. coli* included the detection of six virulence genes. Among these, *hly* and *rfbE* were detected in both WWTPs. This study correlated with that reported by Pillay and Olaniran (2016) and Ram *et al*. (2008), where the *hly* gene was the most prevalent virulence gene. The *hly* gene encodes for intimin, which is responsible for cell lysis and is known to affect renal, leukocytes, and erythrocytes cells (Ker'enyi *et al*., 2005). The *rfbE* gene is responsible for encoding flagella (H) antigens and the synthesis of O antigens. The flagellar protein carries the antigenic determinant(s) for the H antigen, while the O antigen contributes toward major antigenic variability to the cell surface (Wang and Reeves 1998; Wang *et al*., 2000). The least prevalent gene was the *eae* detected only in WWTP II at 8%. This gene is essential in the attachment stage of pathogenesis in the EPEC pathotype, where attachment to Hep-2 epithelial cells within a host is enabled by intimin, an outer membrane protein (Pillay and Olaniran, 2016). Despite the failure to detect *stx1, stx2,* and *fliC* (characteristic of EHEC) in the isolates, studies in South Africa (Pillay and Olaniran, 2016) and Poland (Osińska *et al*., 2018) have reported their presence in untreated and treated wastewater.

The *K. pneumoniae* isolates only harboured one gene, *wabG*, out of the three investigated genes. Though the occurrence of the gene detected in isolates was low in the influent (18%), there was an increase in the pre-chlorinated effluent (50%), showing 10 out of 20 isolates to harbour this gene. This could have resulted from HGT that occurred in the activated sludge as reported in previous studies (Berendonk *et al*., 2015; Rizzo *et al*., 2013b). WWTPs are a hub for HGT due to the favourable conditions they offer (Rizzo *et al*., 2013b).

The *wabG* gene encodes for a GalA transferase enzyme involved in lipopolysaccharide production (Regue *et al*., 2004). The prevalence of this gene harboured by *K. pneumoniae* isolates in clinical settings has been reported at 88 and 88.5%, according to Candan and Aksöz (2015) and Hasani *et al*. (2020) respectively. However, environmental isolates have only been sparsely studied. In a study by Zhang *et al.* (2015), it was reported that increased antibiotic resistance was strongly associated with enlarged carriage of virulence factors in *E. coli* (Zhang *et al.,* 2015), highlighting the difficulties that could arise from attempting to control resistance in pathogenic bacteria. This link could not be established with the genes used for screening in this study, especially with *K. pneumoniae,* since only a small percentage of isolates harboured the virulence genes. This was also reported in a review by Paczosa and Mecsas (2016), where they reported that the carriage and expression of drug resistance do not enhance the virulence of *K. pneumoniae*. It can be deduced that through the presence of these virulence genes, both in *E. coli* and *K. pneumoniae,* the isolates may be pathogenic, even though this study did not further investigate their expressions.

5.5 Conclusions

The findings in this study highlight the importance of WWTPs as a mirror to what might occur and exist in the communities. The investigated WWTPs have shown to be reservoirs of viable carbapenemase-producing *E. coli* and *K. pneumoniae* isolates. A total of 3 carbapenemase genes, NDM-1, OXA-48, and VIM, were detected in both WWTPs, with NDM-1 being the most dominant one. WWTP I, receives wastewater from the local hospital; thus, the *E. coli* isolates from WWTP I showed the highest number of ESBL and carbapenemase genes compared to WWTP II. However, even though many isolates harboured the carbapenemase genes, this did not equate to the expression of these genes. This could be due to other resistance mechanisms such as overproduction of ESBL combined with efflux pumps and porin loss. A few isolates, both *E. coli* and *K. pneumoniae*, harboured virulence genes indicating their possible pathogenicity. However, the findings in this study do not fully reflect the virulence of the isolates since only a limited number of virulence genes were assessed. Therefore, future research should include virulence genes that code for different virulence factors to understand the pathogenicity of the isolates better. Additionally, research around the other carbapenem-resistant mechanisms also needs to be investigated.

