Characterization of *Clostridium* spp. isolated from selected surface water systems and aquatic sediment

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Abstract

Clostridium are ubiquitous in nature and common inhabitants of the gastrointestinal track of humans and animals. Some are pathogenic or toxin producers. These pathogenic *Clostridium* species can be introduced into surface water systems through various sources, such as effluent from wastewater treatment plants (WWTP) and surface runoff from agricultural areas. In a South African context, little information is available on this subject. Therefore, this study aimed to characterize *Clostridium* species isolated from surface water and aquatic sediment in selected river systems across the North West Province in South Africa. To achieve this aim, this study had two main objectives. The first objective focused on determining the prevalence of *Clostridium* species in surface water of the Schoonspruit, Crocodile and Groot Marico Rivers and evaluate its potential as an indicator of faecal pollution, along with the possible health risks associated with these species. The presence of sulphite-reducing *Clostridium* (SRC) species were confirmed in all three surface water systems using the Fung double tube method. The high levels of SRC were correlated with those of other faecal indicator organisms (FIO). WWTP alongside the rivers were identified as one of the major contributors of SRC species and FIO in these surface water systems. These findings supported the potential of SRC species as a possible surrogate faecal indicator. However, limitations of SRC species as FIO were noticed in this study. Furthermore, the results showed that the physico-chemical parameters such as temperature, dissolved oxygen, chemical oxygen demand, nitrates, phosphates and sulphates present in the water had a great effect in the *Clostridium* spp. levels during the warm-rainy season. This was possibly due to non-point source pollution such as surface runoff which promoted eutrophication in parts of these river systems. The second objective of the study was to investigate antibiotic resistance in *Clostridium* species isolated from both surface water and aquatic sediment and the presence of antibiotic resistance gene in these isolates. A total of 67 *Clostridium* isolates obtained from the Schoonspruit and Crocodile Rivers showed resistance against Ampicillin, Tetracycline or Clindamycin. No antibiotic resistant isolates were obtained from the Groot Marico River. The minimum inhibitory concentration (MIC) of 6 antibiotics were determined using the recommended agar dilution method. MIC values of Ampicillin (AMP) ranged from 0.25-2 µg/ml, 0.5 to >256 µg/ml for Tetracycline (TE), 0.25 to >256 µg/ml for Clindamycin (DA), 0.5-16 µg/ml for Amoxicillin (AMX), 0.5-32 µg/ml for Chloramphenicol (C) and 0.5-64 µg/ml for Metronidazole (MTZ). Using these MIC values, resistance profile could be generated for each antibiotic resistant *Clostridium* isolate. These results revealed that Antibiotics such as Amoxicillin and Chloramphenicol were the most effective in inhibiting the growth of antibiotic resistant *Clostridium* species. Whereas the

majority of the isolates showed resistance against Ampicillin and Tetracycline. None of the antibiotics tested for in this study were 100% effective against the *Clostridium* isolates. Furthermore, ten different multi-antibiotic resistant (MAR) phenotypes were also observed across these isolates. The most prevalent one being AMP-TE-DA-MTZ-C-AMX. All the isolates that presented this phenotype were obtained from aquatic sediment, suggesting that aquatic sediment may be a reservoir for antibiotic resistance and MAR *Clostridium* species. Additionally, the presence of several antibiotic resistance genes was also screened for using PCR. One of the genes encoding for macrolide-lincosamide-streptogramin (MLS) (*erm*F), and β-lactam (bla_{TEM}) resistance were not found to be present in any Clindamycin and Ampicillin resistant isolates, respectively. However, several Clindamycin resistant *Clostridium* isolates were found to harbour the *erm*B gene, which also encodes for MLS resistance. Two genes encoding for efflux mechanisms against Tetracycline (*tet*K and *tet*L) were found in the genomes of some of the Tetracycline resistant isolates. Using both Gram and endospore staining, alongside DNA sequencing, 7 *Clostridium* species were identified throughout both studies, which included *Clostridium bifermentans*, *C. perfringens*, *C. sordellii*, *C. baratii*, *C. ghonii*, *C. lituseburense* and *C. dakarense*. Several of these *Clostridium* species are known pathogens and have been associated with severe gastrointestinal diseases, botulism and necrotising gas-gangrene in both humans and animals. To conclude, the data generated revealed the presence of potentially pathogenic *Clostridium* species in both surface water and sediment. The presence of antibiotic resistant genes in environmental *Clostridium* species are also a cause for concern. The expression of these genes could contribute to MAR in these potential pathogenic bacteria. Furthermore, these results highlighted the necessity to screen for other antibiotic resistant pathogens in the aquatic environment and to further investigate the potential sources. Additionally, it is recommended that SRC species should be used as an additional indicator of faecal pollution in surface water systems. Lastly, all these findings indicate that the surface water systems in the North West Province are exposed to various pollutants such as antibiotics and faecal contaminants from runoff and WWTP. This is cause for concern, considering that many rural and informal communities are directly dependent on these water sources and as a result affecting the health of its users, particularly the immune-compromised individuals and livestock.

Keywords: *Clostridium* spp., pathogenic, surface water, aquatic sediment, faecal pollution, antibiotic resistance, antibiotic resistance genes.

Ek dra graag hierdie verhandeling op aan ‘n merkwaardige vrou…

My Ouma, *Altie*.

Psalm 23

“Toe die aarde klaar geskape was en die mens op die aarde kom woon het, het die sewe hoofengele van die hemel vergader om oor ‘n baie ernstige saak te besluit. Hulle moes besluit waar hulle die krag van God kan wegsteek sodat die mens dit nie te gou sal kry en misbruik nie. Die eerste engel het voorgestel dat hulle dit op die maan moet wegsteek. Ja, op die maan het die ander saamgestem. Maar die sewende engel sê: Nee, die mens is slim. Hy gaan eendag weet hoe om op die maan te kom; ons moet ‘n ander plek soek. Op die bodem van die see, waar die see op sy diepste is, stel die tweede engel voor. Ja, sê die ander. Maar die sewende engel keer hulle weer. Die mens is slim, sê hy, op ‘n dag gaan hulle tot op die bodem van die see ook soek. Laat ons dit dan aan die môrester ophang, stel die derde engel voor. Eendag gaan die mense tot by die môrester soek. Toe vra die ander vir die sewende engel waar hy dink hulle dit sal wegsteek? Op die laaste plek waar hulle sal soek, sê hy. Binne in hulself.”

~Dalene Matthee, “*Kringe in ‘n bos*”

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Preface

The work done and discussed in this dissertation for the degree *Magister Scientiae* (M.Sc.) in Microbiology was carried out in the School of Biological Sciences, North-West University (Potchefstroom Campus), South Africa. This study was conducted fulltime during 2015-2016, under the supervision of Prof. Carlos Bezuidenhout and Dr. Charlotte Mienie.

The physico-chemical and general microbiological data form part of a WRC funded research project (K5/2347/3). The candidate was one of the members of the research team that collected some of the data. It was agreed that all participants would use data from the set and it is thus unavoidable that overlaps of the actual data in this dissertation, some M.Sc. dissertations and the WRC final report will exist.

The research done and presented in this dissertation signifies original work undertaken by the candidate and has not been submitted for any degree or examination purposes at this or any other university. Appropriate acknowledgements in the text have been made where the use of work conducted by other researchers have been included.

