Molecular investigation of the chlorine and antibiotic resistance mechanisms of *Escherichia coli* isolated from natural water sources in the Western Cape

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Declaration

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ABSTRACT

Water is used for various purposes and contamination can have severe implications if untreated. One of the most common and cost effective water disinfectants, especially used in developing countries, is chlorine. However, microorganisms have developed different mechanisms in response to environmental stress conditions, such as the viable but non-culturable (VBNC) effects possibly displayed in this study, enabling them to survive. Chlorine may also exert several effects on microorganisms, such as the expression of multi-substrate efflux pumps, decreased membrane permeability and transport inhibition that may lead to chlorine tolerance and antimicrobial resistance. In a descriptive and comparative study, the molecular characteristics of *E. coli* strains isolated from environmental waters in the Western Cape and the possible relationship between chlorination and antimicrobial resistance were investigated.

Water and biofilm samples were exposed to chlorine, as well as efflux pump inhibitor (EPI) concentrations, and surviving *E. coli* strains were tested for their phenotypic characteristics including antimicrobial susceptibility profiles and morphological types. Candidate genes possibly involved in resistance to antimicrobials, disinfection and efflux pumps were detected with polymerase chain reaction (PCR) and sequenced. Sequencing analysis and homology searches were done and *E. coli* strains were typed as either Enteropathogenic *E. coli* strains (EPEC) or Enterotoxigenic *E. coli* strains (ETEC) on the presence of virulence genes.

All water and biofilm sources examined were heavily polluted with *E. coli*, and a high enumeration level of this indicator organism of faecal contamination was recorded. Chlorine tolerance was found to be associated with antimicrobial resistance. Addition of EPI with exposure to chlorine decreased enumeration levels of these organisms, suggesting that efflux pumps may play a role in tolerance to chlorine. Several morphological patterns were described amongst the *E. coli* strains and a change in this was recorded after exposure to chlorine. Highly resistant antibiograms displayed by the isolated strains included *ampC* β-lactamase producing *E. coli* strains and extended spectrum β-lactamases (ESBLs). Amplification of the candidate genes selected for heat-shock, oxidative stress genes and efflux pump were most frequently detected while the structural genes involved in fluoroquinolones (FQs) resistance were detected less frequently in the selected strains. Sequencing of these amplified candidate genes demonstrated various changes in amino acid sequences, including one common

mutational pathway taken by *E. coli* when exposed to stress conditions. Further homology searches of the sequenced candidate genes illustrated similarities in 19 pathogenic and 14 non-pathogenic *E. coli* as well as 3 *Shigella* strains. Detection of virulence genes found three EPEC strains (*bfpA*, *eaeA*), two EPEC (*eaeA*), ten EPEC (*bfpA*) and one ETEC strain (*st*) amongst the isolates.

This study underlines the need for monitoring our water sources, which poses a public health risk due to incomplete chlorination, antimicrobial resistance and the spread of clinically relevant pathogenic strains.

OPSOMMING

Water word vir baie doeleindes gebruik en kontaminasie het verskeie implikasies indien water onbehandeld is. Een van die mees algemeenste en koste-effektiewe ontsmettingsmiddels algemeen gebruik in ontwikkelde lande is chloor. Mikroorganismes het egter verskillende meganismes ontwikkeld in reaksie tot omgewingstres toestande, soos die 'lewendig maar nie-kweekbare effek' moontlik getoon in hierdie studie, en oorleef behandeling met hierdie middel. Chloor kan ook verskeie effekte op mikroorganismes uitoefen, soos die uitdrukking van multi-substraat effluks pompe, afname in membraan deurlaatbaarheid en die inhibisie van transport wat kan lei tot toleransie van chloor en antimikrobiese weerstandigheid. In 'n beskrywende en vergelykende studie is die molekulêre eienskappe van *E. coli* tipes geïsoleer van omgewingswaters in die Wes-Kaap en die moontlike verhouding tussen chlorinasie en antimikrobiese weerstandigheid ondersoek.

Water en biofilm monsters was blootgestel aan chloor sowel as effluks pomp inhibitor (EPI) konsentrasies en *E. coli* tipes wat dit oorleef het was verder getoets vir hul fenotipiese eienskappe insluitend antimikrobiese sensitiwiteits profiele en morfologiese tipes. Kandidaat gene moontlik betrokke by weerstandigheid teen antimikrobiale middels, chloor en effluks pompe was met polimerase kettingreaksie (PKR) geïdentifiseer en die nukleïensuur volgorde is bepaal. Volgorde analises en homologie soektogte was gedoen en *E coli* tipes is getipeer as Enteropatogeniese *E. coli* (EPEC) of Enterotoksigeniese *E. coli* (ETEC) tipes na aanleiding van die teenwoordigheid van virulensie gene.

Al die water en biofilm bronne ondersoek was hoogs besoedeld met *E. coli* en 'n hoë enumerasie vlakke van hierdie indikator organisme vir fekale kontaminasie is beskryf. 'n Verwantskap tussen chloor toleransie en antimikrobiese weerstandigheid is aangetoon. Byvoeging van EPI het 'n afname in die enumerasie vlakke van die organismes na chloor behandeling veroorsaak, wat 'n aanduiding gegee het dat effluks pompe dalk 'n rol in chloor toleransie kan speel. Verskeie morfologiese vorme is beskryf onder die *E. coli* organismes en 'n verandering na blootstelling aan chloor en verskillende antimikrobiese agente is aangetoon. Hoogs weerstandige antibiogramme, insluitend *ampC* β-laktamase produserende *E. coli* tipes en uitgebreide spektrum β-laktamases (ESBL), is beskryf. Amplifisering van die kandidaatgene geselekteer vir hitte-skok, oksidatiewe stres en effluks pomp was mees algemeen, terwyl strukturele gene betrokke by FQs minder waargeneem was in die isolate. Addisionele volgordebepaling van die geamplifiseerde

kandidaat gene het verskeie veranderinge in die aminosuur volgordes gedemonstreer, insluitend een algemene mutasie roete wat deur *E. coli* gevolg word wanneer dit aan stres kondisies blootgestel word. Verdere homologie soektogte van die geenvolgordes het 19 patogeniese en 14 nie-patogeniese *E. coli* tipes, sowel as 3 *Shigella* tipes, geïdentifiseer. In teenstelling, die aantoning van virulensie gene het verder 3 EPEC tipes (*bfpA*, *eaeA*), twee EPEC (*eaeA*), tien (*bfpA*) en een ETEC tipe (*st*) onder die isolate geïdentifiseer.

Hierdie studie bevestig die noodsaaklikheid vir monitering van ons waterbronne, wat dien as ’n publieke gesondheidsrisiko, as gevolg van onvolledige chlorinasie, antimikrobiale weerstandigheid en die verspreiding van klinies relevante patogeniese organismes.

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List of Abbreviations

ABC:	ATP-binding cassette family
Acr:	Acriflavine resistant protein
ADP:	Adenosine diphosphate
AIDS:	Acquired Immunodeficiency Syndrome
ATCC:	American Type Culture Collection
ATP:	Adenosine triphosphate
bfp:	bundle-forming pilus
BGLB:	Brilliant Green Lactose Bile Broth
BLAST:	Basic Local Alignment Sequence Tool
CAF:	Central Analytical Facility
CCCP:	Carbonyl cyanide-m-chlorophenylhydrazone
CFU:	Colony forming unit
CLDT:	Cytolethal distending toxin
CLSI:	Committee for Clinical Laboratory Standards
CTX-M:	Cefotaxime resistant ESBL
DNA:	Deoxyribonucleic acid
DWAF:	Department of Water Affairs and Forestry
eae:	attachment and effacement factor (Intimin)
EAggEC:	Enteroaggregative *Escherichia coli*
EAF:	Adherence factor
E. coli:	*Escherichia coli*
EHEC:	Enterohaemorrhagic *Escherichia coli*
EIEC:	Enteroinvasive *Escherichia coli*
EPEC:	Enteropathogenic *Escherichia coli*
EPI:	Efflux pump inhibitor
ESBLs:	Extended spectrum β-lactamases
ETEC:	Enterotoxigenic *Escherichia coli*
FQs:	Fluoroquinolones
H-antigen:	Flagellar surface antigen
H_2O_2:	Hydrogen peroxide
HAAs:	Halogenated acetic acids
HIV:	Human Immunodeficiency Virus
HOCl:	Hypochlorous acid
IDT:	Integrated DNA Technologies
K-antigen:	Capsular surface antigen

LB:	Luria-Bertani
lt:	Heat-labile toxin
MAR:	Multiple antibiotic resistance
Mast:	Mueller-Hinton sensitivity agar plates
MDR:	Multidrug resistance
MFP:	Membrane fusion protein
MFS:	Major facilitator super family
NCCLS:	National Committee for Clinical Laboratory Standards
NHLS:	National Health Laboratory
NMMP:	National Microbial Monitoring Programme
NTU:	Nephelometric unit
O-antigen:	Somatic surface antigen
O_2:	Oxygen
O_3:	Ozone
OCl^-:	Hypochlorite ion
OD:	Optical Density
OM:	Outer membrane
OMF:	Outer membrane factor
PaβN:	Phe-Arg-β-naphthylamide
PCR:	Polymerase Chain Reaction
PKR:	Polimerase kettingreaksie
QRDR:	Quinolones resistance-determining region
RND:	Resistance-nodulation-division
SABS:	South African Bureau of Standards
SHV-1:	Sulfhydryl variable (β-lactamase which attack narrow-spectrum cephalosporins)
SMR:	Small multidrug resistance
st:	Heat-stable toxin
TAC:	Treatment Action Campaign
TEM-1:	β-lactamase class A (named for a patient called Temoniera)
THMs:	Trihalomethanes
T_m:	Melting point
UPEC:	Uropathogenic *Escherichia coli*
UV:	Ultraviolet
VBNC:	Viable-but-non-culturable
WHO:	World Health Organization

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Chapter One

Introduction

1.1. Background to the problem related to water contamination

Access to clean water is one of the main concerns in many countries, especially the poorer developing ones. This is due to contamination of water sources caused by many factors including agricultural waste, animal excreta, industrial effluent, or sewage disposal.[1] One of the main factors associated with water contamination is sanitation and in many cases, communities with poor access to clean water have failed sanitation facilities which are commonly associated with urban, peri-urban and rural areas.[2] In such areas in South Africa issues such as poverty levels and overcrowded informal settlements contribute to water contamination. Additional factors including minimal and under serviced sanitation works, inadequately maintained sewage systems, shortage of skilled workers and properly designed sanitation treatment plants compounds the problem. It has been shown that when sanitation is poorly managed, large quantities of untreated waste (sewage) which may contain high numbers of microorganisms can be released into various water sources, such as surface water (water that systems pump and treat from sources open to the atmosphere such as rivers, lakes, and reservoirs), groundwater (water that systems pump and treat from natural reservoirs below the earth's surface). Other water sources include raw water (water in its natural state, prior to any treatment for drinking) and wastewater in dams which may act as a large reservoir of human enteric bacteria.[3] Contaminated water sources may contain microorganisms, including bacteria, viruses and protozoa that can be pathogenic to humans or animals.[4] The application of such waters for consumption or irrigation can be undesirable, if not properly disinfected. Diseases caused by contaminated water may vary in severity and clinical presentation such as mild to fatal diarrhea, dysentery, cholera and hepatitis [5] In addition, the effect of waterborne pathogens in a country such as South Africa where HIV/AIDS (Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome) epidemic is severe, may be even more serious.[6]

An important microorganism associated with the quality of water is *E. coli*.[7] If high numbers of *E. coli* are detected in water, it indicates that other microorganisms may be present, including possible pathogenic organisms. The contamination of water with these organisms can be prevented with effective and sustainable strategies. However, if water contaminated with microorganisms is left untreated and consumed for drinking purposes or

food preparation, it may be harmful to the health of the immunocompromised, the sick, the elderly, infants and young children as well as animals.[8] A study on the use of disinfected water an proper water storage and sanitation conditions had shown that diarrhea were reduced by 20-30%.[9] As safe water facilities are a global issue, various countries contributed to a research study of their water sources for the detection of pathogens involved in major orally transmitted infections of high priority. This resulted in the formation of the World Health Organisation (WHO) Water Quality Guidelines, a summary of which are given in Table 1.1.[10] The information presented in this table is given as a guideline only as the microbial content of water sources may differ and should be managed according to the environmental conditions.[11]

1.2. Diseases caused by various microorganisms related to water

One of the most clinically important diseases in humans and animals related to contaminated water is diarrhea. In 2001 it was estimated that across the globe 1.8 billion episodes of childhood diarrhea occurred annually, mostly in developing countries.[12] If not properly managed, each episode of diarrhea can further contribute to malnutrition and growth retardation.[13] In South Africa, diarrhea in infants and young children is still a major cause of morbidity and mortality in both rural and urban populations. Clinical data obtained from the health statistics database of South Africa showed incidence rates for diarrhea amongst children under the age of 5 years per 1000 population of 268.7 in 2005 and 214.9 in 2006.[14] Waterborne diseases can be avoided if proper water management is practiced, which includes regular monitoring of water sources and proper disinfection strategies.

E. coli is often the causative agent of diarrhea. Strains that cause diarrhea, acute gastroenteritis or colitis in humans are referred to as diarrheagenic or enterovirulent *E. coli*. At present there are several recognised classes of enterovirulent *E. coli*, namely ETEC, EPEC, enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EaggEC), diarrhea-associated haemolytic *E. coli* and cytolethal distending toxin (CLDT)-producing *E. coli* (which possesses virulence factors of EPEC or EaggEC).[15] Other serious diseases caused by this pathogen are associated with the urinary tract and wound infections.[16] The capability of these strains causing disease are reflected in the antigenic diversity of these bacteria. They are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles. Each of the six categories has a different pathogenesis and comprises a different set of O:H serotypes. In South Africa, ETEC and EPEC are casual agents in 8-42% of diarrhea incidences.[15]

Therefore, as part of this study the virulence genes of EPEC and ETEC *E. coli* strains in isolates obtained from several water sources were detected. EPEC strains cause either bloody or watery diarrhea and are linked to infant diarrhea.[17] Virulence factors include the genes for the attachment and effacement factor called intimin (*eae*), the bundle-forming pilus (*bfp*) and the EPEC adherence factor (*eaf)* plasmid. Transmission of EPEC is via the faecal-oral route. ETEC strains contain a heat-labile toxin (*lt*) and heat-stable toxin (*st*) or both.[18] It is associated with two major clinical syndromes: traveler's diarrhea and weaning diarrhea in children in developing countries. It causes watery diarrhea, usually without blood or mucus, and is similar to diarrhea caused by *Vibrio cholerae*. Transmission is via faecally contaminated food and water.

A major consequence of water contamination is outbreaks associated with waterborne pathogens. In 2006, the FDA and the State of California reported an *E. coli* O157:H7 outbreak. A batch of spinach tested positive for this EHEC strain and was confirmed to have been treated with water suspected to be the source of contamination. Samples obtained from cattle faeces on one of the nearby ranches, tested positive for EHEC. The genetic fingerprints of EHEC matched the *E. coli* strains that infected 199 people with 3 fatalities confirming the outbreak.[19] In South Africa, Delmas in Mpumalanga, a *Salmonella typhoid* outbreak occurred in 2005, which was speculated to be related to contaminated water.[20] According to the Treatment Action Campaign (TAC), the actual number of deaths from typhoid at Delmas in Mpumalanga was higher than the official figure of three.[21] The media reported a similar outbreak in Mpumalanga in 2007, which affected 648 people since October 22.[22] Bacterial contamination was suspected, but the investigation authorities confirmed chemical pollution as the cause.[22]

Table 1.1. Orally transmitted waterborne pathogens and their significance in water supplies (extracted from WHO Guidelines for Drinking Water Quality 1993) [10]

Pathogen	Health significance	Persistence in water supplies[a]	Resistance to chlorine[b]	Relative infective dose[c]
Bacteria:				
Pathogenic *E. coli*	High	Moderate	Low	High
Salmonella typhi	High	Moderate	Low	High
Other Salmonellas	High	Long	Low	High
Shigella spp.	High	Short	Low	Moderate
Vibrio cholerae	High	Short	Low	High
Yersinia enterocolitica	High	Long	Low	High
Pseudomonas aeruginosa	Moderate	May multiply	Moderate	High (?)
Aeromonas spp.	Moderate	May multiply	Low	High (?)
Viruses:				
Adenoviruses	High	?	Moderate	Low
Enteroviruses	High	Long	Moderate	Low
Hepatitis A	High	?	Moderate	Low
Enterically transmitted Non-A, non-B hepatitis viruses, hepatitis E	High	?	?	Low
Norwalk virus	High	?	?	Low
Rotavirus	High	?	?	Moderate
Small round viruses	High	?	?	Low (?)
Protozoa:				
Entamoeba histolytica	High	Moderate	High	Low
Giardia intestinalis	High	Moderate	High	Low
Cryptosporidium parvum	High	Long	High	Low

[a]Period detected in water at 20 C – Short up to 7 days, Moderate: 7-30 days, Long: >30 days
[b]Water treatment at conventional doses and contact times – Moderate resistance: organisms not completely destroyed
[c]Dose required to cause infection in 50% of healthy adult volunteers. May be as little as one infective unit for some viruses.

1.3. Water quality

Water quality is determined by using both chemical and biological investigations to ensure that the water is optimal for consumption purposes. Water is used for various purposes, such as drinking, cooking, personal hygiene, food production (irrigation and livestock) and others; all of which have different requirements for the determination of their water quality.

1.3.1. Indicator organisms that determine water quality

The routine monitoring of pathogens causing water contamination is usually very complex, expensive and time consuming. It may also be ineffective for the detection of certain pathogens present in low numbers, even if the infective doses associated with these pathogens are very low.[23] To help predict the health risk associated with pathogens present in low or high infective doses, the use of indicator microorganisms are used to monitor the level of water pollution and possible disease outbreaks.

A subgroup of the *Enterobacteriaceae* species provides a biological indicator for faecal pollution and is used for determining the quality of water.[11] No single or universal indicator organism fulfils all the requirements, as listed below. The two indicators most widely used are *Enterococcus* and *E. coli*. Many of the water treatment works, together with the WHO, recommend testing for *E. coli* as an indicator for faecal contamination. This is also the indicator used in this study. The presence of *E. coli* in drinking water is a clear indication of recent faecal contamination, because the organisms do not generally multiply in these waters. The choice of indicator organism usually depends on the water tested, risk of infections, potential source of contamination, cost effectiveness, laboratory facilities and expertise.[23,24] In a study on the use of an indicator organism as a hallmark of faecal pollution of drinking water, [25] Edberg *et al* noted that *E. coli* can be found in all mammalian faeces at concentrations of $>10^9$ organisms per gram, but it does not multiply appreciably in the environment and is therefore the most reliable indicator organism of choice.[24]

E. coli, a commensal gram negative bacteria occurring in the large intestine of animals and humans, fulfils most of the following criteria set out by the Department of Water Affairs and Forestry (DWAF) of South Africa.[4,25]

These criteria are that a suitable indicator organism must:

- be suitable for all water types;

- be present in sewage and polluted waters whenever pathogens are present;
- be present in numbers that correlate with the degree of pollution;
- be present in numbers higher than the pathogens;
- not multiply in the aquatic environment;
- be able to survive in environment as long as pathogens;
- be absent from unpolluted water;
- be detectable by practical and reliable methods; and
- not be pathogenic and safe to work with in the laboratory.

The following section presents a brief summary of the most generally used indicator organisms.

Total coliform bacteria: These include gram-negative bacteria such as *E. coli*, *Citrobacter*, *Enterobacter*, *Klebsiella* and other related bacteria belonging to the family of *Enterobacteriaceae*. They are rod-shaped, non-spore-forming, gram-negative bacteria capable of growth in the presence of bile salts or other surface-active agents. Bacteria produce colonies with a typical metallic sheen within 20-24 hours of incubation at 35°C on Endo agar, a differential and slightly selective culture medium. Coliforms other than *E. coli* will multiply under these conditions. Total coliforms are poor indicators of faecal contamination in water as they are normal inhabitants of soil and water, and can grow in water distribution systems in the absence of faecal contamination. This method is mainly used for evaluation of sanitary quality of drinking water and related waters, e.g. swimming pool water. It is also used for monitoring the efficiency of water treatment and disinfection, as they should not be detected in water sampled after disinfection.[10,23,24]

Thermotolerant coliform bacteria (faecal coliforms): This group comprises of members of the 'total coliform' group mentioned above, which are capable of growth at elevated temperature. It includes all bacteria that produce blue colonies on m-FC agar within 20-24 hours of incubation at 44.5°C. This method is used for the evaluation of the quality of wastewater effluents, river water, seawater at bathing beaches, raw water for drinking water supply, recreational waters as well as irrigation, livestock watering and aquaculture. It is primarily useful as a practical indicator of faecal pollution of bacterial pathogens such

as *Salmonella* spp., *Shigella* spp., *Vibrio cholera*, *Campylobacter jejuni*, *Campylobacter coli*, *Yersinia enterocolitica* and pathogenic *E. coli*. These organisms can be transmitted via faecal-oral route by contaminated or poorly treated waters. This method is more specific for faecal pollution than total coliforms.[10,12,24]

***Escherichia coli*:** *E. coli* is a member of the family *Enterobacteriaceae* and the genus *Escherichia* consists of five species, of which *E. coli* is the most common and clinically most important organism.[15] Like all gram-negative bacteria, it contains a phospholipid bi-layer outer membrane which makes it susceptible to desiccation. The organism possesses various properties and virulence factors that contribute to its pathogenicity. *E. coli* are facultative anaerobic rods, oxidative negative and characterised by the possession of the enzymes β-galactosidase and β-glucuronidase. It grows at 44-45°C on complex media, ferments lactose and mannitol with the production of acid and gas and produces indole from tryptophan. Thermotolerant coliform detection of faecal contamination is a simpler method, but *E. coli* is a better indicator as some environmental coliforms (e.g. some *Klebsiella*, *Citrobacter* and *Enterobacter*) are thermotolerant. These strains can grow at 37°C, but not at 44-45°C, and some do not produce gas. Faecal coliforms that test indole-positive generally consist of only *E. coli* and are almost definitely of faecal origin. This method is therefore a highly specific indicator of faecal pollution which may originate from humans and warm-blooded animals.[10,24,25]

***Enterococci* (faecal streptococci):** Belongs to the genera *Enterococcus* and all possess the Lancefield group D antigen. They are gram-positive cocci found in faeces of humans and animals. They produce a typical reddish colony on m-Enterococcus agar after 48 hours incubation at 35°C. They survive longer in faeces than *E. coli*, but in lower numbers than total or faecal coliforms. *Enterococci* can still be detected after severe dilution of the water. These properties make them the indicator of choice for the indication of certain pathogens that persist and die slowly, e.g. viruses. However, confusion over the identity of these indicators has resulted in few standard optimisations.[10,23,24]

Bacteriophages: Used for the detection of bacterial viruses (phages) in contaminated water. They represent the presence of human viruses in terms of size, structure, and composition and their survival of treatment are more closely regarded than most of the other indicators that are commonly used. The application of coliphages (bacteriophages that infect *E. coli* and certain related species) in water quality assessment is rapidly

gaining ground.

Coliphages: Belongs to the obligate intracellular parasitic microorganisms and do not replicate in environments outside the gut where the host bacterial levels are $<10^4$ CFU/ml or in nutrient-poor environments that do not support growth of the host. Additionally, coliphage lysis of bacteria only occurs in bacterial cultures undergoing exponential growth. Coliphages are useful for indicating public health risk for water consumers and shellfish consumers.[10,23,26]

Somatic coliphages: Infects *E. coli* strains through cell-wall receptors and occur in large numbers in sewage. They can be detected with simple and rapid techniques as an indicator of faecal pollution. PCR methods are commonly used to amplify detectable levels of nucleic acid sequences that are present in low copy numbers of coliphages in the water samples. This approach is fast, specific and more cost effective than the microbiological enumeration methods and cell culturing for virus detection. It cannot determine the infectious state of the organisms, only the presence or absence of the pathogen-specific DNA or RNA sequences.[10,23,25,26]

F-RNA coliphages: Constitute the taxonomic family of *Leviviridae* which contains two genera, *Allolevivirus* (subgroups III and IV) and *Levivirus* (subgroup I and II) based on distinct seriological cross-reactivity. Male-specific coliphages also infects *E. coli* and related hosts and produce fertility fimbrae during the logarithmic phase of growth at temperatures of more than 30°C. These phages will not replicate in natural water environments and are highly specific indicators of faecal pollution. Detection of F-RNA is complicated as it occurs in lower numbers than somatic coliphages in sewage, but their survival and incidence in water environments resemble human viruses even more closely. The subgroup I and IV are isolated from non-human faeces, while subgroups II and III are isolated from human faeces and sewage. The limitations of their use as indicators are their low concentrations in the water environment and therefore enrichment assays are needed for detection. This approach has an inherent bias as a result of differential burst size and infection efficiencies among the subgroups.[10,23,27,28]

Different methods for water analysis with these indicator organisms are available of which the oldest, the multiple tube fermentation (MTF) are still used and applied in this study. This method is divided into the presumptive, confirmed and completed test of the indicator used to determine contamination levels. The MTF test has a major disadvantage as it

takes 3-5 days to complete which is not problematic when experiencing a contamination crisis. This has led to the development of faster and less complex tests such as the membrane filter (MF) technique in which water passed through a filter with small pores to retain microorganisms. The MF technique gives results in a single step. It has good reproducibility and is cost-effective as filters can be transferred between different media and large volumes can be processed. A major disadvantage of this method is that water with high populations of background organisms can cause overgrowth, high turbidity water limits the volumes sampled and sediment or heavy metals can adsorb to the filters and inhibit growth.[4,29]

1.3.2. Standards for monitoring water: reference values

According to international standards,[10] drinking water should contain no *E. coli* organisms, but guidelines are given in Table 1.2 for the ranges of thermotolerant coliforms in drinking water as well as the related effects. Even the presence of *E. coli* organisms in low numbers in the water can serve as a possible risk for diseases, but the risk of transmission of disease starts rising when unacceptable *E. coli* levels are detected in water sources. Water applied for recreational purposes should not contain >400 *E. coli* per 100 ml water. The limit for irrigation water set out by the international standards is 1000 *E. coli* organisms per 100 ml water, while DWAF sets that limit at 2000 *E. coli* organisms per 100 ml water.[4,10] In a previous article it was stated that The National Microbial Monitoring Programme (NMMP) division of DWAF regards a cut-off level of 4000 *E. coli* organisms per 100 ml water for the classification of water as carrying risk of infection. Research on the microbial loads of the rivers in urban areas of South Africa demonstrated that *E. coli* organisms per 100 ml water regularly measure above these standards, e.g. the Plankenburg River (below the dense settlement of Kayamandi) running past Stellenbosch measured 9 200 000 *E. coli* organisms per 100 ml water in January 2006.[2]

The thermotolerant coliforms ranges for water use and releated effects are listed in Table 1.2 and for recreational purposes in Table 1.3. In both drinking and recreational waters, however, the absence of the indicators does not guarantee no risk if the water is consumed.

Table 1.2. Guidelines of thermotolerant coliforms and its related effects[4]

Thermotolerant coliforms (counts / 100 ml water)	Effects: Risk of microbial infection
0 – 10	Slight microbial risk with continuous exposure
10 – 20	Microbial risk with continuous exposure and slight risk with occasional exposure
> 20	Significant and increasing risk of infectious disease transmission. Increased thermotolerant coliforms, the infective dose decrease

Table 1.3. Quality of water used for recreation[4]

Thermotolerant coliforms range	Effects: Health-related risks
0 – 130	Expected risk
130 - 600	Gastrointestinal diseases described in thermotolerant coliforms above
600 – 2000	Gastrointestinal health effects expected in swimmers and bather population. Some health risk if single samples fall in this range, particular if it occurs frequently. Normally 4 out of 5 samples should contain < 600 organisms
> 2000	Increases above this limit, indicates an increased risk of contracting gastrointestinal diseases
0 – 1000	Health risk if extensive intermediate contact

1.4. Disinfection of water for microbial control

Raw water obtained directly from nature is almost in all cases not suitable or safe for human consumption, especially in the areas associated with water contamination

described in section 1.1. To ensure safe or suitable water, we need to treat water with the most effective disinfectant. Disinfection is widely used to prevent the transfer of bacteria, viruses and some protozoa into the water distribution system. Disinfectants used for drinking water include chlorine (and chlorine dioxide, chloramines), ozone and ultraviolet radiation.

1.4.1. Concepts and definitions in water treatment

It was mentioned in section 1.3 that in water treatment the quality is determined by both chemical (organic and inorganic compounds) and biological (microbiological) investigations to ensure that the physical characteristics (taste and odor) of the water is acceptable. The chemical investigations involve inorganic compounds including dissolved salts such as chlorides, which may have resulted from addition of chlorine to water.[23,24] The determination of the organic compounds involves byproducts produced during chlorine disinfection, which may result from the chlorine dosage or pH. However, microbiological safety involves the detection of indicator organisms described in section 1.3.1 during various disinfection strategies and was the method used for determination of water contamination in this study.

The following steps are a short summary of the water purification process: first-line processes (known as 'conditioning of water for disinfection') are mainly coagulation (a metal salt added to raw water to aggregate particles into masses), followed by sedimentation (coagulated particles fall by gravity through water in a settling tank) and filtration (water in sedimentation tank is forced through sand, gravel or charcoal to remove solid particles). Water is then ready for exposure to disinfectants of which chlorine is by far the most commonly used used, due to its affordability and effectivity, especially for small-scale water treatment users, such as farmers and small municipal treatment works.[13]

1.4.2. Disinfection strategies used for water treatment

The primary purpose of the disinfection process in drinking water treatment is the control of waterborne diseases through inactivation of any pathogenic microorganism that may be present in water. This is a similar approach as sterilisation, which completely eliminates or destroys all forms of microbial life[30,31] but is not achievable in the process of making water potable on a large scale. In contrast, sterility of small volumes of water can be achieved by proper boiling of the water.

Disinfection is used for treatment of various water sources and therefore divided into either low, intermediate or high level disinfectants. Low-level disinfectants, such as chlorine, kills most of the vegetative bacteria, some fungi and also some viruses within short exposure times (i.e. <10 minutes).[32] Intermediate-level disinfectants, such as hypochlorite and phenolics may be cidal for tubercle bacilli, most viruses and fungi, but do not necessarily kill bacterial endospores,[33] whereas high-level disinfectants such as glutaraldehyde and hydrogen peroxide are agents required to kill all microorganisms except bacterial endospores after an exposure time shorter than 45 min (used for sterilisation).[32]

Disinfection treatment methods include chlorination, chlorine dioxide, chloramines, ozone and ultraviolet radiation that kill or inactivate most pathogenic organisms except bacterial endospores.[33] Disinfectants should effectively remove the pathogens over a range of chemical and physical conditions; produce a stable and measurable residual, but no byproducts, be easily generated; safe to handle; be suitable for all water types and cost effective. Similar to indicator organisms, no disinfectant meets all these criteria. Therefore, the type of disinfectant applied is dependent on the water source and the pathogens mostly associated with the environment.

1.4.2.1. Chlorine

Chlorine is one of the most commonly used disinfectants worldwide. It is also the most affordable disinfectant, especially in developing countries. It has effective bactericidal properties and short contact times. Chlorine also provides a residual that can be measured after treatment and protects against recontamination and reduces biofilm growth in water distribution systems.[34] It can be easily applied, controlled and monitored. It acts as a strong oxidant that meets most pre-oxidation objectives. However, chlorination of water for disinfection has limitations, including byproduct formation like trihalomethanes (THMs), halogenated acetic acids (HAAs) and brominated organic byproducts. Chlorination is not effective (or only poorly effective) against *Cryptosporidium*, parasitic protozoa and many viruses. Furthermore, it is regarded as a hazardous chemical that requires careful transport.[10] The various forms of disinfectants for water treatment, including chloramine, chlorine dioxide, ozone, ultraviolet radiation are described below and chlorine disinfection in section 1.4.3.

1.4.2.2. Chloramines (Monochloramines)

The disinfectant is formed when chlorine and ammonia are added to water. It is a weaker oxidising agent than chlorine, but very stable and cause less undesirable taste or odors and byproducts (e.g. THMs) than chlorine.[13] It is suitable for all water types and often used as a primary disinfectant with a long contact time (hours). It is effective against bacteria, but ineffective against viruses. The laboratory equipment is simple to handle and the controls for treatment are well developed. Chloramine can persist in a water distribution system, but low levels of chloramines are toxic to various aquatic organisms.

1.4.2.3. Chlorine dioxide

Chlorine dioxide is a reactive gas which cannot be easily stored or transported and therefore have to be generated on-site. Chlorine dioxide is a widely used alternative disinfectant to chlorine. It is more effective than chlorine and chloramines for inactivation of viruses, *Cryptosporidium* and *Giardia*. It is a strong oxidant that reacts with organic material to produce a wide variety of oxidised byproducts that can be effectively removed. It reduces taste and odors, minimises discoloration and is suitable for small-scale water sources and therefore used as a pre-oxidant for disinfection treatment. The laboratory equipment is readily available, but the technology is moderately complex and some still under development. A major disadvantage of this method is that chlorine dioxide is rapidly consumed and volatilised in raw water. The end products, i.e. chloride, chlorite and chlorate ions, as well as residual chlorine dioxide, have been related to previous health risks.[34-36]

1.4.2.4. Ozone

Ozone (O_3) is an unstable gas and generated on-site like chlorine dioxide, but by passing an electric discharge through clean dry air or oxygen. It is a strong oxidant and effectively reduces taste odors, colour, iron and manganese.[34,36] O_3 is more expensive than chlorine and has a low solubility with no residual in water, but is unstable above pH 8.0. Due to its powerful oxidising properties, O_3 can covert bromide into bromine, which can lead to the formation of brominated THMs. These byproducts are in lower concentration than produced during chlorination and therefore not a health risk.[13,23,34]

A secondary disinfectant, usually chlorine, is required, because O_3 does not maintain an adequate residual in water. It has been demonstrated to be an effective disinfectant

against bacteria and *Cryptosporidium* oocysts.[36] The required O_3 doses (4.7 l/min, standard flow rate) are relatively high with shorter contact times and dosages than with chlorine disinfection. Dissolved O_3 concentrations between 0.36 and 2.2mg/L must be achieved to maintain a steady-state.[37] Research conducted by Finch[38] demonstrated that the sequential use of either O_3 or free chlorine followed by monochloramine, was capable of inactivating *Cryptosporidium* oocysts.

1.4.2.5. Ultraviolet radiation

Ultraviolet (UV) radiation, generated by mercury arc lamps, is a non-chemical disinfectant.[35] When UV radiation penetrates the cell wall of an organism, it damages the genetic material and prevents the cell from reproducing. UV has been shown to effectively inactivate many pathogens such as *Cryptosporidium* spp., but has low inactivation for some viruses (for example retroviruses and rotaviruses). A limited amount of disinfection byproducts are formed. UV systems do not involve chemical generation, storage, or handling, but it is not cost effective. It is difficult to monitor the efficacy of UV disinfection and the treatment does not result in residual protection. Another disadvantage is that the turbidity and total suspended solids (TSS) in the wastewater can render UV disinfection ineffective. UV disinfection with low-pressure mercury lamps is not as effective for secondary effluent with TSS levels above 30 mg/L.[39]

1.4.3. Chlorine disinfection

1.4.3.1. Conditions related to chlorine disinfection

Chlorine, previously described in section 1.4.2.1, is highly effective against most pathogens. It is applied in several forms like elemental chlorine (chlorine gas), sodium hypochlorite solution (bleach) and dry calcium hypochlorite. Factors that can influence disinfection efficacy include disinfectant concentration and contact time, temperature, pH, chemical composition of raw and treated waters and the physiological state of microbes. The physiological state of the microorganisms not only influences their response to disinfection,[32] but also their virulence, thereby influencing associated health risks. Previous studies have shown that the dilution of nutrient media and the presence of surfaces of bacterial growth (biofilms) can lead to greater bacterial resistance to disinfection.[40,41] Calculating the optimal contact time of water to be disinfected with chlorine in large water treatment works is therefore a complex exercise.

Free chlorine is the total of the hypochlorous acid and hypochlorite ions expressed in units of milligram per liter (mg/L) or parts per million (ppm). The available chlorine used in disinfection is measured by the oxidising capacity of the hypochlorites.[41] When chlorine is added to the water it hydrolyses rapidly and yields hypochlorous acid (HOCl) and hydrochloric acid. Hydrolysis is completed at a pH >4 with a chlorine dose up to 100 mg/L. HOCl, a weak acid, dissociates further partially into hypochlorite ion (OCl^-) and at pH 7.5, into equal distribution of HOCl and OCl^-. At pH 6.5 about 90% chlorine should be present as HOCl. If the pH is above 9, OCl^- becomes dominant and at pH 7.7, free chlorine consists of approximately 50% HOCl and 50% OCl^-.[42] The pH value of the water is therefore critical to the disinfection treatment. HOCl is an extremely potent bactericidal agent, even at concentrations lower than 0.1mg/L.[43]

Another important factor within water treatment systems is turbidity. Turbidity is an expression of the optical property of water that causes light to be scattered. It is measured by determining the degree of light scattering by particulates present in the samples. A turbidity of 1 nephelometric turbidity unit (NTU) was recommended as guidelines for drinking water by the WHO, and limited to 5 NTU.[13,25] Turbidity in water is caused by the presence of suspended matter, such as clay, silt, organic and inorganic matter, plankton and other microscopic organisms.[44] If the turbidity is too low the chlorine would be oxidized by the water and not the organisms. On the other hand, high turbidity levels have shown to protect microorganisms from the action of chlorine, and to increase the chlorine and oxygen demand for proper water disinfection.[44]

It is recommended that the chlorine contact time should be at least 30 minutes for small-scale users.[13] If too long, microorganisms might repair damage and adapt to the environment. Contact time should only be increased with due cognition of the potential harmful effects of over chlorination,[45] but human health and safety should never be compromised. The residual concentration of free chlorine should not be less than 0.5 mg/L. It has previously been determined that the contact time at a pH of less than 8 and a maximum turbidity of 1 NTU should be 30 minutes.[13,26]

1.4.3.2. Bacterial response to chlorine disinfection

Microbial resistance to chlorine has become a major concern and, if undetected, it has the potential to have enormous implications.[46,47] One investigation on microbial resistance against chlorine compounds indicated that intracellular glutathione, the predominant

intracellular thiol compound in *E. coli* and many other bacteria provides quantitatively important protection of these organisms.[48] A previous study conducted in the United States, showed that *E. coli* that were starved and treated with chlorine were still culturable. In that study it was indicated that *E. coli* O157:H7 developed resistance to chlorine concentrations up to 0.5 mg/L, higher than the detectable disinfectant level mandated for drinking water in the United States.[47] The mechanisms of action of chlorine on microorganisms have been widely investigated, but have not been fully explained.[10,43,49] Several other studies have shown that specific and general damages caused by chlorine exposure seemed to be membrane and protein associated.[43,49] These effects can be genetically investigated by heat shock and *soxRS* systems which become activated *in E. coli* following exposure to stress conditions.[50] When *E. coli* organisms are exposed to chlorine, stimulation of oxidative stress factors come into play, resulting in an oxidative burst.[50] Therefore microbial response to oxidative stress will help in the development of effective disinfection and pathogens control. In this study one approach for detecting bacterial resistance is investigated with the screening of possible candidate genes most likely involved or responsible for resistance towards chlorine disinfection. Candidate genes, such as *soxR*, *soxS*, *grpE*, *osmC* involved in heat-shock or osmotic were selected for this purpose.

1.4.3.3. Chlorine resistance

Chlorine is a powerful antimicrobial substance due to its potential oxidizing capacity.[32] The occurrence of some organisms surviving water treated with chlorination at conventional doses and contact times, and entering the potable water reticulation system in a viable state, is widely referred to as ‘chlorination resistance’ in engineering and medical literature.[51] These organisms surviving such treatments may be pathogenic and have increased health implications. This is not the same concept as is used in antibiotic resistance. While different mechanisms of antimicrobial resistance may be the result of specific target site modifications of the microorganisms against antimicrobial agents, chlorine tolerance might provide a selection pressure for mutations in a multiplicity of the cellular target encoding genes that differ from the concept of antimicrobial resistance. The specifc genes involved in bacterial response to chlorine has not been fully investigated yet, but research in this field have linked heat-shock proteins and *soxRS* regulons to be involved in exposure to hypochlorous acid (strong oxidant of chlorine when added to water).[52] The selection pressure is investigated in this study, with the detection and

sequence of these and others stress response genes that may be involved in the ability of some organisms to survive chlorine disinfection in the form of mutations in these various genes.

Previous research on the impact of chlorination by Dr Jo Barnes concluded the following:

> *"There is increasing concern about the ability of landowners and smaller local authorities in the Western Cape to treat raw water obtained from rivers downstream from dense settlements. Should chlorine resistance be present to any significant degree in organisms occurring in free-flowing waters, this will imply that the ability to disinfect raw water from such polluted rivers is seriously impaired. The presence of chlorine resistant organisms in treated water is an indication that effective water treatment to inactivate pathogens may be ineffective."*[53]

1.5. Biofilms

It has been clearly established that, whether in nature or in industry, most surfaces are colonised by bacterial biofilms.[54] Biofilms are dense bacterial communities attached to a solid surface and surrounded by an exopolysaccharide matrix.[55] Biofilms are formed in response to specific environmental stresses that force physiological changes in bacteria. Organisms occurring in biofilms exist in a highly structured lifestyle and are also generally more resistant to antibiotics and other chemicals than planktonic organisms.[56,57] As a consequence, diseases associated with biofilms are generally chronic and difficult to treat. Persistent infections associated with biofilms include periodontal disease, endocarditis, osteomyelitis, cystic fibrosis and biomaterial-related bacteraemias.[55] Many biofilms were found to significantly protect microorganisms from disinfecting agents[57] including HOCl and monochloramine. It may promote the growth of microorganisms in some water distribution systems.[58] Microorganisms in biofilms is frequently sloughed off pipe surfaces for a variety of reasons.

The ability of total coliforms and *E. coli* to survive in biofilms is of marked significance to the water treatment industry, not only because the detection of these organisms in distribution water gives an incorrect indication of recent faecal contamination, but also because the persistence of these organisms and their release from biofilms may mask true breakthrough events in water treatment. The formation of biofilm in potable water can be limited with proper disinfection efficiency and residual. A previous research investigation[59] concluded that biofilms occurred on various surfaces, including cement coupons and

stainless steel. Increased levels of residual disinfectants varying from hydrogen peroxide (H_2O_2), monochloramine, as well as free chlorine, were not found to prevent the formation of biofilms.