6 CHAPTER 6: SUMMARY AND CONCLUSIONS

6.1 Summary

Carbapenems are antibiotics used as last resort to treat infections caused by Gram-negative bacteria. Additionally, no antibiotics have been developed that have the same spectrum of activity. This study focused on the isolation and characterization of carbapenem-resistant *E. coli* and *K. pneumoniae* from wastewater treatment plants and their receiving surface water bodies. This was motivated by the WHO (2017) statement, which prioritised pathogenic bacteria, among which those resistant to carbapenems needed enhanced research attention. Since carbapenems are antibiotics mostly used in the hospital setting, most research in this area has also been limited to clinical infections, even in South Africa. As proposed by the US EPA, investigating sewage isolates could be more informative to understand the occurrence and dissemination of antimicrobial resistance within the aquatic environment (Boczek *et al*., 2007). Additionally, WWTPs have been reported to be reservoirs for antibiotic resistance (Hoelle *et al*., 2019; Fouz *et al*., 2020). This is due to the presence of a different type of antibiotics and bacterial communities, which may carry and transfer the resistance genes across the bacterial species combined with the nutrient-rich environment favouring bacterial survival (Xu *et al*., 2015; Berendonk *et al*., 2015). Therefore, understanding the role of WWTPs in the development and transmission of these carbapenem-resistant isolates in the environment is of utmost importance as wastewater reuse has become more prominent as a source of water to overcome water shortage in different sectors.

Two WWTPs, mainly treating domestic wastewater, were investigated to assess the occurrence of carbapenem-resistant *E. coli* and *K. pneumoniae* and their virulence factors. Additionally, their

removal within the different stages of treatment processes and the effect on receiving surface water bodies were also studied. The results and conclusion of this study are as follows:

6.1.1 Objective 1

The samples analysed were the influent, aeration/biofilter, secondary effluent, final effluent, upstream and downstream river samples. A total of 120 *E. coli* (WWTP I and II) and 100 *K. pneumoniae* (WWTP II) isolates were obtained. Among these, the CR *E. coli* isolates were only detected in the influent samples, with WWTP I showing a lower percentage (0.01%) compared to WWTP II (0.07%). CR *K. pneumoniae*, on the other hand, was detected in the influent, aeration, and pre-chlorinated effluent samples of WWTP II at 0.34%, 3.03% and 1.33%, respectively. However, it was absent in WWTP I. All isolates (*E. coli* and *K. pneumoniae*) were positive for biochemical tests.

Although the two WWTPs are situated in different locations, the difference in the total *E. coli* count was not significant. The total *K. pneumoniae* isolates were detected at higher concentrations compared to *E. coli*. The two WWTPs harboured CR *E. coli,* and WWTP II harboured CR *K. pneumoniae*, though in small concentrations. Since the WWTPs use different treatment systems, it was noted that the WWTP II with conventional activated sludge system had a slightly higher removal rate (from the influent to the aeration), as compared to the WWTP I with biofilter configuration. The WWTPs were effective in reducing total *E. coli* and *K. pneumoniae* counts, as the removal efficiency was observed at 99,99 and 99.98%, respectively. Complete removal of CR *E. coli* was observed at the aeration and biofilter tanks for WWTP I and II. For CR *K. pneumoniae*, the complete reduction was observed after chlorination as there were no isolates detected in the post-chlorinated effluent samples during any of the sampling events. The upstream and downstream sample analysis further showed that the post-chlorinated effluent had little or

negligible impact on the recipient rivers, as there was no additional microbial load observed in downstream samples compared to the upstream samples.

6.1.2 Objective 2

The presumptive identification of the isolates was performed using biochemical tests. For further confirmation of the isolates PCR, MALDI-TOF, and 16S rRNA sequencing and analysis were conducted on selected isolates. All 120 *E. coli* isolates were successfully identified and verified using both MALDI-TOF and the species-specific PCR. The *mdh* gene used to confirm *E. coli* showed 100% specificity as all isolates were positive. However, for *K. pneumoniae*, MALDI-TOF was only able to detect 8 out of 60 isolates as positives, while the remaining 52 isolates were identified as *K. oxytoca*, *Enterobacter*, *Aeromonas*, unidentified, and others. Using PCR based on two sets of specific primers *(rcsA* and *its*), a positive amplification (57% and 100%, respectively) was achieved. This resulted in sequencing the 60 isolates sent for MALDI-TOF using the *its* amplicons, and 88% isolates were confirmed as *K. pneumoniae* and the remaining 12% as *Enterobacter* species.