Johannes Cornelius Jacobus Fourie
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List of abbreviations

AHC	Agglomerative hierarchical clustering
AMP	Ampicillin
AMX	Amoxicillin
ARG	Antibiotic resistance genes
BLAST	Basic Local Alignment Search Tool
BoNT	Botulinum neurotoxin
C	Chloramphenicol
C/D	Cold-dry
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DA	Clindamycin
dNTPs	Deoxynucleotides
DO	Dissolved oxygen
EDTA	Ethylenediamine-tetra-acetic acid
E-value	Expected *Value*
FDT	Fung double tube
FIO	Faecal indicator organisms
gDNA	Genomic deoxyribonucleic acid
GIT	Gastrointestinal tract
H_2	Hydrogen
ISO	International Organization for Standardization
M.I.C.E.	Minimum inhibitory concentration E-test strips
MAR	Multiple-antibiotic resistant
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MLS	Macrolide-lincosamide-streptogramin
MTZ	Metronidazole
NaCl	Sodium Chloride
NaI	Sodium Iodide
NCBI	National Centre for Biotechnology Information
NO_2^-	Nitrite

NO_3^-	Nitrate
PBP's	Penicillin-binding proteins
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
pH	The co-logarithm of the activity of dissolve hydrogen ions (H^+)
PO_4^-	Phosphates
p-values	Value of probability
RDA	Redundancy analysis
SO_4^{2-}	Sulphate
spp.	Species
SRC	Sulphite reducing *Clostridium*
TDS	Total dissolved solids
TE	Tetracycline
TeNT	Tetanus neurotoxin
Tris	*Tris* (hydroxymethyl) aminomethane
TSC	Tryptose Sulphite Cycloserine
W/R	Warm-rainy
WHO	World Health Organisation
WMA	Water Management Areas
WWTP	Wastewater treatment plants
β	Beta

CHAPTER 1

Literature overview

1.1. Water in South Africa

South Africa is a water scarce country. This situation is exacerbated by climate change, bringing about irregular rainfall patterns across the country (Colvin *et al*., 2013). In turn, this results in extensive wet and dry periods, causing extreme droughts and floods. A scarcity of freshwater is therefore a reality, contributing to ever-growing constraint on South Africa's development, both economic and social (Colvin *et al*., 2013; Amis and Nel, 2011).

Kotze and Rose (2015) illustrated the usage of water in South Africa by various sectors. The agricultural sector consumes the majority of the nation's water reserve, approximately 60%, for irrigation, followed by municipal and domestic needs, with a combined usage of 27%. Other sectors like mining, livestock watering, industrial and power generation utilizes between 2 and 3% each. Currently, there is roughly 15 billion m^3 of water allocated for the whole of South Africa. It is estimated that the demand will rise to 17.7 billion m^3 by 2030 due to industrial and population growth. This by far exceeds the possible limit for water allocation (Colvin *et al*., 2016).

Surface water resources like rivers and dams are used to supply water to urban areas. It is therefore important to ensure that these resources are sustainably managed, monitored and conserved (Colvin *et al*., 2016). Colvin and co-workers (2016) reported that the water quality of several of South Africa's surface water bodies are of concern, showing major deterioration over the past couple of years. It was also stated that many rural and informal settlements are directly dependent on these water sources. This exposes humans and animals using this water to serious health risks. This was confirmed by a community survey done in 2016 which revealed that 10.1% of South Africans don't have access to safe drinking water, showing a 1.3% increase since 2011 (Statistics South Africa, 2016).

Reasons for this ever decline in water quality can be attributed to various factors, the most prevalent being agricultural runoff such as pesticides and fertilisers, sewage effluent from poorly maintained sewage treatment plants, lack of adequate sanitation facilities in rural settlements, discharge of pharmaceutical chemicals in industrial effluent into rivers and acid mine drainage (Colvin *et al*., 2016; Amis and Nel, 2011).

1.2. Water in North West Province

The North West Province is the sixth largest province of the nine in South Africa. It covers around 9.5% of the South African surface area (total area of 116 320 km^2; NWDACE, 2002). Because of the variation in rainfall across the province, ranging from 300-600 mm per annum, it is classified as arid to semi-arid. This makes for a water stressed environment (NWREAD, 2014). Many rivers in the North West Province such as the Schoonspruit, Groot Marico and Mooi River finds its origin from dolomitic eyes. Because of this, ground water (dolomitic eyes) and surface water (rivers) are interrelated. Thus, the water quality and quantity of the one impacts that of the other, and vice versa (NWDACE, 2008). The province encompasses 4 Water Management Areas (WMA): Upper Vaal, Middle Vaal and the Lower Vaal, as well as the Crocodile (West) and Marico (NWREAD, 2008). Like the rest of South Africa, the North West Province struggles with availability of water due to most of the rivers being non-perennial. Also contributing to this situation is the agriculture, urban and mining sectors, collectively demanding 92% of its available water (NWREAD, 2014).

In addition to the serious concerns about water availability in the North West Province, the quality of the surface water in this province is also an issue. This is evident in the rising problem of eutrophication in water systems. This decline in water quality can be attributed to both diffuse and point-source pollution (NWREAD, 2014). Point source pollutants in this province involve acid mine drainage, domestic and industrial effluent (NWREAD, 2008). According to the Department of Water Affairs (2012), wastewater treatment plants (WWTP) in South Arica are still one of the main contributors to this water quality problem, despite all the advances and improvements made in this area. The Green Drop report of 2014 indicated that a total of 27 WWTPs in the North West Province were classified as high risk (DWA, 2014). This means that the final effluent of all these plants do not comply with national standards, and thus resulting in inadequately treated effluent being introduced into the surrounding surface water systems (Abia *et al*., 2015b). The faecal pollutants being discharged into these surface water systems are worrisome, since it poses a public health threat (NWREAD, 2014; Awofolu *et al*., 2007). The main contributors of diffuse pollution are agricultural and storm water runoff (NWREAD, 2008). This means that potentially dangerous compounds and microbes could enter surface water systems. Furthermore, there are not enough monitoring processes in place to identify and control all these pollution factors (NWREAD, 2014). It is therefore vital to develop proper indices for water quality to comprehend the state of the surface water systems.

1.3. Antibiotics in the environment

1.3.1. Water

Antibiotics are used for various reasons, ranging from improving human and animal health by combating infections, to promoting growth in livestock and agriculture (Zhang *et al*., 2009). After administering these antibiotics to humans or animals, they are only partially metabolized. This results in the excretion of active compounds (Kümmerer, 2009). Most sanitation infrastructures, such as wastewater treatment plants, are not equipped to completely remove these compounds, leaving residual amounts in the treated effluent. This effluent is then reintroduced into the environment, contaminating the water systems (Cantas *et al*., 2013; Michael *et al*., 2013; Yang and Carlson, 2004). Agricultural runoff exasperates this situation by flushing out all the antimicrobials present in the top soil, into the water systems (O'Neill, 2016; Kümmerer, 2009). All these factors contribute to the accumulation of antibiotics in the water environment. This increases selective pressure on the aquatic organisms, resulting in the selection and maintenance of antibiotic resistant bacteria and resistance genes (O'Neill, 2016; Graham *et al*., 2014; Alonso *et al*., 2001). This builds a reservoir of antibiotic resistance determinants which can then be transferred between bacteria and subsequently reaching humans through direct or indirect contact (Zhang *et al*., 2009).

1.3.2. Sediment

Studies have shown that antibiotic resistant bacteria and the genes responsible for antibiotic resistance are habitually found in aquatic sediment. This is the result of all the antibiotic compounds released into the water systems and then precipitating in the sediment below (Zhang *et al*., 2009). The use of antibiotics in aquaculture is one of the leading culprits contributing to the increasing concentrations of antibiotics in sediment (Muziasari *et al*., 2016). A study by Pei and colleagues (2006) reported the presence of various antibiotic resistance genes in the sediment of river systems near agricultural and urban settlements. After sorption of antibiotics into the sediment, it becomes more stable and therefore remains active for prolonged periods. Thus, when focusing on the quantity of antibiotics in the environment, the concentration maybe much higher in the sediment than in the surface water (Martinez, 2009).

1.4. *Clostridium* spp.

The *Clostridium* genus was first described by Adam Prazmowski in 1880 and since then, over a 100 bacterial species have been allocated to this genus (Hippe *et al*., 1992; Cato and Stackebrant, 1989). The genus belongs to the Clostridiaceae family within the class

Clostridia and are comprised of highly heterogeneous groups that are phylogenetically fairly large (Willey *et al*., 2011; Public Health England, 2016; Gupta and Gao, 2009).