In another study[56] it was speculated that the mechanism of antibiotic resistance in biofilms constitutes a subpopulation of microorganisms that forms unique and highly protected phenotypic states. This hypothesis was supported by the indication of resistance in newly formed biofilms, even though they were too thin to pose as a barrier against penetration of antimicrobials or metabolic substrates.[57] In addition, antibacterial agents rapidly killed most, but not all, bacteria in the biofilm.[60] Survivors, which consisted of 1% or less of the original biofilm population, persisted despite the continuous exposure to the same antibiotic. In addition, the survival mechanisms can also provide an explanation for reduced susceptibility of biofilms to antibiotics and various disinfectants.[56]

Another resistance mechanism that can be induced in biofilm cells is the alteration of the membrane-protein composition in response to antimicrobial agents.[56] Mutations occurring in *ompB* (a regulator of the genes encoding for the outer membrane (OM) porin proteins *OmpF* and *OmpC*) and in *ompF* affected the permeability and increased the resistance of *E. coli* to a ß-lactam antibiotic. The study suggested that bacteria in a biofilm are indeed living in an environment of increased osmotic stress.[56] Thus, the environmental conditions within the biofilm can lead to alterations within the cell envelope that protect the bacteria from the detrimental effects of antimicrobial agents.[56] The outer membrane protein (OMP), *ompF*, was included in this study to investigate its role in both chlorine and antibiotic resistance.

1.6. Antimicrobial resistance

Microorganisms in water sources already resistant to certain antimicrobials pose a further risk to the efficacy of disinfection applied for water treatment. These resistant factors can be transferred to other organisms and this could have serious implications. Various antimicrobials are described below, as well as two mechanisms (permeability barriers and efflux pumps) involved in antimicrobial resistance.

1.6.1. Bacterial resistance to antimicrobials

Bacterial resistance to antimicrobial agents is a pressing health problem that is experienced worldwide. The concern is that these mechanisms may confer cross-

resistance to other clinically important antibiotics. Multiple antibiotic-resistance, defined as acquired resistance to most of the existing classes of antimicrobial agents, is often encountered in many different pathogens. Microorganisms are infinitely adaptable to environmental changes and have already demonstrated mechanisms of resistance to various biocides (antiseptics, disinfectants and preservatives). A possible link between the resistance strategies employed by bacteria towards antibiotics and biocides is suspected.[61]

An increasing resistance to third-generation cephalosporins amongst *E. coli* and *Klebsiella* spp. is predominantly due to the production of extended spectrum β-lactamases (ESBLs).[59] These enzymes are defined as β-lactamases capable of hydrolysing oxyiminocephalosporins and many of them are inhibited by beta-lactamases inhibitors. They are plasmid-borne enzymes which mostly evolved via point mutations of the classical TEM-1 (Class A β-lactamases called Temoniera), which quickly hydrolyses penicillins, but are not active against the 3rd generation cephalosporins and SHV-1 (Sulfhydryl variable β-lactamases), which attacks narrow-spectrum cephalosporins and all penicillins active against gram-negative bacteria except for temocillin β-lactamases. Other groups are also increasingly encountered, especially CTX-M types (Cefotaxime resistant ESBL), which evolved via the escape and mutation of chromosomal β-lactamases from *Kluyvera* spp. ESBL confers multiple drug resistance, making infections difficult to treat.[59] Currently, over 400 different types of clinically relevant β-lactamases have been described,[62] but the ESBLs have probably had a greater impact than any other group. In a study conducted in Durban, South Africa,[63] it was determined that many ß-lactamases occurred in *Klebsiella pneumoniae* isolates with multiple identical or different gene variants of up to 84 bla_{SHV} and bla_{TEM} gene copies among the 25 *Klebsiella* strains tested. These variants included novel genes with complex and diverse patterns for ESBL production which may complicate treatment with the available antimicrobials. Therefore, any detection of ESBL producing *E. coli* strains or cephalosporins resistance could have severe health implications.

Another antimicrobial class included in this study is the FQ, which are synthetic antimicrobial agents with greater activity against gram-positive and gram-negative bacteria than the older quinolone analogs, such as nalidixic acid and oxolinic acid. One of the resistance mechanisms to FQs involve mutations within the genes encoding resistance to DNA gyrase[64-66] and topoisomerase IV.[64,65,67] Other mutations affecting the accumulation of quinolones include either the expression of porins[64,65] or lipopolysaccharides,[68] or the active efflux of quinolones from the bacterial cell.[65,69] Experimental evidence showed

that higher levels of FQ-resistance can result from both target related and efflux mutations.[70]

It was shown in a study that high-level FQ-resistant *E. coli* isolates displayed multiple antibiotic resistant (MAR) phenotypes that constitutively express the regulatory genes *marA* and *soxS*.[64] The respective genes *marA* is regulated by *marR* and *soxS*, a regulator of the *SoxRS* regulon, which confers increased resistance to chemically unrelated antibiotics by activating or depressing a number of genetic loci in *E. coli.* It is therefore important to determine the genes in commensal, pathogenic and environmental organisms to measure the environmental pool of their resistance. This may lead to a better understanding of the ecology of antimicrobial resistance especially in water systems to prevent related health and economic implications.[71]

1.6.2. Permeability Barriers

Membrane permeability was shown to be the first step involved in bacterial resistance to various antimicrobials. In gram-negative bacteria, the outer membrane acts as a effective barrier and only delays the influx of various antibiotics, detergents and dyes. This form of intrinsic resistance to antimicrobial agents is brought about by efflux pumps, which extrude the drug from the periplasmic space to the environment, enabling the bacterium to survive in the presence of these noxious agents.[72] Disinfectants and certain antibiotics must traverse the outer cell layers to reach their target sites, which are usually present within the microbial cells.[73] In gram-negative bacteria the passage across the outer membrane depends on the chemical nature of the agent. Hydrophilic antibiotics utilise the porin channels (hydrophilic route) and hydrophobic antibiotics entering via the glycerophospholipid bilayers in the outer membrane (hydrophobic route).

1.6.3. Efflux pumps

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment by utilising the energy of the proton motive force. Bacterial antimicrobial efflux transporters have been grouped into four super families, primarily on the basis of amino acid sequence homology.[74] These include the major facilitator super family (MFS, efflux pumps), the ATP-binding cassette family (ABC), the resistance-nodulation-division (RND, efflux pumps) family and small multidrug resistance (SMR) protein family. A fifth family, named the multidrug and toxic compound extrusion

(MATE, efflux pumps) family, has also been identified.

In *E. coli*, seven different proton-dependent MDR pump systems have been identified in biological studies, i.e. *acrAB-tolC*, *emr*, *mdfA*, *tehA*, *emrE*, *acrEF* and *emrD*. A well studied example is the *acrA-acrB-tolC* MDR tripartite pump system[74] of *E. coli*. This complex consists of an MDR pump, a membrane fusion protein (MFP), and an outer membrane factor (OMF).[74] A second component is the outer membrane (OM) protein, *tolC*, which is the required third component for the *acrAB* and *emrAB* drug efflux systems.[75] These efflux pumps are of interest due to their unknown physiological roles and possible contribution to clinical resistance. The genes of the efflux system, *acrAB-tolC* are included in this study to determine their possible involvement in chlorine and antimicrobial resistance.

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Chapter Two

Overall aim of the present study and objectives of the substudies

2.1. Overall aim of the present study

The overall aims of this study were to determine the molecular characteristics of selected *E. coli* strains isolated from environmental waters in the Western Cape and to investigate the possible relationship between chlorination tolerance and antimicrobial resistance.

This study was motivated by the outcome of a research project done towards obtaining my BSc Honours degree in 2005. That research project verified the findings of a previous study conducted by Dr Barnes: that *E. coli* organisms exposed to various concentrations of chlorine demonstrated increased resistance to some widely used antimicrobials.[1,2] As this laid the groundwork for the work presented in this thesis, a summary of the methods and results of that initial study is presented as Chapter Three.[3] This study indicated the need for further research into the characteristics of *E. coli* strains after exposure to chlorine. Antimicrobial resistance profiles were again determined, and different colony morphologies were described before and after exposure to various chlorine concentrations. In order to study the molecular characteristics of selected strains, the presence of candidate genes involved in the response to chlorine exposure and in resistance to specific antimicrobial agents were determined. The amplified candidate genes were then sequenced to illustrate differences between strains isolated before and after exposure to chlorine. Finally, the presence of certain virulence genes was detected in order to establish the pathogenic potential of the *E. coli* strains isolated.

2.2. Objectives of the substudies

The objectives of this study were achieved by doing various substudies. Many of the substudies are linked and the basis of these substudies depended on the outcomes of the previous one. For example: Substudy Three (Chapter Six), in which the phenotypic characteristics of the strains was determined, first needed chlorine resistant strains to be selected by Substudy Two (Chapter Five).

2.2.1. Substudy One:

Sampling strategies and microbiological quantification of *E. coli* organisms in the water sources

Objective 1: To determine the levels of water pollution in selected water sources.

Water and biofilm samples were collected from surface water in rivers, wastewater in dams which are resources for informal settlements, and industrial settings in the Western Cape between January 2006 and December 2007 for the determination of water contamination. This was achieved by using *E. coli* as an indicator organism for faecal contamination of the water sources, and the possible presence of other pathogenic organisms.[4]

Substudy One is described and discussed in Chapter Four.

2.2.2. Substudy Two:

The selection of chlorine tolerant *E. coli* strains

Objective 2: To determine the levels of *E. coli* in the water and biofilm samples exposed to chlorine.

Samples collected for Substudy One were exposed to various chlorine concentrations at different contact times to determine the levels of *E. coli*. The enumeration of organisms up to the highest concentration was considered an indication of chlorination tolerance.

Substudy Two is described and discussed in Chapter Five.

2.2.3. Substudy Three:

Phenotypic characteristics of *E. coli* strains

Objective 3: Identification of different phenotypes of *E. coli* based on their colony morphology and antibiograms before and after exposure to chlorine.

Morphological characteristics of colonies are one of the common methods used to describe and identify microorganisms in the clinical bacteriology laboratory. The *E. coli* strains that survived chlorine treatment in Substudy Two (Chapter Five) were

phenotypically characterised after determination of the morphological appearances of their colonies, and antimicrobial susceptibility patterns. The antimicrobial susceptibility profile of microorganisms is important in clinical microbiology, as doctors base decissions of antimicrobial therapy on these results. With the increasing concern of incomplete disinfection and increasing resistance to antimicrobial drugs, a possible link between the two phenomena is of major importance.

Substudy Three is described and discussed in Chapter Six.

2.2.4. Substudy Four:

The association of an efflux pump involvement in antimicrobial resistance and chlorine tolerance of *E. coli* strains

Objective 4: To identify the effect of an EPI on the susceptibility of *E. coli* isolates exposed to chlorine and selected antimicrobials.

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment by utilising the energy of the proton motive force. To determine whether efflux pumps are involved when *E. coli* are exposed to chlorine and antimicrobials, the inhibition of *acrAB-tolC* efflux pump (RND family transporters for gram-negative bacteria) by an inhibitor was attempted.

Substudy Four is described and discussed in Chapter Seven.

2.2.5. Substudy Five:

Molecular detection of candidate genes involved in antimicrobial resistance and chlorination tolerance

Objective 5: To establish and optimise practical molecular techniques for the amplification of specific candidate genes that is considered to be markers of resistance to certain antimicrobials and chlorine tolerance.

This part of the study was undertaken to investigate the underlying mechanisms involved in chlorine tolerance and antimicrobial resistance observed in the selected *E. coli* strains. The approach used in this substudy was to determine the presence of candidate genes in the strains selected during previous substudies. The candidate genes were selected for

their possible involvement in chlorine tolerance, efflux and other mechanisms of resistance to antimicrobials.

Substudy Five is described and discussed in Chapter Eight.

2.2.6. Substudy Six:

Comparison of the genotypic and phenotypic profiles of selected strains

Objective 6.1.: To compare the gene sequences of candidate genes of selected strains to their phenotypic profiles and to use computational and sequencing methods to confirm that the correct genes were amplified and to detect nucleotide variation.

Microbes adapt in response to detrimental environmental conditions. The exposure of *E. coli* strains to chlorine and antimicrobial agents may lead to the selection for certain gene mutations. The changes within the amino acid sequences could have various implications possibly affecting different environments, including agriculture, health and water purifications systems.

Substudy Six is presented and discussed in Chapter Nine.

2.2.7. Substudy Seven:

Determination of the pathogenic potential of isolated *E. coli* strains

Objective 7: To detect virulence genes of EPEC and ETEC *E. coli* strains in the samples in order to identify and characterise strains of pathogenic potential.

The presence of pathogenic microorganisms within the water sources could have serious health implications, if these waters are used as a supply of drinking water to communities. In addition, the presence of virulence genes in these strains that were pre-selected by their resistance to chlorine would be of grave concern if they enter the water sources with both pathogenic and resistant characteristics.

Substudy Seven is presented and discussed in Chapter Ten.

2.3. Possible impact of the findings of this study

This study attempts to illustrate the effect of water disinfection with various chlorine concentrations on water obtained from various local sources. The outcome of the results aims to contribute to our knowledge of phenotypes and genotypes of *E. coli* strains isolated from water sources. Finally, it illuminates the need for more water surveillance systems to improve disinfection strategies, detect pathogenic strains and maintain the risk for disease outbreaks.

2.4. Ethical approval

Institutional approval for the honours study was granted by the Committee for Human Research of Stellenbosch University. An amended protocol for the MSc research project was re-submitted and approved. (registration number N05/07/111).

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Chapter Three

Background to current study

3.1. Introduction

The preliminary work done towards obtaining my BSc Honours degree in 2005 is described in this chapter as background to the substudies presented in the thesis. As mentioned in Chapter Two, the preliminary research project was chosen to verify the findings of a previous study conducted by Dr Barnes.[1] Many of the rivers in the Boland were heavily polluted with untreated human waste and this raised concerns about the ability of landowners and smaller local authorities to treat raw water obtained from such rivers.

Studies carried out by Barnes[1] in 2003 indicated that *E. coli* organisms that survived various concentrations of chlorine exposure demonstrated increased resistance to some widely used antimicrobials.[2,3] In addition, the development of new antibiotics are very rare and the prevention, as well as the maintenance of sensitivity to antimicrobials, is important to ensure their continued application for therapy.[4]

3.1.1. Aims of the preliminary study

The aims of this preliminary study were to quantify the levels of faecal pollution of the Plankenburg River (Below Kayamandi) and to investigate the role of chlorination in the development of antibiotic resistant *E. coli* organisms from the Plankenburg River.

3.2. Materials and Methods

3.2.1. Study design

The study was a cross-sectional survey conducted with the aim of comparing results to the study previously done by Dr. Barnes.[1] A cross-sectional survey observes some of its target group and bases the overall findings on the views, assuming them to be typical of the whole group. Two water and biofilm samples representative of the Plankenburg River were taken at a particular point in time and the findings of the chlorine exposure and antimicrobial resistance were taken as representative of the overall results of the river.

3.2.2. Isolation of *E. coli* organisms from water and biofilm samples

The samples were collected in exactly the same manner as previous studies to ensure comparability.[2]

3.2.2.1. Collection of water samples

Two water and biofilm samples were obtained from the Plankenburg River, downstream from the dense settlements of Kayamandi (Below Kayamandi) on 4 July 2005. Both samples were collected separately according to the guidelines of the South African Bureau of Standards (SABS),[5] that incorporates the standard methods of the American Public Health Association, American Water Works Association and the Water Environment Federation. This method prescribes that water samples should be taken at mid-stream of the river and where the water depth allowed, at 30 cm depth.[6,7] To ensure countable levels of microorganisms, no sodium thio-sulphanate was present in the sample bottles, as that may neutralise the chlorine and utilise the enzyme, thio-sulphate reductase, to release hydrogen sulphide gas. The water samples were immediately transported to the Medical Microbiology Laboratory of the Tygerberg NHLS Coastal Branch and Stellenbosch University where the analysis was carried out.[2]

3.2.2.2. Collection of biofilm samples

Smooth stones were selected as substrates for the biofilm samples since lumps of cement for instance, are highly alkaline, while decomposing wood hosts different kinds of biofilm communities. Smooth stones are the nearest to inert surfaces that could be obtained. Six stones were taken from the Plankenburg River and bagged in sterile containers.[1]

The stones were sonicated within 15 minutes of collection at the Department of Microbiology at the Stellenbosch campus. The stones were immersed in approximately 100 ml sterile deionised water and sonicated for 6 minutes at a wavelength of 495 – 500 nm to loosen and resuspend the biofilms. The resultant solution was carefully decanted into autoclaved sample bottles and immediately transported on ice to the Medical Microbiology Laboratories of the Tygerberg NHLS Coastal Branch and Stellenbosch University where the analyses were carried out.[2]

3.2.2.3. Chlorine exposure method

A modified method of the standard 5x multiple tube enumeration protocol were followed to assess the organisms isolated from river water and biofilm exposed to chlorine.[8] In the completed test, samples from the positive brilliant green lactose bile broth (BGLB) were not streaked onto Levine's EMB or LES Endo agar and incubated at 37°C for 24 hours, as some methods describe. These agar plates are used for the confirmation of coliforms (which includes Enterobacteriaceae such as *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella* etc.) but needs to be inoculated onto BGLB and nutrient agar and completed with a gram-negative stain. For this study, the completed test included an indole test after positive gas formation in the BGLB tubes for presumptive identification of strains as *E. coli*. Identification was confirmed by a short biochemical test comprising of a negative Simmons citrate sugar and a positive indole test.

Therefore, the exact protocol of Bergey's Manual of Systemic Bacteriology for identification of bacteria were not followed, [9] but the method used was in accordance with the standard operational procedures applied in the microbiological laboratory of the NHLS, Tygerberg Hospital.

All the samples were mixed thoroughly, filtered to remove larger suspended particles, and then divided into equal samples of 225 ml each in clean sterile bottles. Samples were processed in duplicate for accuracy and comparison of the results.

Freshly manufactured Medisure® chlorine powder (Medichem, Tokai, South Africa) was used. The powder was weighed to four decimals of a gram and added to the sample water in the bottles to reach concentrations of total chlorine of 0.0 mg/L, 0.1 mg/L; 0.3 mg/L; 0.4 mg/L, 0.5 mg/L, 0.6 mg/L and 0.7 mg/L with 10 minutes intervals. These concentrations were in accordance to those used by Dr Barnes in previous studies after consultation with the Chief Superintendent of the Cape Town Water Treatment Department. The minimum contact time of 30 minutes for chlorination of small water sources, recommended by the WHO[10] and adopted by many other countries, were followed.[11,12]

After 30 minutes, samples from each of the bottles of water and chlorine mixture were subjected to the standard 5x multiple tube enumeration method to test for faecal coliforms and *E. coli*.[5] First, 10 ml from each sample water bottle (with and without chlorine) was aseptically transferred into each of five sets of 10.0 ml lauryl tryptose broth and then

1.0 ml of sample water (with and without chlorine) was added. Subsequently, 1.0 ml of the sample water (with and without chlorine) was transferred into a 9.0 ml saline blank bottle, constituting a 10^{-1}dilution. One millilitre from this 10^{-1} dilution was transferred to 9.0 ml saline (10^{-2}) and 1.0 ml into each of five test tubes containing 10.0 ml lauryl tryptose broth with Durham tubes. These serial dilutions were continued to complete a range of 10^{-9} dilution. All tubes were then incubated at 37°C for 48 hours.

The bottles of river water and chlorine were left standing for a further hour on the bench (total exposure of 90 minutes). These bottles were initially vortexed and afterwards manually agitated from time to time. After the extra hour, a sample from each bottle was also subjected to the standard 5x multiple tube enumeration method for faecal coliforms and *E. coli* determination as described above. For quality control of lauryl tryptose broth and the presumptive test, *E. coli* as a positive control and *Acinetobacter* as a negative control, were used. The *E. coli* and the *Acinetobacter* strain were clinical isolates identified by the NHLS laboratory at Tygerberg Hospital.

3.2.3. Bacterial counts

A 0.1 ml (100 µl) sample of water was directly taken from each of the river water samples at 30 and 90 minutes exposure to chlorine and inoculated onto a MacConkey agar plate (Diagnostic Media Products, NHLS, Green Point, SA), a blood agar plate (Diagnostic Media Products, NHLS, Green Point, SA) and a Uri-Select agar plate (Diagnostic Media Products, NHLS, Green Point, SA) and incubated aerobically for 24 hours at 37°C. For direct bacterial quantification, a colony count was performed on the different agar plates for quality control of the water samples exposed to chlorine. The same process was repeated for the biofilm samples.

3.2.4. Faecal coliforms and *E. coli* counts

After 48 hours of incubation, samples containing bacteria capable of fermenting lactose with the production of gas were presumed to be faecal coliforms. These positive tubes were labelled and vortexed and 100 µl of positive tube content was inoculated into brilliant green lactose bile broth (BGLB) with Durham tubes. Simultaneously, 100 µl of the positive tube content was inoculated into a corresponding tube containing tryptone water. These were then incubated in a waterbath at 44.5°C for 18-24 hours. After 24 hours incubation, positive samples (those with gas production for the second time) were considered to be

positive for faecal coliforms. Ehrlich reagent (1.0 ml) was added to the corresponding tryptone water tube which reacted with the tryptone and produced a red compound on the surface of the medium if the test was positive. Only those with a positive BGLB tube and corresponding positive tryptone water tube were presumptively identified as containing *E. coli.* The total faecal coliform and *E. coli* counts were calculated according to positive tubes by means of the De Mans Index tables.[6] The number of positive tubes were recorded, given a number on the Index table and multiplied with the dilution factor as well as 100 to determine the number of organisms per 100 ml water.

3.2.5. Cultivation of *E. coli* for biochemical characterisation

One droplet was taken from the positive tubes presumptively identified as containing *E. coli*, inoculated onto Uri-Select and MacConkey agar plates, and incubated for 18-24 hours at 37°C. *E. coli* colonies were characterised as magenta or red in colour with a domed or round shaped appearance. *E. coli* counts were determined by counting the colonies resembling *E. coli.*

3.2.6. Biochemical identification of *E. coli*

UriSelect agar plates (Diagnostic Media Products, NHLS, Green Point, SA) were used to select colonies for further identification. Colonies exhibiting the typical characteristics of *E. coli* on these plates (bright pink colonies with typical morphology described above) were then further identified with a short biochemical test of a negative Simmons citrate and a positive indole test as follows:

A part of a pure colony was inoculated onto Mueller-Hinton broth (Mast, Davies Diagnostics, SA) and further diluted with Mueller-Hinton broth (Mast, Davies Diagnostics, SA) to an optical density (OD) of 0.5 McFarland, indicating a colony forming unit count of 10^6-10^8/ml. A droplet of this was inoculated onto Simmons citrate agar slants (containing sodium citrate as sole carbon source, ammonia as nitrogen source and pH indicator) in order to differentiate *E. coli* from other *Enterobacteriaceae*. The basis of this differentiation is the fact that oxygen (O_2) is removed and carbon dioxide (CO_2) liberated when bacteria oxidise citrate, then combines with sodium and water to form sodium carbonate, which subsequently raises the pH and turn the media into a blue colour, indicating a positive test. *E. coli* will not illicit this reaction, but *E. coli* is indole positive as the amino acid tryptophan is hydrolysed by the enzyme tryptophanase to pyruvic acid, ammonia and indole, which

then accumulates in the media and reacts with Kovac's reagent to produce a red compound on the surface.[13]

3.2.7. Antimicrobial susceptibility determination

In order to ensure the validity of disc sensitivity testing, American Type Culture Collection (ATCC) 25922 and 35218 strains of *E. coli* were used as controls for the quality control of susceptibility testing, and interpreted according to the standards set by the National Committee for Clinical Laboratory Standards (NCCLS). Single *E. coli* colonies isolated from water before and after exposure to chlorine treatment were selected and subjected to antibiotic sensitivity testing, according to the Kirby-Bauer disk diffusion method according to the NCCLS recommendations using Mueller-Hinton broth (Mast, Davies Diagnostics, SA) and Mueller-Hinton sensitivity agar plates (Mast, Davies Diagnostics, SA).[14] Colonies of the selected organisms were inoculated into Mueller-Hinton broth (Mast, Davies Diagnostics, SA) and diluted with Mueller-Hinton broth (Mast, Davies Diagnostics, SA) to an OD of 0.5 McFarland, indicating a colony forming unit count of 10^6-10^8/ml. A sterile cotton swab was used to inoculate this suspension onto Mueller-Hinton agar plates and paper disks (Davies Diagnostics, AB BIODISK, Chemicon International, Inc.) containing the selected antibiotics, were placed onto the seeded agar plates. The seeded plates were allowed to dry for a few minutes and then incubated aerobically for approximately 18-24 hours at 35°C. The zones of inhibition were measured after incubation and the sensitivity/resistance determined according to NCCLS breakpoints.[14]

3.3. Results

3.3.1. Chlorine exposure method

The results of the water and biofilm samples taken at Plankenburg River (Below Kayamandi) were included in this study, as the samples taken above the river contain *E. coli* per 100 ml within the acceptable range. The levels of the isolated *E. coli* organisms before and after exposure to chlorine are illustrated in Tables 3.1. and 3.2.

Table 3.1. The levels of the isolated *E. coli* organisms before and after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water after 30 min	*E. coli* per 100 ml water after 90 min
0.0	7 777000	7 777 000
0.1	1 743 000	134 650
0.3	258 300	21 609
0.4	14 590	0
0.5	199	447
0.6	0	199

Table 3.2. The levels of the isolated *E. coli* organisms from biofilms before and after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water containing biofilm after 30 min	*E. coli* per 100 ml water contaning biofilm after 90 min
0.0	1986	1986
0.1	1986	447
0.3	447	0
0.4	0	0
0.5	0	0
0.6	0	0

Overall, *E. coli* organisms were enumerated across the entire chlorine concentration range as demonstrated in Figures 3.1 and 3.2. In Figure 3.1, after exposure to chlorine for 90 minutes, an anomaly was seen at 0.4 mg/L chlorine where no *E. coli* could be cultured, but at 0.5 mg/L chlorine, a count of 447 *E. coli* per 100 ml was determined. The *E. coli* enumeration levels after exposure to chlorine was compared to previous results of a study done in 2002[2] and this comparison is demonstrated in Figures 3.1 and 3.2.

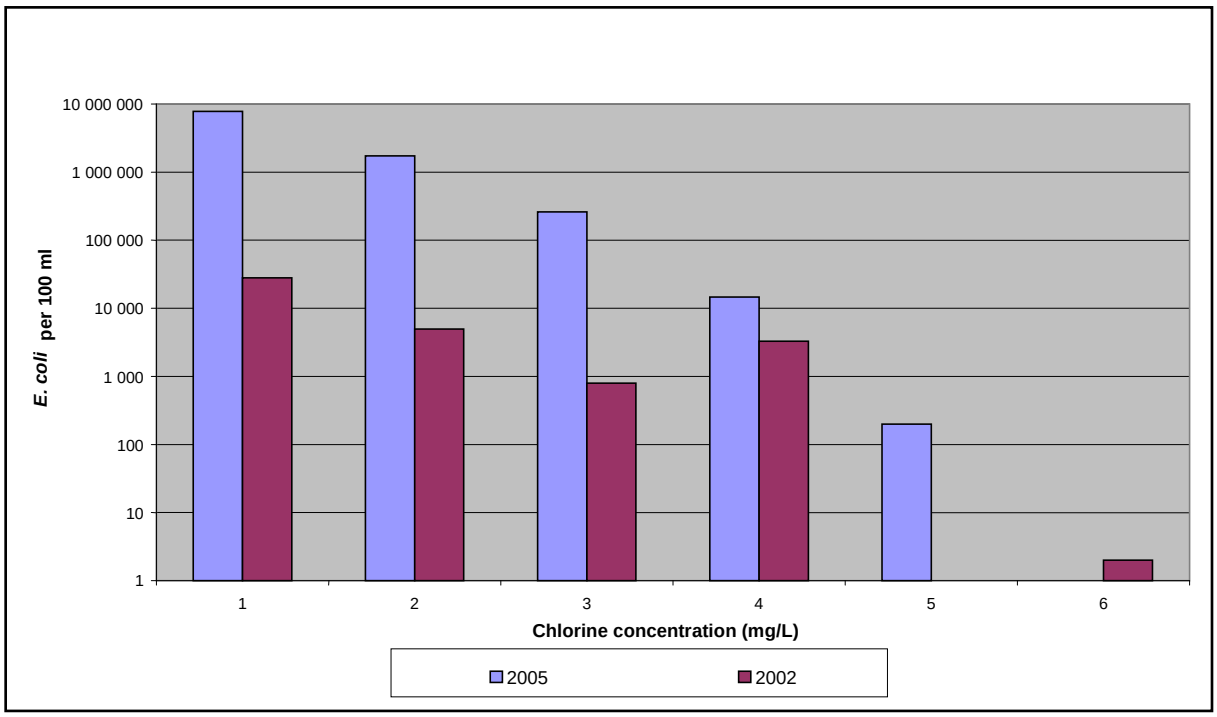

Figure 3.1. Comparison of the enumeration levels of *E. coli* organisms after exposure to chlorine in 2002 and 2005 after a chlorine contact time of 30 minutes[2] The figures are given as logarithmic scale with the chlorine concentrations on the x-axis as 1 (0.0 mg/L), 2 (0.1 mg/L), 3 (0.3 mg/L), 4 (0.4 mg/L), 5 (0.5 mg/L), 6 (0.6 mg/L).

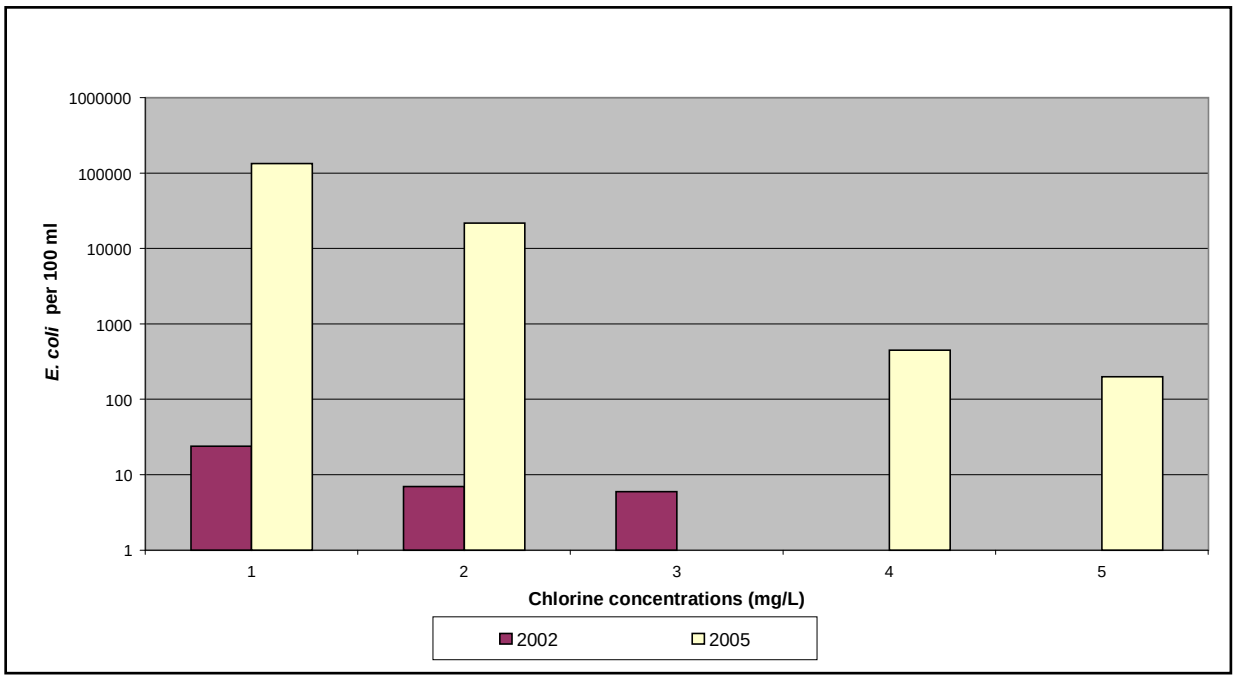

Figure 3.2. Comparison of the enumeration levels of *E. coli* organisms after exposure to chlorine in 2002 and 2005 after a chlorine contact time of 90 minutes[2] The figures are given as logarithmic scale with the chlorine concentrations on the x-axis as 1 (0.0 mg/L), 2 (0.1 mg/L), 3 (0.3 mg/L), 4 (0.4 mg/L), 5 (0.5 mg/L), 6 (0.6 mg/L).

3.3.2. Antimicrobial susceptibility patterns of *E. coli* organisms exposure to chlorine

E. coli strains that were isolated from water and biofilm samples after exposure to various chlorine concentrations were tested for their sensitivity to various selected antimicrobials that are routinely used for the treatment of infections caused by gram-negative microorganisms. The zones of inhibition were measured and the results of sensitivity and resistance to antimicrobials are described in Table 3.3.

Table 3.3. Resistance patterns of selected *E. coli* organisms isolated tested from water and biofilm samples before and after exposure to chlorine

Antimicrobials	*E. coli* strains tested before chlorination (n = 5)	*E. coli* strains tested after chlorination (n = 33)
Ampicillin (AP)	60%	66%
Gentamicin (GM)	15%	18%
Ciprofloxacin (CIP)	0%	30%
Co-amoxiclav (AUG)	5%	6%
Cephalozolin (CZ)	30%	36%
Cotrimoxazole (COT)	40%	48%

Although the numbers of strains in the group of organisms selected before chlorination exposure is very low, it appeared that the *E. coli* organisms were more resistant to the selected antimicrobials after exposure to chlorine. An important observation was the susceptibility pattern of *E. coli* organisms to ciprofloxacin, where the emergence of resistance was most apparent. The antimicrobial resistant patterns of this study reflected the findings of previous research conducted by Dr Barnes in 2002.[3] The results of this study were compared to results obtained from the research by Dr Barnes, and illustrated in Figure 3.3.

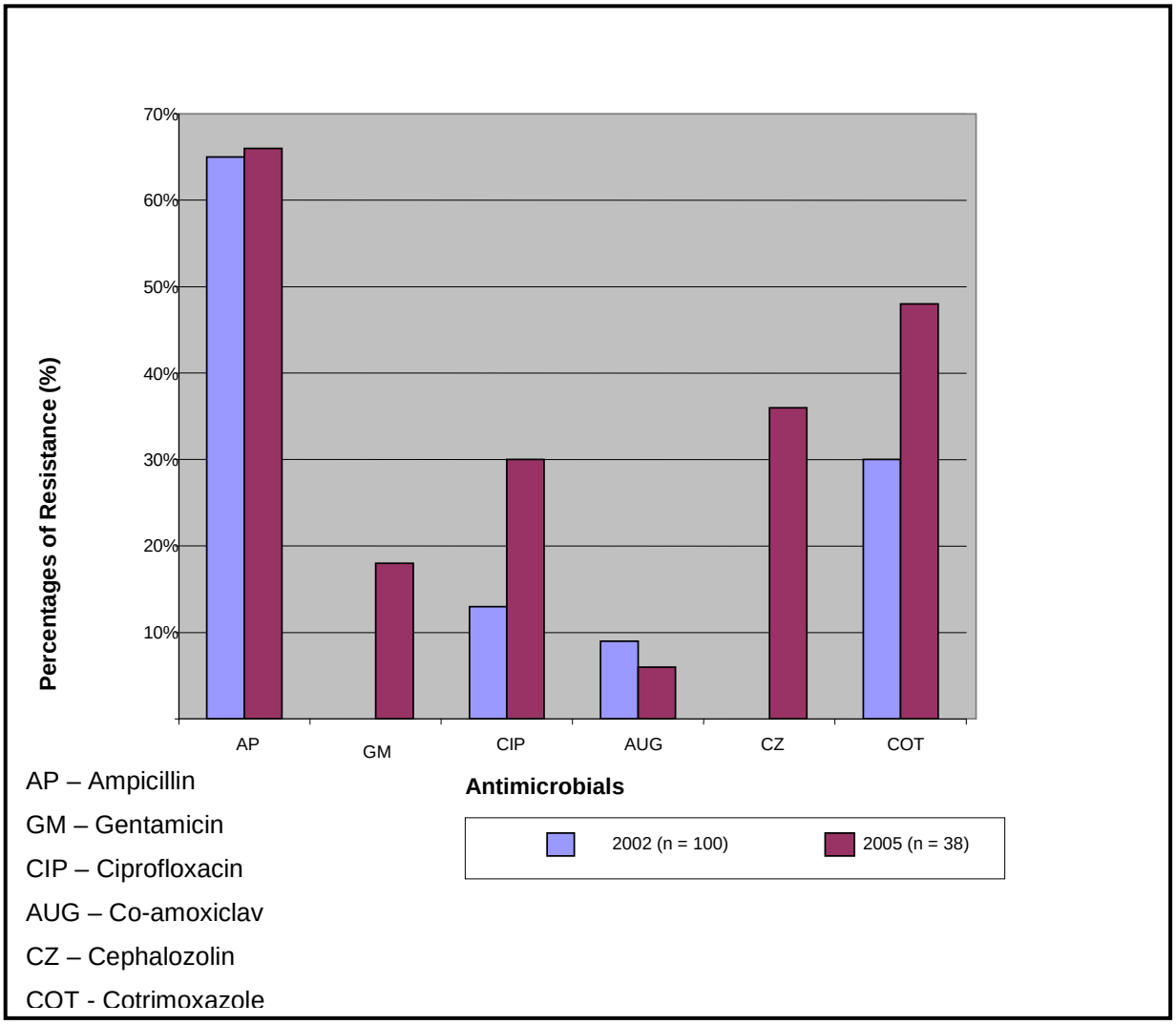

Figure 3.3. Antimicrobial resistance patterns of *E. coli* organisms after exposure to chlorine[3]

3.4. Discussion

3.4.1. Chlorine exposure method

The results of the preliminary study supported previous findings by Dr Barnes[2] and confirmed that water and biofilm samples collected from the Plankenburg River (Below Kayamandi) were contaminated with *E. coli* organisms. After the samples were exposed to chlorine concentrations, *E. coli* organisms were enumerated up to the highest concentrations. This indicates that *E. coli* organisms have adapted to the environment with exposure to chlorine. The anomaly that *E. coli* could not be cultured at a certain chlorine concentration, but re-emerged at higher concentrations has been reported by Barnes on three other occasions.[2] Therefore, inorder to rule out sampling effect, all determinations were done in duplicate for confirmation. The fact that no *E. coli* organisms could be

cultured at a chlorine concentration of 0.4 mg/L could give false positive results if this type of test was performed to determine the most effective concentration for chlorine disinfection of water. It is unclear why more organisms were enumerated at higher concentrations. This pattern of enumeration of *E. coli* has been previous identified as microorganisms appearing to be in a Viable-But-Non-culturable (VBNC) state.[2] The VBNC state is defined as one in which cells are metabolically active, while incapable of undergoing the cellular division required for growth in or on a medium normally supporting growth of that cell.[15], It was further reported that VBNC occur in many gram-negative bacteria, including human-pathogenic *E. coli*. It has been shown that cells entered the VBNC state by one or a combination of environmental stresses, such as starvation, temperature shift and/or exposure to heavy metals.[16]

The comparison of the enumeration levels of *E. coli* organisms exposed to chlorine to the previous results of 2002[2] indicated a significant increase in the total number of *E. coli* organisms per 100 ml water after chlorine exposure. At a chlorine concentration of 0.5 mg/L, 199 *E. coli* organisms per 100 ml water were enumerated in 2005 compared to 0 *E. coli* organisms in 2002, as illustrated in Figure 3.1. A decrease was observed in the levels of *E. coli* organisms in the biofilm sample. The *E. coli* count before chlorine exposure was 1986, while after exposure at 0.3 mg/L chlorine, 447 *E. coli* organisms per 100 ml were enumerated from the biofilm sample. In 2002, *E. coli* ennumeration levels were determined up to 0.6 mg/L chlorine of 199 to 0 *E. coli* organisms per 100 ml in 2005. Possible explanations for this were that the Plankenburg River either contained much higher microbial population in 2005 than 2002, or the chlorine exposure had become more ineffective against the microbial load.

3.4.2. Antimicrobial susceptibility patterns of *E. coli* isolates

A high level of antimicrobial resistance was detected in the *E. coli* that was isolated after exposure to chlorine. Although the number of unexposed organisms tested was very low, it appeared as if an increase of antimicrobial resistance occurred. This opened up the possibility that chlorination may either select or induce changes in antimicrobial resistance in bacterial populations. Armstrong[17] also suggested, without specifying the mechanisms, that stress-tolerant strains selected by chlorination would be more resistant to antimicrobials. Dr Barnes's previous work showed similar resistant patterns for ampicillin in 2002.[3] A higher level of resistance to co-trimoxazole, cephazolin, ciprofloxacin, ceftazidime and gentamicin, but a decrease in augmentin (co-amoxiclav) resistance

patterns were illustrated by this preliminary study. These findings significantly limit the therapeutic options for treatment of microorganisms. Another important factor was that each *E. coli* organism showed resistance to at least two or more antimicrobials, and could therefore be classified as organisms exhibiting MAR. These resistant patterns also suggested that there are definite subpopulations of organisms freely occurring in surface water that may become more resistant to some of the commonly available antibiotics following exposure to certain chlorine concentrations. Although most coliforms may only be harmless indicators of water quality, strains of MAR phenotype that colonise the intestinal tract of humans or animals, could transfer their resistance to intestinal commensals and in turn to drug-sensitive pathogens. The efficient removal of organisms exhibiting MAR phenotypes from sewage before discharge to the environment is therefore important.

3.5. Conclusion

The results from this preliminary study concluded that Plankenburg River (Below Kayamandi) was heavily contaminated with *E. coli* organisms. The study raised questions about the contamination levels of various water sources, as well as the effective chlorine concentration to control microbial contamination. It was also demonstrated that phenotypic profiles, such as morphological and antimicrobial patterns of *E. coli* organisms isolated from water sources exposed to chlorine, are important for diagnostics and research areas. Maybe similar or different morphological patterns exhibit similar or different antimicrobial resistant patterns as well as virulence factors, influencing treatment. Another important issue was the VBNC effect suggested by this study, which may be associated with several mechanisms also contributing to the resistance towards chlorine and certain antimicrobial agents. Since the specific activity of chlorine inactivation is not fully known, *E. coli* organisms may survive chlorine exposure by either their own intrinsic resistance or acquired resistance through gene mutation or horizontal gene transfer from other organisms.

All of the above-mentioned factors contributing to resistance required further investigation and another approach is investigated in the form of my MSc study described in the chapters to follow. To prevent any bias and to demonstrate the problem of water contamination, as observed in the background study, across a wider geographic area, various water sources were included in the MSc study. The microbiological levels using *E. coli* as an indicator organism were determined and the results are demonstrated in

Chapter Four (Substudy One).

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Chapter Four

Sampling strategies and microbiological quantification of *E. coli*

4.1. Introduction

Several disinfectant strategies employed to make water potable, as well as indicator organisms for optimally determining the levels of pollution of water, have been described in Chapter One, Sections 1.3.1 and 1.4.

In the current substudy, a microbiological approach was used to investigate and determine the faecal contamination levels of selected water sources in the Western Cape. The water sources included had various applications, for example the Plankenburg River, which runs through some ecological, agricultural and recreational areas. A high level of microbial pollution in this area may therefore have serious health implications.

The aim of this substudy was to determine the levels of water pollution in selected water sources, such as surface water in rivers, wastewater in dams, which are resources for informal settlements, and industrial settings in the Western Cape.

4.2. Materials and methods

For comparability, the sampling method and enumeration of *E. coli* organisms were similarly executed as in the preliminary study described in Chapter Three, Section 3.2.2, 3.2.2.1 and 3.2.2.2.