6.1.3 Objective 3

To determine the antibiotic resistance profile of the confirmed isolates, the disk diffusion method was used against third-generation cephalosporins and carbapenem antibiotics. The antimicrobial susceptibility tests showed a high prevalence of resistance against cefixime and ertapenem, respectively, both among *E. coli* and *K. pneumoniae* isolates (100%). Resistance to cefixime in *E. coli* from WWTP I and II was 68% and 92%, respectively, and 81% in *K. pneumoniae*. For the third-generation cephalosporins, the isolates were least resistant to ceftazidime. Resistance to

ertapenem *in E. coli* from WWTP I and II was 100% and 95%, respectively, and 100% for *K. pneumoniae*.

The *E. coli* and *K. pneumoniae* isolates showed high resistance against the third-generation cephalosporins and carbapenem antibiotics. This could indicate the multidrug resistance nature of these isolates.

6.1.4 Objective 4

All CR *E. coli* and *K. pneumoniae* isolates harboured both ESBL and carbapenemase genes. The prevalent ESBL genes in *E. coli* were TEM, SHV and CTX-M (100%) in WWTP I and TEM and SHV (100%) in WWTP II. In CR *K. pneumoniae*, TEM (75%) followed by CTX-M (62%) were most prevalent in the influent. A shift occurred in the aeration and pre-chlorinated effluent samples, where TEM and SHV (100%) were prevalent. Similar carbapenemase gene profiles were observed in both WWTPs but at different prevalences for *E coli*. The dominant carbapenemase genes in CR *E. coli* in WWTP I was NDM-1 followed by OXA-48, whereas in WWTP II, the dominant gene was OXA-48 followed by NDM-1. For CR *K. pneumoniae* the dominant gene in the influent was NDM-1 followed by OXA-48; in the aeration, it was NDM-1 and in the pre-chlorinated effluent, only VIM was detected. Though several bacteria harboured carbapenemase genes, only a fraction of 5 (WWTP I) and 19 (WTP II) CR *E. coli*, and 31 CR *K. pneumoniae* (WWTP II) isolates expressed carbapenemase production via the modified Hodge test.

Among the virulence genes analysed, two (*hly* and *rfbE*) out of six genes were detected in *E. coli* in both plants. WWTP II had the highest prevalence of these genes compared to WWTP I. However, among *K. pneumoniae* isolates, only *wabG* was found among the isolates recovered from the influent to the post-chlorinated effluent.

The two WWTPs were observed to be the reservoirs for common ESBL and carbapenemase genes carrying *E. coli* and *K. pneumoniae* isolates. Most of these isolates did not produce the carbapenemase enzyme, indicating that other mechanisms might be responsible for carbapenem resistance in the isolates. In addition, no significant relationship was observed between the identified resistance genes and virulence genes in this study.

6.2 Major conclusions

- The raw influent of the investigated WWTPs highlighted the presence of carbapenem-resistant *E. coli* and *K. pneumoniae.*
- Both WWTPs investigated were efficient in removing these pathogens with a removal efficiency of 100% through the aeration/ biofilter tanks for CR *E. coli*, and chlorination for CR. *K. pneumoniae*.
- The post-chlorinated effluent did not show any significant impact on the recipient rivers with regard to the total bacterial count.
- The biochemical tests, MALDI-TOF, and the primer used for PCR to detect *E. coli* were reliable as all isolated were positively identified. For *K. pneumoniae*, MALDI-TOF and PCR using the *rcsA* gene showed major discrepancies in the confirmation of *K. pneumoniae* isolates compared to other commonly used techniques.
- Amongst the third-generation cephalosporin antibiotics tested for susceptibility, both *E. coli* and *K. pneumoniae* were resistant to cefixime. Amongst the carbapenem antibiotics, the isolates showed high resistance against ertapenem.
- The study provided valuable data on the occurrence of the most popular genes (NDM-1 and OXA-48) responsible for the proliferation of carbapenemase-producing *E. coli* and *K. pneumoniae*.

- The presence of an investigated carbapenemase gene does not equate to the production of the carbapenemase enzyme. Thus, additional resistance mechanisms need to be investigated for a better understanding of carbapenem resistance.
- The isolates obtained from the WWTPs may be of importance due to their composition of clinically relevant resistance and virulence genes.