The majority of Clostridia are unable to grow under aerobic conditions and any exposure to oxygen can be fatal (Hatheway, 1990). Thus, to ensure their survival, *Clostridium* spp. produce endospores. These spores have the ability to withstand prolonged exposure to air and other hostile environmental conditions (Siegrist, 2011; Jones and Keis, 2005). Sporulation is, however, only possible in an anaerobic environment (Hippe *et al*., 1992). There are some species of *Clostridium* that are moderately aerotolerant, for instance *C. carnis*, *C. histolyticum*, *C. tertium* and *C. aerotolerans* (Public Health England, 2016; Hippe *et al*., 1992).

Clostridium spp. are able to grow within a broad range of temperatures, as the genus comprises psychrophilic, mesophilic and thermophilic species (Hippe *et al*., 1992). These species are also restricted to only anaerobic metabolism (Siegrist, 2011; Jones and Keis, 2005). The main role of *Clostridium* spp. in the environment is to break down organic compounds into acids, alcohols, various minerals and large quantities of CO_2 and H_2. This degradation is usually accompanied with a foul-smelling butyric acid odour (Siegrist, 2011; Hippe *et al*., 1992).

The majority of *Clostridium* spp. are Gram-positive and rod-shaped, however there are strains that give Gram-variable/Gram-negative results (Fader, 2015; Brook, 2014). This dissimilarity is usually found in clinical isolates when direct stains are applied, if the culture is incubated for an extended time or if terminal endospores are produced in the species (Brook, 2014). *Clostridium* spp. can be straight or curved shaped rods, ranging between 0.3-1.6 × 1-14 µm and are usually arranged in short chains or pairs (Public Health England, 2016; Hippe *et al*., 1992). There is an exception, namely *Clostridium coccoides*, which is a coccoid rod (Drake *et al*., 2006; Hippe *et al*., 1992). Most of the *Clostridium* spp. are motile due to the presence of a peritrichous flagella, except for *Clostridium perfringens* (Public Health England; 2016).

According to Hippe and associates (1992), a micro-organism could be classified as a *Clostridium* spp. if it is compliant to these four criteria: (1) the micro-organism must be able to produce endospores, (2) is only restricted to anaerobic metabolism, (3) is incapable of dissimilatory sulphates reduction, (4) must be Gram-positive. These criteria made the *Clostridium* genus a depository for numerous organisms (238 described (sub)species). This led to misclassification of species and complications in the taxonomic structure of the genus

Clostridium (Lawson *et al*., 2016; Gupta and Gao, 2009). The 16S rRNA gene sequences of these species were used to revise the *Clostridium* genus, which resulted in 19 defined clusters (Stackebrandt *et al*., 1999). Consequently, only 73 species showed close relation to *Clostridium sensu stricto*, which is the main cluster that was designed based on the type species, *Clostridium butyricum* (Wiegel *et al*., 2006). Even with this revision, the phylogeny of the genus *Clostridium* still remains diverse, indicating the need for further study to improve its taxonomic classification (Wiegel *et al*., 2006; Stackebrandt *et al*., 1999).

1.5. Habitats

Due to the production of resistant spores, *Clostridium* are ubiquitous in nature and can be found in various surroundings ranging from environmental to clinical settings (Hatheway, 1990). As shown in Table 1.1, *Clostridium* species are mainly present in soil, as well as in fresh water systems, sewage, aquatic sediment, fresh produce (milk, fish and meats) and insects (Sathish and Swaminathan, 2009; Haagsma, 1991). Certain *Clostridium* spp., such as *Clostridium perfringens*, are also normal flora in the intestinal tracts of humans and feral animals, which is consistently present in faeces (Siegrist, 2011; Haagsma, 1991).

Table 1.1: *Clostridium* spp. isolated from non-clinical sources (Haagsma, 1991).

Species	**Isolated from**			
	Faeces	**Soil/Water**	**Marine sediment**	**Food**
C. bifermentans	+	+	+	
C. botulinum	+	+	+	+
C. butyricum	+	+		+
C. carnis		+		
C. chauvoei	+			
C. colinum	+			
C. difficile	+			
C. fallax		+		
C. histolyticum		+		
C. novyi	+	+	+	
C. perfringens	+	+	+	+
C. septicum	+	+		
C. sordellii		+		
C. spiroforme	+			
C. sporogenes	+	+		+
C. tetani	+	+		

1.6. Pathogenicity

The *Clostridium* genus is responsible for creating one of the most robust collections of toxigenic micro-organisms in existence (Borriello and Carman, 1988). Because *Clostridium* spp. are so uniformly found, they are often the source for serious illnesses, mediated by the toxins they produce (Mahon and Mahlen, 2015; Hatheway, 1990). Out of all the already identified *Clostridium* spp., the majority of which are non-pathogenic, 25 to 30 are classified as minor pathogens and about 13 species as major pathogens (Sathish and Swaminathan, 2009; Hatheway, 1990). These major pathogens (Table 1.2) frequently cause diseases in humans and animals and can be classified into (A) neurotoxic clostridia, (B) enterotoxic clostridia and (C) histotoxic clostridia (Sathish and Swaminathan, 2009; Borriello and Carman, 1988).

Table 1.2: Number of toxins produced and diseases caused by the major *Clostridium* pathogens (Popoff and Bouvet, 2013; Songer, 2010; Montecucco *et al*., 2006).

Clostridium species	Toxins	Disease	Group
C. argentinense	1	Botulism	Neurotoxic
C. baratii	2	Botulism	Neurotoxic
C. botulinum	3	Botulism	Neurotoxic
C. butyricum	1	Botulism	Neurotoxic
C. tetani	2	Tetanus	Neurotoxic
C. diffiicle	3	Colitis	Enterotoxic
C. spiroforme	1	Enteritis	Enterotoxic
C. chauvoei	4	Gangrene	Histotoxic
C. histolyticum	5	Gangrene	Histotoxic
C. novyi	8	Gangrene	Histotoxic
C. perfringens	14	Gangrene, enteritis	Enterotoxic, Histotoxic
C. speticum	4	Gangrene, enterotoxemia	Enterotoxic, Histotoxic
C. sordellii	4	Gangrene	Enterotoxic, Histotoxic

1.6.1. Neurotoxigenic clostridia

As shown in Table 1.2, there are certain *Clostridium* spp. that have the ability to produce neurotoxins, namely tetanus neurotoxin (TeNT) which is produced by *Clostridium tetani* and

botulinum neurotoxin (BoNT) which is produced by *Clostridium botulinum* (Montecucco *et al*., 2006; Schiavo *et al*., 2000). A *Clostridium tetani* infection can cause tetanus in humans and animals, which results in paralysis with hypertonia of the skeletal muscles (Córdoba *et al*., 2011; Schiavo *et al*., 2000). The BoNT is mainly produced by the organism *Clostridium botulinum*, but other *Clostridium* spp. such as *Clostridium butyricum* and *Clostridium baratii* are also known to produce this neurotoxin (Córdoba *et al*., 2011; Montecucco *et al*., 2006). All these species are known to cause botulism in humans (Montecucco *et al*., 2006).

1.6.2. Enterotoxic clostridia

The gastrointestinal tract (GIT) of mammalians offers an ideal niche for *Clostridium* species, being both anaerobic and rich in nutrients (McClane *et al*., 2006). Some of these species have the ability to produce toxins that have potent effects on the GIT, causing enteric diseases in humans and animals (Songer and Uzal, 2005; Songer, 1996). These types of enteric diseases are relatively common and serious. Although there are numerous enterotoxic *Clostridium* species, the two major contributors to these diseases are *Clostridium difficile* and *Clostridium perfringens* (McClane *et al*., 2006; Songer and Uzal, 2005).

1.6.3. Histotoxic clostridia

Histotoxic clostridia are responsible for a collection of different toxins that contribute interdependently to the symptoms and lesions, both local and systemic. They target various cells like muscle, epithelial, erythrocytes and lymphocytes, and destroy them (Popoff, 2016). This is done by corrupting the intercellular junctions and actin cytoskeleton, along with damaging their cell membranes. Additionally, the *Clostridium* spp. secrete hydrolytic enzymes, which increases the degradation of the soft tissue (Popoff, 2016; Petit *et al*., 1999).