4.2.1. Sampling strategies

The sampling strategy used to collect samples for this study was determined by the geographical setting with respect to water consumption for humans, animals and irrigation purposes. Plankenburg River (Below Kayamandi), Disa River (Imizamo Yethu) and Stiebeuel River (Langrug) are informal settlements that were selected for this substudy, as there were concerns that the main source of pollution in these areas was probably faecally related. A sample from Nietvoorbij Research Project (wastewater effluent at Spier Wine Estate) was included, as this water source originated from an area where poor sanitation facilities were a concern. Finally, a sample from Wellington sewage works was included to evaluate pollution at effluent treatment level.

4.2.2. Water sample collection methodology

The water samples included in this study were all collected exactly as in the preliminary study, described in Chapter Three, Section 3.2.2.1.

4.2.3. Biofilm sample collection method

The biofilm collection method was exactly executed as described in Chapter Three, Section 3.2.2.2.

4.2.4. Summary of the sampling sites and sample collection

4.2.4.1. Site 1 - Plankenburg River

a. Characteristics of Plankenburg River

This river runs along the banks of Stellenbosch and consists of a shallow stream with a slow flow rate during extended dry periods. This frequently low volume of water contributes to the problems of water quality. The first rain normally washes the accumulated surface pollution onto the riverbanks at very high concentrations and will increase the pollution downstream as heavier rainfalls continue. The upper part of the river has water flowing consistently only in the rainy season.[2]

b. Sample Collection

A water and biofilm sample was obtained from the Plankenburg River downstream from the dense settlement of Kayamandi (Below Kayamandi) on 26 June 2006.

4.2.4.2. Site 2 - Stiebeuel River

a. Characteristics of Stiebeuel River

The informal settlement of Langrug is adjacent to Dennegeur property, but separated by an electrified fence. It is situated against a slope, forming one of the foothills of the mountains behind Franschoek. According to Dr Barnes (unpublished data), access to water in the Langrug community is far better than access to sanitation. There is a formal sewage system, but it is not connected to the dwellings within the informal settlement. The imbalance between water use and safe water disposal results in large amounts of dirty water disposed outside the formal sewage system. Large volumes of human excreta are

also deposited in the environment due to the poor state of available toilet facilities. This leads to the accumulation of waste that the rain may then wash down directly into the Stiebeuel River.

b. Sample Collection

One water sample was taken at a ditch adjacent to the dense Langrug informal settlement with run-off water draining into the Stiebeuel River. Another sample was taken below the Franschoek sewage works in the Stiebeuel River. Both samples used in this study were taken on 26 September 2006.

4.2.4.3. Site 3 - Disa River

a. Characteristics of Disa River

Disa River passes through the town of Hout Bay and Imizamo Yethu informal settlement. Several houses and a guest house with septic tanks are situated near the Disa River. Therefore, the river may be polluted with waste or discharges from the surrounding communities.

b. Sample Collection

Two water samples were taken on 17 October 2006 from the Disa River, Imizamo Yethu informal settlement (Hout Bay): one upstream (Disa River about 4 km above the town of Hout Bay) and another one downstream from the dense settlement. A sample from the storm water ditch emanating from the dense settlement of Imizamo Yethu in Hout Bay was taken as the downstream sampling point. Sampling was done at a point in the ditch just before the point of confluence with the Disa River, so that the inflow of fresh water from the river would not affect the reading. The resultant *E. coli* reading thus represent the total concentration that reached the Disa River at that point in time.

4.2.4.4. Site 4 –Nietvoorbij Research Project

Nietvoorbij, which is a research facility situated in Stellenbosch. On 20 November 2006, Dr Barnes sampled water from a winery wastewater effluent at Spier Wine Estate, that formed part of the Nietvoorbij Research Project on irrigation of vegetables with cellular effluent. Inspection of the wine farms indicated leaking of the septic tanks that may have been

disposed into the waste dam. A water sample also taken by her to include in this MSc study.

4.2.4.5. Site 5 - Wellington Sewage Works

a. Characteristics of Wellington Sewage Works

This sewage work supplies water to various areas of Wellington. It was observed that a ditch containing partially treated effluent (possible prior exposure to chlorine) was present at the site. If high levels of the indicator organisms are detected within these effluents, then it may suggest that the treatment was ineffective to maintain the water distribution to its surrounding areas.

b. Sample Collection

Only two biofilm samples were collected so far in this study and they were both obtained from Plankenburg River (Below Kayamandi). Therefore, only a biofilm sample in the form of a stone (an inert substrate) was taken at Wellington Sewage Works on 19 February 2007.

4.2.5. Enumeration of organisms isolated from the water samples

The standard 5x multiple tube enumeration method was followed according to SABS guidelines to test for faecal coliforms and *E. coli*, as described in Chapter Three, Section 3.2.2.3 with no exposure to chlorine to determine the water pollution level with indicator organism, *E. coli*.

4.3. Results

4.3.1. Conditions during sample collection

The physical characteristics of the water and biofilm samples obtained from the water sources were observed and described according to flow speed, turbidity and general appearance during sampling collection. The conditions are summarised in Table 4.1.

4.3.2. Quantification of faecal contamination

The *E. coli* counts before chlorine exposure were determined in water dilutions of all 6

water samples and 2 biofilm samples that were taken from the various sampling sites. The results of water and biofilm samples are illustrated in Tables 4.2. and 4.3.

Table 4.1. Characteristics of water conditions during sample collection

Sample	Date	Time	Water temp (^{o}C) and pH	Turbidity	Flow speed	Remarks
Plankenburg River (Water)	26/06/06	09h05	12 pH 6.5	+++	*	Very dirty Rained <10 mm on 22/06/06. Previous day sunny and warm with minimum of 14^{o}C.
Plankenburg River (Biofilm)	26/06/06	09h15	12 pH 6.5	+++	*	Very dirty
Stiebeuel River (Water before ditch)	26/09/06	09h30	17 pH 7.1	+	*	Appeared clean
Stiebeuel River (Water after ditch)	26/09/06	10h00	16 pH 7.1	++	**	Rained previous day and minimum temperatures still below summer values.
Disa River (Water before settlement)	17/10/06	12h10	16 pH 7.3	+	**	Appeared clean. No recent rain.
Disa River (Water after settlement)	17/10/06	11h50	20 pH 7.3	+++	*	Appeared polluted. Sewage bioslime growing in ditch.
Nietvoorbij Research Project	20/11/06	08h55	20 pH 7.3	+++	*	Few millimeters rain previous day. Temperatures rising.
Wellington Sewage Works	19/02/07	08h40	24 pH 6.5	++	**	Rained recently

+++ (Very turbid), ++ (turbid), + (clear); ** (Medium flow speed), * (Slow flow speed)

Table 4.2. The *E. coli* counts of water sampled at various water sources before exposure to chlorine

Water Sources	Date of Collection	*E. coli* per 100 ml water
Plankenburg River (Below Kayamandi)	26 June 2006	7 777000
Before Stiebeuel River (Langrug informal settlement, Franschoek)	26 September 2006	920 227
Below Stiebeuel River (Langrug informal settlement, Franschoek)	26 September 2006	39 905 600
Imizamo Yethu informal settlement (Before built-up area, Hout Bay)	17 October 2006	446 850
Imizamo Yethu informal settlement (After built-up area, Hout Bay)	17 October 2006	9 178 423
Wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project)	20 November 2006	777 705

Table 4.3. The *E. coli* counts of biofilms obtained at various water sources before exposure to chlorine

Water Sources	Date of Collection	*E. coli* per 100 ml water containing biofilm surviving after 30 min
Plankenburg River (Below Kayamandi)	26 June 2006	446 850
Wellington Sewage Works	19 February 2007	198 671

4.4. Discussion

4.4.1. Conditions during sample collection

The sampling conditions differed at each sampling site, as indicated in Table 4.1. Samples from Plankenburg River and Disa River were turbid with very slow flowing water and indicated high pollution levels. These river conditions are mostly determined by the surrounding sanitation facilities of the dense informal settlements of the nearby areas, as described in Sections 1.1 and 4.2.4. Both turbidity and flow speed are important factors in the determination of the conditions of the water quality, but no measurements of the precise accuracy on the efficacy as an indication of water quality was undertaken in this substudy. Other water sampling conditions can be attributed to previous rainfall, which diluted the water and can disrupt the microenvironment at the bottom of the river or transport the heavily polluted water from one site to another.[1] This may have been the case for the sample taken below the Stiebeuel River. Rainfall plays an important role in the water pollution levels, but this was not investigated in this substudy. The sample from the winery wastewater effluent obtained at Spier Wine Estate as part of the Nietvoorbij Research Project may be contaminated from the sewage winery effluent and can influence the water sample appearance. Some water and biofilm samples appeared clean with slow to medium flow speed, but may contain high counts of microorganisms. Many microorganisms, especially *E. coli* used as the indicator organism, can persist and multiply in water under minimal nutrient and environmental conditions.[2] Similarly, *Helicobacter pylori*, was reported to remain culturable in cold water for up to 2-3 days.[3] The precise effect of this variable as well as others, such as temperature, pH and subculturing, can only be determined by direct comparisons, which was not included in this study approach. However, the quantification of microorganisms present in water sources need to be determined to prevent improper disinfection if such resistant microorganisms are identified.

4.4.2. Quantification of faecal contamination

High *E. coli* counts per 100 ml were obtained from each of the water and biofilm samples included in this substudy. This high count of the indicator organism provides conclusive evidence of recent faecal pollution, as well as an index of environmental effects of sanitation facilities within the immediate environment from where the samples were taken. The method used in this substudy has limitations for the detection of enteric viruses and protozoa that are more resistant to chlorine disinfection. For practical reasons we did

not include the detection of other bacteria, viruses and bacteriophages in this study. However, the absence of *E. coli* does not necessarily indicate the absence of any other pathogenic microorganisms. A previous study[2] found that the inclusion of more than one indicator microorganism, such as bacteriophages and/or bacterial spores, within the water treatment process is highly effective, especially if the source water is known to be contaminated with that microorganisms.

The enumeration of *E. coli* from the biofilm samples were low in comparison with the numbers of *E. coli* organisms detected from the water samples. A possible explanation could be that the samples had different densities of organisms within the biofilms, which is determined by the gradients of nutrient and oxygen availability within these biofilm structures.[4] These gradients can result in differences in the metabolic activities of bacteria, leading to different bacterial variants and stress tolerances.[4] This can have important health implications, especially since antimicrobials primarily target the metabolically active cells and it had been postulated that organisms occurring in a biofilm can have differences in susceptibility to antimicrobial agents.[4]

According to international standards, drinking water should contain no *E. coli* organisms.[5] All of the *E. coli* levels determined in this substudy exceeded both the international and national cut-off levels for classifying water as carrying increased risk for waterborne diseases described in Chapter One, Section 1.3.2.

4.5. Conclusion

The findings from this substudy illustrated that the physical appearance of the water samples cannot be taken as an indicator of clean water, as the water appearances ranged from clean to turbid and contained high numbers of bacteria. This brought about speculation that the water quality was impaired by either the ineffective disinfection treatment or contaminated by the surrounding communities. The need for surveillance and continuous monitoring of the microbiological level of contamination (indicator and specific microorganisms) of water sources are necessary to limit potential health risks.

Since we have now demonstrated that the water sources sampled were heavilly contaminated with faecal coliforms and *E. coli* organisms, the next step was to see what the effect of chlorine exposure, one the most commonly used disinfectants worldwide, would have on this water. Chlorine may be ineffective, as these organisms are present in high numbers that may also be tolerant to its effect. Its effectivity can be demonstrated

by its ability to drastically decrease the microbiological levels of the water and biofilm samples and are described in Substudy Two, presented as Chapter Five.

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Chapter Five

The selection of chlorine tolerant *E. coli* strains

5.1. Introduction

Chlorination is the most widely used disinfectant for microbial control[1] in both drinking water and wastewater processing.[2] It was demonstrated in the preliminary results (Chapter Three) as well as in a previous study[3] that the indicator organism, *E. coli*, isolated from the Plankenburg River, may survive chlorine disinfection up to the highest concentration. Therefore, the main objective of this substudy was to expose the water and biofilm samples included in the study to different chlorine concentrations in order to determine the survival of *E. coli*, and to select a strains for further phenotypic and genotypic characterisation.

5.2. Materials and methods

5.2.1. Isolation of *E. coli* organisms from water and biofilm samples

The water and biofilm samples collected in Chapter Four, Sections 4.2.2 and 4.2.3 were further investigated in this substudy.

5.2.2. Chlorination exposure method

The methodologies for chlorine disinfection was similar to that described in Chapter Three, Section 3.2.2.3.

5.2.3. Direct bacterial plate count from water and biofilm samples

A 0.1 ml (100 µl) sample was taken directly from each of the water and biofilm samples before and after 30 and 90 minutes exposure to chlorine and inoculated onto a MacConkey agar plate (Diagnostic Media Products, NHLS, Green Point, SA) and incubated aerobically for 24 hours at 37°C. For direct bacterial quantification, a colony count was performed on the different agar plates for quality control of the water samples exposed to chlorine. The same process was repeated for the biofilm samples.

5.3. Results

5.3.1. Chlorine exposure of water samples

Overall, *E. coli* organisms were enumerated up to the highest chlorine concentrations. The possible VBNC effect observed and discussed in Chapter Three was again seen amongst the chlorine exposure patterns of *E. coli* organisms. The results are described in Tables 5.1.to 5.4 below.

Table 5.1. The enumeration of the levels of *E. coli* organisms in water taken from Plankenburg River (Below Kayamandi) on 26 June 2006 after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water after 30 min	*E. coli* per 100 ml water after 90 min
0.1	2 398 891	117 608
0.3	446 850	1 987
0.4	19 891	0
0.5	778	1 176
0.6	181 823	0

Table 5.2. The enumeration levels of *E. coli* organisms in water sampled Below Stiebeuel River (Langrug informal settlement, Franschoek) on 26 September 2006 after exposure to various chlorine concentrations.

Chlorine concentration (mg/L)	*E. coli* per 100 ml water after 30 min	*E. coli* per 100 ml water after 90 min
0.1	0	778
0.3	198	169
0.4	0	447
0.5	198	169
0.6	1 696 293	446 850
0.7	447	0

Table 5.3. The enumeration levels of *E. coli* organisms in water sampled from Imizamo Yethu informal settlement (Below built-up area, Hout Bay) on 17 October 2006 after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water after 30 min	*E. coli* per 100 ml water after 90 min
0.1	5 035 098	723 330
0.3	181 823	1 442 696
0.4	198 671	363 988
0.5	911 122	363 988
0.6	820 341	0

Table 5.4. The enumeration levels of *E. coli* organisms in water sampled from a wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) on 20 November 2006 after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water after 30 min	*E. coli* per 100 ml water after 90 min
0.1	44 685	32 597
0.3	92 106	4 469
0.4	19 867	4 027
0.5	19 867	820
0.6	0	0

5.3.2. Chlorine exposure of biofilm samples

Less *E. coli* organisms were isolated from the biofilm samples than the water samples. Nevertheless, *E. coli* organisms were isolated from the biofilms across the entire range of chlorine concentrations. The VBNC effect observed in the water samples, as well as in Chapter Three, was also noted in the *E. coli* organisms isolated from Plankenburg River (Below Kayamandi). These results are tabulated in Tables 5.5 to 5.6.

Table 5.5. The enumeration levels of *E. coli* organisms extracted from biofilms sampled from Plankenburg River (Below Kayamandi) on 26 June 2006 after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water containing biofilm after 30 min	*E. coli* per 100 ml water containing biofilm after 90 min
0.1	44 685	11 818
0.3	44 685	199
0.4	198 670	0
0.5	0	0
0.6	44 685	199

Table 5.6. The enumeration levels of *E. coli* organisms extracted from biofilms sampled at Wellington Sewage Works on 19 February 2007 after exposure to various chlorine concentrations

Chlorine concentration (mg/L)	*E. coli* per 100 ml water containing biofilm after 30 min	*E. coli* per 100 ml water containing biofilm after 90 min
0.1	199	0
0.3	0	0
0.4	0	0

5.3.3. Direct bacterial count from water and biofilm samples

A direct bacterial count was performed on each of the water and biofilm samples for quality control of the disinfection process and are demonstrated in Table 5.7.

Table 5.7. The direct bacterial plate counts of water and biofilm samples exposed to various chlorine concentrations

Sampling Sites	Sample Type (Contact time)	Chlorine concentrations (mg/L)					
		0.0	0.1	0.3	0.4	0.5	0.6
Plankenburg River (Below Kayamandi) 2006	Water (30)	3.80×10^4	3.00×10^4	50×10^2	23×10^2	15×10^2	20×10^2
	Water (90)	3.80×10^4	1.00×10^4	20×10^2	0	15×10^2	0
	Biofilm (30)	32×10^2	12×10^2	12×10^2	22×10^2	0	12×10^2
	Biofilm (90)	32×10^2	9×10^2	6×10^2	0	0	3×10^2
Below Stiebeuel River (Langrug informal settlement Franschoek)	Water (30)	3.20×10^4	0	10×10^2	0	10×10^2	90×10^2
	Water (90)	3.20×10^4	33×10^2	18×10^2	27×10^2	6×10^2	41×10^2
Imizamo Yethu informal settlement (Below built-up area, Hout Bay)	Water (30)	3.50×10^4	2.80×10^4	1.20×10^4	80×10^2	41×10^2	25×10^2
	Water (90)	3.50×10^4	3.00×10^4	2.00×10^4	2.40×10^4	1.10×10^4	1.10×10^4
Nietvoorbij Research Project	Water (30)	4.00×10^4	1.00×10^4	3.50×10^4	82×10^2	80×10^2	0
	Water (90)	4.00×10^4	95×10^2	31×10^2	20×10^2	12×10^2	0
Wellington Sewage Works	Biofilm (30)	88×10^2	44×10^2	1×10^2	0	0	0
	Biofilm (90)	0	0	0	0	0	0

Bacterial count is measured as colony forming units (cfu)

5.4. Discussion

5.4.1. Chlorine exposure of water samples

In this substudy, the enumerated levels of *E. coli* after exposure to various chlorine concentrations from the numerous water and biofilm samples were determined. These levels indicated that *E. coli* organisms were able to withstand exposure to the entire range of chlorine concentrations after both 30 and 90 minutes contact times. Normally, the disinfection method used by small-scale municipal workers includes chlorine concentrations up to 0.5 mg/L with 30 minutes contact time.[3] A previous research study[4] found that a number of bacterial genera found in chlorinated water demonstrated a variety of disinfection resistance patterns after disinfection with free chlorine and monochloramine. The results from this substudy implies that the *E. coli* organisms occurring in the various water sources sampled have the ability to survive exposure to a range of chlorine concentrations at the standard and prolonged contact times, and may render such disinfection treatment ineffective.

The levels of *E. coli* strains determined after chlorine exposure in samples taken during 2006 increased, compared to the results of the preliminary study of 2005 (Chapter Three), as well as previous studies done in 2002.[5] A similar VBNC effect was observed in isolates: no *E. coli* organism was cultured after exposure to chlorine concentrations of 0.4 mg/L (Tables 5.1 and 5.5.). After an increase in chlorine concentration to 0.5 mg/L chlorine, *E. coli* growth emerged again. Pathogenic organisms exhibiting such VBNC states may have an impact on the public health as an increased virulence of stressed bacteria had been illustrated.[6]

The mechanism of this proposed VBNC state with exposure to chlorine is not fully understood. One possible explanation is that most of the microorganisms may have lived in relationships with other organisms and only the free-flowing microorganisms within the water were exposed to chlorine, which resulted in this particular phenotype. Alternatively, some of the *E. coli* organisms may also have a lower threshold for regarding chlorine as toxic or may have activated a cascade of reactions of which active efflux is one, again displaying this phenotype. In addition, particulate matter, such as faecal material, may decrease the biocide activity of chlorine and protect the *E. coli* organisms from the effect of chlorine.

From the results obtained in this substudy it appears that chlorine disinfection of 90 minutes contact time with chlorine concentrations ranging from 0.1 to 0.6 mg/L would be effective for disinfection of the water. It has been shown previously that over-chlorination may lead to the formation of various mutagenic and/or carcinogenic byproducts.[7,8] Although increasing the chlorine concentration would be more effective in decreasing the microorganisms, it may make the water less potable due to an increased likelihood of a chlorine odour and an affect of the taste.[9-11]

5.4.2. Chlorine exposure of biofilm samples

After chlorine exposure, the enumeration of viable organisms seen within the biofilm samples were very low compared to the *E. coli* levels enumerated in the water samples. It was evident from these results that the microorganisms had become more equipped to withstand chlorine exposure. There are many speculations regarding the reaction to chlorine exposure of *E. coli* organisms within biofilms, e.g. that organisms enclosed in biofilms usually grow at different rates and therefore may be more tolerant to chlorine exposure.[12] A comparison of *E. coli* organisms isolated from biofilm samples taken at Plankenburg River (Below Kayamandi) in 2006 showed an increased enumeration levels of *E. coli* strains compared to the preliminary study of 2005 described in Chapter Three and previous studies done in 2002.[3]

In this substudy only the multiple tube enumeration method for water was used to investigate the number of organisms that could still be cultured after exposure to chlorine. Many other techniques not used in this study, are described in the literature, each with its own advantages and disadvantages, as well as specific application for chlorine disinfection. For example, another study[13] used a heterotrophic plate count technique to illustrate the increased survival of bacteria growing on metal surfaces exposed to free chlorine.

Other studies investigating the effectiveness of chlorine to inactivate waterborne bacteria have also illustrated that although disinfectants were present in the distribution systems, microorganisms were still able to survive even under adverse conditions.[4,14] Many microorganisms have the ability to adapt to their changing environmental conditions and have been described to become resistant. Chlorine tolerance has been observed in each of the water samples included in the present study. Some of the water sources contained a higher number of indicator organisms that were tolerant to chlorine exposure. Any

microbial resistance within a water environment is influenced by the concentrations of the various components present. Therefore, previous exposure of any strain to such environmental conditions, including disinfection, may result in more tolerance compared to strains with no prior exposure. Furthermore, the diverse communities existing in biofilms and water sources may also have promoted the formation of such resistance phenotypes.[7]

5.4.3. Direct bacterial plate count from water and biofilm samples

Sufficient *E. coli* growths were obtained from the direct bacterial counts of water and biofilm samples on the selective media. As described in Section 5.2.2, the direct bacterial count of the samples act as an indication of the affectivity of the chlorine disinfection method with the multiple tube enumeration method. This bacterial plate count was a quicker method than the multiple tube enumeration method, but did not give an exact count of the pollution levels as the multiple tube method. The multiple tube method was therefore used for the water pollution level determination over this direct method. The direct bacterial plate counts correlated with the *E. coli* survival rates shown in Tables 5.1 to 5.4 and Tables 5.5 to 5.6. The VBNC effect demonstrated in Chapter Three was also observed in this substudy in the patterns of the direct bacterial plate counts and the enumeration of the *E. coli*. The colonies obtained with the direct bacterial plate count were taken as phenotypic results based on their morphological appearances and stored for further investigation.

5.5. Conclusion

This substudy illustrates that the water sources included were contaminated with *E. coli* strains that have the ability to withstand exposure to various chlorine concentrations. This raises the question of whether or not the standard chlorine disinfection method to inactivate microorganisms is effective to prevent any potential health risk.

It was therefore of interest to study the characteristics of the *E. coli* strains that survived chlorine exposure up to the highest concentrations. The characteristics of these strains may differ from the sensitive strains in both their phenotypic and genotypic profiles. This was investigated by first determining the phenotypic characteristics of the surviving *E. coli* strains as determined by their colony morphological patterns and antimicrobial susceptibility profiles. This study (Substudy Three) is presented as Chapter Six.

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Chapter Six

Phenotypic characterisation of *E. coli* strains

6.1. Introduction

In this substudy, *E. coli* strains were selected from the water and biofilm samples before and after exposure to various chlorine concentrations as described in Chapter Five (Substudy Two), in order to further investigate certain phenotypic characteristics of these strains. These characteristics were based on visual assessment of their colony morphology, as well as determination of antibiotic susceptibility profiles. Finally, single colonies representative of each antibiotic susceptibility pattern and morphological appearances were stored for further investigation.

6.2. Materials and methods

6.2.1. Selection of *E. coli* strains from water and biofilm samples

After identification of strains isolated from water and biofilm samples with the multiple tube enumeration method described in Chapter Four (Substudy One), samples were cultured onto selective agar plates of UriSelect and MacConkey agar (Diagnostic Media Products, NHLS, Green Point, SA), and incubated for 18-24 hours at 37°C. Colonies displaying the typical morphological characteristics of *E. coli* on MacConkey (pink domed-shaped colonies) and UriSelect agar (magenta domed-shaped colonies) were selected and their identification confirmed with a biochemical sugar test to exclude isolates that were not *E. coli*. Selected colonies were inoculated into Mueller-Hinton broth (Mast, Davies Diagnostics, SA) and diluted to an OD of 0.5 McFarland, indicating a colony forming unit count of 10^6-10^8/ml. A droplet of this was inoculated onto Simmons citrate and tryptone water and incubated overnight. *E. coli* was identified by a negative Simmons citrate and a positive spot indole test. Master plates (with single colonies) of UriSelect agar plates were simultaneously prepared from selected colonies to obtain pure growth. Stored *E. coli* isolates from my BSc Honours project in 2005 described in Chapter Three of Plankenburg River (Below Kayamandi) were inoculated onto UriSelect agar plates to obtain single colonies and included for this substudy. Overall 165 colonies identified from the water samples and 60 from the biofilms with varying morphologies were selected, labelled and stored in eppendorf tubes containing 70% of 50 µl glycerol broth (16.8 ml glycerol, 2.5 g

nutrient broth no.2 powder, distilled water made up to 100 ml) for later analysis.

Repeated sub-culturing can influence the colony morphology and antimicrobial susceptibility described in section 6.2.2. Therefore quality control measurements taken to prevent contamination included repeated culturing and separation of colonies to ensure pure growth.

6.2.2. Antimicrobial susceptibility determination

E. coli isolates described in section 6.2.1 were selected for this test based on their morphological types, as well as their ability to survive different chlorine exposure levels. Each isolate was tested in duplicate for quality control. Not all *E. coli* isolates originally selected were tested due to financial restraints and the fact that more than one colony with similar morphological type and chlorine concentration from a sample was included in the original set. Selected isolates (n=137) were sub-cultured onto UriSelect agar plates to obtain single colonies. The Kirby-Bauer disk diffusion method was followed as described by the Committee for Clinical Laboratory Standards (CLSI, previously known as NCCLS) for the antibiotic susceptibility testing. Single *E. coli* colonies selected from the plate were inoculated into Mueller-Hinton broth (Mast, Davies Diagnostics, AB BIODISK, Chemicon International, Inc.) and diluted to an OD of 0.5 MacFarland. The suspension was inoculated onto Mueller-Hinton agar plates and paper disks (Davies Diagnostics, AB BIODISK, Chemicon International, Inc.) containing the selected antimicrobials as listed in Table 6.1, were placed onto the seeded agar plates. The plates were incubated aerobically for 18 - 24 hours at 35°C. The antimicrobial agents tested included those tested in the routine laboratory against the *Enterobacteriaceae*. Additional antibiotics including other FQs (ofloxacin and moxifloxacin) were also tested as resistance to ciprofloxacin have been previously seen amongst *E. coli* organisms isolated from our surface water (Chapter Three), and we were interested to see if this resistance extended to the other drugs in this class.

Resistance mediated by ESBL enzymes are commonly found in *E. coli, Klebsiella pneumonia*e and *Klebsiella oxytoca.*[1] ESBLs are caused by enzymes that mediate resistance to third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone) and monobactams (aztreonam), but have no effect against cephamycins (cefoxitin and cefotetan) or carbapenems (meropenem or imipenem).[2] In this substudy the presence of ESBLs was determined with the double-disk synergy test. This was executed by placing

ceftazidime (for the detection of SHV-5, SHV-12 and TEM β -lactamases), cefotaxime (SHV-2, CTX-M β -lactamases) discs in a circle around a central amoxicillin-clavulanate disk.[3] An additional third generation cephalosporin, cefpodoxime was included, as it was reported to be very sensitive for ESBL detection in *Klebsiella* spp.[4,5] An ESBL was identified by the formation of a so-called 'dumbell' effect between any of the cephalosporin discs and the amoxicillin-clavulanate, which is caused when the clavulanic acid inhibits the effect of the cephalosporinase.

The zones of inhibition were measured after incubation and the sensitivity or resistance determined according to CLSI breakpoints.[2] For the purpose of this study, antimicrobials falling into the 'intermediate' range were recorded as non-susceptible. *E. coli* ATCC 25922 and 35218 strains were included in each batch for quality control of susceptibility testing, and were interpreted according to the CLSI guidelines.[2]

E. coli exhibiting various antibiograms were selected and stored into 10 ml LB-medium (10 g NaCl, 10 g tryptone, 5 g yeast extract, 20 g agar, deionised water made up to 1L) containing ampicillin (50 mg/ml) and incubated for 24 hours at 37°C. LB broth with growth aliquot (850 µl) was added to sterile eppendorf tubes containing 150 µl of 100% glycerol and stored at –80°C until required for later genotypic analysis.

Table 6.1. Antimicrobials selected for the determination of sensitivity patterns of *E. coli* strains[2]

Antibiotics	Concentration of antibiotics in discs(µg)	Interpretation of zone criteria (mm) R I S
Ampicillin (AP)	10	≤13 14-16 ≥17
Amikacin (AK)	30	≤14 15-16 ≥17
Gentamicin (GM)	10	≤12 13-14 ≥15
Trimethoprim-sulfamethaxazole (TS)	1.25 / 23.75	≤10 11-15 ≥16
Ciprofloxacin (CIP)	5	≤15 16-20 ≥21
Moxifloxacin (MOX)	5	≤17 18-20 ≥21
Ofloxacin (OFL)	5	≤12 13-15 ≥16
Co-amoxiclav (AUG)	20/10	≤13 14-17 ≥18
Cephazolin (CZ)	30	≤14 15-17 ≥18
Cefoxitin (FOX)	30	≤14 15-17 ≥18
Cefotaxime (CAZ)	30	≤14 15-17 ≥18
Ceftriaxone (CTX)	30	≤14 15-22 ≥23
Cefpodoxime (CPD)	10	≤17 18-20 ≥21

6.3. Results

6.3.1. Selection of *E. coli* strains from water and biofilm samples

A total of 165 *E. coli* strains were selected on morphological appearances after isolation from the various water sources and exposure to chlorine concentrations. These *E. coli* strains showed some similarities in their colony morphologies and eleven different morphological colony types could be distinguished. The different morphological patterns of the isolated *E. coli* colonies, as identified in this study, are described in Table 6.2, and a number is assigned to each type in order to facilitate further analysis.

Table 6.2. The various morphological colony types associated with *E. coli* strains isolated from various water sources

Description	Morphological type number
Flat, large, magenta colonies	1
Small, dome-shaped magenta colonies	2
Medium, domed-shaped, magenta colonies	3
Medium mucoid magenta, colonies	4
Small, lighter, dome-shaped magenta colonies	5
Light, medium, magenta colonies	6
Pointy red colonies	7
Large, misformed, magenta colonies	8
Large, mucoid, dome-shaped magenta colonies	9
Medium, feathery-type, magenta colonies	10
Large, domed-shaped, magenta colonies	11

The morphological classification of the isolated water and biofilm *E. coli* strains was done according to the morphological colony types listed in Table 6.2. The types of the number of colonies isolated from the various sites were determined and these are presented in the Tables 1.1 (water) and 1.2 (biofilms), attached in Addendum 1. The morphological colony types before and after exposure to chlorine is illustrated in Figures 6.1 (water) and 6.2 (biofilms). In both the water and biofilms samples, the morphological patterns changed after exposure to chlorine.

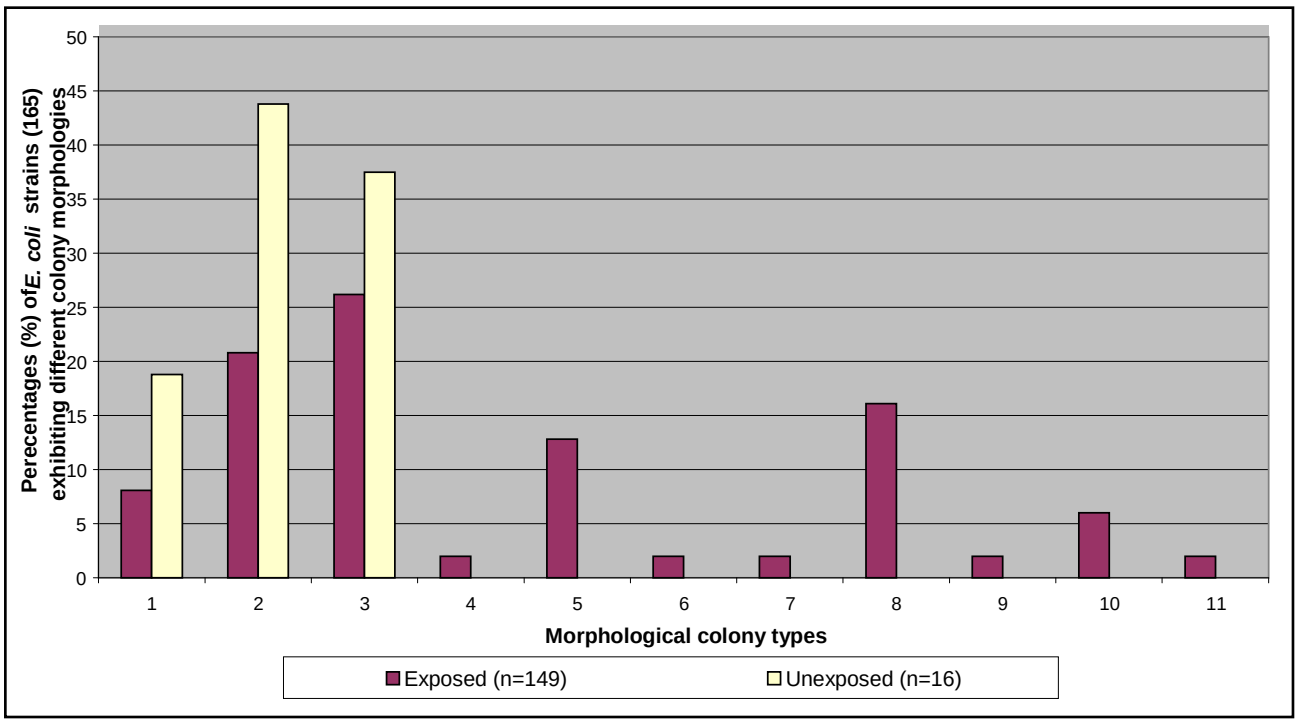

Figure. 6.1. Morphological colony types of *E. coli* strains isolated from water before and after exposure to chlorine

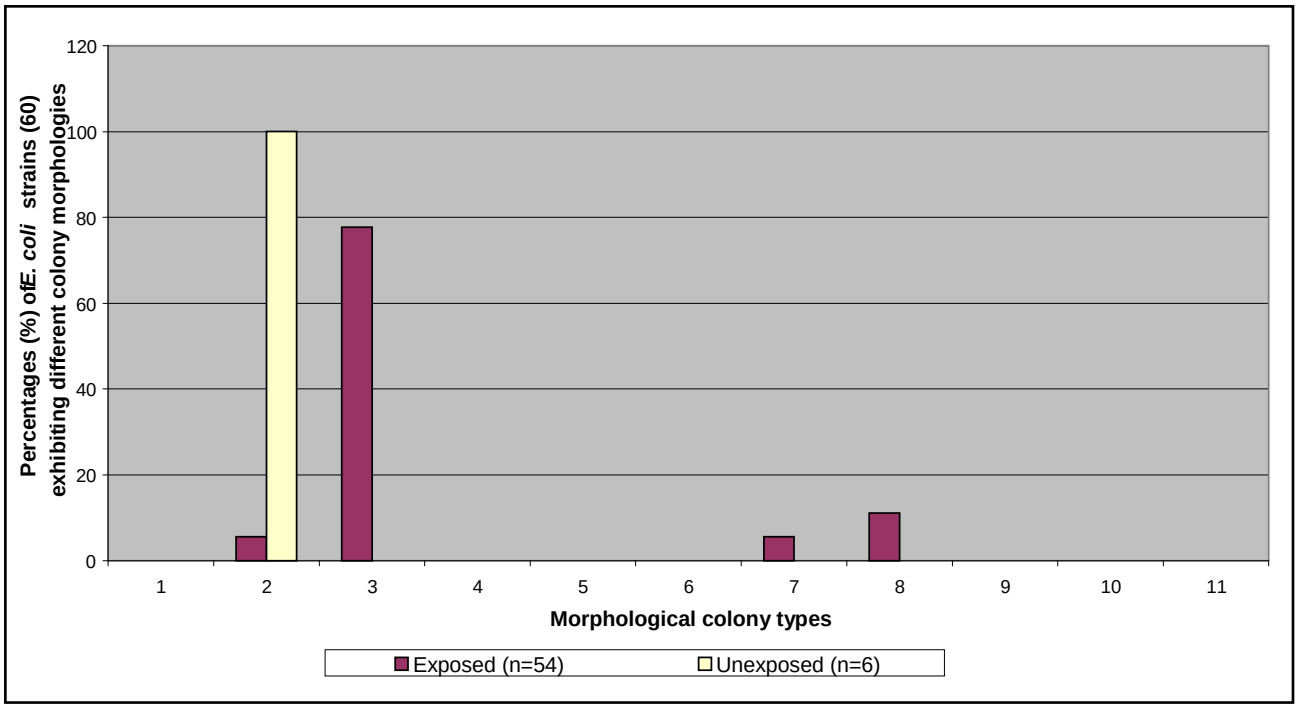

Figure. 6.2. Morphological colony types of *E. coli* strains isolated from biofilm before and after exposure to chlorine

6.3.2. Antimicrobial susceptibility determination

E. coli colonies identified in section 6.3.1 were selected for sensitivity testing according to survival after exposure to different chlorine concentrations and morphological colony

patterns as presented in Figures 6.1 and 6.2, as well as Tables 1.1 and 1.2 in Addendum 1. For quality control purposes all antimicrobial sensitivity testing was done in duplicate. Antibiotic susceptibility patterns of strains with differences in the duplicates were not included in the final results. From the 165 *E. coli* strains selected on morphological type determination, a total of 107 *E. coli* strains isolated from water samples were exposed to various antimicrobials. From the 60 selected *E. coli* strains with specific morphological types and chlorine enumeration levels, 30 isolates from the biofilm sample was included. The *E. coli* strains exhibited various susceptibilities against the antimicrobials tested. Strains that displayed inhibition zones with intermediate sizes were reported as non-susceptible. All strains tested were then classified according to their antimicrobial susceptibility patterns as illustrated in Table 6.3. Antibiograms were numbered according to the spectrum of antibiotic activity (narrow to broad) and grouped to identify resistance to antibiotics of high clinical importance. The antibiograms were arranged in the following order: ampicillin - co-amoxiclav - 1st generation cephalosporins - 2nd generations cephalosporins - third generation cephalosporins - trimethoprim-sulfamethaxazole - aminoglycoside (gentamicin, amikacin) - FQs (ciprofloxacin, ofloxacin). Eight *E. coli* strains were confirmed as ESBL producing strains with the dumbell effect with no cefoxitin resistance and grouped as antibiograms 28 and 32. Several *E. coli* strains exhibited *ampC* producing ß-lactamase phenotypes as indicated with antibiograms 24 to 27.

The antibiogram profiles of the number of colonies selected from the various morphological patterns are illustrated in Tables 2.1 (water) and 2.2 (biofilms) attached in Addendum 2. These antibiogram profiles are summarised and illustrated in Figures 6.3 (water) and 6.4 (biofilms). In both figures the resistance to various antimicrobial increased after exposure to chlorine.