6.3 Future recommendations

Future research could focus on applying emerging/advanced molecular methods for detecting the microbes at lower concentrations than the conventional PCR to understand the occurrence and spread of these microbes in the aquatic environment. Furthermore, the detection of additional carbapenemase genes and virulence genes in future research is recommended to understand the actual proportion of carbapenemase-producing *Enterobacteriaceae* and their virulence nature within the aquatic environment. Additionally, the co-existence of other mechanisms of resistance within the carbapenem-resistant *Enterobacteriaceae* should be explored. Future research to determine the presence of viable but not culturable (VBNC) carbapenem-resistant *Enterobacteriaceae* in these treated effluent and recipient water bodies is hereby recommended as these water bodies are routinely used for agricultural, industrial and household purposes by the local communities. The use of flow cytometric methods for such study to determine the VBNC carbapenem-resistant *Enterobacteriaceae* in this studied aquatic milieu might be novel and contributory to the advocacy by WHO (2017) priority 1. So, it is strongly recommended.

7 CHAPTER 7: REFERENCES

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8 CHAPTER 8: APPENDICES

APPENDIX 1: (Chapter 3)

8.1 Antibiotics preparation

8.1.1 Meropenem

1 mg of the powder antibiotic was weighed in an analytical balance. This was then dissolved in 1 mL of sterile distilled water, mixed, filtered and dispersed in a sterile 2 mL Eppendorf tube. This was stored at -4°C until use.

8.1.2 Cloxacillin

50 mg of the powder antibiotic was weighed in an analytical balance. This was then dissolved in 1 mL of sterile distilled water, mixed, filtered and dispersed in a sterile 2 mL Eppendorf tube. This was stored at -4°C until use.

APPENDIX 2: (Chapter 3)

8.2 Media preparation

8.2.1 *E. coli* media (CHROMagar ECC™)

6.56 g of the powder base was mixed in 199 mL of sterile distilled water in a glass beaker. The dehydrated culture media was stirred until dissolved. This was heated to boil to dissolve the medium completely and regularly stirred. The media was cooled, and a volume of 50 µL meropenem and 800 µL cloxacillin antibiotics was aseptically added. The media was poured into Petri dishes, allowed to solidify and stored at 4°C until use.

8.2.3 *K. pneumoniae* media (Klebsiella *ChromoSelect* Selective Agar Base)

8.12 g of the powder base was mixed in 199 mL of sterile distilled water in a glass beaker. The dehydrated culture media was stirred until dissolved. This was heated to boil to dissolve the medium completely and regularly stirred. The media was cooled, and the rehydrated contents of 1 vial of Klebsiella Selective Supplement. Directly after, a volume of 50 µL meropenem and 800 µL cloxacillin of the antibiotics was aseptically added. The media was poured into Petri dishes, allowed to solidify and stored at 4°C until use.

APPENDIX 3: (Chapter 4)

Results for the confirmation of the 60 presumptive K. *pneumoniae* isolates through MALDI-TOF, PCR and 16rRNA sequencing.

		PCR		
Isolate ID	MALDI-TOF	*its* gene	*rcsA* gene	16S rRNA sequencing
1	*Enterobacter kobei*	+	-	-
2	*Enterobacter kobei*	+	-	-
3	No ID	+	-	+
4	*Enterobacter asburiae / cloacae*	+	-	+
5	No ID	+	-	+
6	*Enterobacter kobei*	+	-	+
7	*Klebsiella pneumoniae*	+	+	+
8	No ID	+	-	+
9	*Klebsiella pneumoniae / variicola*	+	+	+
10	*Aeromonas punctata (caviae)*	+	-	+
11	*Aeromonas punctata (caviae)*	+	-	+
12	*Klebsiella pneumoniae*	+	+	+
13	*Aeromonas punctata (caviae)*	+	-	+
14	*Aeromonas veronii / sobria*	+	-	+
15	*Enterobacter kobei*	+	+	+
16	*Enterobacter asburiae / cloacae / kobei*	+	-	+
17	*Enterobacter kobei*	+	+	-
18	*Enterobacter kobei*	+	-	+