Clostridial myonecrosis, also commonly referred to as gas gangrene, is one of the well-known and aggressive histotoxic infections. This disease occurs as a result of healthy muscle tissue being infected by clostridia and aggravated by decreased blood flow to the surrounding tissue (McClane and Rood, 2001). Characteristics of gas gangrene include tissue necrosis, local edema, toxemia, and gas production. The absence of an inflammatory response has also been observed (Rood, 2006). The cause of gas gangrene is due to *Clostridium perfringens* Type A. However, *Clostridium sordellii, Clostridium septicum*, *Clostridium histolyticum* and *Clostridium novyi* have been involved in 20% of all gas gangrene cases (Popoff, 2016).

1.7. Uses of *Clostridium*

1.7.1. Indicator organism

The use of spore-forming, sulphite-reducing *Clostridium* (SRC) species like *Clostridium perfringens* as an indicator of faecal pollution in water has been studied for several decades (Cabral, 2010). According to Wilson (2005) and Cabral (2010), *Clostridium* spp. are the most dominant of all the anaerobes in the gastrointestinal tract of humans and warm-blooded animals. They are also always present in wastewater (Figueras and Borrego, 2010). Consequently, the presence of anaerobes in surface water environments are usually linked to poorly treated wastewater effluent from wastewater treatment plants (Marcheggiani *et al*., 2008). Clostridia do not replicate in surface water, but has been found to be stable in these aquatic environments due to its spore-forming abilities (Cabral, 2010). These spores are extremely resistant to harsh environmental conditions such as pH and temperature extremes and UV radiation, and most importantly, disinfection treatment processes (Tyagi and Chopra, 2006). Chlorine inactivates most indicator organisms, but is less affective on *Clostridium* spores. Therefore, screening for SRC species can provide an additional margin of safety in water treatment (Figueras and Borrego, 2010). Although SRC species are ubiquitous in sediment, they can still be utilized as indicators of diffuse and point source faecal pollution or even to assess the inactivation of pathogenic protozoans and viruses in water treatment processes (Mubazangi *et al*., 2012; Figueras and Borrego, 2010).

1.7.2. Industrial

Clostridium spp. also has great industrial uses. With the ever-growing demand and cost of fossil fuels like oil, the use of biofuel as an alternative energy source has recently gained worldwide attention (Num and Useh, 2014; Samul *et al*., 2013; Kubiak *et al*., 2012). Species, like *Clostridium acetobutylicum* and *Clostridium beijerinckii,* are just some of the species that can undergo Acetone-butanol-ethanol (ABE) fermentation needed for biofuel production, which utilizes different substrates from mono- or polysaccharides to synthesize solvents like ethanol, acetone and butanol (Num and Useh, 2014; Samul *et al*., 2013). Chemical methods were always used to produce 1,3-propanediol, but nowadays, various species of the *Clostridium* genus are being employed as an alternative for the synthesis (Samul *et al*., 2013; Kubiak *et al*., 2012). *Clostridium diolis*, *Clostridium perfingens*, *Clostridium pasteurianum* and *Clostridium butyricum* are just some of the strains used (Kubiak *et al*., 2012). *Clostridium butyricum*, specifically, has unique qualities not present in the other wild strains, like its low nutrient requirements and high productivity in the production of 1,3-propanediol (Wilkens *et al*., 2012).

1.7.3. Medical

Although *Clostridium* species are primarily known for their pathogenic nature, many of the toxins they produce have shown therapeutic potential for various diseases (Hale *et al*., 2012). With the innovation in recombinant DNA technology, the use of *Clostridium* species has most recently shown promise in cancer therapy. As cancer progresses, tumours are known to develop vasculature which then creates necrotic and hypoxic regions. This environment is ideal for anaerobic bacteria. Anaerobic bacteria such as *Clostridium*, or their endospores, are directly or systematically injected into the hypoxic area of the tumour, ensuing tumour destruction (Staedtke *et al*., 2016; Theys and Lambin, 2015). There have been various *Clostridium* species tried as anti-cancer treatments, namely *Clostridium tetani*, *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Theys *et al*., 1999; Fox *et al*., 1996; Malmgren and Flanigan, 1955). More recently, a non-pathogenic engineered *Clostridium novyi* strain (*C. novyi*-NT) has shown great promise in the pursuit of an anti-cancer treatment, however, there are still challenges to overcome before this type of therapy is approved and applied. Thus, *Clostridium*-mediated anti-cancer therapy can potentially overcome the current disadvantages in systematic treatments and offer an alternative means of eradicating untreatable tumours (Staedtke *et al*., 2016; Theys and Lambin, 2015).

1.8. Problem statement

The *Clostridium* genus encompasses a variety of Gram-positive, anaerobic, opportunistic pathogens which are ubiquitous, reaching from environmental to clinical settings (Hatheway, 1990). Although they form part of the normal flora in the intestinal tracts of humans and feral animals (Siegrist, 2011; Haagsm, 1991), they also cause severe gastrointestinal diseases and infections such as enteritis, botulinum and gas gangrene (Popoff and Bouvet, 2013; Songer, 2010; Montecucco *et al*., 2006). Consequently, the possible presence of *Clostridium* species in surface water systems are alarming, considering the medical consequences. However, several studies have used their presence as an indicator organism to detect possible faecal pollutants and pathogens in aquatic environments (Abia *et al*., 2015b; Mubazangi *et al*., 2012; Vijayavel *et al*., 2009).

Antibiotic resistance in anaerobic bacteria such as *Clostridium* has clinically become more recognised (Hecht, 2004). In contrast to this, very little has been done to examine antibiotic susceptibility and investigate antibiotic resistance genes in environmental isolates (Soge *et al*., 2008). To our knowledge, no studies have focused on the prevalence of antibiotic resistant *Clostridium* species in aquatic environments, particularly its distribution in the surface water systems of South Africa and the North West Province.

Thus, this present study was designed with the main aim to characterize *Clostridium* species that were isolated from surface water and aquatic sediment obtained from selected surface water systems in the North West Province, South Africa. To achieve this aim, the study had two major objectives with each having had its own specific objectives. The first objective was to determine the prevalence of *Clostridium* species in surface water of selected river systems in the province and evaluate its potential as an indicator of faecal pollution, along with the possible health risks associated with these species. The second objective was to investigate antibiotic resistance in *Clostridium* species isolated from surface water and aquatic sediment obtained from the same river systems and the presence of antibiotic resistance genes in the genomes of these isolates.

1.9. Areas under investigation

1.9.1. Schoonspruit River

The Schoonspruit River (Figure 1.1) forms part of the Middle Vaal Water Management Area (WMA) and encompasses an area of 325 km^2, with the majority being characterized as wetland habitat (DWAF, 2007). The water quality of this river system is impacted by various anthropogenic activities which include mining and agriculture. The diamond digging and gold mining activities around the Klerksdorp area are the key contributors to the decline in the Schoonspruit River (Colvin and Burns, 2011; DWAF, 2004). Dolomite springs present in the upper regions of the catchment feeds the Schoonspruit River. These springs are under great pressure as a result of its use for irrigation, exceeding its recharge (NWREAD, 2008). Little information is available on the state of the Schoonspruit River, however, a study by Molale (2012) revealed a high presence of faecal contamination in this river. Furthermore, several parts of the Schoonspruit River has been reported as eutrophic (DWAF, 2009). This is troublesome, since this river is also an important source of water for irrigation and urban necessities for the Ventersdorp area (DWAF, 2004).