Table 6.3. Antibiogram profiles as described by 'non-susceptible' antibiotics

Antimicrobials that tested as 'non-susceptible'	Antibiogram numbers
Sensitive to all antimicrobials	1
(AP)	2
(TS)	3
(GM)	4
(CIP)	5
(AP + TS)	6
(AP + GM)	7
(AP + AUG)	8
(AP + TS + GM)	9
(AP + TS + CIP)	10
(AP + AUG + TS)	11
(AP + AUG + GM)	12
(AP + AUG + AK)	13
(AP + TS + GM + CIP)	14
(AP + CZ + TS + AK)	15
(AP + AUG + TS + GM)	16
(AP + AUG + TS + AK)	17
(AP + AUG + GM + AK)	18
(AP + AUG + CZ + GM)	19
(AP + AUG + CZ +TS)	20
(AP + AUG + CZ + CIP)	21
(AP + AUG + CZ + OFL)	22
(AP + AUG + CZ + FOX + TS + OFL)	23
(AP + AUG+ 3rd Cef + FOX)	24
(AP + AUG + 3rd Cef + FOX + GM)	25
(AP + AUG + 3rd Cef + FOX + TS + GM)	26
(AP + AUG + 3rd Cef + FOX + TS + GM + CIP)	27
(AP + AUG +3rd Cef)	28
(AP + AUG + 3rd Cef + TS)	29
(AP + 3rd Cef + GM + AK)	30
(AP + AUG + 3rd Cef + TS + GM)	31
(AP + AUG + 3rd Cef + CIP)	32

AP = Ampicillin; AK = Amikacin; AUG = Co-amoxiclav; CIP = Ciprofloxacin; GM = Gentamicin; 3rd Cef = third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone); CZ = Cephalozolin; FOX = Cefoxitin; OFL = Ofloxacin; TS = Trimethoprim-sulfamethaxazole

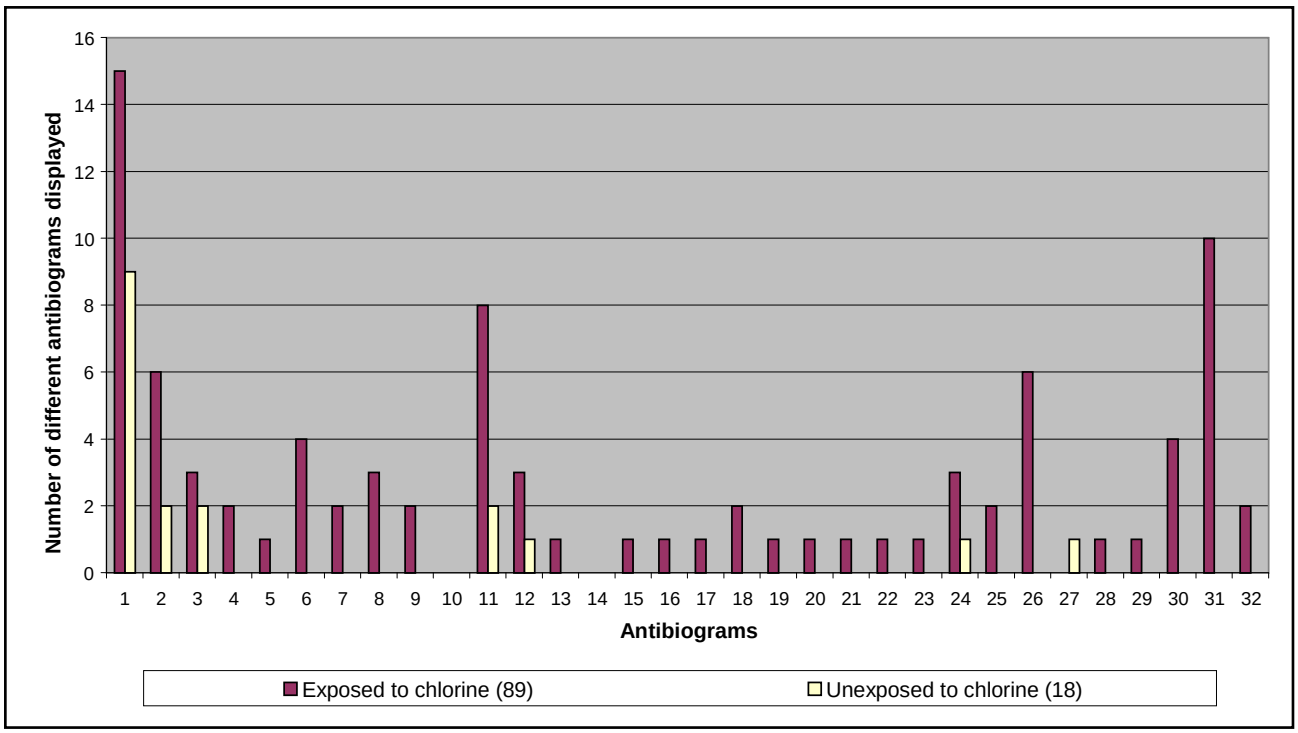

Figure. 6.3.. Antibiograms of *E. coli* strains isolated from water samples before and after exposure to various chlorine concentrations and contact times

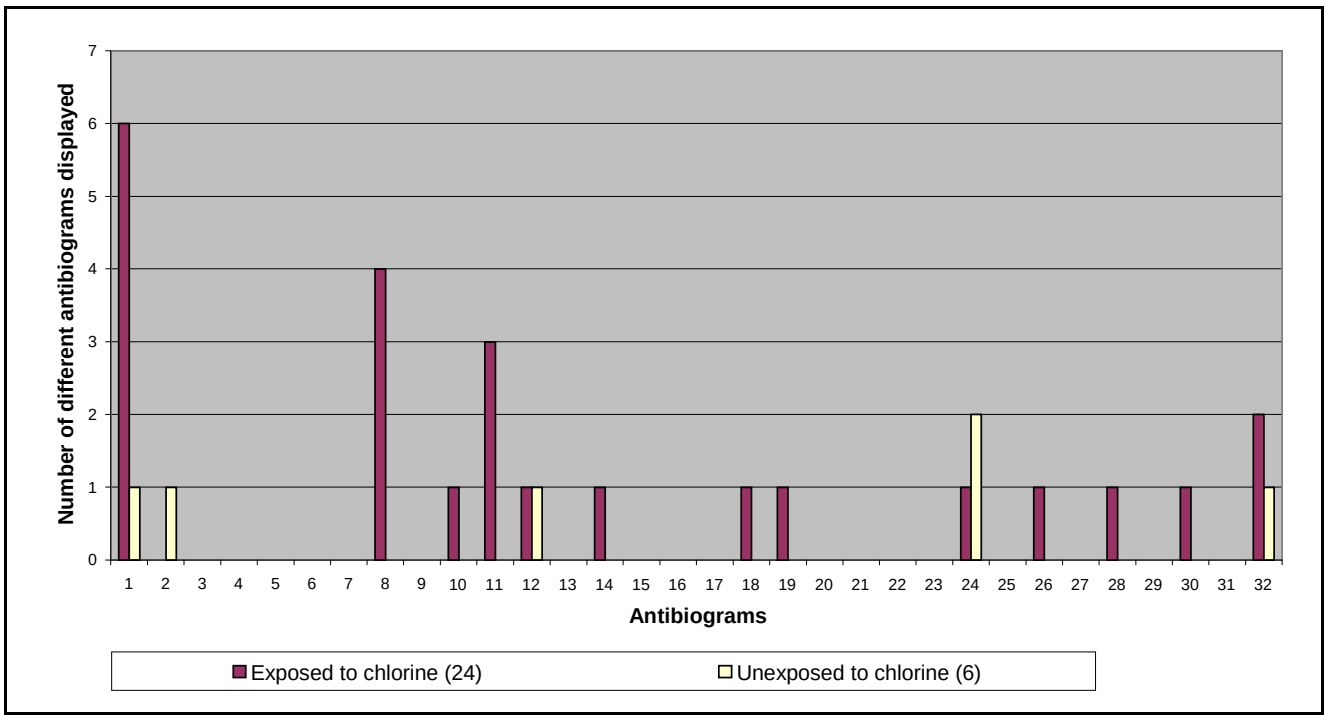

Figure. 6.4. Antibiograms of *E. coli* strains isolated from biofilm samples before and after exposure to various chlorine concentrations and contact times

A summary of the cumulative resistance displayed by the *E. coli* strains against antimicrobials before and after exposure to chlorine is presented in Table 6.4. The strains that displayed no lack of sensitivity to any of the antimicrobials tested, is excluded from

this summary and therefore the numbers are lower than in the previous analysis. In both the exposed and unexposed groups, loss of sensitivity was most frequently seen to ampicillin, co-amoxiclav, trimethoprim-sulfamethaxazole and the aminoglycosides (gentamicin, amikacin). A high level of resistance to the third generation cephalosporins was also noted in both the exposed and unexposed isolates.

Table 6.4. Percentage of non-susceptible *E. coli* strains isolated from the various water and biofilm sources

Antimicrobials	*E. coli* strains tested before chlorination (n = 15)	*E. coli* strains tested after chlorination (n = 91)
Ampicillin (AP)	46%	57%
Amikacin (AK)	33.3%	12%
Gentamicin (GM)	20%	14%
Trimethoprim-sulfamethaxazole (TS)	46%	39.5%
Ciprofloxacin (Cip)	0%	15%
Co-amoxiclav (AUG)	40%	21.9%
Cefoxitin (FOX)	20%	8.7%
Ceftriaxone (CTX)	20%	17.5%
Cefotaxime (CAZ)	20%	18.6%
Cephazolin (CZ)	13.3%	19.7%
Cefpodoxime (CPD)	13.3%	7.6%
Moxifloxacin (MOX)	0%	0%

Statistical analysis of the non-sensitivity to antimicrobials exhibited by the *E. coli* strains before and after exposure to chlorine (Table 6.4) were conducted, but were limited by the low number of strains in the pre-exposure group. The reason for these low numbers in this analysis was mainly due to the selection process of the strains described in section 6.2.1 and 6.2.2. The most common antibiogram profile exhibited by this group of isolates was number 1 (sensitive to all antimicrobials tested). The analysis of the antimicrobials and chlorination demonstrated that only ciprofloxacin ($p = 0.0318$) indicated a significant difference in their resistance patterns. No statistical differences in resistance were observed for antimicrobials, such as ampicillin, amikacin, co-amoxiclav, trimethoprim-sulfamethaxazole, cefoxitin, gentamicin, cefotaxime, ceftazidime, cephazolin, cefpodoxime

and moxifloxacin ($p > 0.05$).

6.4. Discussion

6.4.1. Selection of *E. coli* strains from water and biofilm samples

It was established in Substudy One (Chapter Four) that all the water sources tested were heavily contaminated with faecal coliforms and *E. coli* strains. In Substudy Two (Chapter Five), water and biofilm samples were further exposed to various chlorine concentrations to determine the chlorine tolerance of these strains. Isolated *E. coli* strains from water and biofilm samples were confirmed with biochemical identification tests and stored for further analysis. In this substudy, *E. coli* strains were cultured from the stored isolates onto selective media where several different morphological types were observed. Eleven different morphological types were identified amongst the strains; with some similar to the general phenotype, but certain *E. coli* colonies differed in appearance, as shown in Table 1.1 (Addendum 1). All the different morphological types described were seen across the range of isolated *E. coli* strains from the various water sources treated with chlorine, as illustrated in Table 1.2 (Addendum 1). The *E. coli* strains unexposed to chlorine displayed only morphological type number 1, 2 and 3, illustrated in Figures 6.1 and 6.2. Morphological types 1, 2 and 3 were commonly associated with *E. coli* strains isolated from the water samples exposed and unexposed to chlorine. Other morphological types were further associated with strains exposed to chlorine. Only morphological type 2 was exhibited by the *E. coli* strains isolated from the biofilm samples exposed and unexposed to chlorine. Morphological type 1 (flat, large, magenta colonies) was not exhibited. The reason for the fewer morphological types can be explained by the lower number of biofilm sources included in this study and the enumeration levels (Chapter Five), which demonstrated decreased *E. coli* levels after exposure to chlorine.

Figure. 6.1 illustrates that increased chlorine concentrations changed the colony morphology of the strains compared to the unexposed samples. The morphological patterns of the *E. coli* strains isolated from the biofilm samples, illustrated in Figure 6.2, showed similar results, indicating that chlorine exposure also affected the colony morphologies of these strains.

The phenomenon of colony morphology variation associated within the same organism is well known by experienced laboratory workers, and also in the literature. This

phenomenon has also been described in other organisms. In a study of *Pseudomonas aeruginosa* isolated from a wastewater sample exposed to 30 mg/L chlorine treatment, Macauley *et al* showed that two unique colony types dominated.[6] One of these colonies had a volcanic morphology, while the other was irregular to globular lobe in shape, compared to the round-shaped colonies in the unexposed samples. In a research study with *Pseudomonas aeruginosa* as indicator organisms, it was reported that colonies with a mucoid phenotype was associated with enhanced survival of the organism within chlorinated water. The researchers showed an increased chlorine sensitivity in *P. aeruginosa* after removal of the slime.[7]

Campylobacter jejuni is another microorganism that change its phenotype from the curved, spiral morphology to a coccoid form when exposed to suboptimal conditions.[8] In a clinical study, 18 *E.coli* strains were isolated from patients with bacteriuria, and characterised as mucoid lactose fermenting colonies.[9] The phenotypic and biochemical characterisation further demonstrated that 10 of these 18 isolates contain the virulence factor for serum resistance, which was determined with the serum bactericidal assay. The mucoid characteristic of an organisms is caused by a capsule, which confers serum and phagocyte resistance to some *E.coli* strains.[10] Phenotypic characteristics of a capsule is also an important virulence factor for many organism, including uropathogenic *E. coli* strains (UPEC).[10]

Furthermore, phenotypic characterisation is an important initial classification tool for identifying microorganisms and any changes in these patterns can affect its applications, especially if results are used as identification of causative agents or quality control purposes in food environment.[11]

6.4.2. Antimicrobial susceptibility determination

E. coli strains exhibiting specific morphological phenotypes described in section 6.3.1 were selected and exposed to a panel of antimicrobial agents to determine their resistance patterns. Thirty two different antibiograms were described across the selected *E. coli* strains exposed and unexposed to chlorine, as recorded in Tables 2.1 and 2.2 (Addendum 2). The *E. coli* strains selected in this study displayed multiple antibiotic resistance patterns against various antibiotics, including trimethoprim-sulfamethoxazole, ampicillin and ciprofloxacin, which are of clinical importance as they are used for treatment of bacillary dysentery caused by *Shigella* spp. and enteroinvasive *E. coli* organisms.[12] These

phenotypes were displayed by *E. coli* strains isolated from different water samples both before and after exposure to chlorine illustrated in Figures 6.3 and 6.4.

In general the *E. coli* strains isolated from the water sources displayed various antibiotic resistance patterns before and after chlorine exposure. The *E. coli* strains were already resistant to some of the selected antimicrobials, but after chlorine treatment, a shift in the antibiogram profiles was observed. A statistically significant increase resistance to ciprofloxacin was seen in the *E. coli* strains.

Third generation cephalosporin resistance in *E. coli* and *Klebsiella* species occurs via one of two resistance mechanisms, either Class A ESBL (classical ESBL) or Class C ESBL (AmpC). Class A ESBL is typically plasmid-mediated, whereas Class C ESBL is typically chromosomal, although numerous exceptions to this rule have been reported.[13] Eight ESBL producing strains of the selected *E. coli* strains were confirmed with the demonstration of the dumbell effect.

A study done in the United Kingdom on the comparison of various screening methods for the detection of ESBL mediated by enzymes (TEM and SHV) in ESBL positive *E. coli* and *Klebsiella* strains showed 91% sensitivity in the double-disk synergy test with cephalosporins, including cefpodoxime.[4] The findings of this substudy suggest that cefpodoxime can be used to detect ESBL producing strains but it did not enhance the sensitivity of screening with cefotaxime and ceftazidime.

Resistance to fluoroquinolones in *Enterobacteriaceae* is a growing concern in many clinical settings.[14] The selected *E. coli* strains demonstrated resistance against all of the antibiotics, except moxifloxacin in both the exposed and unexposed samples. This could be due to the fact that moxifloxacin is one of the newer FQs and not available for use in the public health service. Exposure of the surrounding communities to this antimicrobial where the samples were collected was therefore minimal; this may be the reason why no resistance was detected.

A previous study conducted in the United States also indicated that *E. co*li isolates taken from clinical and surface water sources displayed resistance to ciprofloxacin and other antimicrobials.[14] In wastewater sources[12,15] they found *E. coli* resistance of less than 2% to ciprofloxacin and 3% to gentamicin, while both antimicrobials displayed less than 1% resistance in surface waters.[16,17] Another study on the occurrence of antibiotic resistance in *E. coli* organisms isolated from combined sewage tanks and municipal wastewater

treatment plants in Ontario, Canada, showed that antimicrobial resistance was most prevalent in *E. coli* organisms isolated from the untreated sewage tanks.[18] The antimicrobial susceptibility patterns of twelve isolates in that study showed resistance to ciprofloxacin and gentamycin with increased resistance to amoxicillin, ampicillin, and penicillin. *E. coli* strains with multiple antibiotic resistant phenotypes were observed in their study,[18] as well as in this substudy, which illustrates inherent mechanisms to maintain resistance within water sources against certain antimicrobials and the need for chlorination to prevent the spread of more resistant strains.

E. coli strains isolated from the water and biofilm sources were identified according to their chlorine enumeration levels (Chapter Five), morphological colony types and antibiograms (this substudy). From the 106 *E. coli* strains that displayed resistance to antimicrobials, 15 were unexposed to chlorine and 91 exposed. Both exposed and unexposed *E. coli* strains showed the highest antimicrobial resistance against ampicillin and trimethoprim-sulfamethoxazole. Resistance to the narrow spectrum β-lactam drugs, such as ampicillin, comes as no surprise since *ampC* β-lactamases (cephalosporinases) are naturally produced by a variety of enterobacterial species (*Salmonella* spp., *Klebsiella pneumoniae* etc.).[19,20] However, gram--negative bacteria showing resistance to third generation cephalosporins have emerged as a challenge in the acute and long-term care setting.[21,22] These organisms that produce ESBL or contained the *ampC* β-lactamase gene can spread rapidly, especially in a hospital setting.[21] Therefore, the presence of broad spectrum β-lactamase producing organisms within the environmental samples is a major concern.

6.5. Conclusion

This substudy showed that exposure of water and biofilm samples to chlorine changed their morphological colony appearance compared to unexposed samples. Antimicrobial susceptibility testing of these morphologically different *E. coli* strains characterised various antibiograms including *ampC* β-lactamase producing *E. coli* strains, ESBLs as well as multiple resistance to other antimicrobials frequently used in the treatment of community acquired infections. The presence of such resistant organisms in water sources that are able to survive incomplete disinfection can have severe health implications. The statistical comparison of these resistance patterns and chlorine exposure illustrated a link between ciprofloxacin and chlorine resistance.

It was described in section 1.4.2.3 that chlorine may exert several effects on *E. coli,* such as the expression of multi-substrate efflux pumps, decreased membrane permeability and transport inhibition. All of the above could lead to increased resistance to antimicrobial substances as observed in this substudy. Therefore, the addition of an efflux pump inhibitor (EPI) to some of the water samples was performed in an attempt to demonstrate the involvement of efflux pumps in antimicrobial resistance and chlorine tolerance. This pilot study (Substudy Four) is presented in Chapter Seven.

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Chapter Seven

Efflux pump involvement in antimicrobial resistance and chlorine tolerance of *E. coli* strains

7.1. Introduction

It was illustrated in Chapter Five that *E. coli* strains have the ability to survive chlorine treatment. The further antibiotic susceptibility test confirmed that chlorine exposure influenced the resistance patterns of some *E. coli* strains. Efflux pumps are one of the mechanisms implicated in resistance and its possible involvement in antimicrobial and chlorine resistance of the isolated *E. coli* strains in this study was of interest. Chlorine was shown to diffuse through the cell wall to inactivate an organism, thereby also causing dysfunction in the internal enzymes.[1] As described in Chapter One, Section 1.6.3., the *acrAB-tolC* efflux pump system is associated with gram negative organisms and is maximally active when the bacteria are exposed to stress conditions, such as nutrient-poor medium and osmotic shock.[2] Several studies have been conducted on the up-regulation and over-expression of these efflux pumps, to investigate their involvement in stress conditions.[3-5] Results revealed that efflux pumps can be effective in exporting toxic substrates, but can cause harm to the organism, as nutrients and metabolic intermediates were also shown to be lost with efficient efflux.[3]

Therefore the objective of this substudy was to identify the effect of an EPI on the number of *E. coli* colonies that could be cultured after exposure to chlorine, and on resistance to selected antibiotics.

7.2. Materials and methods

This was a pilot study to test if the effect of an EPI could be reversed to prove its role in decreasing the enumeration of *E. coli* strains in water exposed to chlorine. Water and biofilm samples were taken from Plankenburg River (Below Kayamandi) for exposure to chlorine at an exposure time of 60 minutes. An exposure time of 60 minutes was applied in this substudy, as it was observed in Chapter Five that after 30 minutes contact time with the chosen range of chlorine concentrations, high *E. coli* counts were still evident. Increasing the contact time to 90 minutes with the same chlorine concentrations markedly decreased the *E. coli* ennumeration levels with no indication of the point at which the

numbers started to increase or decrease.

As this was experimental, the addition of carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) was not investigated in the other water and biofilms samples exposed to chlorine, which could have given a broader description of the influence of the EPI to chlorination. Other EPIs were also not included in order to identify the best possible inhibitor for specific addition with chlorine, which limits the findings of this substudy.

7.2.1. Chlorine and EPI exposure

An EPI, CCCP with a final concentration of 100 mg/L; (Sigma-Aldrich Corp., St. Louis, USA), which inhibits the efflux function of *emrAB* and *acrAB-tolC* multidrug pump in *E. coli,*[6] was directly added to the water and biofilm samples. Chlorine concentrations of 0.0 mg/L, 0.1 mg/L; 0.3 mg/L; 0.4 mg/L, 0.5 mg/L and 0.6 mg/L were used as previously described . The EPI and chlorine concentrations were then added to the water and biofilm samples and incubated at room temperature for a contact time of 60 minutes.[7] The 5x multiple tube enumeration method was executed, as described in Chapter Three, Section 3.2.2.3, to determine the effect of EPI on the survival of *E. coli*.

A positive control, *E. coli* DHM1[8] [*F-*, *cya-854*, *recA1*, *endA1*, *gyrA96* (*Nalr*), *thil*, *hsdR17*, *spoT1*, *rfbD1*, *glnV44* (AS)] was used to illustrate the effect of EPI. Sub-cultures of this strain were obtained from the Faculty of Health Sciences, Department of Biomedical Sciences, Division of Molecular Biology & Human Genetics (Stellenbosch University). This strain was streaked onto Luria-bertani (LB) agar plates and incubated at 37°C overnight. Single colonies were inoculated into LB broth and incubated at 37°C, overnight to obtained sufficient growth (OD_{600} = 1.00). An aliquot of this culture was added to deionized water to a volume of 55 ml. This was exposed to EPI (100 mg/L) and various chlorine concentrations and incubated for 60 minutes. Direct bacterial counts were determined on LB agar plates as described in Chapter Five, Section 5.2.3.

7.2.2. Antimicrobial susceptibility testing with EPI

E. coli strains (n=96) were directly selected from the enumeration plates after exposure to chlorine and EPI as described in Section 7.2.1, and cultured in a Mueller-Hinton broth up to an optical density of 0.5 MacFarland for the determination of their antibiograms. CCCP was then added to the suspensions to a concentration of 100 mg/L. This was incubated at room temperature for approximately 60 minutes, in accordance with the method

described by Saenz *et al.*[9]. Antimicrobial susceptibility was then determined as described in Chapter Six (Substudy Three), Section 6.2.2.

E. coli ATCC 25922 was used as a positive control.[10] Sub-cultures of the strain was obtained from the Faculty of Health Sciences, Department of Biomedical Sciences, Division of Molecular Biology & Human Genetics (Stellenbosch University).

7.3. Results

7.3.1. Chlorine and EPI exposure

A direct comparison of the *E. coli* ennumeration levels after chlorine exposure without EPI (Chapter Five) could not be made due to the different contact time used in this substudy. The results of the ennumeration levels of *E. coli* isolated from the water and biofilm samples included in this substudy are presented in Table 7.1. It was noted that the levels of *E. coli* organisms isolated from the biofilm sample was higher than in those isolated from the water sample.

Table 7.1. *E. coli* organisms isolated from water and biofilm samples exposed to various chlorine and EPI concentrations for 60 minutes

Chlorine concentration (mg/L)	*E. coli* per 100 ml water with 100 mg/L of EPI	*E. coli* per 100 ml water containing biofilm with 100 mg/L of EPI
0.0	778	778
0.1	1 987	4 469
0.3	0	1 986
0.4	0	4 685
0.5	0	0
0.6	0	199
0.7	0	0

7.3.2. Antimicrobial susceptibility testing with EPI

A total of 96 *E. coli* colonies were selected after exposure of water and biofilm samples to chlorine in addition to CCCP as described in Section 7.2.1 to obtain their antibiograms. Various susceptibility patterns against the antimicrobials tested were exhibited by 89 of

the isolates. The antibiograms of 7 strains were not included, due to contamination and differences in the antibiogram of the duplicates. Strains that displayed inhibition zones with intermediate sizes were interpreted as 'non-susceptible'. All strains tested were then classified according to their antimicrobial susceptibility patterns, as defined in Chapter Six (Substudy Three), Section 6.3.2 and listed in Table 7.2.

Table 7.2. Antibiogram profiles as described by 'non-susceptible' antibiotics

Antimicrobials that tested as 'non-susceptible'	Antibiogram numbers	Number of strains exhibiting antibiograms
Sensitive to all antimicrobials	1	31
(AP)	2	6
(TS)	3	7
(AP + TS)	6	15
(AP + AUG)	8	6
(AP + TS + GM)	9	3
(AP + TS + CIP)	10	3
(AP + AUG + TS)	11	3
(AP + AUG+ 3rd Cef + FOX)	21	6
(AP + AUG + 3rd Cef - FOX + TS + GM)	23	6
(AP, TS, CTX, CAZ)	27	3

AP = Ampicillin; AUG = Co-amoxiclav; CIP = Ciprofloxacin; GM = Gentamicin; 3rd Cef = third generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone); CTX = Ceftriaxone; FOX = Cefoxitin; CAZ = Ceftazidime; TS Trimethoprim-sulfamethaxazole (TS)

The antibiograms of the isolates before and after exposure to chlorine are presented in Table 7.3. The antibiograms of the isolate beforeand after addition of the EPI addition of the EPI are presented in Table 7.3. A summary of these antibiograms are illustrated in Figures 7.1 (water) and 7.2 (biofilms). In both figures resistant antibiogram profiles were displayed by *E. coli* strains before and after exposure to the EPI.

Table 7.3. Antimicrobial susceptibility patterns of selected *E. coli* strains before and after exposure to chlorine and EPI concentrations

Chlorine exposure, 60 minutes contact time	Exposure to efflux pump inhibitors (EPI)	Antibiogram number
0.0 mg/L (W)	No	1
0.0 mg/L (W)	Yes	1
0.0 mg/L (B)	No	1
0.0 mg/L (B)	Yes	10
0.1 mg/L (B)	No	1
0.1 mg/L (B)	Yes	1
0.1 mg/L (B)	No	10
0.1 mg/L (B)	Yes	21
0.1 mg/L (W)	No	23
0.1 mg/L (W)	Yes	23
0.1 mg/L (W)	No	3
0.1 mg/L (W)	Yes	1
0.3 mg/L (W)	Yes	1
0.3 mg/L (W)	No	6
0.3 mg/L (W)	Yes	24
0.3 mg/L (W)	No	24
0.4 mg/L (W)	No	1
0.4 mg/L (W)	Yes	1
0.4 mg/L (B)	No	3
0.4 mg/L (B)	Yes	1
0.5 mg/L (W)	No	1
0.5 mg/L (W)	Yes	10
0.5 mg/L (W)	No	2
0.5 mg/L (W)	Yes	6
0.5 mg/L (W)	No	2
0.5 mg/L (W)	Yes	1
0.6 mg/L (B)	No	2
0.6 mg/L (B)	Yes	1
0.6 mg/L (B)	No	21
0.6 mg/L(B)	Yes	21
0.7 mg/L (W)	No	2
0.7 mg/L (W)	Yes	1

W = water; B = biofilm

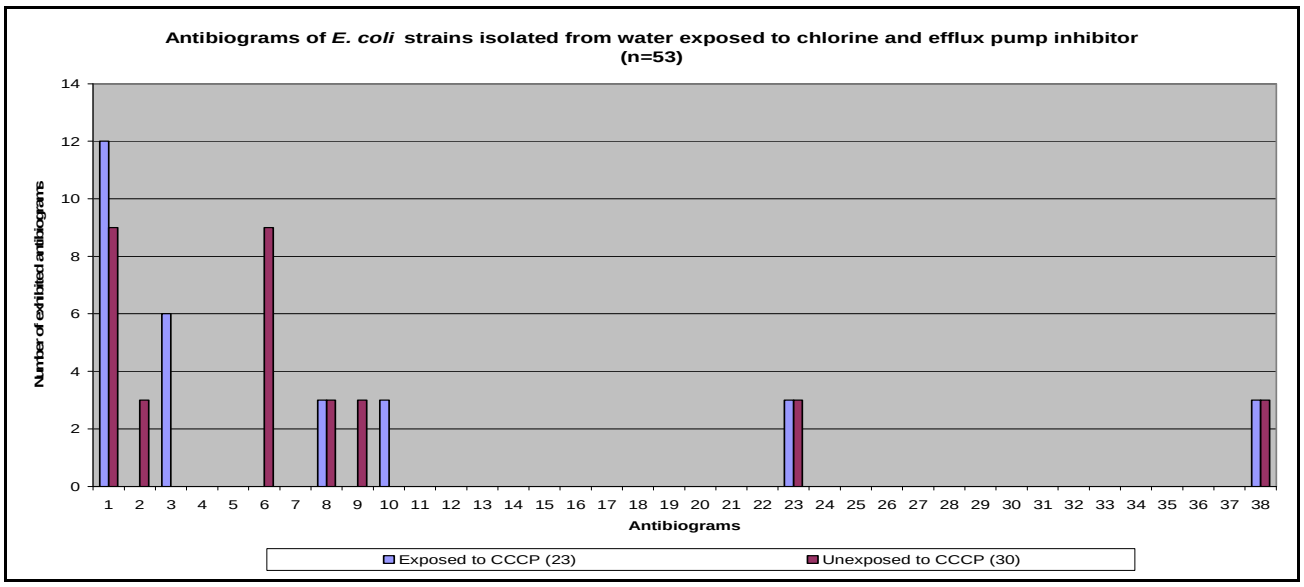

Figure 7.1. Antimicrobial susceptibility patterns of *E. coli* strains isolated from water before and after exposure to chlorine and EPI

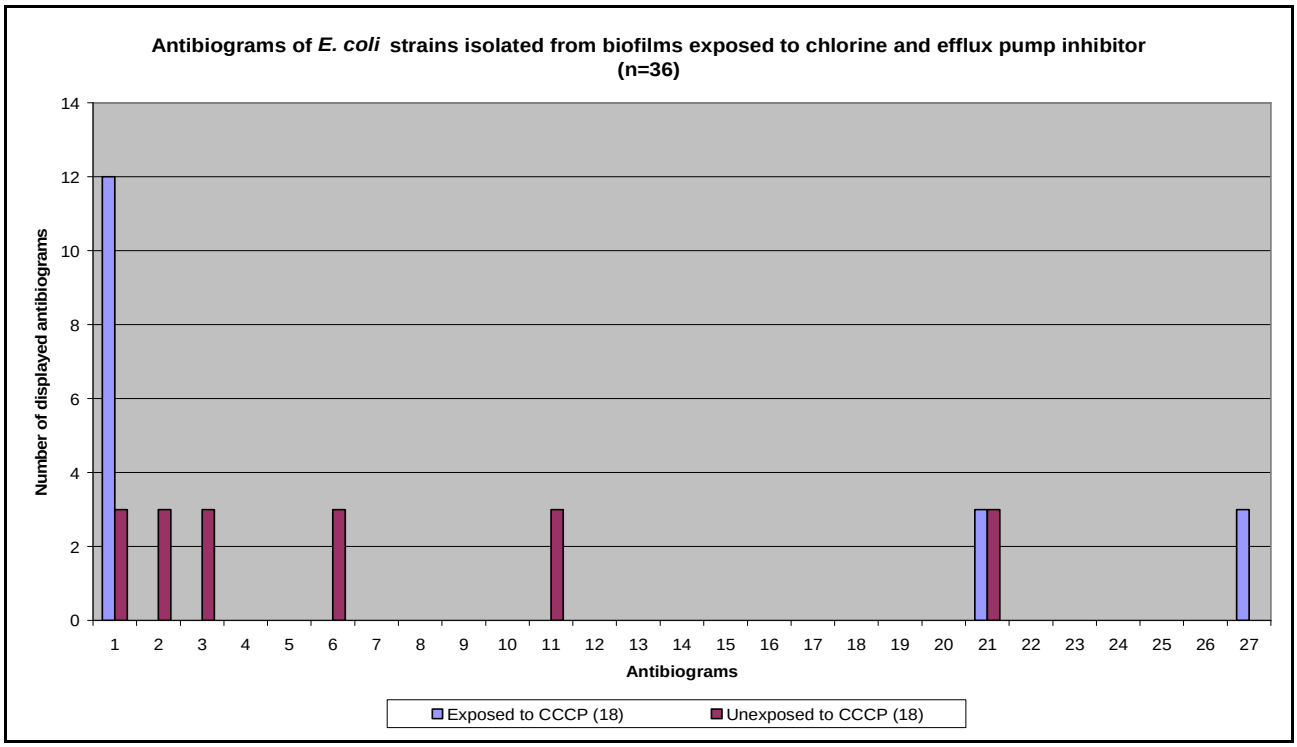

Figure 7.2. Antimicrobial susceptibility patterns of *E. coli* strains isolated from biofilms before and after exposure to chlorine and EPI

After exposure of *E. coli* strains to EPI and testing for antimicrobial susceptibility, it was observed that the antibiograms of 10 strains changed. A summary of the cumulative resistance exhibited by these 10 *E. coli* strains against antimicrobials before and after exposure to 100 mg/L of CCCP (an EPI) is illustrated in Table 7.4. This was done to

determine whether efflux pumps were involved in resistance to certain antimicrobials. No change in ciprofloxacin resistance patterns of *E. coli* strains exposed to CCCP was observed.

Table 7.4. The antimicrobial resistant patterns of *E. coli* strains before and after exposure to EPI (CCCP)

Antimicrobials	Number of antimicrobial resistant *E. coli* strains isolated before CCCP exposure (n = 10)	Number of antimicrobial resistant *E. coli* strains Isolated after CCCP exposure (n = 10)
Ampicillin (AP)	6	4
Trimethoprim-sulfamethaxazole (TS)	5	3
Ciprofloxacin (CIP)	2	2
Co-amoxiclav (AUG)	0	1
Ceftriaxone (CTX)	0	1

7.4. Discussion

7.4.1. Chlorine and EPI exposure

A variety of structurally similar or dissimilar compounds can be transported by efflux pumps, including as substrate specific compounds.[3,11] The use of the EPI, CCCP, blocks the energy dependent efflux pump and increases the cytoplasmic levels of the chemical or compound. The purpose of this substudy was to investigate the effect of an EPI on the ennumeration levels of *E. coli* exposed to chlorine, and on resistance to antimicrobials. *E. coli* strains isolated from the water sample survived up to a chlorine concentration of 0.1 mg/L with addition of CCCP after a contact time of 60 minutes. In the biofilm samples, *E. coli* could be isolated up to a chlorine concentration of 0.6 mg/L . The phenomenon of a possible pattern of an VBNC state was observed in isolates from the biofilm samples as seen in Substudy Two (Chapter Five) with exposure to chlorine only. This indicates that more tolerant *E. coli* strains were present in the biofilm sample within this substudy. It seemed that the addition of an EPI decreased the enumeration levels of *E. coli* strains

exposed to chlorine in the water sample. This does not exclude the involvement of other mechanisms in survival within such environments. More sensitive methods, such as specific measurements for the accumulation of chlorine by the *E. coli* cells with a spectrofluorometer (detection of fluorescence), would have given a better result on the affectivity of the EPI during chlorine exposure.[12] Due to the unavailability of the equipment needed for this methodology, the test was not performed. The direct bacterial count of the positive control decreased gradually in colony count after addition of EPI and chlorine concentrations (data not shown). This could be explained with the fixed inoculum size added to the deionised water which was not the same for the water and biofilm samples obtained from Plankenburg River (Below Kayamandi).

7.4.2. Antimicrobial susceptibility testing with EPI

Efflux systems contributing to antimicrobial resistance have been described in many organisms, including *E. coli*[11] and *Salmonella typhimurium.*[3] The selected *E. coli* strains within this substudy exhibited 11 different antimicrobial susceptibility profiles compared to the 32 types identified in Chapter Six (Substudy Three), Section 6.3.2. This may be because only one water source was included and a decrease in the *E. coli* numbers that survived was observed in this substudy compared to Chapter Six (Substudy Three).

A previous study on the regulation of efflux pumps with the addition of an EPI with exposure time of 5 minutes to maximum of 60 minutes showed an increase in the sensitivity to some antimicrobials.[13] The antibiograms of 10 *E. coli* strains in this substudy were changed after exposure to an EPI for antimicrobial susceptibility testing. These include decreased resistance in the number of isolates to ampicillin and trimethoprim-sulfamethaxazole. No significant reversal of antibiotic resistances in *E. coli* could be demonstrated in this substudy. In contrast, the antimicrobial resistance patterns of co-amoxiclav and ceftriaxone were increased. The addition of the EPI also did not change the susceptibility patterns of the selected *E. coli* strains to ciprofloxacin. Another explanation for ciprofloxacin resistance in *E. coli* strains may be low permeability (slow uptake of the drug into the bacterial cell)[2,13] or resistance mechanisms involving mutations within the genes encoding resistance to DNA gyrase[14-16] and DNA topoisomerase IV. The findings within this substudy of no change in the resistance of ciprofloxacin suggest that the method was not sensitive enough or that other mechanisms beside efflux might be involved in ciprofloxacin resistance.

7.5. Conclusion

The ennumeration levels of *E. coli* strains isolated from the water sample decreased after addition of the EPI to the water, but not for the biofilm sample. Various antibiotic susceptibility profiles were produced by the selected *E. coli* strains after addition of the EPI with a change in the antibiograms of 10 of these isolates. This substudy could not indicate an influence on the resistance to ciprofloxacin by the addition of the EPI.

The phenotypic antimicrobial resistance profiles of the *E. coli* strains were determined after exposure to chlorine and this ranged from sensitive to highly resistant. The association between chlorine tolerance and acquired antimicrobial resistance as determined by the colony morphological profiles and antibiograms of the *E. coli* strains in Substudy Three, required further clarification. Since the specific mechanisms of chlorine tolerance were not previously identified, candidate genes involved in antibiotic resistance and chlorine tolerance, including genes encoding for efflux pumps, were detected in selected strains by PCR method. The next substudy describes these methods and results.

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Chapter Eight

Molecular detection of candidate genes involved in antimicrobial resistance and chlorine tolerance

8.1. Introduction

The results of Substudy Two (Chapter Five) demonstrated that *E. coli* strains survived chlorine treatment and displayed a VBNC phenotype. In Substudy Three (Chapter Six) the strains displayed various morphological patterns as well as antimicrobial resistant phenotypes. The results suggested that the ability of these *E. coli* strains to survive chlorine exposure and antimicrobial resistance may be intrinsically related. A previous study on the effect of culturable and non-culturable cells showed that the proteins involved in non-culturable cells demonstrated increased and irreversible oxidative damage, which affects various bacterial compartments and proteins.[1] The specific microbial response to chlorine exposure is not fully understood yet, but chlorine does exert various disruptive effects on microorganisms, including inhibition of protein biosynthesis.[2] Other effects involve damage of the chromosomal DNA[3], inhibition of membrane-mediated active transport processes and uncoupling of oxidative phosphorylation.[4]

For the purpose of this substudy, candidate genes were selected based on their function in response to stress conditions such as chlorine disinfection or exposure to antimicrobials as described in a review by Russell *et al* on microbial response to stress conditions.[5] Possible mechanisms involved in microbial resistance such as efflux and targeted genes for uptake of substances were also considered.

The aim of this substudy was to establish and optimise practical molecular techniques for the amplification of specific candidate genes selected for consideration as markers of resistance to disinfection and antimicrobials.

8.2. Materials and methods

8.2.1. Criteria and selection of *E. coli* strains for detection of candidate genes

For this substudy, *E. coli* strains were selected based on their previously described phenotypic characteristic which includes the *E. coli* enumeration levels after chlorine

exposure (Chapter Five), morphological colony types (Chapter Six) and antibiogram profiles (Chapters Six and Seven). *E. coli* isolates stored as described in Chapter Six (Substudy Five), Section 6.2.1 and 6.2.2 as well as Chapter Seven (Substudy Six), Section 7.2.1 and 7.2.2 were sub-cultured to obtain pure growth. Strains were selected if they displayed highly resistant antibiograms (Chapters Six and Seven) and those antibiograms with changed resistance after addition of an EPI (Chapter Seven). The selected *E. coli* strains used for detection of candidate genes are listed in Table 3.1 of Addendum 3.

Candidate genes were selected based on their functions and possible involvement in antimicrobial, chlorine or efflux resistance. The genes were obtained from a database (www.ecogene.org) for *E. coli* strains. These candidate genes for PCR amplification are grouped into chlorine, antibiotic and efflux pump genes and are shown in Tables 8.1, 8.2 and 8.3, respectively.

Table 8.1. Candidate gene selected for possible involvement in chlorine tolerance[6-9]

Candidate genes	**Function**
soxS	Regulatory gene for superoxide stress response. Induce 9-protein sox regulon when superoxide levels increase. Redox regulon genes are induced following exposure to chlorine
ompF	OMP forms passive diffusion pores which allow small molecular weight hydrophilic materials to cross the OM
osmC	Involved in defense against oxidative stress caused by exposure to organic hydroperoxides
soxR	Regulatory gene for superoxide stress response
grpE	Participates actively in the response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins, in association with *dnaK*
dnaK	Heat-shock gene involve/induced following exposure to chlorine

Table 8.2. Candidate gene selected for involvement in antimicrobial resistance [6-8,10-13]

Candidate genes	Function	Antimicrobial affected
ampC	Chromosomally encoded *AmpC* β-lactamases confer β-lactam antibiotic resistance	Beta lactams
parC	Structural genes. Topoisomerase IV subunit A is essential for chromosome seggregation. It performs the decatenation events required during replication of a circular DNA molecule	FQs
gyrA	Structural genes. DNA gyrase, negatively supercoils the closed circular double-stranded DNA in an ATP-dependent manner and also catalyzes the interconversion of other topological isolmers of double-stranded DNA rings.	FQs
ctx-m-9	Detection of ESBL mediated by enzymes. It hydrolyze cefotaxime and Ceftriaxone	Broad spectrum cephalosporins
marA	Controls the expression of the *acrAB* efflux pump	Tetracycline and other antimicrobials

Table 8.3. Criteria of candidate gene selection for possible involvement in efflux[6,9,13,14]

Candidate genes	Function	PCR amplification for resistance against
acrA	Membrane fusion protein of *AcrAB* efflux system	Tetracycline, ciprofloxacin, FQs, β-lactams and novobiocin
tolC	Required for proper expression of outer membrane protein involved in efflux	Various antimicrobials
emrA	Regulatory gene or mutation of efflux components	Various antimicrobials
acrR	Regulatory gene or mutation of efflux components	Various antimicrobials

8.2.2. Bacterial strains used as positive control for Polymerase Chain Reaction (PCR) of candidate genes

In a previous article by Santos *et al* it was demonstrated that *E. coli* DHM1[15] [*F-*, *cya-854*, *recA1*, *endA1*, *gyrA96* (*Nalr*), *thil*, *hsdR17*, *spoT1*, *rfbD1*, *glnV44* (AS)] and *E. coli* BTH101 [*F-*, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* (Strr), *hsdR2*, *mcrA1*, *mcrB1*] strains were suitable positive controls for the screening of all the candidate genes included and used as positive controls in this study.[16] Sub-cultures of these strains were obtained from the Faculty of Health Sciences, Department of Biomedical Sciences, Division of Molecular Biology & Human Genetics (Stellenbosch University).

8.2.3. DNA preparation for PCR

Deoxyribonucleic acid (DNA) was prepared for PCR using two different methods. Bacterial pelleting was initially used, but resulted in ineffective PCR amplification. It was followed by the bacterial lysate method, which yielded better PCR amplifications.

8.2.3.1. Bacterial pelleting of *E. coli* colonies for PCR

Strains selected in Section 8.2.1 were inoculated into Luria-Bertani broth containing ampicillin and incubated at 35℃ for 18-24 hours. One ml of the solution was transferred into an eppendorf tube and spun down for 5 minutes at 16000 x g. The supernatant was

discarded and 50 µl de-ionised water added to the bacterial pellet and stored at 4℃. [17]

8.2.3.2. Crude DNA lysate preparation

Strains selected in Section 8.2.1 were also inoculated onto Uri-Select and MacConkey agar plates. Cultured colonies were picked up with a toothpick and swirled into 150 µl de-ionized water in an eppendorf tube and incubated in a waterbath at 80℃ for 2-5 minutes. Eppendorf tubes containing crude DNA lysates were spun down for 5 minutes at 16000 x g and crude DNA lysate supernatant transferred and stored at 4℃. [18]

8.2.4. DNA quantification of the bacterial lysate and pellet

DNA concentrations of the bacterial lysate and pellet prepared in Sections 8.2.2.1 and 8.2.2.2 were measured with a Nanodrop ND-1000 Spectrophotometer V3.1.0 (Nanodrop Technologies, Inc., Wilmington, DE, USA). The absorbance was read at 260 nm and 280 nm. The purity of the DNA was determined using the ratio of the two readings between A260 and A280.

8.2.5. Polymerase Chain Reaction

8.2.5.1. Characteristics of primers

For this substudy, unique primer pairs (oligonucleotides) were designed, which included sequences of 200 base pairs (bp) before and after the complete candidate gene sequences, obtained from Ecogene database (www.ecogene.org) using DNAMAN Version 4.0, Lynnon Biosoft, Copyright©1994-97 software. The different oligonucleotides were determined by parameters, such as GC content (nitrogenous bases bound by 3 hydrogen bonds on the DNA molecule as either guanine (G) or cytosine (C)) of 40-60% and Melting point (Tm of 50-55℃). Primers were obtained from I ntegrated DNA Technologies (IDT), Inc., Coralville, IA, USA as described in Table 8.4. The first set of primers were designed for candidate genes were selected for chlorine tolerance, the grey coloured part for antibiotic resistance and the others for efflux pump resistance.