19	*Enterobacter kobei*	+	+	-
20	*Aeromonas punctata (caviae)*	+	-	+
21	*Citrobacter freundii / braakii*	+	+	+
22	*Raoltella ornithinolytica*	+	-	+
23	No ID	+	+	+
24	*Klebsiella pneumoniae*	+	+	+
25	*Pseudomonas putida*	+	+	+
26	*Klebsiella oxytoca*	+	+	+
27	*Aeromonas punctata (caviae)*	+	-	+
28	*Klebsiella pneumoniae*	+	+	+
29	No ID	+	-	+
30	*Citrobacter freundii*	+	+	+
31	*Aeromonas punctata (caviae)*	+	+	+
32	*Enterobacter kobei*	+	+	-
33	*Klebsiella pneumoniae*	+	-	+
34	*Pseudomonas putida*	+	+	+
35	*Enterobacter kobei*	+	-	+
36	*Aeromonas punctata (caviae)*	+	+	+
37	*Enterobacter kobei*	+	+	+
38	*Aeromonas punctata (caviae)*	+	+	+
39	*Klebsiella pneumoniae*	+	+	+
40	*Enterobacter asburiae / cloacae*	+	+	+
41	*Enterobacter asburiae / cloacae*	+	+	+
42	*Klebsiella oxytoca*	+	+	+
43	*Enterobacter kobei*	+	+	-

44	*Enterobacter asburiae / cloacae*	+	+	+
45	*Enterobacter asburiae / cloacae*	+	+	+
46	*Enterobacter kobei*	+	+	-
47	*Klebsiella oxytoca*	+	+	+
48	*Klebsiella pneumoniae*	+	+	+
49	No ID	+	-	+
50	*Enterobacter asburiae / cloacae*	+	-	+
51	*Aeromonas punctata (caviae)*	+	-	+
52	*Aeromonas punctata (caviae)*	+	+	+
53	*Aeromonas punctata (caviae)*	+	-	+
54	*Aeromonas punctata (caviae)*	+	+	+
55	*Aeromonas punctata (caviae)*	+	+	+
56	*Klebsiella oxytoca*	+	-	+
57	*Enterobacter asburiae / cloacae*	+	+	+
58	No ID	+	+	+
59	*Aeromonas punctata (caviae)*	+	-	+
60	No ID	+	-	+

+: positive; -: negative; No ID: not identified by MALDI-TOF

APPENDIX 4: (Chapter 4)

The susceptibility of the isolates was determined according to the CLSI standards as indicated below;

Class	Antimicrobial agent	Disk content	Interpretive categories and zone diameter breakpoints		
			S	I	R
Third-generation cephalosporins	Cefixime	5 µg	≥ 19	16 - 18	≤ 15
	Cefotaxime	30 µg	≥ 26	23 - 25	≤ 22
	Ceftazidime	30 µg	≥ 21	18 - 20	≤ 17
Carbapenem	Ertapenem	10 µg	≥ 22	19 - 20	≤ 18
	Meropenem	10 µg	≥ 23	20 - 22	≤ 19
	Doripenem	10 µg	≥ 23	20 - 22	≤ 19
	Imipenem	10 µg	≥ 23	20 - 22	≤ 19

S: susceptible, I: intermediate; R: resistant

APPENDIX 5: (Chapter 4)

Total *E. coli* and K. *pneumoniae* counts (CFU/100 mL) in the post-chlorinated effluent and river samples; upstream and downstream.

	WWTP I *E. coli*			**WWTP II *E. coli***			**WWTP II *K. pneumoniae***		
	US	PCE	DS	US	PCE	DS	US	PCE	DS
Jan	2,8	1,9	2,6	2,9	2,5	2,9	3,5	3,3	3,4
Feb	2,8	2	2,5	3	2,6	2,8	3,4	3,5	3,3
Mar	2,7	2,1	2,5	3	2,7	2,6	3,5	3,4	3,5
Apr	2,7	2,2	2,6	2,8	2,4	2,7	3,5	3,4	3,4
May	2,7	2	2,5	3	2,5	2,8	3,4	3,3	3,3
Jun	2,8	2	2,5	2,7	2,4	2,7	3,4	3,3	3,3
Jul	2,7	2,1	2,6	2,9	2,4	2,6	3,4	3,4	3,3
Aug	2,7	2,3	2,6	2,9	2,3	2,8	3,6	3,6	3,5
Sept	2,7	2	2,6	3	2,6	2,7	3,5	3,6	3,4
Oct	2,3	2,2	2,3	2,8	2,6	2,8	3,5	3,5	3,5
Mean	2,7	2,1	2,5	2,9	2,5	2,7	3,5	3,4	3,4
SD	0,14	0,12	0,09	0,1	0,12	0,09	0,06	0,11	0,08

WWTP: wastewater treatment plants; US: upstream; PCE: post-chlorinated effluent; DS: downstream