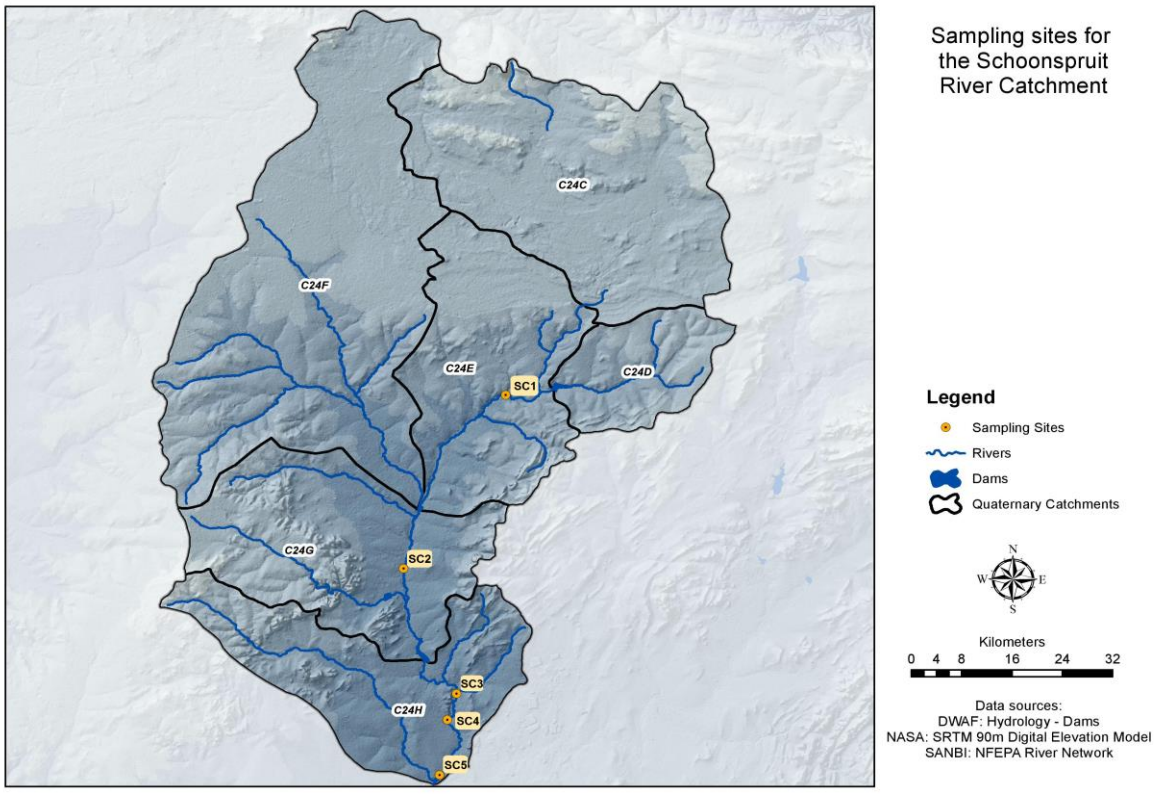

Figure 1.1: Geographical illustration of the Schoonspruit River system. The five sampling points are indicated on the map (SC1-SC5).

1.9.2. Crocodile River

The Crocodile River, Figure 1.2 below, has a catchment of 29 349 km^2 and forms part of the Crocodile (West) and Marico Water Management Area (WMA). This catchment is predominantly situated within the North West Province, with some part reaching Gauteng and the Limpopo Province (DWA, 2012). The Crocodile River is one of many stressed river systems in South Africa. This is mainly due to the increase of industrial and urban developments in this catchment, all of which are reliant on its water. The problem is further exacerbated by fluctuating weather patterns. All of these aspects result in a water shortage (DWA, 2012; DWAF, 2008). To relief some of this strain, water from the Upper Vaal WMA is relayed to the Crocodile River (DWA, 2012; DWAF, 2008). According to the River Health Programme (DEAT, 2005), the Crocodile River is in a very poor state, with elevated levels of organic pollutants and incidences of eutrophication in parts of the river. The Crocodile River is being predominantly exploited for agricultural, mining, industrial and urban uses (DWA, 2012; DAET, 2005). These activities are also responsible for diffuse pollution in the Crocodile River. Furthermore, point source pollution like sewage spills and treated waste also contributing to the decline in water quality in this river system (DWAF, 2007; NWREAD, 2014).

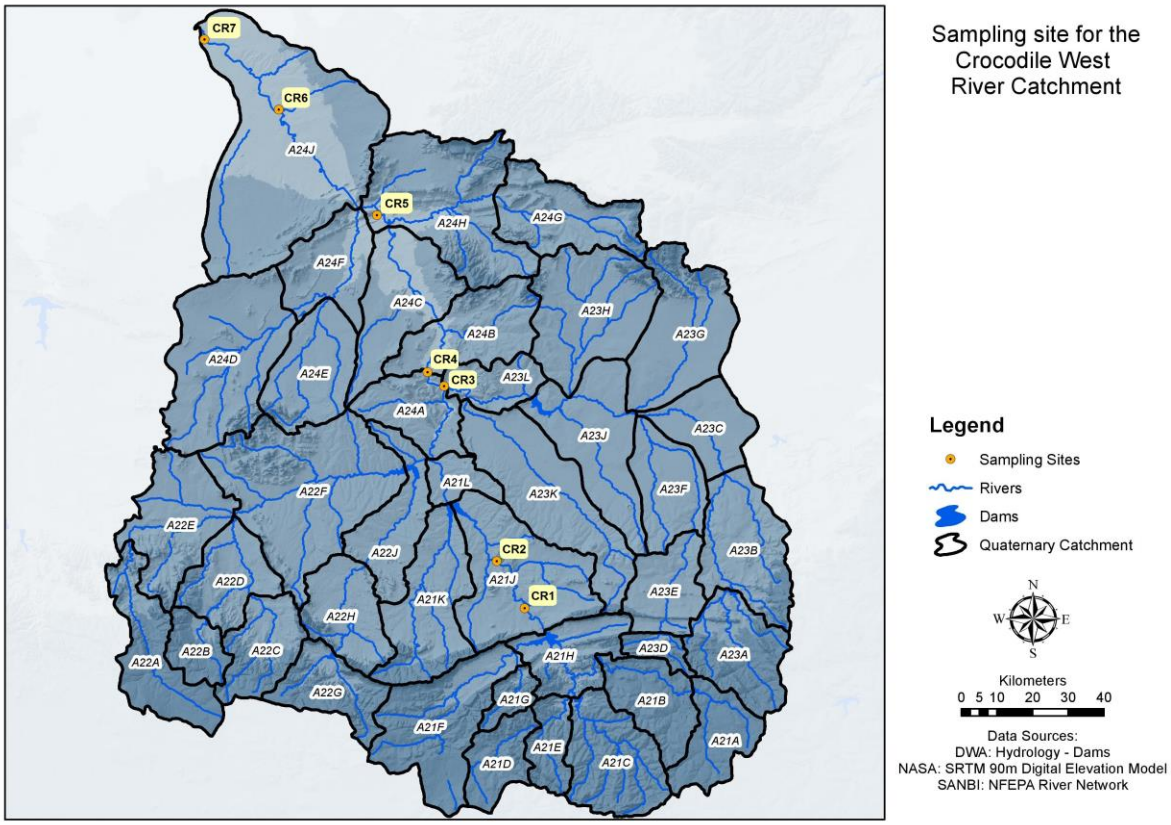

Figure 1.2: Geographical illustration of the Crocodile River system. The seven sampling points are indicated on the map (CR1-CR7).

1.9.3. Groot Marico River

Like the Crocodile River, the Groot Marico River (Figure 1.3) forms part of the Crocodile (West) Marico Water Management Area (WMA) (DWA, 2012). This river catchment covers an area of 12 049 km^2 and is similar to the Schoonspruit River since both these rivers originate from dolomite eyes (DWA, 2012; NWREAD, 2008). According to the River Health Programme (DAET, 2005), the overall state of the Groot Marico River is good, with no organic pollutant being present in the surface water. This is mainly due to the area surrounding the Groot Marico River being very much undeveloped, with no major towns nearby. However, a few farms and smaller rural settlements are present upstream from the river (NWREAD, 2008; DEAT, 2005). Additionally, this river is most probably the lone source of water for these residents (NWREAD, 2014). The little anthropogenic activity present results in natural vegetation and cattle grazing to be predominate around this area (NWREAD, 2008). The surface water of upper regions of the Groot Marico River is exploited for commercial irrigation and livestock watering. Consequently, these activities have started affecting the river system. Runoff from agricultural areas introduces fertilisers, insecticides and herbicides into the system, and there is also additional pressure on the dolomitic eye to restore the water volume (NWREAD, 2008; DEAT, 2005).