Table 8.4. Characteristics of primers of candidate genes for PCR

Genes	Gene Size	PCR fragment (Including 200 bp before and after sequence)	Primer sequence, 5'-3'
soxS	324 bp	724 bp	F:AGCAGCGCTTTAATGCGG R:GCCAGGGATGGTTCTTTGC
ompF	1089 bp	1489 bp	F:GGCAGTGGCAGGTGTCATAA R:GAAGGGAAGTCCGCTATCAGG
osmC	432 bp	832 bp	F:TTTAAGCCCACAGGAGAGCAA R:ACAACGCATCAGGCATTCAC
soxR	465 bp	865 bp	F:CGGCTGGTCAATATGCTCG R:TGGCGAAGAAACGTCGGT
grpE	594 bp	994 bp	F:GCTTCCCTTGAAACCCTGAAA R:GAGAGTGTGGCGGAGTAACGA
dnaK	1917 bp	2317 bp	F:CACAACCACATGACCGAA R:CTAGATGAATGCACGGGCG
ampC	1134 bp	1534 bp	F:TAAATCCGGCCCGCCTAT R:AACGACCAGAAATGCAGCTGT
parC	752 bp	1152 bp	F:CGACGCGGCAGATAATGTAGT R:CTTGTAAGCGGGAGGAAA
gyrA	2628 bp	3028 bp	F:GCGATCTCTTCGTGGTCACG R:GCCAAACTTTACCGTGCCC
Ctx-m-9	474 bp	874 bp	F:GCAGATAATACGCAGGTG R:CGGCGTGGTGGTGTCTCT
marA	390 bp	790 bp	F:CCATCAATTAGTTGGCCAGGA R:CAGTGACGTTGTCACGTT
acrA	1194 bp	1594 bp	F:TTTCGTGCCATATGTTCGTGA R:GAGGCGGAGATCGTTACTGC
acrB	3150 bp	3550 bp	F:AAGTGCGTCCTGGTGTCCCA R: ACAATCAGTATTTTTTATGAA
tolC	1482 bp	1882 bp	F:CGCGCTAAATACTGCTTCACC R:TGCCTTACGTTCAGACGGG
emrA	1173 bp	1573 bp	F:AAATGACTGGCCAGCTCG R:CTGTTGCATCACACGCACC
acrR	648 bp	1048 bp	F:CCTCGAGTGTCCGATTTCAAA R:CGCAAGAATATCACGACGCA

bp = base pairs; F = Forward primer; R = Reverse primer

8.2.5.2. PCR amplification of genes

PCR amplification was performed with a final volume of 50 µl in 0.2 ml thin-walled tubes. The primers used for PCR amplification are listed in Table 8.2.5.1.1. Each PCR reaction contained 1x Go Taq Flexi Buffer; 200 µM of deoxynucleoside triphosphate; 1.5 mM $MgCl_2$; 50 pM forward and reverse primers; and 0.01 U of Go Taq DNA polymerase (Promega, Madison, Wisonsin, USA). Template DNA (3.0 µl) was added to 47.0 µl of the each reaction. The reagents within the tubes were thoroughly mixed and placed into a Thermocycle (Applied Biosystems GeneAmp® PCR System 9700).

The PCR conditions for each gene consisted of an initial denaturation step at 94℃ for 2 min, followed by 40 cycles of DNA denaturation at 94℃ for 30s, primer annealing at 55℃ for 30s, and primer extension at 68℃ for 30s with a extra 30s for every 500 bp product and a final extension step at 68℃ for 10 min was a dded. Changes in extension time for the amplification of candidate genes by PCR are listed in Table 8.5.

Table 8.5. Optimization of the extension time for amplification of candidate genes

Gene	Primer extention time (s)	Product size (bp)
soxR	30	465
soxS	30	324
osmC	30	432
ompF	90	1089
dnaK	60	1917
grpE	30	594
ampC	90	1134
gyrA	180	2628
parC	60	752
marA	60	390
acrA	60	1194
tolC	60	1482
acrR	60	648
emrA	60	1173

s = seconds

8.2.6. Agarose Gel Electrophoresis

Approximately 0.8g agarose (LE Analytic Grade; Promega, Madison, Wisonsin, USA) were weighed and added to 100 ml TAE (0.04 M Tris acetate, 0.001M EDTA). Five µl of ethidium bromide (0.5 µg per ml; Promega, Madison, Wisonsin, USA) was added to the melted agarose gel. Eight µl aliquots of PCR products was added to 2 µl of 6x loading buffer (Promega, Madison, Wisconsin, USA) and loaded into the wells of the gel. Five µl of 1 kb Molecular weight marker (Promega, Madison, Wisonsin, USA) was added to identify the product size. The gel was run at 8 V/cm for 2 hours at room temperature. The first set of candidate genes were selected for chlorine resistance, the grey coloured part for antibiotic resistance and the others for efflux pump resistance.

After electrophoresis, the gel was removed and DNA bands were visualised with the Syngene™ GeneGenius computer system (a Division of synoptics LTD., Cambridge, United Kingdom).

8.3. Results

8.3.1. Amplification of candidate genes

A total of 100 *E. coli* strains as described in Table 3.1 (Addendum 3) were selected from water sources from this study as well as isolates from my BSc Honours project described in Chapter Three, were included for amplification of the various candidate genes. Several PCR methods were optimised for all selected candidate genes to obtain the best possible results.

In Figure 8.1, *grpE* and *soxS* were amplified in *E. coli* strains isolated from water and biofilms from Plankenburg River (Below Kayamandi) in 2006. The gene product with fragment size corresponding to 594 and 324 bp according to the 1 kb molecular marker (M, Promega, Madison, Wisconsin, USA) in lane 1 were seperated on a 0.8% agagraose gel (Promega, Madison, Wisconsin, USA). Gene *grpE* was detected in all the isolates except lane 3 and 4 while heat-shock gene, *soxS* was detected in all isolates except lane 15. *E. coli DHM1*, labelled with P were loaded in lane 7 and 13. Negative controls (no DNA) were included for quality control of the PCR reactions and labelled with N were loaded in lane 14. The other candidate genes detected were demonstrated similarly as Figure 8.1.

Figure 8.1. Amplification of candidate genes *grpE* (lanes 2-7) and *soxS* (lanes 8-16) that may play a role in chlorine tolerance. Lanes: 1 (M); lane 2 (P5); lane 3 (P10); lane 4 (B4); lane 5 (B5); lane 6 (P22) ; lane 7 (P); lane 8 (P5); lane 9 (P10); lane 10 (B6) ; lane 11 (B5); lane 12 (P22); lane 13 (P); lane 14 (N); lane 15 (P1); lane 16 (M)

Candidate genes participating in efflux pumps, which were previously described in Chapters One and Seven as important mechanisms for microorganisms survival, were not all identified in the *E. coli* strains tested with the amplification of candidate genes involved in the *AcrAB-TolC* pumps. The other genes participating in the efflux system, e.g. *acrA* was successfully amplified except *acrB* gene. The results of *acrR* as well as *emrA* gene, that produced various non-specific products are demonstrated in Table 8.6.

In Figure 8.2, the presence of *tolC* was simultaneously detected in all *E. coli* strains taken from water and biofilm samples taken from Plankenbrug River (Below Kayamandi) in 2005. These gene products are illustrated in lanes 1-9. The presence of *ompF*, which was detected in all the *E. coli* strains obtained from water and biofilm samples taken from WS1 Plankenburg River (Below Kayamandi) in 2005, is illustrated in lanes 10-15. The gene product with fragment size corresponding to 1482 (*tolC*) and 1089 bp (*ompF*) according to the 1 kb molecular marker (M, Promega, Madison, Wisconsin, USA) in lane 16 were seperated on a 0.8% agagraose gel (Promega, Madison, Wisconsin, USA). These *E. coli* strains all displayed resistance to some of the antimicrobials tested in Chapter Six after exposure to various chlorine concentrations and in this study, efflux genes and outermembrane protein were shown to be present.

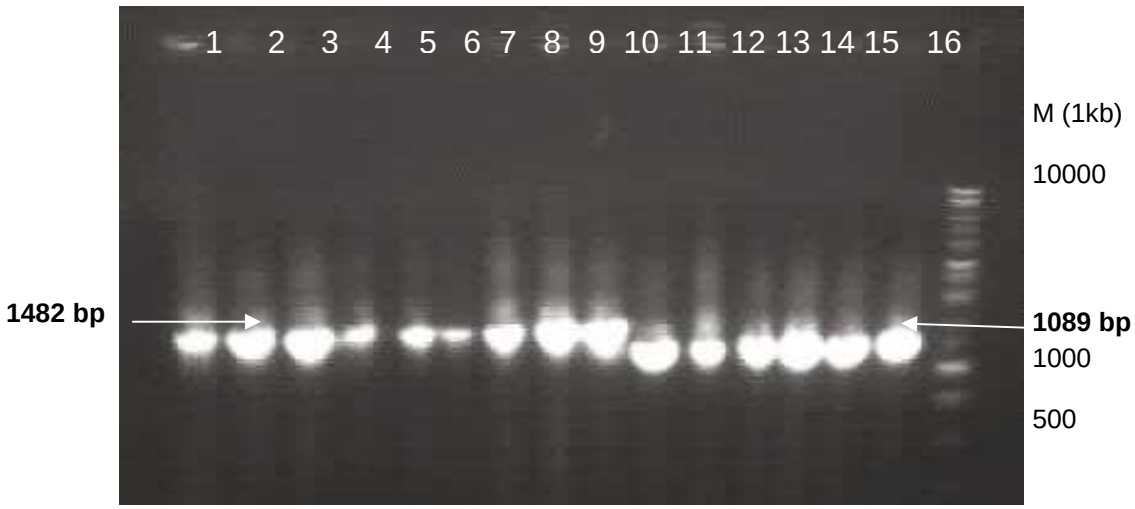

Figure 8.2. Amplification of candidate genes involved in efflux, *tolC* (lanes 1-9) and *ompF* (lanes 10-15) possibly involved in chlorine tolerance. Lanes1 (K1); lane 2 (K9); lane 3 (BK1); lane 4 (K13); lane 5 (K14); lane 6 (K12); lane 7 (K6); lane 8 (K10); lane 9 (P); lane 10 (K1); lane 11 (K9); lane 12 (BK1); lane 13 (K13); lane 14 (K14); lane 15 (PP); lane 16 (M)

In this substudy fluoroquinolones were included and one mechanism for resistance are mutations in targets DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) genes. The presence of *gyrA* was detected in a few samples isolated from WS1 and BS1, Plankenburg River (Below Kayamandi) in 2005 demonstrated in Table 3.1 (Addendum 3). The percentage for *ampC*, *marA,* and *parC* detection in the candidate genes are presented in Table 8.6. It was noted that antimicrobial gene *parC* was detected in more isolates than the *gyrA* gene. Detection of possible ESBLs was unsuccessful in amplifying this candidate gene fragment in any of the isolates including the positive controls used in this substudy.

A summary of each of the candidate genes detected in the 100 selected *E. coli* strains are described and presented in Table 8.6. The first set of candidate genes were selected for chlorine resistance, the grey coloured part for antibiotic resistance and the others for efflux pump resistance. Possible genes with similar functions were grouped and the presence within the same organisms are demonstrated in Table 8.7.

Table 8.6. Percentages of candidate genes detected in *E. coli* strains isolated from various water and biofilm samples

Candidate Genes	Plankenburg River (Below Kayamandi)		Plankenburg River (Below Kayamandi)		Nietvoorbij Research Project	Stiebeuel River (Langrug informal settlement, Franschoek)	Imizamo Yethu informal settlement (HoutBay)
Year	2005 (WS1)	2005 (BS1)	2006 (WS2)	2005 (BS2)	2006 (WS5)	2006 (WS3)	2006 (WS4)
E. coli Isolates	14	1	33	13	14	12	13
osmC	64%	100%	94%	92%	64%	25%	100%
grpE	57%	100%	70%	85%	43%	92%	100%
soxS	64%	100%	70%	85%	71%	25%	100%
soxR	57%	100%	60%	62%	79%	33%	100%
dnaK	0%	0%	48%	69%	0%	67%	100%
ompF	36%	100%	52%	54%	50%	42%	100%
ampC	50%	100%	33%	15%	14%	50%	0%
gyrA	29%	100%	15%	8%	7%	0%	0%
parC	0%	0%	6%	0%	50%	42%	100%
emrA	0%	0%	58%	92%	00%	83%	31%
marA	29%	100%	52%	69%	0%	42%	100%
acrA	64%	100%	6%	23%	7%	58%	100%
tolC	57%	100%	52%	62%	50%	67%	100%
acrR	36%	0%	60%	62%	0%	92%	100%

Table 8.7. Analysis of candidate genes detected simultaneously in *E. coli* strains isolated from various water and biofilm samples

Candidate Genes	WS1 (n=14)	BS1 (n=1)	WS2 (n=33)	BS2 (n=13)	WS3 (n=13)	WS4 (n=13)	WS5 (n=14)
soxR + soxS	57%	100%	52%	62%	23%	92%	64%
dnaK + grpE	0%	0%	45%	62%	62%	100%	0%
dnaK + grpE + ompF + osmC + soxR + soxS	0%	0%	30%	62%	0%	100%	0%
acrA + tolC	36%	100%	3%	23%	46%	100%	7%
acrA + tolC + acrR	21%	0%	3%	23%	46%	0%	38%
ompF + acrA + tolC	21%	100%	3%	15%	23%	0%	36%
acrA + tolC+ emrA	0%	0%	3%	23%	38%	23%	0%
gyrA + parC	0%	0%	3%	0%	0%	0%	7%
gyrA + parC + emrA	0%	0%	3%	0%	0%	0%	0%
ampC + marA	14%	100%	27%	15%	38%	0%	0%

WS1, BS1 = Plankenbrug River (Below Kayamandi in 2005); WS2, BS2 = Plankenbrug River (Below Kayamandi in 2006); WS3 = Stiebeuel River (Langrug informal settlement, Franschoek); WS4 = Imizamo Yethu informal settlement (After built-up area, Hout Bay); WS5 = Nietvoorbij Research Project.

8.4. Discussion

8.4.1. Amplification of candidate genes involved in chlorine tolerance

The candidate genes included for the possible detection of chlorine tolerance were present in many of the *E. coli* strains tested except for *dnaK* (not detected in isolates from WS5) as illustrated in Table 8.6. In this substudy it was noted that the 13 strains selected from WS4 showed the presence of all the heat-shock genes and other candidate genes included for chlorine tolerance. The strains tested survived up to the highest chlorine concentrations,

exhibited a variety of morphological patterns and had non-susceptible antibiogram profiles. These results suggest that the candidate genes may have participated in the microbial response to chlorine exposure. A study by Greenberg *et al*., demonstrated that the induction of *soxRS* system confers low-level MAR.[20] Both candidate genes *soxR* and *soxS* was present in more than 50% of the isolates from each of the water sources, illustrated in Table 8.7. A large variety of sub-lethal environmental stresses, including chlorine disinfection, can trigger the expression of these heat-shock proteins, *soxR* and *soxS*.[6] Additionally, it has been shown that the *soxRS* regulatory system is a two-component system involved in the adaptive response of *E. coli* to superoxide stress.[6,7] A study by Nikajima *et al*., illustrated that the induction of *soxRS* system confers resistance to HOCl, mercury and organic solvents.[21]

Other genes involve in heat-shock proteins include the molecular chaperones such as *dnaK* and *grpE* which was included in this substudy. Additional function are the ATPase activity of *dnaK* which are control by *dnaJ* (accelerates *dnaK*) and *grpE* (promotes nucleotide exchange of ATP and ADP) to bind and release substrate proteins.[22-24] The gene, *grpE* was detected in isolates from WS2 and BS2 except samples P10 and B6 as illustrated in Figure 8.1. Isolate P10, described in attached Addendum 3 survived chlorine exposure of 0.1 mg/L and exhibited antibiogram profile 32. This isolate was confirmed as an ESBL. Isolate B4 (Addendum 3) was isolated from a biofilm sample and survived chlorine exposure of 0.3 mg/L after 90 minutes contact time and exhibited antibiogram profile 26 (*ampC* producing β-lactamases). The absence of this gene in these resistant isolates can be attributed to the presence or overexpression of other candidate genes involved in stress conditions. Since both gene *grpE* and *dnaK* are molecular chaperones that help the cell to survive heat and high temperatures inactivation, were detected in the *E. coli* strains.[25] Both these genes were only detected in strains isolated from WS2, BS2, WS3 and WS4 demonstrated in Table 8.7.

Various substances, such as chlorine, must traverse the cell membrane for entry into the cell. It was described in Chapter One, Section 1.4 that different mechanisms are used to facilitate uptake of which the OM permeability is one. This mechanism is regulated by the balance of porin proteins and the diffusion channels (route for passage of small hydrophilic compounds).[19,26,27] OMP, *ompF* was included as one of the candidate genes in this substudy as it forms passive diffusion pores which allow small molecular weight hydrophilic materials to cross the OM. This *ompF* gene was detected in *E. coli* organisms

isolated from both water and biofilm samples exposed to various antimicrobial agents and chlorine disinfection. Alterations of the membrane proteins are another resistance mechanism that can be induced in both planktonic and biofilm cells in response to antimicrobial agents such as β-lactams.[28,29] In a subsequent substudy the changes in the gene sequences of *ompF* and the possible decrease in permeability of the cell to various compounds will be investigated.

Overall, all the candidate genes selected for their possible involvement in chlorine tolerance was detected in some of the isolated strains taken from WS2, BS2 and WS4 shown in Table 8.7. Microbial response to various stress conditions has always been associated with intrinsic defence mechanisms and is a key factor in the development of resistant strains that can lead to serious public health threats.

8.4.2. Amplification of candidate genes involved in antimicrobial resistance

In Chapter Six (Substudy Three) it was demonstrated that 60% of the selected *E. coli* strains (n=89) isolated from the water samples and exposed to chlorine were resistant to ampicillin. Twenty percent of the unexposed isolates (n=18) taken from the water samples were ampicillin resistant. In contrast, isolates obtained from the biofilms demonstrated 10% resistance to ampicillin in the strains unexposed to chlorine and 0% resistance to those exposed. In this substudy, *ampC* were detected in 29 *E. coli* isolates and the antibiogram profiles of 23 of these *E. coli* strains were ampicillin resistant with ESBLs and ampC producing β-lactamase phenotypes illustrated in Addendum 3. Four isolates displayed sensitive patterns with the other two as trimethoprim-sulfamethaxazole resistant phenotypes. In contrast, the *ampC* gene was not detected in any of the *E. coli* strains selected from WS4. The antibiogram profiles of these isolates demonstrated in Chapter Six (Substudy Three) were all ampicillin resistant, indicating that the amplification method was not as sensitive for detection or that other mechanisms contributed to their exhibited resistant profiles. Candidate genes *ampC* and *marA* were detected in similar strains isolated from WS1, BS1, WS2, BS2 and WS3 demonstrated in Table 8.7. Both these genes were seen amongst 17% of the isolates demonstrated in Table 8.7 and all displayed a resistant phenotype to more than one antimicrobial.

ESBLs have been reported in most of the members of the Enterobacteriaceae. CTX-M-type-β-lactamases are typical ESBLs with the *ctx-m-9* group of these enzymes,

including *ctx-m-14,* and are found worldwide in species of Enterobacteriaceae. Additionally, almost all of the *ctx-m-9* genes are found in *E. coli*. The PCR method failed to detect any *ctx-m-9* genes within *E. coli* strains isolated in this substudy, including the positive control. This gene was also not detected in *E. coli* strains that exhibited and ESBL phenotype. This may illustrate that the method for detecting this gene was not optimal or that unknown mutations might have occurred in the primer target region preventing annealing. No detection of this gene could also suggest that no *ctx-m-9* type ESBL phenotype was present in the *E. coli* isolated included in this substudy, but perhaps other ESBL types, such as TEM or SHV ESBLs.[30,31] Phenotypic differentiation of organisms producing CTX-M- β-lactamases from other types of ESBLs can be difficult but molecular identification requires an extensive range of candidate genes that continues to evolve. The detection of specific ESBL producing strains was not an aim of this study and therefore not investigated further.

Fluoroquinolones (FQs), which are antimicrobials used for the treatment of community-acquired and nosocomial infections, were investigated in this substudy. The two genes were detected in *E. coli* strains exposed to chlorine and tested against various antimicrobials, including FQs. From the 100 isolates selected for this substudy demonstrated in Addendum 3, 10 isolates had only the structural genes, *gyrA,* while in 25 other isolates *parC* gene was present. The PCR method detected 5 *gyrA* and 2 *parC* genes in isolates from WS2. Antibiogram profiles of only two of these *E. coli* strains isolated from WS2 displayed resistance to ciprofloxacin. The gene *gyrA* were not detected in many of the isolates obtained from WS3, WS4 and WS5 while the *parC* gene was detected in more of the isolates obtained from these sites. Only one *E. coli* strain from WS3 showed resistance to ciprofloxacin. Only one *E. coli* strains obtained from WS2 and one strain from WS5 had both the structural genes *gyrA* and *parC*. Both these strains displayed non-susceptible antibiograms type 11 and 30, but was sensitive to ciprofloxacin. Both the phenotypes and genotypes displayed by the *E. coli* strains as well as detection of these structural genes responsible for DNA gyrase (primary target)[32-34] and DNA topoisomerase IV(secondary target)[32-35] in some of the *E. coli* strains indicates that other factors such as efflux, which has been associated with FQs-resistance, may be involved. Therefore, the detection of only *parC* in certain isolates can be further investigated with DNA-DNA hybridization which involves detection of these genes with probes.

The involvement of decreased accumulation of FQs in *E. coli* mutants with a multiple-antibiotic-resistance phenotype was shown to be due to the reduced expression of

ompF gene and the overexpression of active efflux systems.[36-38] No expression studies were conducted in this substudy but the *ompF* gene was detected in 26 of these *E. coli* strains with one or both of the structural genes for detection of FQ-resistance. The presence of *gyrA* + *parC* + *emrA* was detected in one isolated from WS2 illustrated in Table 8.7 which displayed a non-susceptible antibiogram type 11 and sensitivity to ciprofloxacin.

The presence of *marA* gene was detected in 49 of the selected isolates as demonstrated in Addendum 3 except those taken from WS5. In contrast, the antibiogram profiles of many of the isolates from WS5 displayed multiple resistant phenotypes described in Chapter Six. Only 39 of the strains with the detection of the *marA* gene displayed resistance to more than one antimicrobial, suggesting the involvement of other mechanisms for expression of this phenotype.

8.4.3. Amplification of candidate genes involved in efflux pumps

Efflux pumps are transport proteins involved in the extrusion of toxic substrates from within cells into the external environment by utilising the energy of the proton motive force.[14] Efflux pumps are a major component of the bacterial cell. It was also found that the activity of efflux pumps could contribute to the modulation of the antimicrobial resistance, particularly quinolones.[39] The pilot study conducted in Chapter Seven (Substudy Four) on a water and biofilm samples taken from WS2, demonstrated that the addition of an EPI was effective in decreasing the *E. coli* enumeration levels after exposure to chlorine, as well as reversed some resistant antibiogram profiles of the isolates.

In this substudy, both regulatory (*soxR, soxS, acrR*) and efflux pump genes (*acrA, tolC*) were found in 13 of the strains and may suggest their involvement in survival of exposure to chlorine and antimicrobial treatment. *AcrAB*, which encodes for an efflux pump and responsible for maintaining low levels of antibiotics and other toxin, is one of the genes induced by *soxS*.[40] The efflux pump gene, *acrA* of the *acrAB* efflux system in *E. coli* acts as a transporter for tetracycline, ciprofloxacin, FQs, β-lactams and novobiocin. This gene was detected in many of the *E. coli* strains, except a isolate K6 obtained from Plankenburg River (Below Kayamandi) in 2005 which survived chlorine treatment of 0.1 mg/L and displayed an antibiogram with resistance to (ampicillin + co-amoxiclav + 3rd generation cephalosporins + cefoxitin + trimethoprim-sulfamethaxazole + gentamicin). Other mechanisms seemed to be involved in antimicrobial resistance against these organisms.

The presence of *acrA* + *tolC* was detected in similar isolates taken from each of the water sources demonstrated in Table 8.7. Detection of *acrA* + *tolC* + *acrR* in similar isolates were detected in several of the isolates except those isolated from BS1 and WS4. The detection of *ompF* + *acrA* + *tolC* was also seen in isolates illustrated in Table 8.7. Again, no expression studies were done, but the involvement of the regulatory gene (*acrR*, *soxS*) and decreased expression of the OMP (*ompF*) resulted in overexpression of the efflux pump system, *acrAB-tolC*.[13,38,41]

Various optimisation strategies were applied for the detection of *acrB* in the *E. coli* strains. *AcrB* was one of the largest genes amplified within this substudy with a fragment size of 3150 bp. The annealing primer conditions were incompatible with the T_m of the forward (T_m of 61.5℃) and reverse primers (T_m of 43.1℃). This difference resulted in poor specificity of each primer and contributed to PCR failure of this *acrB* gene. The OMP, *tolC*, required for proper expression of efflux in the acrAB-tolC pump was detected in 62 *E. coli* isolates. The isolates survived exposure to various chlorine concentrations and displayed sensitive to highly resistant antibiograms. These results suggest that this efflux pump could be involved in exposure to these conditions.

8.4.4. Factors influencing the effectivity of the PCRs

The aim of this substudy was to establish and optimise PCR methods for the detection and amplification of various candidate genes isolated from water sources in the Western Cape. To our knowledge no prior study was done on the screening of candidate genes involved of antimicrobial and chlorine tolerance of *E. coli* strains isolated from water and biofilm sources. Therefore, new amplification strategies had to be designed and optimised. The *E. coli* genome is extensively studied and it was not difficult to obtain the candidate gene sequences. For this study, the entire candidate gene had to be amplified to further screen for any changes in the nucleotides sequences and 200 nucleotide sequences flanking the gene were included. Primers were needed to be designed for these flanking sequences that did not bind to any other part of the sequence, which may have led to non-specific products. Although this was attempted, it was always a concern that environmental differences of the water sources could result in modification of the sequences that may lead to non-specific binding of primers. Non-specific products were therefore a main concern and this did occur in some of the samples. Each PCR method needed to be optimised by consideration of the gene length, melting point and annealing temperatures of primers to ensure optimal functioning of the method. Risk of contamination in

preparation of a sample for PCR conditions for each gene amplification was another concern. The slightest compromise in any of these could lead to contamination and PCR failure, which happened. The PCR amplifications of the candidate genes was successful with quality controlling of the extracted DNA, appropriate enzyme conditions and a sterile working environment.

8.5. Conclusion

PCR methods for detection of possible candidate genes involved in antimicrobial resistance, chlorine tolerance and efflux were established and optimised. Most of these candidate genes were present in the *E. coli* strains, but whether they changed due to the antimicrobial and chlorine exposure will only be determined from the sequencing results presented in the following substudy. Both phenotypic and genotypic information are important, especially for antibiotic resistance as seen with the FQs. No expression studies were included in this study but the detection of several genes in similar isolates suggest the involvement of secondary mechanisms, such as regulatory genes (*acrR*, *marA*, *soxS*), decreased expression of the OMP (*ompF*), antimicrobial resistance (β-lactams) and overexpression of the efflux pump system as described previously. These results indicate that, due to the increased complexity of antimicrobial resistance in gram-negative organisms, effective surveillance with both phenotypic and genotypic analyses is needed.

If amino acid changes occur, especially in the conserved and structural regions of a gene, it can influence the functioning of that gene when exposed to adverse conditions. To determine whether the exposure of the strains to various chlorine and efflux pump inhibitor (EPI) concentrations had an effect on the function of the gene, sequencing of these candidate genes were done. This is described in Substudy Six (Chapter Nine).

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Chapter Nine

Comparison of the genotypic and phenotypic profiles of selected strains

9.1. Introduction

Microorganisms have the ability to adapt in response to detrimental environmental conditions. The *E. coli* strains isolated in this study survived various concentrations of chlorine treatments described in Substudy Two (Chapter Five) with different morphological types (Chapter Six) and antibiotic susceptibility patterns (Chapter Six). After addition of an EPI in Substudy Four (Chapter Seven), strains continued to display the phenotypes described above. In *E. coli* strains both intrinsic and acquired resistance was demonstrated to various antimicrobials due to their efflux pumps.[1] Several candidate genes were selected and detected with PCR in Substudy Five (Chapter Eight) for their possible involvement in the phenotypes observed when *E. coli* strains were exposed to chlorine and various antimicrobials. In this substudy, candidate genes were sequenced and differences in the DNA and protein sequences were analysed.

The aim of this substudy was to compare the gene sequences of candidate genes of selected strains to their phenotypic profiles and to use computational and sequencing methods to confirm that the correct genes were amplified and to detect nucleotide variation.

9.2. Materials and methods

9.2.1. Preparation of PCR products for sequencing

Thirty-nine *E. coli* strains were selected after detection of the various candidate genes demonstrated in Substudy Five (Chapter Eight). Selection criteria were similar to those described in Chapter Eight, Section 8.2.1. From these strains forty-eight candidate genes were amplified with PCR and their size confirmed according to the molecular marker, as illustrated in Chapter Eight, Section 8.3.1. Afterwards, PCR products were purified from the agarose and PCR reaction with the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, Wisconsin, USA), as well as the QIAquick Gel and Extraction Kit protocol (QIAGEN Inc., USA) according to the manufacturers instructions. These methods

are described in section 9.2.1.1 and 9.2.1.2.

9.2.1.1. Clean-up from the gel bands

The PCR product was excised from the agarose gel with a clean sharp scalpel and placed into a microcentrifuged tube. The gel slice was weighed in the microcentrifuged tube and the mass recorded. One volume of the membrane binding solution (10 mM potassium acetate, pH 5.0; 16.5 mM EDTA, pH 8.0) was added per 10 mg of gel slice. Samples were vortexed and incubated at 50-65℃ until gel slices were completely dissolved.

The dissolved gel mixture from agarose gel and PCR reaction was transferred into a minicolumn with a collection tube, incubated at room temperature for 1 minute and centrifuged at 16 000 x g for 1minute. The flowthrough was discarded from the collection tube and the minicolumn re-inserted into the same collection tube. 95% Ethanol was added to the Membrane Wash Solution (10 mM potassium acetate, pH 5.0; 16.5 mM EDTA, pH8.0) and mixed. This was then added to the minicolumn with a collection tube and centrifuged at 16 000 x g for 1 minute. The flowthrough was discarded and the minicolum re-inserted into the same collection tube again. Addition of Membrane Wash Solution was repeated and centrifuged at 16 000 x g for 5 minutes. The flowthrough was discarded and the minicolum re-inserted into the same collection tube and re-centrifuged for 1 minute with the microcentrifuged lid opened (to allow evaporation of ethanol). Thereafter, the minicolumn was carefully removed and inserted into a clean 1.5 ml microcentrifuge tube. The column was discarded with the flowthrough. Approximately 50 µl of nuclease-free water was added to the minicolumn within the clean microcentrifuge tube and incubated for 1 minute at room temperature. The minicolumn within the clean microcentrifuge tube was centrifuged at 16 000 x g for 1 minute. Thereafter, the minicolumn was discarded and the DNA captured in the clean microcentrifuge tube was stored at 4°C or –20°C (longer storage period).

9.2.1.2. Clean-up directly from the PCR reaction

An equal volume of Membrane Binding Solution (10 mM potassium acetate, pH 5.0; 16.5 mM EDTA, pH 8.0) was added to samples obtained directly from the PCR reaction. A SV Minicolumn was inserted into a Collection tube and processed as described in section 9.2.1.1.

9.2.1.3. DNA purification with QIAquick Gel and Extraction Kit protocol

Only DNA from the PCR reaction was cleaned up. Approximately 5 volumes of Buffer PBI (Binding buffer, ensures optimal salt concentrations and pH for adsorption of DNA to the membrane) were added to 1 volume of the PCR reaction and mix. A QIAquick column (QIAGEN Inc., USA) was placed in the 2 ml collection tube provided. The sample was applied to the column and centrifuged at 13 000 x g for 30-60 s to bind the DNA. The flowthrough was discarded and the QIAquick column was placed into the same tube. 96-100% Ethanol was added to Buffer PE (Ethanol and Tris buffer, for washing of the DNA) and 0.75 ml of mixture added to the QIAquick column and centrifuged for 30-60 s to wash away impurities. The flowthrough was discarded and QIAquick column replaced in the same tube. The column was further centrifuged in a 2 ml collection tube for 1 min. Each QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. Elution buffer, EB (10 mM Tris-Cl, pH 8.5) or water (50 µl) was added to the center of the QIAquick membrane and centrifuged for 1 min to elute the pure DNA.

9.2.2. Primer preparation for sequencing

DNA sequencing primers listed in Tables 9.1 to 9.3 were designed for each of the candidate genes with Web Primer software programmer (SGD^{TM} pages Database Copyright© 1997-2008). Multiple sequencing primers were designed to obtain results of the entire candidate gene. Sequencing was done at Central Analytical Facility (CAF, Stellenbosch University) to determine the nucleotide sequences of the amplified genes.

Table 9.1. Characteristics of sequencing primers of purified candidate genes that may be involved in chlorine tolerance

Genes	Primer sequence, 5'-3'
soxS (324 bp)	F-1ATGTCCCATCAGAAAATT R-1TCAATGTTAAGCGGCTGG F-2AGACCTTCTCCCGCGTTT R-2TTACAGGCGGTGGCGATA
ompF (1089 bp)	F-1AACTTATTGACGGCAGTGGC R-1GCAGCATATCGGTGTAACCC F-2TTAAATACGCTGACGTTGGTTC R-2TTTTGTTGAAGTAGTAGGTTGCG F-3TAGCACACCTCTTGTTAAATGC R-3TGCAAGACGTGAGATTGCTC
osmC (432 bp)	F-1TGACAATCCATAAGAAAG R-1TTTTTCGCCTTCAAAACG F-2AAGAGTGAAGTTGCGGTG R-2TTACGATTTCAACTGGTA
soxR (465bp)	F-1ATGGAAAAGAAATTACCC R-1GCCAGCGGAATGCCAATA F-2ATACGTTAAGTGCGAAAG R-2TAGTTTTGTTCATCTTCC
grpE (594 bp)	F-1ATGAGTAGTAAAGAACAG R-1CTTCTGGCTCAACTGCCT F-2ACAAATTCGCGCTGGAGA R-2TCCGGGTTAGCTTTATCA F-3AGGTAACGTACTGGGCAT R-3TTAAGCTTTTGCTTTCGC
dnaK (1917 bp)	F-1CGTTTAGATGGGTAAAATAATTGG R-1GTACGGTTGCCAGTGCCTT F-2CGTATCGCTGGTCTGGAAGT R-2TGGCATACGAGTCTGACCAC F-3CTGAAAGTTGCACTGCAGGA R-3CGTTCAGACCAGAAGAAGCC F-4ATGCTGACGGTATCCTGCAC R-4TTTAAATTGCCCCTAGATGAATG

Table 9.2. Characteristics of sequencing primers of purified candidate genes for antimicrobial resistance

Genes	Primer sequence, 5'-3'
ampC (1134 bp)	F-1TAACGCATCGCCAATGTAAA R-1GGTTGCGAGATGTAATAGTGTGA F-2TCAAGTTAAGCGATCCCACA R-2GCAATTTTATTGTCACTGCCA F-3GATATGTATCAGGGCCTGGG R-3GAACTGCAGGCAACGACC
parC (752 bp)	F-1ATGAGCGATATGGCAGAG R-1TGACCATCAACCAGCGGA F-2TGAAGCGGAAATTATCAC R-2TGGTAGCGAAGAGGTGGT F-3CGGTGAGCAGAGTGAACT R-3CTTTCACCGCCGCTTTGA F-4CGTATGTTGATGTTCCCG R-4TTACTCTTCGCTATCACC
gyrA (2628 bp)	F-1CGCGGCTGTGTTATAATTTGC R-1TCAACGAAATCGACCGTCTCT F-2TGATAACTATGACGGCACGG R-2CTTCCACGCGTTTTTCTTTT F-3CGTCCACGAAATTCCGTATC R-3TTGTCACCGAACTGTTCACG F-4GCGGAACTGTTGCGTATTCT R-4GTTCGTCCTGCTCCAGCG F-5CGCGTCTATTCGATGAAAGTT R-5CACTGCCGTCGATGGTATC
marA (390 bp)	F-1ATGTCCAGACGCAATACT R-1ATCCGTTGCAGGTGCCAT F-2ATGGCTTCGAGTCGCAAC R-2CTAGCTGTTGTAATGATT

Table 9.3. Characteristics of sequencing primers of purified candidate genes for efflux pump resistance

Genes	Primer sequence, 5'-3'
acrA (1194 bp)	F-1TTTGACCATTGACCAATTTGA R-1AGCCAGAGCCTGATCGTACT F-2ATATCGCGCAATTGACGGTG R-2GAAGATAGCGCGTAGGGTGA F-3TTAAGTTCCCGCAGGACG R-3CGGGCGATCGATAAAGAAAT
tolC (1482 bp)	F-1CTGCTTCACAAGGAATG R-1TTAACTGCAAGGACGCACTG F-2GTGCAGATTACACCTATAGCAACG R-2CGTTTTTCGGCTTCTTTCAG F-3GAACTGGCTGCGCGTAAT R-3CGTACCGACCGAGTAGCC F-4TCAGTAGCATTAACGCCTACAAAC R-4CCCATCAGAATAGAGGATGGC
acrR (648 bp)	F-1ATGGCACGAAAAACCAAA R-1GATGTGTTGGCGCGTTTC F-2GTGACAGAACGGCGT R-2TTGTGGAATATAATCTCC F-3ATGTATCTCCTGTGCCCC R-3 TTATTCGTTAGTGGCAGG
emrA (1173 bp)	F-1TTTTTGGCAAGCAGGTCG R-1TTTCGCGAGGGCGATTT F-2CTTCCAGCGTTCGCCAAA R-2TTTCTGGTCCAGTTCGATACG F-3GTTCTCACTGCTTCCAGCG R-3 CGAAAGAAGTGATTACCCACG

9.2.3. Sequence analysis

PCR fragments of candidate genes were sequenced as described in Section 9.2.2. The overlapping sequence contigs of each of the candidate genes were assembled with

Sequencher 4.7 (2006 Gene Codes Corporation, Ann Arbor, Mich.) and checked against the reference strain. The consensus sequence of each gene was exported in various formats including fasta format for further analysis. DNA sequences were converted into the gene sequence with prefix .seq for analysis with DNAMAN Version 4.0, Lynnon Biosoft, Copyright©1994-97 for translation into amino acid sequences. These amino acid sequences with their reference strain were saved in one text file and entered into ClustalX (version 1.8) multiple sequence alignment program for comparison between the similar genes and the published genome sequence of *E. coli* K-12.[2,3] The differences in the amino acid sequences of the candidate genes were compared to the reference gene.[4] A modified table obtained from Current Protocols describes the physical characteristics of amino acids commonly found in proteins (such as hydrophobicity) are summarised in Table 9.4, which is a summary of[5]:

- From highly polar amino acids to non-polar amino acids or *vice versa*.
- Complex structured amino acids (F, Y, W, R, H) to simple amino acids (G, A, L, V, P) or *vice versa*.
- Tyrosine and asparagines with their bulky side chains within α-helices destabilised α-helices.
- Glycine within α-helices (destabilises the helical structures since rotation around it's α-carbon is unconstrained).
- Proline within α-helices (least common amino acid because it's rigid cyclic side chain which disrupts the right-handed helical conformation by occupying the space that a neighbouring residue of the helix would otherwise occupy).
- Glutamate in the interior part of the protein decreases the substitution for nonpolar amino acids.

Table 9.4. Physical characteristics of the amino acids [5]

Amino acids	3-letter code	1-letter code
Highly Hydrophobic		
Isoleucine	Ile	I
Phenylalanine	Phe	F
Valine	Val	V
Leucine	Leu	L
Methionine	Met	M
Less hydrophobic		
Tryptophane	Trp	W
Alanine	Ala	A
Glycine	Gly	G
Cysteine	Cys	C
Tyrosine	Tyr	Y
Proline	Pro	P
Threonine	Thr	T
Serine	Ser	S
Highly hydrophilic		
Histidine	His	H
Glutamate	Glu	E
Asparagine	Asn	N
Glutamine	Gln	Q
Aspartate	Asp	D
Lysine	Lys	K
Arginine	Arg	R

9.2.4. Homology Searches of the candidate genes

Homology searches were done with the nucleotide sequences determined in this substudy using the program Basic Local Alignment Search Tool (BLAST) algorithms program available at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/blast/).[6] Candidate genes homology searches ranging from 70-100% on identity to the results on the database was included.

9.3. Results

9.3.1. Sequencing Analysis

The 48 candidate genes included in this substudy and demonstrated in Table 3.1 in Addendum 3 were not all successfully sequenced. No sequencing results were obtained for gene, *emrA.* Sequences of 40 isolates characterised by peaks with little to no background interference were selected for further analysis. DNA sequences of the candidate genes were converted into amino acid sequences and short as well as bad sequences were excluded. Amino acid sequences of 29 genes were aligned and characteristics of these genes are illustrated in Table 9.4 and multiple alignment demonstrated in Figures 4.1 to 4.13 in Addendum 4. Homologous regions as well as variations were observed amongst the sequenced results of the candidate genes compared with the reference gene.[3]

9.3.2. Homology searches of the candidate genes

Homology searches of 36 sequenced candidate genes with sufficient nucleotide sequences were determined. Sequences were compared to databases on similarity to known sequencing data previous identified. These nucleotide sequences were BLAST on NCBI and the results of the candidate genes are illustrated in Table 9.5.

Table 9.5. Characteristics of the candidate genes included for multiple sequence alignment

Candidate Genes	Source	Chlorine exposure and contact time	Isolate Number	Phenotypes (Morphological Colony Types & Antibiogram profiles)
acrA59	WS5	0.3 mg/L	N4	8; 19
acrA90	WS2	0.3 mg/L	P18	2; 26
tolC11	WS5	0.3 mg/L	N4	8; 19
tolC32	WS4	0.5 mg/L (90min)	I8	3; 31
tolC65	WS2	0.1 mg/L (90min)	P5	4; 11
acrR21	BS2	0.3 mg/L	B3	3; 10
acrR25	WS4	0.6 mg/L (90min)	I13	1; 31
acrR43	WS2	0.1 mg/L	P7	1; 22
marA10	WS2	0.3 mg/L (90)	P15	1; 11
marA31	WS4	0.6 mg/L (90min)	I13	1; 31
gyrA37	WS1	0.5 mg/L (90min)	K13	3; 19
gyrA38	WS2	0.1 mg/L	P6	1; 21
parC21	WS3	0.3 mg/L	S7	3; 32
parC39	WS2	0.1 mg/L (90min)	P5	4; 11
ampC17	WS2	0.1 mg/L (90min)	P5	4; 11
ampC89	WS5	0.3 mg/L (90 min)	N4	8; 19
soxR12	WS5	0.4 mg/L (90 min)	N9	10; 13
soxR67	WS2	0.1 mg/L	P7	1; 22
soxS8	BS2	0.6 mg/L (90min)	B12	3; 24
soxS68	WS2	0.4 mg/L	P24	2; 28
osmC5	WS2	0.1 mg/L	P10	2; 32
osmC9	WS4	0.5 mg/L (90min)B	I9	3; 31
grpE9	WS2	0.1 mg/L (90min)	P5	4; 11
grpE19	WS4	0.4 mg/L (90min)	I8	3; 31
grpE22	WS5	0.4 mg/L (90min)	N12	5; 31
ompF18	WS2	0.3 mg/L	P17	1; 26
ompF50	WS3	0.4 mg/L (90min)	S9	8; 24
dnaK1	BS2	0.3 mg/L (90min)	B4	3; 26
dnaK29	WS4	0.5 mg/L	I7	2; 26

min = minutes. WS2, BS2 = Plankenbrug River (Below Kayamandi in 2006); WS1= Plankenbrug River (Below Kayamandi in 2005); WS3 = Stiebeuel River (Langrug Informal Settlement, Franschoek); WS4 = Imizamo Yethu informal settlement (After built-up area, Hout Bay); WS5 = Nietvoorbij Spier Project.