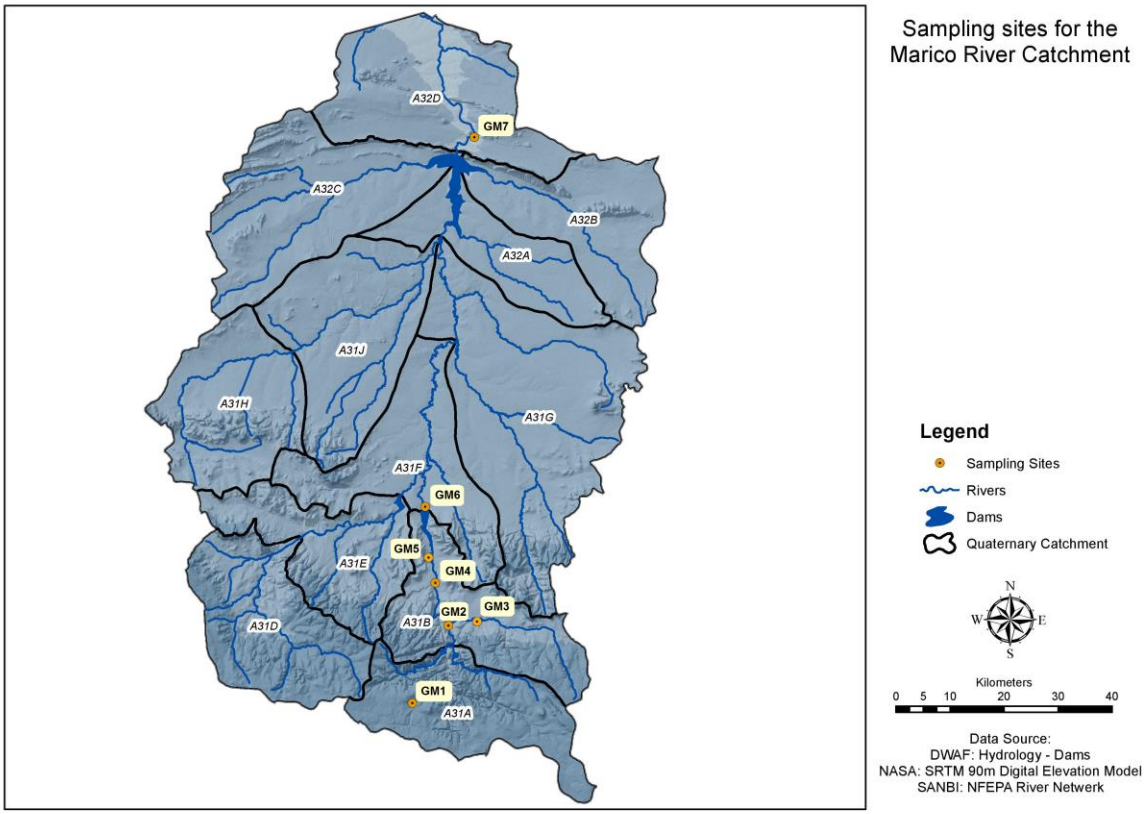

Figure 1.3: Geographical illustration of the Groot Marico River system. The seven sampling points are indicated on the map (GM1-GM7).

1.10. Methodology for isolation and culturing anaerobic *Clostridium* species

1.10.1. Tryptose sulphite cycloserine (TSC) Agar

There are different growth media available for isolating and enumerating *Clostridium* species, however, tryptose sulphite cycloserine (TSC) Agar has been found to yield the best results (Barrios *et al.*, 2013; Burger *et al*., 1984). Tryptose sulphite cycloserine Agar was first formulated in 1971 by Harmon and associates and is based on the reduction of sulphite, present in the media, to sulphide by anaerobic sulphite-reducing *Clostridium* (SRC) species (Barrios *et al*., 2013). Tryptose, yeast extract and soya peptone provide the essential vitamins and nutrients for SRC species to develop. Furthermore, sulphite-reducing indicators such as ferric ammonium citrate and sodium metabisulphite results in distinct black SRC colonies (HiMedia, 2015). With the addition of D-cycloserine, the growth of other facultative anaerobes is inhibited (Harmon *et al*., 1971). Although the International Organization for Standardization (ISO) recommends the use of TSC agar in the enumeration of *Clostridium* species in foodstuffs, various studies also support its use in isolating *Clostridium* species from environmental and clinical sources (Leja *et al*., 2014; Mubazangi *et al*., 2012; Kotsanas *et al*., 2010).

1.10.2. Fung double tube

The Fung double tube (FDT) was developed in 1980 as a means of culturing and enumerating obligate anaerobes such as *Clostridium* species (Barrios *et al*., 2013). The FDT consists of 2 tubes, one test tube with a small diameter which is then inserted into a larger screw-capped test tube (Vijayavel *et al*., 2009). This unique method achieves anaerobiosis without any additional atmospheric generators or chambers, through simply forming a thin agar medium layer between the 2 tubes and leaving minimum headspace (Barrios *et al*., 2013). The FDT method has shown to deliver better results than that of traditional methods, is also more convenient, cost effective and time efficient (Fung, 2013; Barrios *et al*., 2013; Ruengwilysup *et al*., 2009). The FDT, in combination with TSC agar, have shown to be a very reliable method in isolating an enumeration *Clostridium* species from surface water and sediment (Vijayavel and Kashian, 2014; Vijayavel *et al*., 2009). Furthermore, it is currently the fastest method in detecting the presence of faecal bacteria in water, delivering results within 6 hours of incubation (Fung, 2013).

1.11. Antibiotic susceptibility testing of anaerobes

The purpose of performing susceptibility testing is to assess the response that bacteria have to antibiotics (Schuetz, 2014). Minimum inhibitory concentration (MIC) is the lowest concentrations of an antibiotic that inhibits the growth of an organism and is usually tested at

a 2-fold serial dilution (Schuetz, 2014; Coyle *et al*., 2005). There are various means of determining MIC values, for instance agar dilution, broth microdilution and commercial E-test strips (Brook *et al*., 2013). However, the Clinical and Laboratory Standards Institute (2014) recommends the agar dilution method, especially when working with anaerobes such as *Clostridium* species. This method is seen as the gold standard of which all other methods are measured against (Schuetz, 2014). It involves the agar medium in each plate being supplemented with a different concentration of the antibiotic tested against (Coyle *et al*., 2005). Also, the results obtained can be reproduced, which is a key advantage of this method (Rennie *et al*., 2012).

1.12. Molecular techniques

It has been reported that anaerobic bacteria, including *Clostridium*, are at present poorly characterized, with only 50-75% being adequately characterized. (Garcia *et al*., 2014; Song, 2005). This is mainly due to the conventional anaerobic bacteriological methods and phenotype tests available being insufficient. In addition, these types of analyses are complicated, laborious, expensive and not always reliable, for it is based on dated taxonomy (Valones *et al*., 2009).

The development of molecular methods, such as Polymerase Chain Reaction (PCR), have brought immense benefits and achievements in molecular biology (Valones *et al*., 2009). PCR has been described as an essential component in the molecular diagnostics of bacteria (Song, 2005). Furthermore, this method has been found to be very specific, sensitive and rapid, with no complex cultivation and added confirmation requirements (Romprè *et al*., 2002). Ever since its development in 1984, it has led to great scientific advances such as gene expression, genome sequencing and molecular genetics studies (Valones *et al*., 2009).

Various studies have described the use of PCR for the identification of bacteria by amplifying and sequencing the 16S rRNA gene (Jenkins *et al*., 2012; Petti, 2007). This gene is targeted because it is universally present in all prokaryotes (Jenkins *et al*., 2012). The 16S rRNA gene contains highly conserved sequences that are the same in all bacteria, but also numerous variable regions which are genus or species specific. Therefore, PCR primers can be designed to target the conserved regions of 16S rRNA genes, thereby amplifying the variable sequences of the gene (Jenkins *et al*., 2012; Song, 2005). These sequences can also be used to identify and characterize possible novel species, along with assessing relationships between bacteria (Song, 2005; Sacchi *et al.*, 2002).

1.13. Chapter summary

The literature overview revealed that water availability is a major problem in the North West Province, with the demand of water available exceeding the supply (NWREAD, 2014). Furthermore, diffuse and point source pollution result in the deterioration of surface water quality in this province (Colvin *et al*., 2016). Chemical compounds, pathogenic bacteria along with faecal contaminants are introduced into river systems through runoff from agricultural and industrial areas, as well as from the discharge of poorly treated effluent of WWTPs (Pandey *et al*., 2014; DWA, 2012; Kümmerer, 2009). Subsequently, all these pollution sources have been identified as contributors to the introduction of antibiotics and antibiotic resistant bacteria into the aquatic environments (O'Neill, 2016; Graham *et al*., 2014; Alonso *et al*., 2001). This is cause for concern, since many informal and rural settlements directly rely on these surface water systems (Colvin *et al*., 2016).

With known characteristics such as being able to grow under anaerobic conditions, across a broad range of temperatures and formation of tough endospores, *Clostridium* species can survive and flourish in various environments (Siegrist, 2011; Jones and Keis, 2005; Hippe *et al*., 1992). As shown in the literature, *Clostridium* species are ubiquitous, being present in various environmental sources such as soil, sediment, and water, to the gastrointestinal track and faeces of humans and animals (Siegrist, 2011; Haagsm, 1991). Furthermore, this genus encompasses a collection of toxigenic bacteria which causes serious infections and diseases through their ability to produce neuro-, entero-, and/or histotoxins (Sathish and Swaminathan, 2009; Borriello and Carman, 1988). However, *Clostridium* species have been shown in literature to be beneficial in various areas such as biofuel production and cancer therapy, as well as its use as an indicator organisms for determining the presence of pathogens and faecal pollution in water (Staedtke *et al*., 2016; Num and Useh, 2014; Mubazangi *et al*., 2012).

CHAPTER 2

Prevalence of sulphite-reducing *Clostridium* species, the potential health risks and its use as a faecal pollution indicator in selected surface water systems in the North West Province

2.1. Research rationale

The ongoing decline of surface water quality in South Africa and in particular the North West Province is a major problem and cause for concern. Both diffuse and point-source pollution are responsible for this decline (NWREAD, 2014). All these pollution sources enter the various river systems across the province, introducing pathogens and undesirable compounds from WWTPs, faecal matter as well as fertilizers and pesticides from agriculture (NWREAD, 2014; Awofolu *et al*., 2007). Also, during rainy occasions, the river systems are flushed with pathogens present in these surrounding areas due to watershed and sub-surface flow (Pandey *et al*., 2014). The presence of these pathogens and pollutants could potentially threaten the health of residents in the vicinity of the water sources as well as animals that drink this water.

The World Health Organisation (WHO, 2008) recommends the use of indicator organisms to determine the safety and quality of water. However, indicator organisms can serve various purposes, depending on the problem at hand (Abia *et al*., 2015b; Ashbolt *et al*., 2001). According to Ashbolt and associates (2001), they can function as process indicators (micro-organisms that shows how effective treatment processes are), faecal indicators (indicates faecal pollution) and index/model indicators (indicates the presence of pathogens). A number of indicator organisms are commonly employed to screen for faecal pollution in surface water, including *Escherichia coli*, faecal streptococci, total and faecal coliforms (Abia *et al*., 2015b; Griffin *et al*., 2001). However, several studies have found shortcomings among these faecal indicator organisms (FIO) (Figueras and Borrego, 2010; Ferguson *et al*., 1997). This shows that the current FIO are flawed and relying on just one FIO could be insufficient (Wu *et al*., 2011; Tyagi and Chopra, 2006).

Several studies have supported the use of sulphite-reducing *Clostridium* (SRC) species as a FIO (Abia *et al*., 2015b; Mubazangi *et al*., 2012; Vijayavel *et al*., 2009; Fujioka and Shizumura, 1985; Sartory, 1985). The genus *Clostridium* mostly comprises of opportunistic pathogens and have been associated with various human and animal diseases (Payment *et al*., 2002). Anaerobic SRC species, such as *Clostridium perfringens*, are commonly found in

faeces of both human and warm-blooded animals, but also in wastewater (Mubazangi *et al*., 2012; Siegrist, 2011). They have the ability to produce endospores which are highly resistant to wastewater treatment processes and harsh environmental conditions (Tyagi and Chopra, 2006; Davies *et al*., 1995). Although they cannot reproduce in aquatic environments, SRC species will remain present in the environment for a longer period than conventional FIO, making it a suitable indicator for both past and recent faecal pollution (Graziano *et al*., 2007; Davies *et al*., 1995). Additionally, SRC species have also been proven useful as model indicators to determine the presence of pathogenic protozoans and viruses, such as *Giardia* cysts and *Cryptosporidium* oocysts (Mubazangi *et al*., 2012; Tyagi and Chopra, 2006). Besides the WHO recommendation to use SRC species, such as *Clostridium perfringens*, as a suitable faecal indicator for water quality assessments, the European Union and the State of Hawaii have also adopted SRC species as an additional indicator for water quality assessment (Mubazangi *et al*., 2012; Griffin *et al*., 2001).

In a South African context, water quality is determined by measuring the physico-chemical parameters and indicator organisms such as *Escherichia coli*, faecal streptococci, total and faecal coliforms (DWAF, 1996). Even though there have been numerous studies done across the country that support the use of SRC species as a faecal indicator in sediment and wastewater, few studies have investigated its presence and potential impacts on surface water (Abia *et al*., 2015a; Mubazangi *et al*., 2012; Potgieter *et al*., 2006; Sartory, 1988).

Thus, the aim of this study was to determine the presence of sulphite-reducing *Clostridium* spp. in selected surface water systems in the North West Province and evaluate its use as a faecal pollution indicator, as well as the potential health risks associated with these micro-organisms. The specific objectives included: (I) to evaluate the use of sulphite-reducing *Clostridium* species as a faecal pollution indicator; (II) to determine if there are correlations between the levels of *Clostridium* species and the physico-chemical parameters influenced by seasonal variation through RDA; (III) to identify the *Clostridium* species isolated using Gram reaction, endospore staining and 16S rDNA sequencing; (IV) and to evaluate the potential associated health risks *Clostridium* species can cause.

2.2. Material and methods

2.2.1. Preparation of media and broth

Selective growth media were used, namely Reinforced Clostridia agar (Oxoid; UK) and tryptose sulphite cycloserine (TSC) agar (Oxoid; UK). Agar is a solid medium and was prepared according to the manufacturer's instructions. The TSC media was used for the cultivation and enumeration of *Clostridium perfringens* and other sulphite-reducing

Clostridium species. *Clostridium* spp. produces black colonies in the media as a result of the sulphite reduction indicators, sodium metabisulphite and ferric ammonium citrate. Reinforced Clostridia agar (Oxoid; UK) was used to purify the isolates by streak plating.

2.2.2. Sampling

Three surface water systems were investigated in the North West Province, South Africa, namely the Crocodile River, Groot Marico River and the Schoonspruit (Figures 1.1-1.3 in Section 1.9). A total of 19 sites (Crocodile = 7 sites; Groot Marico = 7 sites; Schoonspruit = 5 sites) were sampled. Water from these sites were collected during the warm and rainy season, between March and May, and again during the dry and cold season, June to August, in 2015 and 2016. Samples were collected aseptically (Molale, 2012) and immediately placed on ice. Laboratory analysis of the samples took place within 8 hours of sampling. Coordinates and names of all the sites are listed in Table C1 in Appendix C.