Table 9.6. Homology searches of the candidate genes

Candidate Genes	Source	Isolate Number	Score (bits)	Identity (%)	Strains	Accession Link (PubMed)
acrA59	WS5	N4	941	98	*E. coli* W3110	Gene ID: AP003585.1
acrA90	WS2	P18	942	98	*E. coli* W3110	Gene ID: AP003585.1
tolC11	WS5	N4	944	98	*E. coli* W3110	Gene ID: AP003585.1
tolC32	WS4	I8	961	97	*E. coli* 536	Gene ID: ZP03043238.1
tolC34	WS2	P24	522	96	*E. coli* E22	Gene ID: 4190687
tolC65	WS2	P5	958	97	*E. coli* W3110	Gene ID: AP003585.1
acrR21	BS2	B3	415	98	*E. coli* str. K12 substr. MG1655	Gene ID: 945516
acrR12	WS4	I9	416	99	*E. coli* str. K12 substr. MG1655	Gene ID: 945516
acrR25	WS4	I13	423	100	*E. coli str. K12 substr. MG1655*	Gene ID: 945516
acrR43	WS2	P7	425	100	*Shigella boydii Sb227*	Gene ID: YP_406904.1
marA10	WS2	P15	267	100	E. coli APEC 01	Gene ID: 4493407
marA31	WS4	I13	620	93	*E. coli* APEC 01	Gene ID: 4493407
gyrA37	WS1	K13	1754	99	*E. coli* str. K12 substr. MG1655	Gene ID: 946614
gyrA30	WS5	N4	1753	99	*E. coli* CFT073	Gene ID: 1038282
gyrA38	WS2	P6	1754	99	*E. coli* str. K12 substr. MG1655	Gene ID: 946614
parC21	WS3	S7	518	87	*E. coli* B7A	Gene ID: ZP 03029364.1
parC39	WS2	P5	401	94	*E. coli* B7A	Gene ID: ZP 03029364.1
ampC17	WS2	P5	758	95	*E. coli* CFT073	Gene ID: 1037511
ampC89	WS5	N4	640	76	*E. coli* CFT073	Gene ID: 1037511
ampC36	WS1	K3	416	94	*E. coli* HS	Gene ID: 5593543
soxR12	WS5	N9	313	100	*E. coli* O157:H7 EDL933	Gene ID: 960061
soxR67	WS2	P7	298	96	*E. coli* O157:H7 EDL933	Gene ID: 960061
soxR82	WS3	S5	179	91	*E. coli* CFT073	Gene ID: 1039198
soxS8	BS2	B12	213	99	*E. coli* O157:H7 EDL933	Gene ID: 960062
soxS68	WS2	P24	220	100	*E. coli* O157:H7 EDL933	Gene ID: 960062
osmC5	WS2	P10	294	100	*E. coli* str. K12 substr. MG1655	Gene ID: 946043
osmC9	WS4	I9	292	99	*E. coli* str. K12 substr. MG1655	Gene ID: 946043
grpE9	WS2	P5	377	100	*E. coli* CFT073	Gene ID: 1038282
grpE19	WS4	I8	377	100	*E. coli* O157:H7 EDL933	Gene ID: 957103
grpE22	WS5	N12	376	100	*E. coli* O157:H7 EDL933	Gene ID: 957103
ompF18	WS2	P17	623	98	*Shigella sonnei* Ss046	Gene ID: YP_309906.1
OmpF20	WS2	P7	664	92	Shigella flexneri 2a str. 301	Gene ID: NP_706848.1
ompF50	WS3	S9	664	92	*E. coli* str. K12 substr. MG1655	Gene ID: 945554
dnaK1	BS2	B4	1244	99	*E. coli* O157:H7 EDL933	Gene ID: 956673
dnaK29	WS4	I7	1235	98	*E. coli* O157:H7 EDL933	Gene ID: 956673
dnaK20	WS2	P2	1240	98	*E. coli* HS	Gene ID: 5594568

9.4. Discussion

9.4.1. Sequencing analysis of candidate genes

As described in section 9.3.1, 36 nucleotide sequences of the candidate genes were obtained from sequencing with peaks containing little background interference. These sequences were further manually edited to exclude any incorrect results due to overlapping peaks. Nucleotide sequences were converted into amino acid and the open reading frame (ORF) with the least amount of stopcodons were exported for alignments. Similar candidate genes were grouped and aligned with the reference gene.[3] Amino acid sequences of candidate genes that did not aligned and resulted in corrupt sequences were excluded. Amino acid sequences of 29 candidate genes aligned properly and are illustrated in Figures 4.1 to 4.13 (Addendum 4). Various changes were observed in the amino acid sequences of these genes within secondary structures that could affect its function. The sequencing results of the possible candidate genes involved in antimicrobial, chlorine and efflux resistance are discussed below.

9.4.2. Factors influencing the effectivity of sequencing

Some difficulties were experienced which resulted in the failure of sequencing of certain genes. One was the template DNA concentrations, which could have been contaminated or contained high salt concentration resulting in poor sequencing. The PCR clean-up from the agarose was another factor, as one band on the agarose gel from a PCR product did not always guarantee a good sequencing result. A second concern was the application primers. The PCR primers were initially used for sequencing and resulted in poor nucleotide sequences. More specific primers were then designed on the gene nucleotide order on the size of the amplified gene fragments according to the molecular weight marker. This was not effective for all the candidate genes, as non-specific amplification occurred during PCR and some of the gene fragments were lost during the clean-up steps. All of the above mentioned factors resulted in poor sequencing and failure at the beginning stages and thereafter, in a few isolates. The correct primer concentrations and sufficient PCR product was needed to ensure accurate sequencing. A positive control for sequencing of the individual genes was not initially included to correlate and quality control of the sequencing. Sequences had to be manually edited as sequences overlapped or had chromatograms with the width of the base of the peaks begun to approach ¼ of the peak's height. Other sequences contained very large peaks at the beginning of the sequence

which may mask the true sequence. The peak underneath the large sequences was manually edited as the correct bases. Poor sequences with large initial peaks followed by flat sequence could be a cause of the concentrations and quality of the template or primer. The purification and sequencing of such sequences were repeated. Results of genes containing sequences with multiple peaks at a single position were re-cultured for PCR amplification and then prepared for sequencing. Sequencing analysis with Sequencer V3.0 program worked well, but human error in base calling can also occur.

9.4.3. Candidate genes sequenced for chlorine tolerance

The candidate genes selected for their possible involvement in chlorine resistance were detected in 50% of the *E. coli* strains, as demonstrated in the previous substudy (Table 8.6.) and sequenced in this substudy. In Chapter One, Section 1.3.3.2 it was described that specific and general damages caused by chlorine exposure of the microorganisms were not fully explained in the literature.[7,8] Heat-shock genes involved in the defence mechanism of *E. coli* strains against H_2O_2 were demonstrated to be involved in chlorine resistance.[7,9] In addition the oxidative response in *E. coli* strains are mediated by the *soxRS* regulon that protects the cell.

Three genes for *soxR* were sequenced and after translation into proteins only two amino acid sequences of these *soxR* genes were included for multiple sequence alignment demonstrated in Figure 4.8 (Addendum 4). No change was observed in the amino acid sequence of *soxR*12 compared to the reference *soxR* gene of *E. coli* K12. This *E. coli* strain was isolated from a wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) after exposure to 0.4 mg/L chlorine of 90 minutes contact time and displayed a morphological type 10 phenotype with an antibiogram profile 13 as illustrated in Substudy Three (Chapter Six).

The amino acid sequence of *soxR67* had various changes compared to the reference *soxR* gene. These amino acid changes occurred outside the major secondary regions but can have an impact on the strain as some changes were from highly hydrophilic amino acids Aspartate (D) to highly hydrophobic properties Phenylalanine (F), described in section 9.2.3. This isolate were obtained from Plankenburg River (Below Kayamandi) after exposure of 0.1mg/L chlorine and displayed a morphological type 1 with an antibiogram profile, 21 illustrated in Substudy Three (Chapter Six).

The other gene involved in the *soxRS* regulon is *soxS*, which was successfully amplified. Two amplified *soxS* genes selected for sequencing and converted into amino acid sequences and aligned with the reference *soxS* gene of *E. coli* K12. No changes were observed in the amino acid sequence of *soxS*68 from a *E. coli* strain which was isolated from a water sample obtained from Plankenburg River (Below Kayamandi) after exposure to 0.6 mg/L chlorine illustrated in Substudy Three (Chapter Six). This strain displayed an antibiogram profile number 28 and morphological type 2. The other *soxS8* gene selected had a deletion of Arginine (R) at amino acid 58 which is a highly hydrophilic amino acid that can easily substitute other non-polar amino acids. This *E. coli* strains was isolated from a biofilm sample obtained from Plankenburg River (Below Kayamandi) after exposure 0.6 mg/L of chlorine and 90 minutes exposure time and displayed an antibiogram profile 24 and morphological type 3.

All three amplified *grpE* genes selected were sequenced and the results are demonstrated in Figure 4.11 (Addendum 4). These *E. coli* strains were obtained from different water sources and demonstrated one change at amino acid 44. This change from Valine (V) to Isoleucine (I) occurred within a secondary structure, α–helix and are also a common mutational pathway used by *E. coli* organisms. Gene *grpE*9 of a *E. coli* strain isolated from a water sample obtained from Plankenburg River (Below Kayamandi) after exposure to 0.1 mg/L chlorine for 90 minutes, displayed an antibiogram profile number 11 and morphological type 4 illustrated in Substudy Three (Chapter Six). Gene *grpE*19 was sequenced from a strain obtained from water sampled at Imizamo Yethu informal settlement (After built-up area, Hout Bay) after exposure to 0.4 mg/L chlorine for 90 minutes. This strain displayed an antibiogram profile number 31 (confirmed ESBL) and morphological type 3 illustrated in Substudy Three (Chapter Six). Gene *grpE*22 was sequenced from a strain isolated from water sampled at a wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) after exposure to 0.4 mg/L chlorine for 90 minutes. This strain displayed an antibiogram profile number 31 (confirmed ESBL) and morphological type 5 illustrated in Substudy Three (Chapter Six). The function of these genes includes cellular protection and homeostatic response to cope with stress-induced damage in proteins.

Candidate gene, *dnaK* was detected in many of the *E. coli* isolates and three genes selected for sequencing. Two of these 3 *dnaK* genes were successfully sequenced and aligned with the reference gene and results are illustrated in Figure 4.10 (Addendum 4). Changes were observed in the amino acid sequence of *dnaK1* sequenced from an *E.*

coli strain which was isolated from a water sample obtained from Plankenbrug River (Below Kayamandi) after exposure to 0.3 mg/L chlorine and 90 minutes contact times. This strain displayed an antibiogram profile number 26 and morphological type 3 illustrated in Substudy Three (Chapter Six). The amino acid sequence of *dnaK*29 which was sequenced from a *E. coli* strain isolated from water obtained from Plankenburg River (Below Kayamandi) after exposure to 0.6 mg/L chlorine displayed an antibiogram profile number 26 and morphological type 2 illustrated in Substudy Three (Chapter Six). The amino acid changes observed in *dnaK1* were at positions 92 from Lysine (K) to Methionine (M), 202 from Isoleucine (I) to Lysine (K) and 624 from Aspartate (D) to Glutamine (Q). An amino acid changed at 625 from Aspartate (D) to Asparagine (N) occurred only in *dnak*29. Both *dnaK*1 and *dnaK*29 genes had amino acid changes at 432 from Asparagine (N) to Phenylalanine (F), an insertion at 433 from Glycine (G) and 434 from Glutamine (Q) to Threonine (T). Other amino acid changes within both these genes were at position 435 from Serine (S) to Lysine (K), 436 from Alanine (A) to Asparagine (N) and 437 from Valine (V) to Methionine (M). Many heat-shock proteins (HSP) in *E. coli* and other bacteria are molecular chaperones (e.g. DnaK, GroEL and their cohorts) or ATP-dependent proteases (e.g. Lon, ClpAP).[3] The HSP70 region in *dnaK* ranges from amino acid 4 to 600 assists in protein folding, assembly, transport, repair and turnover under stress conditions.[3] All the changes within the amino acid sequence of *dnaK* genes were within this HSP70 protein. None of these changes were common mutational pathways taken by *E. coli* strains during evolution. These changes were from highly hydrophilic amino acids to hydrophobic amino acids and *vice versa* and could have serious implications on the functioning of the proteins encoded by this gene.

One of the targets of HOCl seems to be associated with the membrane and proteins of the organism.[10] In a previous study it was shown that alterations of the membrane-proteins were another resistance mechanism that can be induced in both planktonic and biofilm cells in response to antimicrobial agents like β-lactams.[11,12] Three *ompF* genes were amplified and two amino acids were included on the multiple alignment results illustrated in Figure 4.12 (Addendum 4). The OMP, *ompF*, had various changes in the amino acid sequences illustrated in Addendum 4. Amino acid changes in *ompF*50 were at position 46 from Glycine (G) to Valine (V), 58 from Glycine (G) to Cysteine (C) and deletions from 279 to 302. This gene was detected in an *E. coli* strain isolated from the Stiebeuel River after chlorine treatment of 0.4 mg/L chlorine. The strain displayed a morphological type 8 and antibiogram 24 illustrated in Substudy Three (Chapter Six). In *ompF18* the amino acid

changed at position 65 from Leucine (L) to Proline (P), 69 from Glycine (G) to Lysine (K), 86 from Asparagine (N) to Threonine (T), 110 from Leucine (L) to Phenylalanine (F) and amino acid insertions at 249. The gene, *ompF*18 was detected in a *E. coli* strain isolated from Plankenburg River (Below Kayamandi) after treatment of 0.3 mg/L chlorine and displayed a morphological type 1 and antibiogram 26 (*AmpC* β-lactamase). The amino acid changes within *ompF*50 occurred within β-strand 57-59 and the transmembrane 275-287. Changes within *ompF*18 occurred within the transmembrane 62-74, 77-91 and 102-112. Again the amino acid was from hydrophilic to hydrophobic properties with one complex change from Leucine (L) to Phenylalanine (F). The substitution of Leucine (L) to Proline (P) is one of the least common amino acids within a gene as it disrupts the conformation and may influence the gene function.

When *E. coli* strains are exposed to chemical and physical stresses, such as heat, oxidative agents or osmotic shock, they undergo a global programmed modification of gene expression, which leads to resistance.[13,14] A gene involved in osmotically stress, *osmC* was amplified in many *E. coli* strains illustrated in Table 8.6 in Substudy Five (Chapter Eight). Four genes were selected and two successfully sequenced. No changes were observed in *osmC*5 with one amino acid change *osmC9* at position 32 from glutamine (Q) to arginine (R). This change occurred within a β-strand and was from one highly hydrophilic amino acid to another. The amino acid sequence of *osmC*5 was sequenced a *E. coli* strain isolated from a water sample obtained from Plankenbrurg River (Below Kayamandi) after exposure to 0.1 mg/L chlorine. This strain displayed an antibiogram profile number 32 and morphological type 2 illustrated in Substudy Three (Chapter Six). In contrast, *osmC*9 with one amino acid change was isolated from a water sample obtained from Imizamo Yethu informal settlement (After built-up area, Hout Bay) after exposure to 0.5 mg/L chlorine and 90 minutes contact time. This strain displayed an antibiogram profile number 31 and morphological type 3 illustrated in Substudy Three (Chapter Six).

9.4.4. Candidate genes sequenced for antimicrobial resistance

The *E. coli* strains isolated from water and biofilm samples in this study survived exposure to various chlorine concentrations and displayed various antimicrobial resistant patterns, including ESBLs and *ampC* β-lactamases. As described in Chapter One, Section 1.6.1, antimicrobial resistance phenotypes of microorganisms can be required via lateral genetic transfer, as well as the occurrence of *de novo* mutations within the bacterial genome.

Antimicrobial resistant *E. coli* strains, arising either through phenotypic adaptation, genetic alteration or genetic acquisition, can result in treatment failure, should infections occur associated with these organisms, which may lead to severe health implications.[15]

In this study the focus was on resistance to FQs in *E. coli* strains. As described in Chapter One, Section 1.6.1, FQs resistance is primarily caused by mutations in the structural genes, topoisomerase II (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*).[16,17] One mutation causing quinolone resistance occurs primarily in a highly conserved region called 'quinolones resistance-determining region' (QRDR) of the DNA gyrase (nucleotide positions 248 and 259/260) and topoisomerase IV (nucleotide 238/239 and 250/251) of alterations at Ser-83 and Asp-87.[18] The QRDR is a highly conserved region but alterations have been identified in a variety of species including *Campylobacters* spp., *E. coli* spp., *Salmonella* spp. and *Shigella* spp.[19] Candidate genes, *gyrA* and *parC* were amplified in Substudy Five (Chapter Eight) and three of each of the PCR products were purified and sequenced. Sequencing results were successful for two of each of the PCR products, aligned with the reference gene and the results are illustrated in Figures 4.4 and 4.5 (Addendum 4). No substitutions occurred in the QRDR regions of *gyrA* and *parC* sequenced in this substudy. The amino acid sequence of *gyrA*37 was sequenced from a *E. coli* strain obtained from water sampled at Plankenburg River (Below Kayamandi) in 2005 after exposure to 0.5 mg/L chlorine and 90 minutes contact time. This strain displayed an antibiogram profile number 19 and morphological type 3 illustrated in Substudy Three (Chapter Six). In contrast, *gyrA*38 was sequenced from a *E. coli* strain obtained from a water sample at Plankenburg River (Below Kayamandi) in 2006 after exposure to 0.1 mg/L chlorine. This strain displayed an antibiogram profile number 21 and morphological type 1 illustrated in Substudy Three (Chapter Six). This antibiogram profile included resistance to ciprofloxacin (CIP), a fluoroquinolone.

The amino acid sequence of *parC*21 was sequenced from a *E. coli* strain obtained from water sampled at Stiebeuel River (Langrug informal settlement, Franschoek) after exposure to 0.3 mg/L chlorine and 90 minutes contact time. This strain displayed an antibiogram profile number 32 and morphological type 3 illustrated in Substudy Three (Chapter Six). The antibiogram of *parC*21 included resistance to ciprofloxacin (FQ). In contrast, *parC*39 was sequenced from a *E. coli* water sample obtained from Imizamo Yethu informal settlement (After built-up area, Hout Bay) after exposure to 0.1 mg/L chlorine and 90 minutes contact time. In Figure 4.5 (Addendum 4) it was illustrated that the multiple alignments of *gyrA* and *parC* had various amino acid changes across the

entire chain. No changes occurred within the active sites of *gyrA* at amino acid 122 but within the other secondary structures such as the turns, β-strands and α-helices. For *parC*, no change occurred at its active site at 120 but within the DNA topoisomerase IV domain from 27-467. This strain displayed an antibiogram profile number 11 and morphological type 4 illustrated in Substudy Three (Chapter Six). The determination of FQ-resistance linked to *gyrA* and *parC* in this QRDR of *Shigella dysenteriae* Type 1, had no mutations in these genes.[20] Thus other factors may be involved in controlling FQ-resistance in *Shigella dysenteriae* Type 1 and possibly the *E. coli* strains isolated in this study.

Fluoroquinolone resistance in *E. coli* is primarily caused by mutations in the structural genes for *gyrA* and *parC* genes as well as mutation affecting the regulatory genes, *marA*, *soxS* and *rob*.[21-24] Four of amplified PCR products of *marA* gene was selected for sequencing of which two were successful. One of these genes, *marA*10 was sequenced from a *E. coli* strain obtained from water sampled at Plankenburg River (Below Kayamandi) after exposure to 0.3 mg/L chlorine and 90 minutes contact time. The phenotypic results of this strain displayed a morphological pattern number 1 and antibiogram profile number 11 illustrated in Substudy Three (Chapter Six).

The other gene, *marA*31 was sequenced from an *E. coli* strain taken from water sample obtained from Imizamo Yethu informal settlement (After built-up area, Hout Bay) after exposure to 0.6 mg/L chlorine and 90 minutes contact time. This strain displayed an antibiogram profile number 31 and morphological type 1 illustrated in Substudy Three (Chapter Six).

The multiple sequence alignment of the two *marA* genes sequenced are illustrated in Figure 4.6 (Addendum 4). Only one substitution occurred in *marA*10 at 128 of Serine to Asparagine (N). This change is from one hydrophilic amino acid to another more hydrophilic one. Several amino acid changes demonstrated in Figure 4.6 occurred in *marA*31 which were within the helix-turn-helix regions (31-52 and 79-102) and β-strand (37-39). Two stopcodons occurred at amino acid position 98 and 124 within this gene and can render it non-functional. Another function of this regulatory gene, *marA* involves the activation of the *AcrAB-tolC* efflux system.

In Substudy Three (Chapter Six), it was demonstrated that 46% of the *E. coli* strains isolated before chlorine exposure (n=15) were resistant to ampicillin, while 57% of the 91 strains selected after chlorine treatment showed resistance. Four of the amplified *ampC*

genes described in Substudy Five (Chapter Eight) were selected and two were successfully sequenced. Multiple alignments of these two genes are illustrated in Figure 4.7 (Addendum 4). Several amino acid changes occurred in *ampC*17 and *ampC*89 which are within the helix regions (22-39, 79-81, 186-193, 209-214, 296-301 and 362-376) and β-strands (42-50, 53-63 and 315-321). Amino acid insertions also occurred at positions 230 and 293. The amino acid sequence of *ampC*17 was sequenced from a *E. coli* strain isolated from water sample obtained from Plankenburg River (Below Kayamandi) after exposure to 0.1 mg/L chlorine and 90 minutes contact time. This strain displayed an antibiogram profile number 11 and morphological type 4 illustrated in Substudy Three (Chapter Six). The amino acid sequence of *ampC*89 was sequenced from a *E. coli* strain isolated from water sample obtained from a wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) after exposure to 0.3 mg/L chlorine and 90 minutes contact time. This strain displayed an antibiogram profile number 19 and morphological type 8 illustrated in Substudy Three (Chapter Six). Both these *E. coli* strains displayed ampicillin resistance as well as the other *ampC* gene which was excluded on having too many stopcodons in its ORF and changes in their amino acid sequences. This sequencing results indicated that the *E. coli* strains were stressed and responded by taking one of the most frequent mutational pathways which led to an amino acid change from Leucine (L) to Valine (V).

9.4.5. Candidate genes sequenced for efflux pump resistance

In this substudy genes involved in efflux pump system of *E. coli* strains, *AcrAB-tolC*, described in Chapter One, Section 1.6.3 were investigated. From the results of Substudy Six (Chapter Eight), only *acrA* and *tolC* were successfully amplified and sequenced in this substudy. Efflux pumps are one important mechanism of antimicrobial resistance in microorganisms.[1] It was previously shown that a synergistic action between efflux pumps and the OM were seen in *E. coli* strains displaying MAR.[1] Analysis of candidate genes detected simultaneously in the *E. coli* strains isolated from various water and biofilm samples (Table 8.3.1.2) demonstrated that *acrA* + *tolC* + *ompF* were present in 15 of the isolates.

Alignment of the two sequenced *acrA* genes was highly similar in amino acid sequence to the reference gene, as demonstrated in Figure 4.1 (Addendum 4). In both these sequences, only an insertion at amino acid 201 was observed. This insertion involved amino acids of GIKFPQ which was not within a DNA binding site or secondary

structure but involved amino acids with high physical characteristics, described in Table 9.2.3.1. These amino acid are highly hydrophilic [Lysine (K), Glutamine(Q)] and hydrophobic [Isoleucine (I), Phenylalanine (F)] that can substitute other amino acids easily. Phenylalanine (F) is a highly complex amino acids and can substitute simple amino acids [Glycine (G), Proline (P)] easily. Glycine (G) and Proline (P) destabilises and disrupts helical structures. This gene functions in the efflux mechanism of antimicrobials such as tetracycline, ciprofloxacin, FQs, β-lactams and novobiocin described in Table 8.2.1.3 (Chapter Eight). One of the two genes successfully sequenced, A*cra*59 originated from an *E. coli* strain that was isolated from wastewater effluent at Spier Wine Estate (Nietvoorbij Research project) after chlorine exposure of 0.3 mg/L and displayed a morphological type 8 with an antibiogram 19. The other gene, *acrA90* was sequenced from an *E. coli* strain taken from Plankenburg River (Below Kayamandi) that displayed a morphological type 2 and antibiogram 26. These *E. coli* strains exhibited resistant phenotypes to antimicrobials of which resistance is mediated by efflux and although the amino acid sequences changes were not in secondary structures, the changes involved highly structured amino acids and may suggest the involvement of efflux.

Four *tolC* genes were sequenced and three analysed after conversion into amino acid sequences. Alignment of these three *tolC* genes demonstrated various changes compared to the reference gene in Figure 4 (Addendum 4). These changes occurred within the signal sequence (1-22), α-helices (243-263, 319-385) and β–strand (302-316) as well as deletions from amino acid 451 to 454. *TolC*11 was sequenced from an *E. coli* strain that was isolated from wastewater effluent at Spier Wine Estate (Nietvoorbij Research project) after chlorine exposure of 0.3 mg/L and displayed a morphological type 8 with an antibiogram 19. The other gene, *tolC*32 was detected and sequenced from an *E. coli* strain taken from Imizamo Yethu informal settlement (After built-up area, Hout Bay) that displayed a morphological type 3 and antibiogram 31. TolC65 was detected from an *E. coli* strain which was isolated from Plankenburg River (Below Kayamandi) after chlorine exposure of 0.1 mg/L and which displayed a morphological type 4 with an antibiogram 11. These were all resistant phenotypes displayed by the *E. coli* strains with amino acid changes with highly hydrophilic or hydrophobic properties that can contribute to the gene function in efflux. These results correlate with a study where it was found that the disruption of the efflux gene encoding for the *MexB* pump in *Pseudomonas aeruginosa*, displayed antimicrobial resistance to β-lactams, chloramphenicol, FQs and tetracycline which indicated that resistance was mediated by efflux.[25]

Four of the regulatory gene *acrR* was sequenced and three of these genes were further aligned and illustrated in Figure 4.2 (Addendum 4). Several substitutions, as well as deletions, were seen in the amino acid sequence. All of the changes were outside the DNA binding domains. *AcrR*25 was detected and sequenced from an *E. coli* strain taken from Imizamo Yethu informal settlement (After built-up area, Hout Bay) that displayed a morphological type 1 and antibiogram 31 (ESBL). *AcrR*43 was detected from an *E. coli* strain that was isolated from Plankenburg River (Below Kayamandi) after chlorine exposure of 0.1 mg/L and displayed a morphological type 1 with an antibiogram 22 (OFL-resistant). Two stopcodons were observed at amino acid 201 and 203 of *acrR21*. This gene was detected in an *E. coli* strain taken from a biofilm samples from Plankenbrug River after exposure to 0.3 mg/L chlorine and also displayed morphological type 3 with an antibiogram pattern 10 (CIP–resistant). This gene regulates the function of the *AcrAB-TolC* efflux pump to export various antimicrobials. Therefore mutations within these genes in strains that exibited multiple antibiotic resistance suggest the involvement of efflux in antimicrobial resistance. Piddock *et al* showed that additional resistance due to efflux was due to over-expression of genes such as *acrR, marA,* resulting in a MAR phenotype.[26]

In this substudy the candidate genes sequenced for their possible involvement in antimicrobial, chlorine and efflux resistance, had changes in the amino acid sequences. Changes seen on protein level of the *E. coli* organisms indicated adaptation to the conditions at hand. Although we can only speculate on the severity of the effect of these changes, this data showed that antimicrobial, chlorine and efflux may play a role in *E. coli* strains and is phenotypically related with various changes on genotypic level. In this substudy no specific protein expression studies were done to evaluate the effect of the antimicrobial and chlorine conditions on the strains. Therefore, we can only speculate on the impact of these amino acid changes within the coding regions and secondary structures.

9.4.6. Homology searches of candidate genes

Different *E. coli* and some *Shigella* strains were identified with the sequence homology searches of the NCBI database based on their bit scores, e-value and identity. Bit score is derived from the raw sequence alignments which were determined by a statistical scoring system. E-value is the number of different alignments with the bit score. The lower the e-value, the more significant the score. Identity is defined as the percentage of the extent to which two sequences are invariant. In this substudy, homology searches of the query

strain were only included if the e-value were 0.

Eight of the strains were similar in sequence homology to *E. coli* O157:H7 EDL933 (EHEC) illustrated in Table 9.3.2.1. These strains were isolated from Plankenburg River (Below Kayamandi in 2005 and 2006), Nietvoorbij Reseach Project and Imizamo Yethu informal settlement (After built-up area, Hout Bay). This strain (substrain) is considered a reference strain of O157:H7, which was first isolated during an outbreak in 1982. This is a highly pathogenic organism and can cause haemorrhagic colitis and food poisoning. The O157:H7 refers serotype reflects the specific antigenic markers found on the surface of the cell for attachment and effacement to cells in the large intestine.[27] This EHEC strain is not commonly isolated from water sources within South Africa, and therefore the identification of this strain based on the sequence similarity is a concern. In South Africa, a survey for *E coli* 0157:H7 in selected samples of sewage, river water, ground beef and milk was done. In this report[28], named 'Occurrence in water sources of *E coli* 0157-H7 and other pathogenic *E coli* strains' of February 2003, the authors described a new enrichment-IMS-selective agar procedure for detection of this strain. At least one *E coli* O157:H7 strain was successfully isolated from a sewage sample and may at least in part be due to observed shortcomings of the final selective cultivation procedure of this isolation procedure. In this study the media used were not specifically geared towards culturing.*E. coli* O157:H7 and may have been less sensitive to detect this pathogen.

Non-pathogenic *E. coli* strain K12 substrain MG1655 was identified in eight of the strains isolated from Plankenburg River (Below Kayamandi in 2005 and 2006), Stiebeuel River (Langrug informal settlement, Franschoek) and Imizamo Yethu informal settlement (After built-up area, Hout Bay). This is a wild-type *E. coli* with very little genetic manipulation except for the curing (removal) of bacteriophage lambda and the F-plasmid.[3] Four strains isolated from Plankenburg River (Below Kayamandi in 2006), and Nietvoorbij Research Project were identified as *E. coli* W3110. This strain is closely related to MG1655, a descendant of K-12 (W1485) which was derived from the original K-12 strain (EMG2). More of a wild-type strain than MG1655 and lacks the fertility factor (F^+ plasmid) of the original *E. coli* K-12 (EMG2).[29] Additional two strains isolated from Plankenburg River (Below Kayamandi in 2005 and 2006) were identified as *E. coli* HS. This strain (HS; serotype O9) is a human commensal that was originally isolated from a laboratory scientist at Walter Reed Army Institute of Research in 1978 (Levine, 1978). Strain HS colonises the human gastrointestinal tract but does not cause disease.[30]

Another *E. coli* strain identified based on sequence similarity included *E. coli* CFT073. This was found in five of the strains isolated from Plankenburg River (Below Kayamandi in 2005 and 2006), Stiebeuel River (Langrug informal settlement, Franschoek) and Nietvoorbij Research Project. This strain (CFT073; O6:K2:H1) is uropathogenic *E. coli* (UPEC) and one of the most common causes of non-hospital-acquired urinary tract infections.[31] One of the major differences from EHEC is the loss of specific virulence factors such as the type III secretion system and plasmid- and phage-associated pathogenicity islands (PAI). The pathogenicity of UPEC strains include fimbrial operons, genes for production of Pap pili and other virulence factors that allow colonisation of the urinary tract.[31] One isolate obtained from Imizamo Yethu informal settlement (After built-up area, Hout Bay) was identified as *E. coli* 536. This strain (536; O6:K15:H31) is a uropathogenic *E. coli* (UPEC) that was isolated from a patient with acute pyelonephritis (inflammation of the kidney and pelvis). It is a model organism of extraintestinal *E. coli*, commonly found in non-hospital-acquired urinary tract infections (UTI) and UPEC strains.[32]

Two strains isolated from Plankenburg River (Below Kayamandi in 2005 and 2006) and Imizamo Yethu informal settlements (After built-up area, Hout Bay) were identified as *E. coli* APEC01. This strain (serovar O1) is an avian pathogenic strain and causes respiratory, blood and enteric infections primarily in poultry.[33] Nucleotide sequences of one of the strains isolated from Plankenburg River (Below Kayamandi in 2006) were identified as *E. coli* E22. This is an EPEC strain of *E. coli* that produces a number of virulence factors including the locus of enterocyte effacement (LEE) type III secretion system PAI that is responsible for producing secreted effector proteins that cause the typical attachment/effacing (A/E) lesions of the epithelial layer.[34]

E. coli B7A was identified in two strains, one isolated from Stiebeuel River (Langrug informal settlement, Franschoek) and one from Plankenburg River (Below Kayamandi) in 2006. This is an ETEC strain (O148:H28:CS6:LT+:ST+) which cause travelers' diarrhea. This strain colonises the small intestine via pili, and releases two virulence toxins, heat-stable enterotoxin (ST+) and heat-labile enterotoxin (LT+).[34]

It comes as no surprise that three of the *E. coli* gene sequences showed similarities to the *Shigella boydii* Sb227, *Shigella sonnei* Ss046 and *Shigella flexneri* 2a str. 301species, as these two strains are bacteriologically closely related.[35] *Shigella* species are associated with diseases like dysentery and food poisoning. *Shigella sonnei* is mostly associated with dysentery in industrialised countries, less severe than other types of *Shigella,* and

causing mild diarrhea and dehydration. *Shigella sonnei* 1 strain Ss046 was also first isolated in China during a epidemic in the 1950s.[36] *Shigella flexneri* is another major cause of shigellosis in industrialised countries and is responsible for endemic infections. *Shigella flexneri* 2a str. 301 was isolated in 1984 from a patient in Beijing, China. It is closely related to pathogenic *E. coli* with additional insertion in its sequences and a virulence plasmid (pCP301).[37] *Shigella boydii* is a uncommon species except in India, where it was first isolated. Only 18 serotypes are known and they express a diverse range of toxins in addition to a *Shigella*-specific toxin. Infection with this agent often progresses to clinical dysentery. *Shigella boydii*4 strain Sb227 was isolated from stool samples in China in the 1950s.[38]

Both pathogenic and non-pathogenic strains were identified to be present in the water sources. One can now speculate that littering and faecal waste disposal were ways in which these strains ended up in the rivers sampled in this study. As these rivers are a source of drinking water, irrigation and also recreational purposes amongst others, the findings of this substudy might indicate a possible health risk to the water users.

9.5. Conclusion

The sequencing analysis of the candidate genes selected for chlorine tolerance illustrated various changes in the amino acid sequence including one common mutational pathway taken by the *E. coli* strain when exposed to stress conditions. Amino acid sequences of the candidate genes for antimicrobial resistance demonstrated various changes in the structural genes *gyrA* and *parC* but not the QRDR of the isolated *E. coli* strains within this study, even in those isolates that displayed a FQ-resistant phenotype. Sequencing analysis of candidate genes detected in *E. coli* strains involved in efflux pumps also had various amino acid substitutions. These strains displayed various resistant antibiogram profiles which indicated that resistance was mediated by efflux. In this substudy we can only speculate on the impact of these amino acid changes within the coding regions and secondary structures of the genes. Specific protein expression studies are needed to understand the specific effect of chlorine, antimicrobial agents and EPI on *E. coli* strains. Such experiments will demonstrate which genes are activated or repressed under the conditions, which may assist us in optimising these treatments.

Homology searches of the sequenced candidate genes identified several pathogenic strains that may cause severe infections. The fact that the *E. coli* strains isolated in this study displayed highly resistant phenotypic and variable genotypic profiles which could

be functionally or structurally related, as well as sequence similarity to different strains, indicate that the water sources studied were contaminated with highly pathogenic strains. To investigate this further, EPEC and ETEC strains were selected from the isolated *E. coli* strains and specific virulence genes were detected, as demonstrated in the following substudy.

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Chapter Ten

Determination of the pathogenic potential of isolated *E. coli* strains

10.1. Introduction

The water sources included in this study were heavily contaminated with faecal coliforms and *E. coli* strains. These *E. coli* strains demonstrated the ability to survive chlorine exposure up to the highest concentrations and displayed various morphological colony types. The data illustrated that the *E. coli* strains changed their morphological colony appearance as they were exposed to increased concentrations of chlorine Antimicrobial susceptibility test were performed on the selected *E. coli* strains and displayed various non-susceptible antibiograms including *ampC*-β lactamases and ESBLs. The *E. coli* strains displayed resistance to various antimicrobials before chlorine exposure with an increased resistance after chlorine exposure. Molecular detection for the possible candidate genes involved in these conditions were detected sequenced and BLAST against the database for similarity to known strains. These homology search results identified several pathogenic and non-pathogenic *E. coli* and *Shigella* strains. The pathogenic strains may have a serious impact on health if given the opportunity to infect humans in sufficient inoculum sizes, or even in lower numbers in the case of immune compromised individuals. Examples of waterborne disease outbreaks (e.g. diarrhea) and mortality in humans from the literature has been described in Chapter One, Section 1.2.1. This further motivated the investigation for determination of the pathogenic ability of these isolated *E. coli* strains.[1]

Several methods for detection of microorganisms within environmental samples using PCR have been developed.[2] One such microbial source tracking method includes ribotyping, which genetically match (DNA fingerprinting) the potential source to isolates from humans and many different animals within the area.[3]

No specific source tracking, as determined with ribotyping, was executed in this study as no humans or animals samples were included in this substudy. The six major classes of enterovirulent *E. coli* strains described in the literature were narrowed down to the detection of only EPEC and ETEC strains. In South Africa, ETEC and EPEC are casual agents in 8-42% of diarrhea incidences.[4] Therefore, the aim of this substudy was to detect virulence genes of EPEC and ETEC strains in the samples in order to identify and

characterise strains of pathogenic potential.

10.2. Materials and methods

10.2.1. Criteria and selection of *E. coli* strains for detection of virulence genes

In this substudy, *E. coli* strains selected for PCR as previously described in Substudy Five (Chapter Eight), Section 8.2.1 were included for strain-typing. Stored DNA samples of the selected *E. coli* strains used for detection of candidate genes as listed in Table 8.1 (Addendum 3) were screened for the detection of virulence genes of EPEC and ETEC strains.

10.2.2. Bacterial strains used as positive control for PCR of virulent genes

E. coli (Migula) Castellani & Chalmers ATCC 43887 [B170 (011:NM)], which has the virulent genes *eaeA* and *bfp* and *EAF* plasmid, as well as *E. coli (Migula) Castellani & Chalmers ATCC* 31705 [H10407 (078:NM)] containing toxic genes for *lt* and *st* for ETEC were the positive controls for strain-typing. All the strains were obtained from ATCC (Boulevard, Manassas, USA). The detection for specific virulence of EHEC strains was not included due to logistic constraints even though both EPEC and EHEC strains harbour the *eaeA,* but the major virulence factor in EHEC strains is *stx* (shiga toxin).

10.2.3. Polymerase Chain Reaction (PCR)

10.2.3.1. Characteristics of primers

Primers used for detection of virulent genes were obtained from previously published studies[6,7] and are listed in Table 10.1. One hundred *E. coli* isolates as attached in Addendum 3 were screened for virulent genes for identification as either EPEC or ETEC strains.

Table 10.1.Characteristics of primers for virulence gene detection[5,6]

Genes	Product Size (bp)	Primer Sequence, 5'-3'	Reference	Gene Description
eaeA	376	F-1:CACACGAATAAACTGACTAAAATG R1:AAAAACGCTGACCCGCACCTAAAT	Nguyen *et al*., 2005	(EPEC). Attachment and effacement gene
st	147	F1:TTAATAGCACCCGGTACAAGCAGG R-1:CTTGACTCTTCAAAAGAGAAAATTAC	Matar *et al*., 2002	(ETEC). Heat-stable toxin gene
lt	322	F-1:TCTCATTGTGCATACGGAGC R-1:CCATACTGATTGCCGCAAT	Matar *et al*., 2002	(ETEC). Heat-labile toxin gene
bfpA	367	F-1:TTCTTGGTGCTTGCGTGTCTTTT R-1:TTTTGTTTGTTGTATCTTTGTAA	Nguyen *et al*., 2005	(EPEC). Bundle-forming pilus gene

10.2.3.2. PCR detection of virulence genes

PCR amplification was performed with a final volume of 50 µl in 0.2 ml thin-walled tubes. The primers used for PCR amplification are listed in Table 10.2.3.1.1. Each reaction contained 1x Go Taq Flexi Buffer; 200 µM of deoxynucleoside triphosphate; 50 pM forward and reverse primers; and 0.01 U of Go Taq DNA polymerase (Promega, Madison, Wisconsin, USA). Template DNA (3.0 µl) was added to 47.0 µl of the each reaction. The reagents within the tubes were thoroughly mixed and placed into a Thermocycle (Applied Biosystems GeneAmp® PCR System 9700).For the detection of the virulent genes in *E. coli* strains isolated from water and biofilm samples, PCR optimizations were done with $MgCl_2$ titration, illustrated in Table 10.2. In addition the PCR conditions for melting point and extension time of each gene were changed and are listed in Table 10.3.

Table 10.2. $MgCl_2$ titration for amplifications

Gene	$MgCl_2$ (1.5 mM)	Product size (bp)
eaeA	3.5	376
bfpA	3.5	367
lt	2.0	322
st	2.0	147

Table 10.3. Primers and conditions used in PCR for amplification of virulence *E. coli* genes[5,6]

Gene	Cycling conditions
eaeA and *bfpA*	Denaturation at 94℃ for 2 min, 30 cycles at 94℃ f or 30s, primer annealing at 47℃ for 30s and primer extension at 6 8℃ for 1 min, and final extension at 68℃ for 10 min.
st and *lt*	Denaturation at 95℃ for 5 min, 30 cycles at 95℃ f or 1 min, primer annealing at 50℃ for 1 min and primer extension at 72℃ for 1 min, and final extension at 72℃ for 10 min.

min = minutes; s = seconds

10.2.4. Agarose Gel Electrophoresis

A 0.8% agarose gel (LE Analytic Grade; Promega, Madison, Wisconsin, USA) and 1 kb molecular weight marker (Promega, Madison, Wisconsin, USA), as described in Substudy Five, Section 8.2.6, were used. Syngene™ GeneGenius computer system (a Division of synoptics LTD., Cambridge, United Kingdom) was used to visualise the gene fragments.

10.3. Results

10.3.1. Amplification of the virulence genes

A total of 100 *E. coli* strains, as described in Table 3.1 (Addendum 3), were selected for amplification of the virulent genes. Several PCR methods with $MgCl_2$, extension time and T_m of the individual gene amplifications were optimised. Virulence genes for EPEC and ETEC were detected in 16 of the *E. coli* strains and are presented in Table 10.4. The other strains tested negative for carrying virulence for EPEC and ETEC strains.

Table 10.4. Characteristics and detection of the virulence genes in the selected *E. coli* strains

***E.coli* strain**	**Virulent gene**	**Sampling site**	**Isolate Number**	**Chlorine exposure and contact time**	**Phenotypes (Morphological Types & Antibiogram profiles)**
EPEC	*eaeA*	WS1	BK1	0.6 mg/L (30min)	1; 30
EPEC	*eaeA*	WS2	P32	0.6 mg/L (30min)	3; 1
EPEC	*bfpA*	WS2	P15	0.1 mg/L (90min)	4; 11
EPEC	*bfpA*	WS2	P18	0.3 mg/L (30min)	1; 26
EPEC	*bfpA*	WS2	P29	0.5 mg/L (30min)	1; 3
EPEC	*bfpA*	WS2	P30	0.5 mg/L (90min)	2; 25
EPEC	*bfpA*	WS1	K7	0.1 mg/L (30 min)	3; 29
EPEC	*bfpA*	WS1	K10	0.3 mg/L (90 min)	2; 2
EPEC	*bfpA*	WS1	K12	0.5 mg/L (30 min)	2; 11
EPEC	*bfpA*	WS1	K14	0.5 mg/L (90 min)1	3; 30
EPEC	*bfpA*	WS5	N3	0.1 mg/L (A9)	1; 30
EPEC	*bfpA*	WS5	N8	0.4 mg/L (A4)	8; 7
EPEC	*eaeA* + *bfpA*	WS1	K10	0.3 mg/L (90min)	2; 2
EPEC	*eaeA* + *bfpA*	WS2	P4	0.1 mg/L (30min)	1; 1
EPEC	*eaeA* + *bfpA*	WS2	P24	0.4 mg/L (30min)	1; 27
ETEC	*st*	WS3	S7	0.3 mg/L (30min)	3; 32

min = minutes. WS2 = Plankenbrug River (Below Kayamandi in 2006); WS1= Plankenbrug River (Below Kayamandi in 2005); WS3 = Stiebeuel River (Langrug Informal Settlement, Franschoek); WS5 = Nietvoorbij Spier Project.

Figure 10.1 illustrates that *eaeA,* one of the virulence gene for EPEC was detected in the one *E. coli* strains isolated from a biofilm sample, as shown in lane 3. All the genes were electrophoretically confirmed according to band size of 376 bp with a 1 kb molecular marker (M, Promega, Madison, Wisconsin, USA) in lane 1 on a 0.8% agarose gel (Promega, Madison, Wisconsin, USA). In lane 2, *E. coli ATCC* 43887 (EPEC) was

used as the positive control and is labelled as EP1. Negative controls (no DNA) were included for the quality control of the PCR reactions and are labelled with N in lane 9 and *E. coli ATCC* 31705 (ETEC) labelled as EP2 in lane 10.

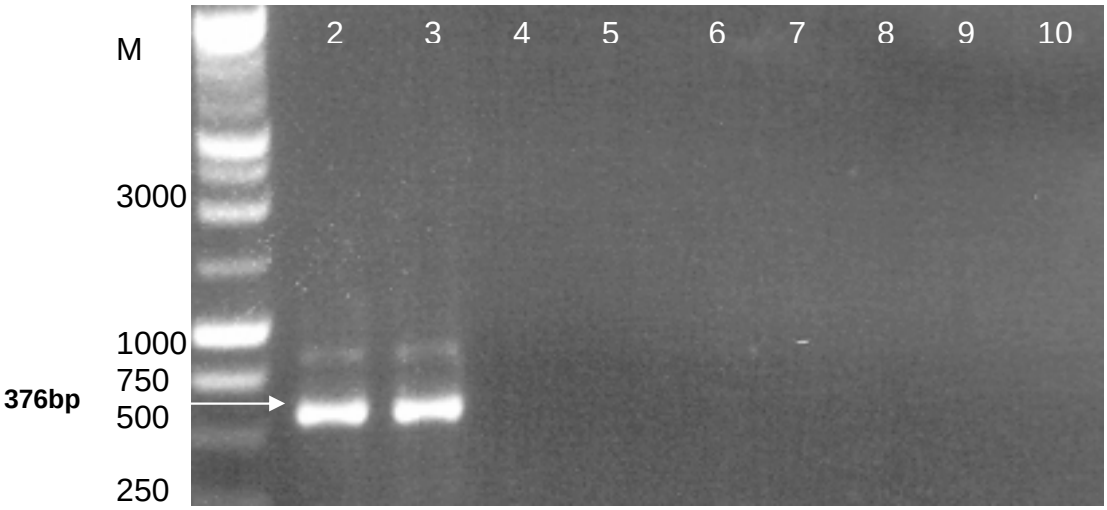

Figure 10.1 Amplification of *eaeA* gene (376 bp) for detection of EPEC strains. Lane 1 (M); lane 2 (EP1); lane 3 (BK1); lane 4 (K1); lane 5 (K2); lane 6 (K3); lane 7 (K4); lane 8 (K5); lane 9 (N); lane 10 (EP2).

Figure 10.2 illustrates that *bfpA,* one of the virulence gene for EPEC, was detected in *E. coli* strains isolated from a water sample as shown in lanes 6 and 7. All the genes were electrophoretically confirmed according to band size of 367 bp with a 1 kb molecular marker (M, Promega, Madison, Wisconsin, USA) in lane 1 on a 0.8% agarose gel (Promega, Madison, Wisconsin, USA). In lane 5, *E. coli ATCC* 43887 (EPEC) was used as a positive control and is labelled as EP1. Negative controls (no DNA) were included for the quality control of the PCR reactions and are labelled as N in lane 10.

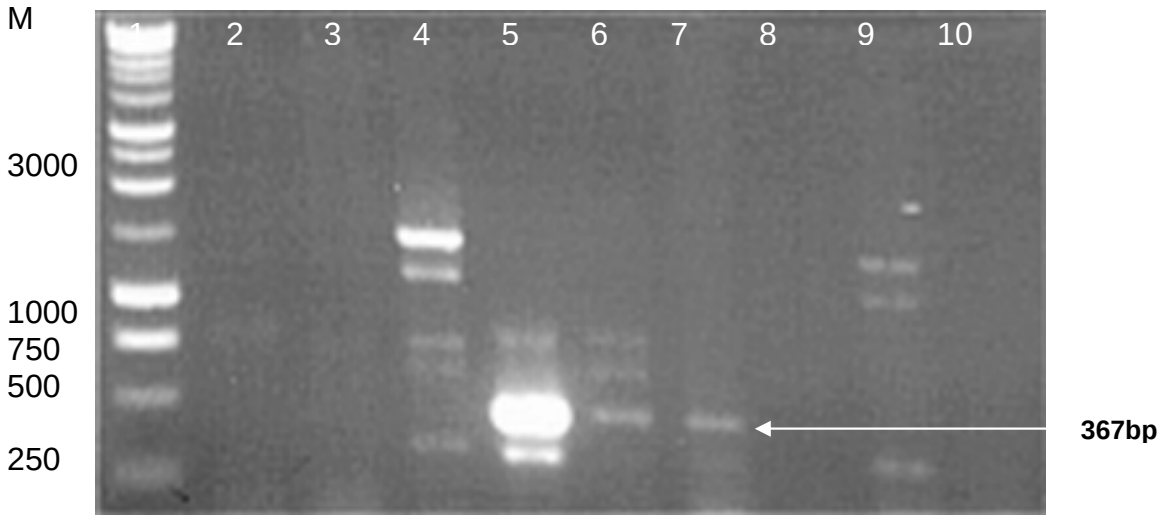

Figure 10.2. Amplification of *bfpA* gene (367 bp) for detection of EPEC strains. Lane 1 (M); lane 2 (P3); lane 3 (P6); lane 4 (P4); lane 5 (EP1); lane 6 (P5); lane 7 (P18); lane 8 (K6); lane 9 (K7); lane 10 (N).

10.4. Discussion

10.4.1. Amplification of the virulence genes

In the previous study strains were identified on homology searches and 16 *E. coli* strains were idenfied as non-pathogenic *E. coli* strains. Virulence is the quality of a pathogen to overcome defense mechanisms and become resistant to conditions of stress. It has been described that in South Africa, ETEC and EPEC are casual agents in 8-42% of diarrhea incidences.[4] In Chapter One, Section 1.2.1 it was also noted that in South Africa, diarrhea in infants (EPEC) and young children (ETEC) is still a major cause of morbidity and mortality in both rural and urban populations.

From the 100 *E. coli* strains screened for the presence of virulence genes of EPEC and ETEC, 16 of these strains harboured one or both of the virulence genes. Three *E.coli* strains with both *eaeA* and *bfpA* genes illustrated in Table 10.1, indicated a PAI typical of EPEC strains. These isolates with both of the virulence genes were isolated from water sampled at Plankenburg River in 2005 and 2006. Their phenotypic profile included chlorine survival up to 0.4 mg/L with various morphological types as well as susceptible and non-susceptible antibiograms. In the previous substudy, the nucleotide alignment of one *E. coli* strain P24 isolated from Plankenburg River (Below Kayamandi in 2006), was identified as *E. coli* E22 on a sequence homology search and was confirmed with the detection of both virulence genes in this substudy. This is an EPEC strain of *E. coli* that produces a number

of virulence factors including the LEE type III secretion system PAI that is responsible for typical attachment/effacing (A/E) lesions of the epithelial layer.[4]

Two isolates contained the *eaeA* gene coding for EPEC strains. One of these strains was isolated from a biofilm sample obtained at Plankenburg River (Below Kayamandi) in 2005 that survived 0.6 mg/L chlorine and displayed an antibiogram pattern 30, which was confirmed as an ESBL. The other strain was isolated from a water sample taken at Plankenburg River (Below Kayamandi) in 2006 that survived 0.6 mg/L chlorine and displayed a sensitive antibiogram profile.

The other virulent gene, *bfpA* coding for EPEC strains was detected in 13 of the *E. coli* isolates. *BfpA* is a structural gene encoding the *bfp* that produced fimbrae under certain culture conditions. Ten (10%) of the isolates were positive for detection of *bfpA* gene with no *eaeA* gene in the same isolate. These strains survived across the entire chlorine concentration range with various morphological types and antibiogram profiles. This was in accordance with a previous study[6] that detected 26% *bfpA* among *E. coli* isolates from river water sources and sediments in rural Venda communities in South Africa.

EPEC strains are further characterised as either atypical or typical strains with association of a common locus, LEE.[7] Typical EPEC strains are only associated with humans and bear the EPEC adherence factor (EAF) plasmid.[8] Atypical EPEC are closely related to Shiga toxin-producing *E. coli* (STEC) strains in genetic characteristics, serotypes, production of shiga-toxins (Stx), reservoir, and other epidemiologic aspects. STEC strains are asymptomatic in animals, but human infections may lead to haemorrhagic colitis, haemolytic uraemic syndrome, or death.[9] In industrialised countries, atypical EPEC strains have become a more frequent cause of diarrhea than typical EPEC, and the same shift may be occurring in Brazil.[10,11] In an investigation by Trabulsi *et al*, they described that a large number of Stx-negative, *eae*-positive strains coding for typical and atypical EPEC-like strains are different in O serogroups of EPEC strains and need further investigation for identification of these pathogenic strains.[10]

Since the aim of this substudy was to determine the pathogenic ability of *E. coli* strains as either EPEC or ETEC on detection of virulence genes, the differention between specific EPEC strains as atypical and typical were not included. The detection of the virulence gene encoding for EHEC was excluded in this study and distinguishing whether the two isolates containing only *eaeA* gene as atypical EPEC or EHEC strains cannot be stated. In

the previous substudy eight strains were identified as *E. coli* O157:H7 EDL933 on a sequence homology illustrated in Table 9.6.

Although the study design does not allow qualitative comparisons, the occurrence of ETEC strains in the water sources appeared to be low in comparison to the EPEC strain. One isolate harboured a virulent gene for ETEC determination. Neither *lt* or *st* genes were detected in any of the isolates. The isolate coding for *st* for confirmation as ETEC strain was isolated from the Stiebeuel River (Langrug informal settlement, Franschoek). The phenotypic profile of this strain S7, survived exposure to chlorine of 0.3 mg/L and displayed a morphological colony type 3 with a antibiogram profile 32. In the previous substudy two strains were identified as *E. coli* B7A on a similarity search on the NCBI database of which one was S7. This is an ETEC strain (O148:H28:CS6:LT+:ST+) which cause travelers' diarrhea.[4]

In another South African study conducted in Venda, 4% of the *E. coli* strains possessed both the virulent genes.[6] In another investigation on the occurrence of pathogenic *E. coli* strains in children with and without diarrhea in Maputo, Mozambique, stool samples were analysed and 18 ETEC, 16 EPEC, 3 EIEC strains were detected with PCR. Six (four with and two without diarrhea) of these ETEC strains encoded for both *lt* and *st* virulent genes. Additionally, the presence of only the *st* virulent gene was detected in three ETEC strains and 9 strains contained the *lt* virulent gene. These results are indicative of the variability of the pathogenic profile in ETEC strains.[12] Virulent genes *lt* and *st* can be encoded together or separately on the Ent plasmids.[13]

10.5. Conclusion

Water sources included in this study contained EPEC and ETEC strains carrying both or one of the virulence genes. These water sources are occasionally used for domestic and recreational purposes and might be a risk to the end users. Continuous screening for potentially pathogenic *E. coli* strains are needed for microbial characterisation and public health protection.

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Chapter Eleven

Overall Conclusion

The various substudies contributed towards the overall aim of this study, which was to determine the molecular characteristics of selected *E. coli* strains isolated from environmental waters in the Western Cape and to investigate the possible relationship between chlorination and antimicrobial resistance. The results illustrated the possible involvement of certain genes in environmental adaptation of *E. coli* strains to chlorine and certain antimicrobials.

11.1. Microbiological quantification of *E. coli* organisms in the water sources

The water sources included in this study was polluted with faecal coliforms and *E. coli* strains. Despite the apparent clear appearance of some of the water the enumeration test revealed the presence of high numbers of bacteria. These faecal coliforms and *E. coli* levels per 100 ml regularly exceeded the standards set out by the DWAF of South Africa for drinking and recreational water. Although the specific sources of contamination was not determined, it was likely that ineffective disinfection treatment or water contamination by the surrounding communities played a role. Several limitations are also associated with microbiological approaches as they often lack specific pathogen detection and require cell culturing, which is time consuming and labour intensive as experienced with the multiple tube enumeration method used in this study. The results of this substudy emphasised the need for continuous monitoring of the microbiological levels of water is necessary to detect contamination and limit potential health risks. A recommendation would be to apply microbiological techniques that include more than one indicator organism and if possible, specific organisms associated with the water source tested. Approaches like these would broaden the screening of contamination and decrease testing time.

11.2. Chlorine exposure

It was again confirmed by this study that *E. coli* strains isolated from the various water and biofilm samples had the ability to survive exposure to various chlorine concentrations and contact times. These strains further displayed possible VBNC states making them more resistant and detection difficult with conventional methods. As chlorine is one of the most

cost-effective disinfectants, especially used by small-scale municipalities and poorer countries, inactivation of strains with this VBNC growth form may result in severe problems including water contamination with high levels of *E. coli* as observed in this study. Although the specific detection of VBNC microorganisms were not investigated in this study, one way to overcome this problem may be the application of different disinfectant strategies with chlorine as the final disinfectant to decrease the microbial load of the water sources. In this study it was noted that *E. coli* strains were enumerated after 30 minutes chlorine contact time and displayed a possible VBNC pattern and we recommend that the application of shorter contact times like 10 minutes would be more effective in understanding the survival of these organisms under low chlorine concentrations.

11.3. Phenotypic characterisation of *E. coli* strains

Microorganisms have different mechanisms in response to environmental stress conditions and in this study it was showed that exposure of water and biofilm samples to chlorine changed their morphological appearance compared to unexposed samples. Different and similar morphological types were produced that can be of importance to possibly assist as determinants for characterisation of water previously disinfected or other stress conditions. The antimicrobial susceptibility tests illustrated several antibiograms including *ampC* β-lactamase producing *E. coli* strains, ESBLs as well as MAR to other antimicrobials frequently used in the treatment of community acquired infections. A high rate of resistance was especially displayed to ampicillin and trimethoprim-sulfamethaxazole. Antimicrobial resistance patterns displayed in this study is of major concern as it includes resistance to common therapies such as trimethoprim-sulfamethaxazole, which is used in prophylaxis for HIV-opportunistic infections. The presence of such resistant organisms in water sources that are able to survive incomplete disinfection can have severe health implications. A recommendation from this substudy would be an awareness of atypical phenotypes that may be cultured from specimens subjected to environmental stress, and to include inoculation onto different agar plates for detection of the organism and comparison of their morphological colony apearance. In addition, the trends in antimicrobial susceptibility should be followed by regular surveillance.

11.4. Efflux pump involvement in antimicrobial resistance and chlorine tolerance of *E. coli* strains

The addition of an EPI to the water sample decreased the enumeration levels of *E. coli* strains but this effect was not illustrated in the biofilm samples. Further addition of EPI to the antimicrobial susceptibility test, changed the antibiograms of 10 *E. coli* strains from resistant to sensitive profiles. FQs, one of the antimicrobials included in this study and of which one of the mechanisms of resistance is mediated by efflux, showed no change after addition of the EPI. This pilot study illustrated that the addition of an EPI with chlorine treatment can decrease the *E. coli* survival and change antimicrobial resistance to some extent. Specific test involving other EPI with broader spectrums of inhibitions and EPI concentrations for effective inhibition of the strains exposed to antimicrobials and chlorine were not determined but would be important to identify the optimal standards to decrease antimicrobial resistance and chlorine survival.

11.5. Molecular detection of candidate genes involved in antimicrobial resistance and chlorine tolerance

In this study PCR methods for the detection of candidate genes selected for their possible involvement in antimicrobial resistance, chlorine tolerance and efflux were established and optimised. Candidate genes involved in heat-shock, oxidative stress genes and efflux pump genes were most frequently detected in the isolates. In more than 50% of the *E. coli* strains both *soxR*+*soxS* were detected and this *soxRS* regulatory system is known to be triggered when the strain is exposed to sublethal environmental conditions. Structural genes involved in fluoroquinolone resistance were detected in 10 *E. coli* strains with only *gyrA* and in 25 *E. coli* strains only *parC* was detected. Three of these strains exhibited resistance to ciprofloxacin. These results indicate that, due to the increased complexity of antimicrobial resistance in gram-negative organisms, effective surveillance with both phenotypic and genotypic analyses is needed for screening of genes as possible markers for resistance in isolates exposed to similar environmental conditions.

11.6. Comparison of the genotypic and phenotypic profiles of selected strains

The sequencing analysis of the candidate genes selected for chlorine resistance illustrated various changes in the amino acid sequence including one common mutational

pathway taken by the *E. coli* strain when exposed to stress conditions. No changes were observed in the QRDR region for confirmation of FQ-resistance in the structural genes *gyrA* and *parC* especially in isolates that displayed a FQ-resistant phenotype, illustrating that other mechanisms contributed to resistance.

In addition, homology searches of the sequencing results of the candidate genes were done and illustrated similarities to 19 pathogenic and 14 non-pathogenic *E. coli* as well as 3 *Shigella* strains on the NCBI database. These pathogenic strains include *E. coli O157:H7* EDL933 which cause haemorrhagic colitis and food poisoning and *E. coli* CFT073 that causes non-hospital-acquired urinary tract infections. Sequencing is expensive and databases is not available for all types of organisms but it would be highly recommended to include homology searches as a presumptive test for determining strains for typing, in research studies, as it gives a broader range of possible strains present.

Protein expression studies were not done to evaluate the effect of these conditions on the strains and we can therefore only speculate on whether these amino acid changes could influence the function of the proteins it encodes. A further recommendation would be employ specific protein expression and knock-out studies as well as cloning of these candidate genes into multicopy plasmid to obtain a mutator phenotype when overexpressed under the phenotypic conditions described in this study. Such experiments will demonstrate whether the candidate genes are activated or repressed under these conditions, and assist in optimization of these treatments from a microbiological perspective.

11.7. Determination of the pathogenic potential of isolated *E. coli* strains

Virulence genes for determination of EPEC and ETEC were detected in certain isolates. Strain-typing identified three EPEC strains (*bfpA*, *eaeA*), two EPEC (*eaeA*), ten EPEC (*bfpA*) and one ETEC strain (*st*). Although detection was low, these strains cause diarrhea in infants (EPEC) and young children (ETEC), which is still a major cause of morbidity and mortality in both rural and urban populations. Therefore, to ensure public health safety and monitoring of biological threats, continued surveillance is needed for the identification of *E. coli* pathotypes and other related pathogens. The bacterial genome is extremely dynamic and their ability to acquire genetic elements, such as PAI and virulence factors is a concern that could only be detected by strain typing.

11.8. Possible impact of the findings of this study

It was illustrated in this study that the disinfection method employed by most of the small-scale municipalities seemed to be ineffective as microorganisms isolated from environmental sources had the ability to survive various chlorine concentrations. Phenotypic and genotypic characteristics of *E. coli* strains highlighted the importance of the presence of these organisms in our surface water sources, and these characteristics can assist water treatment works as well as the health care environment with treatment of such *E. coli* strains. The general outcome of this study is to again highlight the importance of continuous surveillance and monitoring of the microbiological content of our water sources, re-evaluate various disinfectant strategies and to screen for various pathogenic strains to prevent potential outbreaks with resistant pathogens down the line.

Addendum 1

Table 1.1 Morphological colony patterns of *E. coli* strains isolated from water samples

Source	Chlorine exposure & contact time	Morphological type number	Isolate Number
Plankenburg River (Below Kayamandi) in 2006	0.0 mg/L	1	P1
	0.0 mg/L	2	P2
	0.1 mg/L (30min)	3	P3
	0.1 mg/L (30min)	1	P4
	0.1 mg/L (90min)	4	P5
	0.1 mg/L (a)	1	P6
	0.1 mg/L (30min)	1	P7
	0.1 mg/L (30min)	2	P8
	0.1 mg/L (1)	2	P9
	0.1 mg/L (30min)	2	P10
	0.3 mg/L (30min)	2	P11
	0.3 mg/L (2)	1	P12
	0.3 mg/L (90min)	6	P13
	0.3 mg/L (90min)	1	P14
	0.3 mg/L (90min)	1	P15
	0.3 mg/L (1)	2	P16
	0.3 mg/L (2)	2	P17
	0.3 mg/L (30min)	2	P18
	0.4 mg/L (30min)	7	P19
	0.4 mg/L (30min)	1	P20
	0.4 mg/L (2)	2	P21
	0.4 mg/L (30min)	2	P22
	0.4 mg/L (90min)	2	P23
	0.4 mg/L (30min)	2	P24
	0.4 mg/L (30min)	2	P25
	0.4 mg/L (30min)	2	P26
	0.5 mg/L (30min)	3	P27
	0.5 mg/L (30min)	8	P28
	0.5 mg/L (30min)	1	P29
	0.5 mg/L (90min)	2	P30
	0.5 mg/L (90min)	9	P31
	0.6 mg/L (30min)	3	P32
	0.6 mg/L (90min)	11	P33
	0.0 mg/L (90min)	2	P34
	0.1 mg/L (1)	8	P35
	0.3 mg/L (30min)	8	P36
	0.3 mg/L (90min)	6	P37
	0.3 mg/L (90min)	4	P38
	0.3 mg/L (1)	3	P39

	0.3 mg/L (90min)	3	P40
	0.3 mg/L (90min)	3	P41
	0.3 mg/L (3)	8	P42
	0.3 mg/L (4)	8	P43
	0.4 mg/L (1)	11	P44
	0.4 mg/L (3)	8	P45
	0.4 mg/L (4)	8	P46
	0.4 mg/L (3)	3	P47
	0.4 mg/L (4)	3	P48
	0.5 mg/L (1)	10	P49
	0.5 mg/L (30min)	3	P50
	0.5 mg/L (90min)	3	P51
	0.5 mg/L (3)	8	P52
	0.5 mg/L (4)	8	P53
	0.6 mg/L (1)	10	P54
	0.6 mg/L (2)	10	P55
	0.6 mg/L (3)	8	P56
	0.6 mg/L (4)	8	P57
	0.1 mg/L (90min)	3	P58
	0.1 mg/L (a)	3	P59
	0.5 mg/L (30min)	3	P60
	0.6 mg/L (3)	3	P61
	0.6 mg/L (3)	2	P62
	0.6 mg/L (4)	3	P63
	0.7 mg/L (1)	8	P63
	0.7 mg/L (2)	8	P64
	0.0 mg/L(1)	1	P65
Disa River, Imizamo Yethu informal settlement (Below built-up area, Hout Bay) in 2006	0.0 mg/L(A)	1	I1
	0.0 mg/L(1)	2	I2
	0.0 mg/L (A1)	2	I3
	0.0 mg/L(2)	2	I4
	0.5 mg/L (90min) (B2)	2	I5
	0.5 mg/L (90min) 2	2	I6
	0.5 mg/L	2	I7
	0.5 mg/L (90min)	3	I8
	0.5 mg/L (90min) (B)	3	I9
	0.5 mg/L (90min) 1	1	I10
	0.5 mg/L (90min) (B1)	2	I11
	0.6 mg/L (90min) (B2b)	2	I12
	0.6 mg/L (90min)	1	I13
	0.1 mg/L (1)	10	I14
	0.1 mg/L (2)	10	I15
	0.1 mg/L (1)	3	I16
	0.1 mg/L (2)	3	I17
	0.6 mg/L (30min)	10	I18
Nietvoorbij	0.0 mg/L (2)	1	N1

	0.1 mg/L (B10)	2	N2
	0.1 mg/L (A9)	2	N3
	0.3 mg/L (90min)	8	N4
	0.3 mg/L (A9-3)	7	N5
	0.4 mg/L (2)	10	N6
	0.4 mg/L (30min)	2	N7
	0.4 mg/L (A4)	8	N8
	0.4 mg/L (90min)	10	N9
	0.4 mg/L (1)	2	N10
	0.4 mg/L (A94L)	2	N11
	0.4 mg/L (90min)	5	N12
	0.6 mg/L (30min)	5	N13
	0.6 mg/L (A6)	1	N14
	0.6 mg/L	3	N15
	0.4 mg/L (3)	3	N16
	0.0 mg/L (1)	3	N17
	0.0 mg/L (2)	3	N18
	0.4 mg/L	5	N19
	0.4 mg/L	5	N20
	0.6 mg/L (30min)	3	N21
	0.6 mg/L (A6)	3	N22
	0.6 mg/L (30min)	3	N23
	0.4 mg/L (30min)	2	N24
	0.4 mg/L (30min)	3	N25
	0.1 mg/L	5	N26
	0.3 mg/L (1)	8	N27
	0.3 mg/L (2)	8	N28
	0.3 mg/L (1)	3	N29
Stiebeuel River (Langrug informal settlement, Franschoek) in 2006	0.0 mg/L(1)	2	S1
	0.0 mg/L	2	S2
	0.0 mg/L	3	S3
	0.1 mg/L (30min)	2	S4
	0.1 mg/L (30min)	8	S5
	0.1 mg/L (30min)	5	S6
	0.3 mg/L (30min)	3	S7
	0.3 mg/L (30min)	8	S8
	0.4 mg/L (90min)	8	S9
	0.5 mg/L (30min)	1	S10
	0.5 mg/L (90min)	2	S11
	0.6 mg/L (30min)	8	S12
	0.6 mg/L (90min)	8	S13
	0.7 mg/L(30min)	3	S14
	0.0 mg/L (30min)	3	S15
	0.1 mg/L (30min)	2	S16
	0.0 mg/L (1C1)	5	S17
	0.5 mg/L (30min)	5	S18

	0.5 mg/L (30min)	3	S19
	0.3 mg/L (30min)	3	S20
	0.3 mg/L (30min)	3	S21
	0.5 mg/L (30min)	3	S22
	0.5 mg/L (90min)	3	S23
	0.6 mg/L (90min)	8	S24
	0.6 mg/L (90min)	3	S25
	0.7 mg/L (1)	5	S26
	0.7 mg/L (2)	5	S27
	0.7 mg/L (30min)	5	S28
Plankenburg River (Below Kayamandi) in 2005	0.0 mg/L	3	K1
	0.1 mg/L (90 min)	3	K2
	0.1 mg/L (90 min)	5	K3
	0.1 mg/L (1)	2	K4
	0.1 mg/L (2)	2	K5
	0.1 mg/L (4)	5	K6
	0.1 mg/L (30 min)	3	K7
	0.3 mg/L (30 min)	3	K8
	0.3 mg/L (1)	5	K9
	0.3 mg/L (90 min)	2	K10
	0.4 mg/L (1)	2	K11
	0.5 mg/L (30 min)	2	K12
	0.5 mg/L (90 min)	3	K13
	0.5 mg/L (90 min)1	3	K14
	0.1 mg/L (90 min)	5	K15
	0.3 mg/L (90min) (2)	5	K16
	0.4 mg/L	5	K17
	0.6 mg/L (30min)	5	K18
	0.6 mg/L (90min)	9	K19
	0.0 mg/L	5	K20
	0.6 mg/L (1)	5	K21
	0.6 mg/L (30min)	2	K22
	0.6 mg/L (30min)	3	K23
	0.0 mg/L	3	K24

Table 1.2. Morphological colony patterns of *E. coli* strains isolated from biofilm samples

Source	Chlorine exposure & contact time	Morphological type number	Isolate Number
Plankenburg River (Below Kayamandi) in 2006	0.0 mg/L (B)	2	B1
	0.1 mg/L(1) + EPI	3	B2
	0.3 mg/L (30min)	3	B3
	0.3 mg/L (90min)	3	B4
	0.4 mg/L (B) + EPI	3	B5
	0.5 mg/L (30min) + EPI	3	B6

	0.6 mg/L (30min)	3	B7
	0.6 mg/L (90min)	2	B8
	0.6 mg/L (B)	3	B9
	0.6 mg/L (90min)	2	B10
	0.6 mg/L (90min)	8	B11
	0.6 mg/L (90min)	3	B12
	0.3 mg/L (B)	7	B13
	0.0 mg/L (B)	2	B14
	0.1 mg/L(30min)	3	B15
	0.1 mg/L(1)	7	B16
	0.1 mg/L(B)	8	B17
	0.3 mg/L(30 min)	3	B18
	0.0 mg/L	3	B19
	0.0 mg/L	8	B20
	0.3 mg/L (B)	3	B21
	0.3 mg/L (30min)	3	B22
	0.3 mg/L (30min)	3	B23
	0.3 mg/L (90min)	3	B24
	0.4 mg/L	3	B25
	0.4 mg/L (30 min)	3	B26
	0.4 mg/L (30 min)	7	B27
	0.4 mg/L (30 min)	8	B28
	0.5 mg/L (30min)	3	B29
	0.5 mg/L (30min)	3	B30
	0.6 mg/L (30min)	3	B31
	0.6 mg/L (30min)	3	B32
	0.0 mg/L	2	B33
Wellington Sewage Works in 2006	0.0 mg/L	2	W1
	0.0 mg/L	2	W2
	0.1 mg/L (30min)	3	W3
	0.1 mg/L (30min)	3	W4
	0.3 mg/L (30min)	3	W5
	0.3 mg/L (30min)	3	W6
	0.3 mg/L (30min)	3	W7
	0.3 mg/L (30min)	3	W8
	0.3 mg/L (30min)	2	W9
	0.1 mg/L (30min)	3	W6
Plankenburg River (Below Kayamandi) in 2005	0.6 mg/L (30min)	3	BK1
	0.6 mg/L (30min)	2	BK2
	0.0 mg/L	2	BK3
	0.1 mg/L (30min)	3	BK4
	0.1 mg/L	3	BK5
	0.1 mg/L	3	BK6
	0.1 mg/L	3	BK7
	0.3 mg/L (90min)	8	BK8
	0.3 mg/L (90min)	3	BK9
	0.3 mg/L	3	BK10
	0.4 mg/L	3	BK11
	0.5 mg/L	3	BK12

	0.5 mg/L	3	BK13
	0.3 mg/L	3	BK14
	0.3 mg/L (90min)	3	BK15
	0.3 mg/L (90min)	2	BK16
	0.4 mg/L	3	BK17

Addendum 2

Table 2.1. Antimicrobial susceptibility patterns of *E. coli* strains isolated from water samples

Source	Chlorine exposure and contact time	Antibiograms	Isolate Number
Plankenburg River (Below Kayamandi) in 2006	0.0 mg/L	1	P1
	0.0 mg/L	1	P2
	0.1 mg/L (30min)	11	P3
	0.1 mg/L (30min)	11	P4
	0.1 mg/L (90min)	11	P5
	0.1 mg/L (a)	21	P6
	0.1 mg/L (30min)	22	P7
	0.1 mg/L (30min)	24	P8
	0.1 mg/L (1)	30	P9
	0.1 mg/L (30min)	32	P10
	0.3 mg/L (30min)	1	P11
	0.3 mg/L (2)	1	P12
	0.3 mg/L (90min)	1	P13
	0.3 mg/L (90min)	3	P14
	0.3 mg/L (90min)	11	P15
	0.3 mg/L (1)	23	P16
	0.3 mg/L (2)	26	P17
	0.3 mg/L (30min)	26	P18
	0.4 mg/L (30min)	1	P19
	0.4 mg/L (30min)	1	P20
	0.4 mg/L (2)	1	P21
	0.4 mg/L (30min)	11	P22
	0.4 mg/L (90min)	11	P23
	0.4 mg/L (30min)	28	P24
	0.4 mg/L (30min)	26	P25
	0.4 mg/L (30)	30	P26
	0.5 mg/L (30min)	1	P27
	0.5 mg/L (30min)	1	P28
	0.5 mg/L (30min)	3	P29
	0.5 mg/L (90min)	25	P30
	0.5 mg/L (90min)	26	P31
	0.6 mg/L (30min)	1	P32
	0.6 mg/L (90min)	2	P33
	0.0 mg/L	24	P34
	0.1 mg/L (1)	12	P35
	0.3 mg/L (30min)	1	P36
Disa River, Imizamo Yethu informal settlement	0.0 mg/L(A)	1	I1
	0.0 mg/L(1)	1	I2
	0.0 mg/L (A1)	1	I3

	0.0 mg/L(2)	2	I4
	0.5 mg/L (90min) (B2)	9	I5
	0.5 mg/L (90min) 2	12	I6
	0.5 mg/L	26	I7
	0.5 mg/L (90min)	31	I8
	0.5 mg/L (90min) (B)	31	I9
	0.5 mg/L (90min) 1	31	I10
	0.5 mg/L (90min) (B1)	31	I11
	0.6 mg/L (90min) (B2b)	9	I12
	0.6 mg/L (90min)	31	I13
	0.0 mg/L (2)	3	N1
	0.1 mg/L (B10)	2	N2
	0.1 mg/L (A9)	30	N3
	0.3 mg/L (30min)	19	N4
	0.3 mg/L (A9-3)	25	N5
	0.4 mg/L (2)	4	N6
	0.4 mg/L (30min)	7	N7
	0.4 mg/L (A4)	8	N8
	0.4 mg/L (90min)	13	N9
	0.4 mg/L (1)	18	N10
	0.4 mg/L (A94L)	2	N11
	0.4 mg/L (90min)	31	N12
	0.6 mg/L (30min)	1	N13
	0.6 mg/L (A6)	24	N14
	0.6 mg/L (A6M)	1	N15
	0.4 mg/L (3)	1	16
	0.0 mg/L (1)	1	N17
	0.0 mg/L (2)	1	N18
Stiebeuel River (Langrug informal settlement, Franschoek) in 2006	0.0 mg/L(1)	3	S1
	0.0 mg/L	27	S2
	0.0 mg/L	1	S3
	0.1 mg/L (30min)	3	S4
	0.1 mg/L (30min)	8	S5
	0.1 mg/L (30min)	12	S6
	0.3 mg/L (30min)	32	S7
	0.3 mg/L (30min)	31	S8
	0.4 mg/L (90min)	24	S9
	0.5 mg/L (30min)	6	S10
	0.5 mg/L (90min)	31	S11
	0.6 mg/L (30min)	31	S12
	0.6 mg/L (90min)	31	S13
	0.7 mg/L (30min)	20	S14
	0.0 mg/L	11	S15

	0.0 mg/L (1C1)	11	S16
	0.0 mg/L	12	S17
	0.5 mg/L (30min)	15	S18
	0.5 mg/L (30min)	16	S19
Plankenburg River (Below Kayamandi) in 2005	0.0 mg/L	1	K1
	0.1 mg/L (90 min)	5	K2
	0.1 mg/L (90 min)	6	K3
	0.1 mg/L (1)	7	K4
	0.1 mg/L (2)	8	K5
	0.1 mg/L (4)	26	K6
	0.1 mg/L (30 min)	29	K7
	0.3 mg/L (30 min)	1	K8
	0.3 mg/L (1)	2	K9
	0.3 mg/L (90 min)	2	K10
	0.4 mg/L (1)	6	K11
	0.5 mg/L (30 min)	11	K12
	0.5 mg/L (90 min)	19	K13
	0.5 mg/L (90 min)1	30	K14
	0.1 mg/L (90 min)	2	K15
	0.3 mg/L (90 min) (2)	6	K16
	0.4 mg/L	1	K17
	0.6 mg/L (30 min)	11	K18
	0.6 mg/L (90 min)	4	K19
	0.0 mg/L	2	K20
	0.6 mg/L (1)	17	K21

Table 2.2. Antibiograms of *E. coli* strains isolated from biofilm samples

Source	Chlorine exposure & contact time	Antibiograms	Isolate Number
Plankenburg River (Below Kayamandi) in 2006	0.0 mg/L (B)	32	B1
	0.1 mg/L(1) + EPI	1	B2
	0.3 mg/L (30min)	10	B3
	0.3 mg/L (90min)	26	B4
	0.4 mg/L (B) + EPI	32	B5
	0.5 mg/L (30min) + EPI	1	B6
	0.6 mg/L (30min)	1	B7
	0.6 mg/L (90min)	1	B8
	0.6 mg/L (B)	19	B9
	0.6 mg/L (90min)	1	B10
	0.6 mg/L (90min)	2	B11
	0.6 mg/L (90min)	24	B12
	0.3 mg/L (B)	28	B13
	0.0 mg/L (1)	2	B14
	0.1 mg/L (30min)	1	B15

	0.1 mg/L(1)	11	B16
	0.1 mg/L(B)	32	B17
	0.3 mg/L (30min)	11	B18
	0.0 mg/L	1	B19
	0.0 mg/L	24	B20
Wellington Sewage Works in 2006	0.0 mg/L	12	W1
	0.0 mg/L	24	W2
	0.1 mg/L (30min)	8	W3
	0.1 mg/L (30min)	12	W4
	0.3 mg/L (30min)	8	W5
	0.3 mg/L (30min)	8	W6
	0.3 mg/L (30min)	18	W7
	0.3 mg/L (30min)	11	W8
	0.3 mg/L (30min)	14	W9
Plankenburg River (Below Kayamandi) in 2005	0.6 mg/L (30min)	30	BK1

Addendum 3

Table 3.1. *E. coli* isolates used for amplification and sequencing of the canadidate genes

Source	Chlorine exposure and contact time	Phenotypes (Morphological Types & Antibiogram profile)	Isolate Number	*soxR*	*soxS*	*grpE*	*dnaK*	*ompF*	*osmC*	*acrA*	*tolC*	*acrR*	*ampC*	*gyrA*	*parC*	*marA*	*emrA*	Isolates Sequenced
WS2	0.0 mg/L	1; 1	P1	X		X			X			X					X	
	0.0 mg/L	2; 1	P2	X	X	X	X	X	X		X	X	X	X		X	X	X
	0.1 mg/L (30min)	3; 11	P3	X	X	X	X	X	X		X	X				X	X	
	0.1 mg/L (30min)	1; 11	P4		X	X	X		X									
	0.1 mg/L (90min)	4; 11	P5	X	X	X	X	X	X		X	X	X	X	X	X	X	X
	0.1 mg/L (a)	1; 21	P6	X	X	X		X	X		X	X				X	X	
	0.1 mg/L (30min)	1; 22	P7	X	X	X		X	X		X	X				X	X	X
	0.1 mg/L (30min)	2; 24	P8	X	X	X		X	X		X	X	X			X	X	
	0.1 mg/L (1)	2; 30	P9	X	X	X	X	X	X		X	X	X			X	X	
	0.1 mg/L (30min)	2; 32	P10	X				X	X		X	X	X			X	X	X
	0.3 mg/L (30min)	2; 1	P11						X						X			
	0.3 mg/L (2)	1; 1	P12						X									
	0.3 mg/L (90min)	6; 1	P13		X	X	X		X				X	X				X
	0.3 mg/L (90min)	1; 3	P14	X	X	X	X	X	X		X	X				X	X	
	0.3 mg/L (90min)	1; 11	P15	X	X	X	X	X	X		X	X				X	X	X
	0.3 mg/L (1)	2; 23	P16	X	X	X	X		X			X	X				X	
	0.3 mg/L (2)	2; 26	P17		X	X		X	X		X	X	X			X	X	X
	0.3 mg/L (30min)	2; 26	P18	X	X	X			X	X		X						X
	0.4 mg/L (30min)	7; 1	P19		X	X	X		X									
	0.4 mg/L (30min)	1; 1	P20						X									X
	0.4 mg/L (2)	2; 1	P21						X									
	0.4 mg/L (30min)	2; 11	P22		X				X									
	0.4 mg/L (90min)	2; 11	P23	X	X	X			X									
	0.4 mg/L (30min)	2; 28	P24	X	X	X	X	X	X		X	X	X	X		X	X	X

	0.4 mg/L (30min)	2; 26	P25	X	X	X	X	X	X		X	X	X			X	X	X
	0.4 mg/L (30min)	2; 30	P26	X	X	X	X	X	X		X	X	X			X	X	
	0.5 mg/L (30min)	3; 1	P27				X		X									
	0.5 mg/L (30min)	8; 1	P28					X	X		X	X		X		X	X	
	0.5 mg/L (30min)	1; 3	P29		X	X	X		X									
	0.5 mg/L (90min)	2; 25	P30	X	X	X		X	X	X	X	X				X	X	
	0.5 mg/L (90min)	9; 26	P31	X	X	X	X	X	X		X	X				X	X	
	0.6 mg/L (30min)	3; 1	P32															
BS2	0.0 mg/L (B)	2; 32	B1	X	X	X	X	X	X		X	X	X			X	X	X
	0.1 mg/L(1) + EPI	3; 1	B2	X	X	X	X	X	X		X	X				X	X	
	0.3 mg/L (30min)	3; 10	B3	X	X	X	X	X	X		X	X				X	X	
	0.3 mg/L (90min)	3; 26	B4	X	X		X	X	X	X	X	X	X	X		X	X	X
	0.4 mg/L (B) + EPI	3; 32	B5	X	X	X	X	X	X		X	X				X	X	
	0.5 mg/L (30min) + EPI	3; 1	B6		X	X			X								X	
	0.6 mg/L (30min)	3; 1	B7		X	X			X									
	0.6 mg/L (90min)	2; 1	B8														X	X
	0.6 mg/L (B)	3; 19	B9	X	X	X	X	X	X	X	X	X				X	X	
	0.6 mg/L (90min)	2; 1	B10			X			X								X	
	0.6 mg/L (90min)	8; 2	B11		X	X	X		X							X	X	
	0.6 mg/L (90min)	3; 24	B12	X	X	X	X		X	X	X	X				X	X	X
	0.3 mg/L (B)	7; 28	B13	X	X	X	X	X	X		X	X				X	X	X
WS4	0.0 mg/L(A)	1; 1	I1	X	X	X	X	X	X	X	X	X			X	X		
	0.0 mg/L(1)	2; 1	I2	X	X	X	X	X	X	X	X	X			X	X		
	0.0 mg/L (A1)	2; 1	I3	X	X	X	X	X	X	X	X	X			X	X		
	0.0 mg/L(2)	2; 2	I4	X	X	X	X	X	X	X	X	X			X	X		X
	0.5 mg/L (90min) (B2)	2; 9	I5	X	X	X	X	X	X	X	X	X			X	X	X	
	0.5 mg/L (90min) 2	2; 12	I6	X	X	X	X	X	X	X	X	X			X	X		
	0.5 mg/L	2; 26	I7		X	X	X	X	X	X	X	X			X	X		X
	0.5 mg/L (90min)	3; 31	I8	X	X	X	X	X	X	X	X	X			X	X		X
	0.5 mg/L (90min) (B)	3; 31	I9	X	X	X	X	X	X	X	X	X			X	X		X
	0.5 mg/L (90min)	1; 31	I10	X	X	X	X	X	X	X	X	X			X	X		

	0.5 mg/L (90min) (B1)	2; 31	I11	X	X	X	X	X	X	X	X	X			X	X	X	
	0.6 mg/L (90min) (B2b)	2; 9	I12	X	X	X	X	X	X	X	X	X			X	X		
	0.6 mg/L (90min)	1; 31	I13	X	X	X	X	X	X	X	X	X			X	X	X	X
WS5	0.0 mg/L (2)	1; 3	N1	X	X	X		X	X		X				X			
	0.1 mg/L (B10)	2; 2	N2	X	X			X	X									
	0.1 mg/L (A9)	2; 30	N3	X	X	X			X						X			
	0.3 mg/L (90min)	8; 19	N4	X	X	X			X	X	X		X	X	X			X
	0.3 mg/L (A9-3)	7; 25	N5	X	X													
	0.4 mg/L (2)	10; 4	N6	X	X										X			
	0.4 mg/L (30min)	2; 7	N7	X	X	X		X	X		X							X
	0.4 mg/L (A4)	8; 8	N8	X	X				X									
	0.4 mg/L (90min)	10; 13	N9	X	X			X			X				X			X
	0.4 mg/L (1)	2; 18	N10	X														
	0.4 mg/L (A94L)	2; 2	N11	X				X	X						X			
	0.4 mg/L (90min)	5; 31	N12		X	X		X			X							X
	0.6 mg/L (30min)	5; 1	N13		X	X			X		X		X		X			
	0.6 mg/L (A6)	1; 24	N14					X			X							
WS3	0.0 mg/L(1)	2; 3	S1			X						X	X		X	X	X	
	0.0 mg/L	2; 27	S2	X						X	X							
	0.0 mg/L (1C)	3; 1	S3			X	X			X	X	X					X	
	0.1 mg/L (30min)	2; 3	S4								X	X	X					
	0.1 mg/L (30min)	8; 8	S5	X	X	X	X			X		X	X		X	X	X	X
	0.1 mg/L (30min)	5; 12	S6			X						X					X	
	0.3 mg/L (30min)	3; 32	S7			X		X	X	X	X	X	X		X	X	X	X
	0.3 mg/L (30min)	8; 31	S8	X	X	X	X			X	X	X					X	
	0.4 mg/L (90min)	8; 24	S9			X	X	X	X	X	X	X	X		X	X	X	X
	0.5 mg/L (30min)	1; 6	S10	X	X	X	X										X	X
	0.5 mg/L (90min)	2; 31	S11			X	X	X	X	X	X	X					X	
	0.6 mg/L (30min)	8; 31	S12			X	X		X			X	X		X	X	X	
	0.6 mg/L (90min)	8; 31	S13			X	X		X		X	X						
WS1	0.0 mg/L	3; 1	K1	X	X	X		X	X	X	X		X					X

	0.1 mg/L (90 min)	3; 5	K2					X	X	X	X	X						
	0.1 mg/L (90 min)	5; 6	K3			X			X	X			X					X
	0.1 mg/L (1)	2; 7	K4							X								
	0.1 mg/L (2)	2; 8	K5						X									
	0.1 mg/L (4)	5; 26	K6	X	X				X	X	X	X	X					X
	0.1 mg/L (30 min)	3; 29	K7	X	X	X				X		X		X		X		
	0.3 mg/L (30 min)	3; 1	K8							X				X		X		X
	0.3 mg/L (1)	5; 2	K9		X	X		X	X		X		X					
	0.3 mg/L (90 min)	2; 2	K10	X	X	X			X		X	X	X					
	0.4 mg/L (1)	2; 6	K11	X	X													
	0.5 mg/L (30 min)	2; 11	K12	X	X	X			X	X	X	X						
	0.5 mg/L (90 min)	3; 19	K13	X	X	X		X			X		X	X		X		X
	0.5 mg/L (90 min)1	3; 30	K14	X	X	X		X	X	X	X		X	X		X		
BS1	0.6 mg/L (30min)	3; 30	BK1	X	X	X		X	X	X	X		X	X		X		X

min = minutes. E = Efflux pump inhibitor. WS2, BS2 = Plankenbrug River (Below Kayamandi in 2006); WS1, BS1 = Plankenbrug River (Below Kayamandi in 2005); WS3 = Stiebeuel River (Langrug informal settlement, Franschoek); WS4 = Imizamo Yethu informal settlement (After built-up area, Hout Bay); WS5 = Nietvoorbij Research Project. X = Detected. Empty cells = Not Detected

Addendum 4

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acrA90          MNKNRGFTPLAVVLMLSGSLALTGCDDKQAQQGGQQMPAVGVVTVKTEPLQITTELPGRT
EG11703_1       MNKNRGFTPLAVVLMLSGSLALTGCDDKQAQQGGQQMPAVGVVTVKTEPLQITTELPGRT
acrA59          MNKNRGFTPLAVVLMLSGSLALTGCDDKQAQQGGQQMPAVGVVTVKTEPLQITTELPGRT
                ************************************************************

acrA90          SAYRIAEVRPQVSGIILKRNFKEGSDIEAGVSLYQIDPATYQATYDSAKGDLAKAQAAAN
EG11703_1       SAYRIAEVRPQVSGIILKRNFKEGSDIEAGVSLYQIDPATYQATYDSAKGDLAKAQAAAN
acrA59          SAYRIAEVRPQVSGIILKRNFKEGSDIEAGVSLYQIDPATYQATYDSAKGDLAKAQAAAN
                ************************************************************

acrA90          IAQLTVNRYQKLLGTQYISKQEYDQALADAQQANAAVTAAKAAVETARINLAYTKVTSPI
EG11703_1       IAQLTVNRYQKLLGTQYISKQEYDQALADAQQANAAVTAAKAAVETARINLAYTKVTSPI
acrA59          IAQLTVNRYQKLLGTQYISKQEYDQALADAQQANAAVTAAKAAVETARINLAYTKVTSPI
                ************************************************************

acrA90          SGRIGKSNVTEGALVQNGQAQGIKFPQDGTLEFSDVTVDQTTGSITLRAIFPNPDHTLLP
EG11703_1       SGRIGKSNVTEGALVQNGQAQ------DGTLEFSDVTVDQTTGSITLRAIFPNPDHTLLP
acrA59          SGRIGKSNVTEGALVQNGQADGIKFPQDGTLEFSDVTVDQTTGSITLRAIFPNPDHTLLP
                ********************:     *********************************

acrA90          GMFVRARLEEGLNPNAILVPQQGVTRTPRGDATVLVVGADDKVETRPIVASQAIGDKWLV
EG11703_1       GMFVRARLEEGLNPNAILVPQQGVTRTPRGDATVLVVGADDKVETRPIVASQAIGDKWLV
acrA59          GMFVRARLEEGLNPNAILVPQQGVTRTPRGDATVLVVGADDKVETRPIVASQAIGDKWLV
                ************************************************************

acrA90          TEGLKAGDRVVISGLQKVRPGVQVKAQEVTADNNQQAASGAQPEQSKS
EG11703_1       TEGLKAGDRVVISGLQKVRPGVQVKAQEVTADNNQQAASGAQPEQSKS
acrA59          TEGLKAGDRVVISGLQKVRPGVQVKAQEVTADNNQQAASGAQPEQSKS
                ************************************************
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Figure 4.1. Alignment of amino acid sequences of *acrA* detected in *E. coli* strains isolated from wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) and Plankenburg River (Below Kayamandi) water source in comparison with the reference gene sequence. Asterisk s indicate identical residues.

```
acrR25          ------------------------------TRQHILDVALRLFSQQGVSSTSLGEIAKAA
EG12116_1       -------------------MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAA
acrR43          MVHTFTNVCKSNACKFHEHMARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAA
acrR21          -------------------MARKTKQEAQETRQHILDVALRLFSQQGVSSTSLGEIAKAA
                                             ******************************

acrR25          GVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTV
EG12116_1       GVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTV
acrR43          GVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTV
acrR21          GVTRGAIYWHFKDKSDLFSEIWELSESNIGELELEYQAKFPGDPLSVLREILIHVLESTV
                ************************************************************

acrR25          TEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADLMTR
EG12116_1       TEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADLMTR
acrR43          TEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADLMTR
acrR21          TEERRRLLMEIIFHKCEFVGEMAVVQQAQRNLCLESYDRIEQTLKHCIEAKMLPADLMTR
                ************************************************************

acrR25          RAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCPTLRNPATNE
EG12116_1       RAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLCPTLRNPATNE
acrR43          RAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYLLGPT--------
acrR21          RAAIIMRGYISGLMENWLFAPQSFDLKKEARDYVAILLEMYXPXPHA-------
                ****************************************   *
```

Figure 4.2. Alignment of amino acid sequences of *acrR* detected in *E. coli* strains isolated from Disa River and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
tolC65  MKKLCSILINLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAF
tolC32  MKKLCSILIGLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAF
tolC11  MKKLCSILIGLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAF
EG11009_MKKLLPILIGLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAF
        **** .***.****************************************

tolC65  EKINEARSPLLPQLGLGADYTYSNGYRDANGINSNATSASLQLTQSIFDM
tolC32  EKINEARSPLLPQLGLGADYTYSNGYRDANGINSQATSASLQLTQSIFDM
tolC11  EKINEARSPLLPQLGLGADYTYSNGYRDANGINSNATSASLQLTQSIFDM
EG11009_EKINEARSPLLPQLGLGADYTYSNGYRDANGINSNATSASLQLTQSIFDM
        **********************************:*****************

tolC65  SKWRALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNVLNAIDVLSYTQA
tolC32  SKWRALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNVLNAIDVLSYTQA
tolC11  SKWRALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNVLNAIDVLSYTQA
EG11009_SKWRALTLQEKAAGIQDVTYQTDQQTLILNTATAYFNVLNAIDVLSYTQA
        ************************************************

tolC65  QKEAIYRQLDQTTQRFNVGLVAIXDVQNARAQYDTVLANEVTARNNLDNA
tolC32  QKEAIYRQLDQTTQRFNVGLVAIXDVQNARAQYDTVLANEVTARNNLDNA
tolC11  QKEAIYRQLDQTTQRFNVGLVAIXDVQNARAQYDTVLANEVTARNNLDNA
EG11009_QKEAIYRQLDQTTQRFNVGLVAITDVQNARAQYDTVLANEVTARNNLDNA
        ********************** *************************

tolC65  VEQLRQITGNYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARL
tolC32  VEQLRQITGNYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARL
tolC11  VEQLRQITGNYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARL
EG11009_VEQLRQITGNYYPELAALNVENFKTDKPQPVNALLKEAEKRNLSLLQARL
        ************************************************

tolC65  SQDLAREQIRQAHDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSN
tolC32  SQDLAREQIRQAHDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSN
```

```
tolC11  SQDLAREQIRQAHDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSN
EG11009_SQDLAREQIRQAQDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSN
        ************:************************************

tolC65  MGQNKVGLSFSLPIYQGGMVNSQVKQAQYNFVGASEQLNSAHRSVVQTVR
tolC32  MGNNKVGLSFSLPIYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVR
tolC11  MGQNKVGLSFSLPIYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVR
EG11009_MGQNKVGLSFSLPIYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVR
        **:**********************************:***********

tolC65  SSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTT
tolC32  SSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTT
tolC11  SSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTT
EG11009_SSFNNINASISSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTT
        **************************************************
tolC65  LYNAKQELANARYNYLINQLNIKSALGTNNEQDLLALNNALSKPVSTNPE
tolC32  LYNAKQELANARYNYLINQLNIKSALGTLNEQDLLALNNALSKPVSTNPE
tolC11  LYNAKQELANARYNYLINQLNIKSALGTLNEQDLLALNNALSKPVSTNPE
EG11009_LYNAKQELANARYNYLINQLNIKSALGTLNEQDLLALNNALSKPVSTNPE
        *************************** ********************

tolC65      ----ATPEQNAIADGYAPDSPAPVVQQTSARTTTSNGHNPFRN---
tolC32      ----ATPEQNAIADGYAPDSPAPVVQQTSARTTTSNGHNPFRNRRN
tolC11      ----ATPEQNAIADGYAPDSPAPVVQQTSARTTTSNGHNPFRN---
EG11009_    NVAPQTPEQNAIADGYAPDSPAPVVQQTSARTTTSNGHNPFRN---
                 ************************************
```

Figure 4.3. Alignment of amino acid sequences of *tolC* detected in *E. coli* strains isolated from Disa River, wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
gyrA38  MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLY
EG10423 MSDLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLY
gyrA37  MSSLAREITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLY
        **.**************************************************

gyrA38  AMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVNNDTIVRMAQPFSLR
EG10423 AMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVY-DTIVRMAQPFSLR
gyrA37  AMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVY-DTIVRMAQPFSLR
        **********************************  *************

gyrA38  YMLVDGQGNFGSIDGDSAAAMRYTEIRLAKIAHELMADLEKETVDFVDNY
EG10423_YMLVDGQGNFGSIDGDSAAAMRYTEIRLAKIAHELMADLEKETVDFVDNY
gyrA37  YMLVDGQGNFGSIDGDSAAAMRYTEIRLAKIAHELMADLEKETVDFVDNY
        **************************************************

gyrA38  DGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHNLTEVINGCLAYID
EG10423_DGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHNLTEVINGCLAYID
gyrA37  DGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHNLTEVINGCLAYID
        **************************************************

gyrA38  DEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKVYIRARAEVE
EG10423_DEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKVYIRARAEVE
gyrA37  DEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKVYIRARAEVE
        **************************************************

gyrA38  VDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKD
EG10423_VDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKD
gyrA37  VDAKTGRETIIVHEIPYQVNKARLIEKIAELVKEKRVEGISALRDESDKD
        **************************************************

gyrA38  GSRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKD
EG10423_GMRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKD
gyrA37  GMRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKD
        * **************************************************

gyrA38  IIAAFVRHRREVVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRH
EG10423 IIAAFVRHRREVVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRH
gyrA37  IIAAFVRHRREVVTRRTIFELRKARDRAHILEALAVALANIDPIIELIRH
        **************************************************

gyrA38  APTPAEAKTALVANPWQLGNVAAMLERAGDDAARPEWLEPEFGVRDGLYY
EG10423_APTPAEAKTALVANPWQLGNVAAMLERAGDDAARPEWLEPEFGVRDGLYY
gyrA37  APTPAEAKTALVANPWQLGNVAAMLERAGDDAARPEWLEPEFGVRDKLYY
        ********************************************* ***

gyrA38  LTEQQAQAILDLRLQKLTGLEHEKLLDEYKELLDQIAELLRILGSADRLM
EG10423_LTEQQAQAILDLRLQKLTGLEHEKLLDEYKELLDQIAELLRILGSADRLM
gyrA37  LTEQQAQAILDLRLQKLTGLEHEKLLDEYKELLDQIAELLRILGSADRLM
        **************************************************

gyrA38  EVIREELELVREQFGDKRRTEITANSADINLEDLITQEDVVVTLSHQGYV
EG10423_EVIREELELVREQFGDKRRTEITANSADINLEDLITQEDVVVTLSHQGYV
gyrA37  EVIREELELVREQFGDKRRTEITANSADINLEDLITQEDVVVTLNHQGYV
        *******************************************.*****

gyrA38  KYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRV
EG10423_KYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRV
gyrA37  KYQPLSEYEAQRRGGKGKSAARIKEEDFIDRLLVANTHDHILCFSSRGRV
        **************************************************
```

```
gyrA38  YSMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVTEFEEKVKVFMA
EG10423_YSMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVTEFEEGVKVFMA
gyrA37  YSMKVYQLPEATRGARGRPIVNLLPLEQDERITAILPVTEFEEGVKVFMA
        ****************************************** ******

gyrA38  TANGTVKKTVLTEFNRLRTAGKVAIKLVDGDELIGVDLTSGEDEVMLFSA
EG10423_TANGTVKKTVLTEFNRLRTAGKVAIKLVDGDELIGVDLTSGEDEVMLFSA
gyrA37  TANGTVKKTVLTEFNRNRTAGKVAIKLVDGDELIGVDLTSGEDEVMLFSA
        *************** ********************************

gyrA38  EGKVVRFKESSVRAMGCNTTGVRGIRLGEGDKVVSLIVPRGDGAILTATQ
EG10423_EGKVVRFKESSVRAMGCNTTGVRGIRLGEGDKVVSLIVPRGDGAILTATQ
gyrA37  EGKVVRFKESSVRAMGCNTTGVRGINNGEGDKVVSLIVPRGDGAILTATQ
        *************************. ***********************

gyrA38  NGYGKRTAVAEYPTKSRATKGVISIKVTERNGLVVGAVQVDDCDQIMMIT
EG10423_NGYGKRTAVAEYPTKSRATKGVISIKVTERNGLVVGAVQVDDCDQIMMIT
gyrA37  NGYGKRTAVAEYPTKSRATKGVISIKVTERNGLVVGAVQVDDCDQIMMIT
        **************************************************

gyrA38  DAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVAEPVDEEDLD
EG10423_DAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVAEPVDEEDLD
gyrA37  DAGTLVRTRVSEISIVGRNTQGVILIRTAEDENVVGLQRVAEPVDEEDLD
        **************************************************

gyrA38  TIDGSAAEGDDEIAPEVDVDDEPNEE
EG10423_TIDGSAAEGDDEIAPEVDVDDEPEEE
gyrA37  TIDGSAAEGDDEIAPEVDVDDEPEEE
        ************************:**
```

Figure 4.4. Alignment of amino acid sequences of *gyrA* detected in *E. coli* strains isolated from wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
EG10686_parC_MSDMAERLALHEFTENAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMS
parC39       MSDMAERLALHEFTENAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMS
parC21       MSDMAERLALHEFTENAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMS
             **************************************************

EG10686_parC_ELGLNASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLV
parC39       ELGLNASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLV
parC21       ELGLNASAKFKKSARTVGDVLGKYHPHGDSACYEAMVLMAQPFSYRYPLV
             **************************************************

EG10686_parC_DGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSELGQGTADWVPNFDGT
parC39       DGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSELGQGTADWVPNFDGT
parC21       DGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSELGQGTADWVPNFDGT
             **************************************************

EG10686_parC_LQEPKMLPARLPNILLN--------------------------------
parC39       LQEPKMLPARLPNILLNGTTGIAVGRPSGPTTLDQLLDIVQGPDYPTEAE
parC21       LQEPKMLPARLPNILLN--------------------------------
             *****************

EG10686_parC_--------------------------------------------------
parC39       IITSRAEIRKIYENGRGSVRMRAVWKKEDGAVVISALPHQVSGARVLEQI
parC21       --------------------------------------------------

EG10686_parC_--------------------------------------------------
parC39       AAQMRNKKLPMVDDLRDESDHENPTRLVIVPRSNRVDMDQVMNHLFATTD
```

```
parC21       --------------------------------------------------

EG10686_parC_--------------------------------------------------
parC39       LEKSYRINLNMIGLDGRPAVKNLLEILSEWLVFRRDTVRRRLNYRLEKVL
parC21       --------------------------------------------------

EG10686_parC_--------------------------------------------------
parC39       KRLHILEGLLVAFLNIDEVIEIIRNEDEPKSLHNRRYRRADGIYPVRHRR
parC21       --------------------------------------------------

EG10686_parC_-------GTTGIAVGMATDIPPHNLREVAQAAIALIDQPKTTLDQLLDIV
parC39       LPNILLNGTTGIAVGMATDIPPHNLREVAQAAIALIDQPKTTLDQLLDIV
parC21       -------GTTGIAVGMATDIPPHNLREVAQAAIALIDQPKTTLDQLLDIV
                    ****************************************

EG10686_parC_QGPDYPTEAEIITSRAEIRKIYENGRGSVRMRAVWKKEDGAVVISALPHQ
parC39       QGPDYPTEAEIITSRAEIRKIYENGRGSVRMRAVWKKEDGAVVISALPHQ
parC21       QGPDYPTEAEIITSRAEI-KIYENGRGSVRMRAVWKKEDGAVVSPLCCRR
             ****************** ********************** .   ::

EG10686_parC_VSGARVLEQIAAQMRNKKLPMVDDLRDESDHENPTRLVIVPRSNRVDMDQ
parC39       VSGARVLEQIAAQMRNKKLPMVDDLRDESDHENPTRLVIVPRSNRVDMDQ
parC21       WAGYAALKAMISTRR---------------------------------
              :*  .*: : :  *

EG10686_parC_VMNHLFATTDLEKSYRINLNMIGLDGRPAVKNLLEILSEWLVFRRDTVRR
parC39       VMNHLFATTDLEKSYRINLNMIGLDGRPAVKNLLEILSEWLVFRRDTVRR
parC21       --------------------------------------------------

EG10686_parC_RLNYRLEKVLKRLHILEGLLVAFLNIDEVIEIIRNEDEPKPALMSRFGLT
parC39       RLNYRLEKVLKRLHILEGLLVAFLNIDEVIEIIRNEDEPKPALMSRFGLT
parC21       --------IIKR-------------------------------------
                      ::**

EG10686_parC_ ETQAEAILELKLRHLAKLEEMKIRGEQSELEKERDQLQGILASERKMNNL
parC39        ETQAEAILELKLRHLAKLEEMKIRGEQSELEKERDQLQGILASERKMNNL
parC21        --------------------------------------------------

EG10686_parC_ LKKELQADAQAYGDDRRSPLQEREEAKAMSEHDMLPSEPVTIVLSQMGWV
parC39        LKKELQADAQAYGDDRRSPLQEREEAKAMSEHDMLPSEPVTIVLSQMGWV
parC21        --------------------------------------------------

EG10686_parC_ RSAKGHDIDAPGLNYKAGDSFKAAVKGKSNQPVVFVDSTGRSYAIDPITL
parC39        RSAKGHDIDAPGLNYKAGDSFKAAVKGKSNQPVVFVDSTGRSYAIDPITL
parC21        ----------------------VVKGKSNQPVVFVDSTGRSYAIDPITL
                                    .*************************

EG10686_parC_ PSARGQGEPLTGKLTLPPGATVDHMLMESDDQKLLMASDAGYGFVCTFND
parC39        PSARGQGEPLTGKLTLPPGATVDHMLMESDDQKLLMASDAGYGFVCTFND
parC21        PSARGQGEPLTGKLTLPPGATVDHMLMESDDQKLLMASDAGYGFVCTFND
              **************************************************

EG10686_parC_ LVARNRAGKALITLPENAHVMPPVVIEDASDMLLAITQAG-------RML
parC39        LVARNRAGKALITLPENAHVMPPVVIEDASDMLLAITQAG-------RML
parC21        LVARNRAGKALITLPENAHVMPPVVIEDASDMLLAITQAGRMLMFPGRML
              ************************************       ***

EG10686_parC_MFPVSDLPQLSKGKGNKIINIPSAEAARGEDGLAQLYVLPPQSTLTIHVG
parC39       MFPVSDLPQLSKGKGNKIINIPSAEAARGEDGLAQLYVLPPQSTLTIHVG
parC21       MFPVSDLPQLSKGKGNKIINIPSAEAARGEDGLAQLYVLPPQSTLTIHVG
             **************************************************
```

```
EG10686_parC_KRKIKLRPEELQKVTGERGRRGTLMRGLQRIDRVEIDSPRRASSGDSEE
parC39       KRKIKLRPEELQKVTGERGRRGTLMRGLQRIDRVEIDSPRRASSGDSEE
parC21       KRKIKLRPEELQKVTGERGRRGTLMRGLQRIDRVEIDSPRRASSGDSEE
             *********************************************
```

Figure 4.5. Alignment of amino acid sequences of *parC* detected in *E. coli* strains isolated from Stiebeuel River and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
EG11434_marA_MSRRNTDAITIHSILDWIEDNLESPLSLEKVSERSGYSKWHLQRMFKKET
marA10       MSRRNTDAITIHSILDWIEDNLESPLSLEKVSERSGYSKWHLQRMFKKET
marA31       MSRRNTDAITIHSILDWIEDNLESPLSLEKVSERSGYSIWHLPRMFKKET
             ************************************ *** *******

EG11434_marA_GHSLGQYIRSR-KMTEIAQKLKESNEPILYLAERYGFESQQTLTRTFKNY
marA10       GHSLGQYIRSR-KMTEIAQKLKESNEPILYLAERYGFESQQTLTRTFKNY
marA31       GHSLGQYIRSRRKMTEIAQKLKESNEPILYLAERYGFKSQQTLTRTFXNY
             *********** *************************:********* **

EG11434_marA_FDVPPHKYRMTNMQGESRFLHPLNHYNS------
marA10       FDVPPHKYRMTNMQGESRFLHPLNHYNNLITQRH
marA31       FDVPPPKYRMTNMQGESRFLHPLYRYNN------
             ***** ***************** :**.
```

Figure 4.6. Alignment of amino acid sequences of *marA* detected in *E. coli* strains isolated from Disa River and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
EG10040_ampC_MFKTTLCALLITASCSTFAAPQQINDIVHRTITPLIEQQKIPGMAVAVIY
ampC17       MFKTTLCALLITASCSTFAAPQQINDIVHRTITPLIEQQKIPGMAVAVIY
ampC89       MFKTTLCALLITASCSTFAAPQQINDIVHRTITPVLEQQKIPGMAVAVIY
             **********************************::**************

EG10040_ampC_QGKPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIK
ampC17       QGKPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIK
ampC89       QGKSYYFTWGYADIAKKQPVTQQTLFELGSVSKTFT--------------
             ***.*******************************

EG10040_ampC_LSDPTTKYWPELTAKQWNGITLLHLATYTAGGLPLQVPDEVKSSSDLLRF
ampC17       LSDPTTKYWPELTAKQWNGITLLHLATYTAGGLPLQVPDEVKSSSDLLRF
ampC89       --------------------TLLHLATYTAGGLPLQVPDEVKSSSDLLRF
                                 ****************************

EG10040_ampC_YQNWQPAWAPGTQRLYANSSIGLFGALAVKPSGLSFEQAMQTRVFQPLKL
ampC17       YQNWQPAWAPGTQRLYANSSIGLFGALAVKPSGLSFEQAMKTRVFQPLKL
ampC89       YQNWQPAWAPGTQRLYANSSIGLFGALAVKPSGLSFEQAMQTRVFQPLKL
             ****************************************:*********

EG10040_ampC_NHTWINVPPAEEKNYAWGYREGKAVHVSP----------------------
ampC17       NHTWINVPSAEEKNYAWGYREGKAVHVSP----------------------
ampC89       NHTWINVPPAEEKNYAWGYREGKAVHVSPGALDAEAYGVKQPLKLNHTWI
             ********.*******************

EG10040_ampC_------------------------GALDAEAYGVKSTIEDMARWVQSNLK
ampC17       ------------------------GALDAEAYGVKSTIEDMARWVQSNLK
ampC89       NVPXAEEKNYAWGYREGKAVHVSPGALDAEAYGVKSTIEDMARWVQSNLK
                                     **************************

EG10040_ampC_PLDINEKTLQQGIQLAQSRYWQTGDMYQGLGWEMLDWP------------
ampC17       PLDINEKTLQQGIQLAQSRYWQTGDMYQGLGWEMLDWPGKFLTSSFXGNA
ampC89       PLDINEKTLQQGIQLAQSRYWQTGDMYQGLGWEMLDWX-WNLTSSXLGNA
             ***********************************

EG10040_ampC_----VNPDSIINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGFG
ampC17       GL-AGIPDIIINGSDNKIALAARPVKPITPPTPAVRASWVHKTGATGGFG
ampC89       GLGQVNPDIIINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGFG
                   ** ****************.**********************

EG10040_ampC_SYVAFIPEKELGIVMLANKNYPNPARVDAAWQILNALQ------------
ampC17       SYVAFIPEKELGIVMLANKNYPNPARVAAAWQILNALQNFIGSEFSDLFS
ampC89       SYVAFIPEKELGIVMLANKNYPNPARVAAAWQILNALQ------------
             *************************** **********

EG10040_ampC_--------------------
ampC17       AFPCCHLHLEKNQGNIYAPA
ampC89       --------------------
```

Figure 4.7. Alignment of amino acid sequences of *ampC* detected in *E. coli* strains isolated from wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisk s indicate identical residues.

```
EG10957_soxR_MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYK
soxR_12      MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYK
soxR67       MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYK
             **************************************************

EG10957_soxR_RDVLRYVAIIKIAQRIGIPLATIGEAFGVLPEGHTLSAKEWKQLSSQWRE
soxR_12      RDVLRYVAIIKIAQRIGIPLATIGEAFGVLPEGHTLSAKEWKQLSSQWRE
soxR67       RDVLRYVAIIKIAQRIGIPLATIGEAFGVLPEGHTLSAKEWKQLSSHWRE
             **************************************************:***

EG10957_soxR_ELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGARLLE
soxR_12      ELDRRIHTLVALRDELDGCIGCGCLSRSDCPLRNPGDRLGEEGTGARLLE
soxR67       ELDRCIHTLVALLFELNACIGCGCLSRSDCPLRNPGDRLGEEGTGARLLE
             **** *******  **:.*******************************

EG10957_soxR_DEQN
soxR_12      DEQN
soxR67       DEQN
             ****
```

Figure 4.8. Alignment of amino acid sequences of *soxR* detected in *E. coli* strains isolated from wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
EG10958_soxS_MSHQKIIQDLIAWIDEHIDQPLNIDVVAKKSGYSKWYLQRMFRTVTHQTL
soxS68       MSHQKIIQDLIAWIDEHIDQPLNIDVVAKKSGYSKWYLQRMFRTVTHQTL
soxS8        MSHQKIIQDLIAWIDEHIDQPLNIDVVAKKSGYSKWYLQRMFRTVTHQTL
             **************************************************

EG10958_soxS_GDYIRQRRLLLAAVELRTTERPIFDIAMDLGYVSQQTFSRVFRRQFDRTP
soxS68       GDYIRQRRLLLAAVELRTTERPIFDIAMDLGYVSQQTFSRVFRRQFDRTP
soxS8        GDYIRQR-LLLAAVELRTTERPIFDIAMDLGYVSQQTFSRVFRRQFDRTP
             ******* ****************************************

EG10958_soxS_SDYRHRL
soxS68       SDYRHRL
soxS8        SDYRHRL
             *******
```

Figure 4.9. Alignment of amino acid sequences of *soxS* detected in *E. coli* strains isolated from Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
dnaK1           MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVT
dnaK29          MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVT
EG10241_1       MGKIIGIDLGTTNSCVAIMDGTTPRVLENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVT
                ************************************************************

dnaK1           NPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGDAWVEVKGQKMAPPQISAEVL
dnaK29          NPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFMIIAADNGDAWVEVKGQKMAPPQISAEVL
EG10241_1       NPQNTLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGDAWVEVKGQKMAPPQISAEVL
                ****************************** ****************************

dnaK1           KKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYG
dnaK29          KKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYG
EG10241_1       KKMKKTAEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYG
                ************************************************************

dnaK1           LDKGTGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYL
dnaK29          LDKGTGNRTIAVYDLGGGTFDKSIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYL
EG10241_1       LDKGTGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYL
                ******************** ************************************

dnaK1           VEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKV
dnaK29          VEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKV
EG10241_1       VEEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNIKV
                ************************************************************

dnaK1           TRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQKKVAEFFGKE
dnaK29          TRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQKKVAEFFGKE
EG10241_1       TRAKLESLVEDLVNRSIEPLKVALQDAGLSVSDIDDVILVGGQTRMPMVQKKVAEFFGKE
                ************************************************************

dnaK1           PRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGIETMGGVMTTLIAKNTTIPT
dnaK29          PRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGIETMGGVMTTLIAKNTTIPT
EG10241_1       PRKDVNPDEAVAIGAAVQGGVLTGDVKDVLLLDVTPLSLGIETMGGVMTTLIAKNTTIPT
                ************************************************************

dnaK1           KHSQVFSTAEDFGTKNMTIHVLQGERKRAADNKSLGQFNLDGINPAPRGMPQIEVTFDID
dnaK29          KHSQVFSTAEDFGTKNMTIHVLQGERKRAADNKSLGQFNLDGINPAPRGMPQIEVTFDID
EG10241_1       KHSQVFSTAEDN-QSAVTIHVLQGERKRAADNKSLGQFNLDGINPAPRGMPQIEVTFDID
                ***********   . :****************************************

dnaK1           ADGILHVSAKDKNSGKEQKITIKASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQG
dnaK29          ADGILHVSAKDKNSGKEQKITIKASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQG
EG10241_1       ADGILHVSAKDKNSGKEQKITIKASSGLNEDEIQKMVRDAEANAEADRKFEELVQTRNQG
                ************************************************************

dnaK1           DHLLHSTRKQVEEAGDKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLM
dnaK29          DHLLHSTRKQVEEAGDKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLM
EG10241_1       DHLLHSTRKQVEEAGDKLPADDKTAIESALTALETALKGEDKAAIEAKMQELAQVSQKLM
                ************************************************************

dnaK1           EIAQQQHAQQQTAGADASANNAKDDDVVDAEFEEVKDKK
dnaK29          EIAQQQHAQQQTAGADASANNAKQNDVVDAEFEEVKDKK
EG10241_1       EIAQQQHAQQQTAGADASANNAKDDDVVDAEFEEVKDKK
                ***********************:*:**************
```

Figure 4.10. Alignment of amino acid sequences of *dnaK* detected in *E. coli* strains isolated from Disa River and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisk s indicate identical residues.

```
grpE9           ------------------------------MSSKEQKTPEGQAPEEIIMDQHEEIEAVEP
grpE19          -----------------------------------PEGQAPEEIIMDQHEEIEAVEP
EG10416_1       ------------------------------MSSKEQKTPEGQAPEEIIMDQHEEIEAVEP
grpE22          MSSKEQKTKTETDPHNKRSRDECEKNAEKFMSSKEQKTPEGQAPEEIIMDQHEEIEAVEP
                                                **********************

grpE9           EASAEQVDPRDEKIANLEAQLAEAQTRERDGILRVKAEMENLRRRTELDIEKAHKFALEK
grpE19          EASAEQVDPRDEKIANLEAQLAEAQTRERDGILRVKAEMENLRRRTELDIEKAHKFALEK
EG10416_1       EASAEQVDPRDEKVANLEAQLAEAQTRERDGILRVKAEMENLRRRTELDIEKAHKFALEK
grpE22          EASAEQVDPRDEKIANLEAQLAEAQTRERDGILRVKAEMENLRRRTELDIEKAHKFALEK
                ************:***********************************************

grpE9           FINELLPVIDSLDRALEVADKANPDMSAMVEGIELTLKSMLDVVRKFGVEVIAETNVPLD
grpE19          FINELLPVIDSLDRALEVADKANPDMSAMVEGIELTLKSMLDVVRKFGVEVIAETNVPLD
EG10416_1       FINELLPVIDSLDRALEVADKANPDMSAMVEGIELTLKSMLDVVRKFGVEVIAETNVPLD
grpE22          FINELLPVIDSLDRALEVADKANPDMSAMVEGIELTLKSMLDVVRKFGVEVIAETNVPLD
                ************************************************************

grpE9           PNVHQAIAMVESDDVAPGNVLGIMQKGYTLNGRTIRAAMVTVAKAKA
grpE19          PNVHQAIAMVESDDVAPGNVLGIMQKGYTLNGRTIRAAMVTVAKAKA
EG10416_1       PNVHQAIAMVESDDVAPGNVLGIMQKGYTLNGRTIRAAMVTVAKAKA
grpE22          PNVHQAIAMVESDDVAPGNVLGIMQKGYTLNGRTIRAAM--------
                ************************************
```

Figure 4.11. Alignment of amino acid sequences of *grpE* detected in *E. coli* strains isolated from Plankenburg River (Below Kayamandi), Disa River and wastewater effluent at Spier Wine Estate (Nietvoorbij Research Project) in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
EG10671_1       MMKRNILAVIVPALLVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKGNGENSYGGNGDM
ompF18          MMKRNILAVIVPALLVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKGNGENSYGGNGDM
ompF50          MMKRNILAVIVPALLVAGTANAAEIYNKDGNKVDLYGKAVGLHYFSKVNGENSYGGNCDM
                ********************************************* ********* **

EG10671_1       TYARLGFKGETQINSDLTGYGQWEYNFQGNNSEGADAQTGNKTRLAFAGLKYADVGSFDY
ompF18          TYARPGFKXETQINSDLTGYGQWEYTFQGNNSEGADAQTGNKTRLAFAGFKYADVGSFDY
ompF50          TYARLGFKGETQINSDLTGYGQWEYNFQGNNSEGADAQTGNKTRLAFAGLKYADVGSFDY
                **** *** ****************.***********************:**********

EG10671_1       GRNYGVVYDALGYTDMLPEFGGDTAYSDDFFVGRVGGVATYRNSNFFGLVDGLNFAVQYL
ompF18          GRNYGVVYDALGYTDMLPEFGGDTAYSDDFFVGRVGGVATYRNSNFFGLVDGLNFAVQYL
ompF50          GRNYGVVYDALGYTDMLPEFGGDTAYSDDFFVGRVGGVATYRNSNFFGLVDGLNFAVQYL
                ************************************************************

EG10671_1       GKNERDTARRSNGDGVGGSISYEYEGFGIVGAYGAADRTNLQEAQPLGNGKKAEQWATGL
ompF18          GKNERDTARRSNGDGVGGSISYEYEGFGIVGAYGAADRTNLQEAQPLGNGKKAEQWATGL
ompF50          GKNERDTARRSNGDGVGGSISYEYEGFGIVGAYGAADRTNLQEAQPLGNGKKAEQWATGL
                ************************************************************

EG10671_1       KYDANNIYLAANYGETRNATPITNKFTNTSGFANKTQDVLLVAQYQFDFGLRPSIAYTKS
ompF18          KYDANNIYLAANYGETRNATPITNKFTNTSGFANKTQDVLLVAQYQFDFGLRPSIAYTKS
ompF50          KYDANNIYLAANYGKTRNATPITNKFTNTSGFANKTQDVLLVAQSQFDFGLRPSIAYTKS
                **************:***************************** ***************

EG10671_1       KAKDVEGIG-------------------------------------------------
ompF18          KAKDVEGIGDVDLVNYLKGMPFMMKRNILAVIVPALLVAGTANAAEIYNKDGNKVDLYGK
ompF50          KAKDVEGIG-------------------------------------------------
                *********
```

```
EG10671_1       ------------------------------------------------------------
ompF18          AVGLHYFSKGNGENSYGGNGDMKPVYGGNGDMTYARLGFKGETQINSDLTGYGQWEYNFQ
ompF50          ------------------------------------------------------------

EG10671_1       ------------------------------------------------------------
ompF18          GNNSEGADAQTGNKTRLAFAGLKYADVGSFDYGRNYGVVYDALGYTDMLPEFGGDTAYSD
ompF50          ------------------------------------------------------------

EG10671_1       ------------------------------------------------------------
ompF18          DFFVGRVGGVATYRNSNFFGLVDGLNFAVQYLGKNERDTARRSNGDGVGGSISYEYEGFG
ompF50          ------------------------------------------------------------

EG10671_1       ------------------------------------------------------------
ompF18          IVGAYGAADRTNLQEAQPLGNGKKAEQWATGLKYDPDNIYLAATYGGTRNATPITNKFTN
ompF50          ------------------------------------------------------------

EG10671_1       ----------------------------DVDLVNYFEVGATYYFNKNMSTYVDYIINQI
ompF18          TSGFPNKTQDVLLVAQYQVDKAKDVEGIGDVDLVNYFEVGATYYFNKNMSTYVDYIINQI
ompF50          ------------DVIWELLKKAKDVEGIGDVDLVNYFEVGATYYFNKNMSTYVDYIIN--
                                          ****************************

EG10671_1       DSDNKLGVGSDDTVAVGIVYQF
ompF18          DSDNKLGVGSDDTVAVGIVYQF
ompF50          ----------------------
```

Figure 4.12. Alignment of amino acid sequences of *ompF* detected in *E. coli* strains isolated from Plankenburg River (Below Kayamandi) and Stiebeuel River in comparison with the reference gene sequence. Asterisks indicate identical residues.

```
EG10680_osmC_MTIHKKGQAHWEGDIKRGKGTVSTESGVLNQQPYGFNTRFEGEKGTNPEE
osmC5         MTIHKKGQAHWEGDIKRGKGTVSTESGVLNQQPYGFNTRFEGEKGTNPEE
osmC9         MTIHKKGQAHWEGDIKRGKGTVSTESGVLNQRPYGFNTRFEGEKGTNPEE
              ********************************:*****************

EG10680_osmC_LIGAAHAACFSMALSLMLGEAGFTPTSIDTTADVSLDKVDAGFAITKIAL
osmC5         LIGAAHAACFSMALSLMLGEAGFTPTSIDTTADVSLDKVDAGFAITKIAL
osmC9         LIGAAHAACFSMALSLMLGEAGFTPTSIDTTADVSLDKVDAGFAITKIAL
              ************************************************

EG10680_osmC_  KSEVAVPGIDASTFDGIIQKAKAGCPVSQVLKAEITLDYQLKS-----
osmC5          KSEVAVPGIDASTFDGIIQKAKAGCPVSQVLKAEITLDYQLKSAXXRX
osmC9          KSEVAVPGIDASTFDGIIQKAKAGCPVSQVLKAEITLDYQLKS-----
               ****************************************
```

Figure 4.13. Alignment of amino acid sequences of *osmC* detected in *E. coli* strains isolated from Disa River and Plankenburg River (Below Kayamandi) in comparison with the reference gene sequence. Asterisks indicate identical residues.