2.2.3. Physico-chemical parameters

The physical water quality parameters, such as pH, temperature (°C), salinity (ppm) and total dissolved solids (ppm), were measured on site using the Oakton PCS testr™ 35 waterproof field multi-parameter probe (Thermo Fisher scientific, US) and dissolved oxygen (mg/L) was measured with a multi-parameter probe (Eutech Instruments, Singapore), both according to the manufacturer's instructions. The chemical parameters, such as chemical oxygen demand (COD), Nitrate (NO_3^-), Nitrite (NO_2^-), Phosphates (PO_4^-) and Sulphate (SO_4^{2-}) were measured in mg/L in the laboratory with the use of the HACH DR 2800™ instrument (HACH, Germany).

2.2.4. Determining Colony Forming Units (CFU) using Fung double tube method

A modified version of the Fung double tube method, as described by Barrios and co-workers (2013), was used and each water sample was analysed in triplicate. A capped test tube (16 mm x 125 mm; Pyrex) was filled with 7 ml of double strength *Clostridium perfringens* agar base and autoclaved. When the test tubes containing the liquefied media cooled down to approximately 50°C, one (1) ml of sample and 32 µl of the TSC supplement containing D-cycloserine (Oxoid; UK) were mixed with the liquefied agar. An autoclaved inserter test tube with a diameter of 8 mm was inserted into the Pyrex test tube and sealed with the cap. This created favourable anaerobic conditions. The test tube was then incubated for 6 hours (to prevent overgrowth) at 44°C. The black colonies were counted and documented as CFU/ml water. Tubes with more than 300 colonies were considered as too numerous to count and for statistical analysis, these tubes were given a value of 300 colonies (White *et al*., 2010).

2.2.5. Isolation of *Clostridium* species

The *Clostridium* spp. were isolated from the Fung double tubes by emptying the content of the tubes into an empty sterile petri dish. Sterile wooden picks were used to pierce the black colonies in the agar and streaked onto TSC agar plates. The plates were then placed in an AnaeroJar (AG0025; Oxoid), with an AnaeroGen sachet (AN0025; Thermo Scientific) and anaerobic indicator (BR0055B; Oxoid). The plates were incubated for 24 hours at 44°C. To ensure purity, these isolates were subcultured a total of three times on Reinforced Clostridia agar.

2.2.6. Primary phenotypical characterization

Gram staining of each isolate was done to determine morphology and whether the isolate was Gram-positive or Gram-negative. Bacterial smears were made from overnight cultures and then stained with a drop of 1% Gram's crystal violet of 60 seconds, rinsed with water, stained with a drop of Gram's iodine again for 60 seconds, destained with acetone alcohol and lastly counterstained with Gram's safranin for 60 seconds and rinsed with water (Pandolfi and Pons, 2004). The Schaeffer and Fulton's method for endospore staining was also performed to determine if the isolates produced endospores (Salle, 1973). According to Stackebrant and Rainey (1997), the expected results for *Clostridium* spp. are rod shaped, Gram-positive and produce endospores.

2.2.7. DNA isolation

DNA was isolated by means of colony-PCR, as described by Jordaan and Bezuidenhout (2016). In short, a single colony was carefully collected with a sterile wooden pick and resuspended in 10 µl of Milli-Q® water in a sterile 1.5 ml Eppendorf tube. The tube was then placed in a microwave for 2 min at 1 000 W, centrifuged for 90 seconds at 13 400 rpm and immediately placed on ice.

2.2.8. PCR amplification

A PCR was then performed where 2 µl of the supernatant of the process described in Section 2.2.7, was used as the DNA template. The PCR reaction was performed in a final volume of 25 µl, which consisted of (a) 12 µl 2X PCR Master Mix (contains 0.4 mM of each dNTPs, 0.05 U/µl Taq DNA Polymerase, reaction buffer and 4 mM $MgCl_2$) (Fermentas Life Science, US), (b) 9 µl Nuclease free water (Fermentas Life Sciences, US), (c) 1 µl of each primer (0.4 µM) and (d) 2 µl of the DNA template. The 27F (5'- AGA GTT TGA TCM TGG CTC AG- 3') and 1492R (5'- GG TTA CCT TGT TAC GAC TT- 3') (Inqaba Biotec; SA) 16S rDNA primers as published in Jiang and colleagues (2006) were used and has an amplicon size of about 1 465 bp (Weisburg *et al*., 1991). Using the ICycler Thermocycler (Bio-Rad,

US), the reaction started with a denaturing step of 94°C for 2 minutes, where after 35 cycles commenced. These cycles consisted of denaturing at 94°C for 30 seconds, annealing at 54°C for 60 seconds and extension at 72°C for 1 minute. The reaction was concluded by an additional extension at 72°C for 5 minutes.

2.2.9. Agarose gel electrophoresis

Gel electrophoresis was performed on PCR products. Five microliters (5 µl) of PCR product was mixed with 2 µl 6x Orange Loading Dye (Thermo Scientific; US) containing GelRed (Biotium, US) and loaded on a 1.5% (w/v) agarose gel. A 1 kb molecular weight marker (Fermentas; US) was used to confirm the size of the individual bands. Electrophoresis was performed at 80 V for 45 minutes using a Bio-Rad electrophoresis system for the determination of the product quality.

2.2.10. Sequencing and identification

All PCR products that were successfully amplified, were purified to remove all residual primer dimers by using a silica resin as described by Li and co-workers (2010). Briefly, (a) 150 µl of 6 M NaI was added to the 25 µl Sequencing PCR product, inverted and (b) 10 µl silica matrix (0.2 g Silica dioxide (Sigma-Aldrich; US) in 2 ml Milli-Q® water) was added and incubated at room temperature (28°C) for 5 min. (c) The mixture was then centrifuged for 15 seconds at 13 400 rpm and supernatant was removed by pipetting. (d) The pellet was then washed with 500 µl Washing Buffer (50 mM NaCl; 10 mM Tris pH 7.5, 2.5 mM EDTA, 50% v/v Ethanol and Milli-Q® water for a final volume of 50 ml). Both step C and D were then repeated, (e) centrifuged again for 1 min, afterwards removing all the supernatant by pipetting. (f) The pellet was then left to air dry for 5 min, (g) resuspended in 20 µl Milli-Q® water and then centrifuged for 2 min at 13 400 rpm. (h) The DNA eluate was then transferred to a new sterile PCR tube. DNA quantity and quality was determined by a NanoDrop 1000 Spectrophotometer (Thermo Scientific; US).

A Cycle Sequencing BigDye Terminator Kit (Zymo Research, US) was used to perform the sequencing PCR. The reaction mix consisted of: 4 µl 1:10 dilution Ready Reaction Premix (2.5x), 2 µl BigDye Sequencing Buffer (5x), 3.2 pmol 27F/1492R primer (Inqaba Biotech, SA), 1 µl Template DNA (10 ng) and 12 µl of nuclease free water (Fermentas Life Sciences, US) to make up a final volume of 20 µl. Cycling conditions for the Thermocycler (Bio-Rad, US) were set at 96°C for 1 minute initial denaturation that was followed by 25 cycles of 96°C for 10 seconds; 50°C for 5 seconds and 60°C for 4 minutes. A Zymo Research DNA-Sequencing Clean up kit™ (Zymo Research, US), was used to purify the sequencing PCR products following the manufacturer's protocol.

Amplicons were sequenced in-house, using an ABI 3130 Genetic Analyser (Applied Biosystems, UK). Chromatograms were obtained and then viewed in Geospiza Finch TV software (version 1.4; http://www.geospiza.com/ftvdlinfo.html). The sequences were then compared to known sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) and also to the EzTaxon database (http://www.ezbiocloud.net) to determine the identity of the amplified sequences.

2.2.11. Statistical analysis

All the statistical analyses regarding the physico-chemical parameters and levels of *Clostridium* spp. data (averages, standard deviation, minimum and maximum values) were done in Microsoft Office Excel 2013 and was a representation of triplicates. The data collected of both the physico-chemical and microbiological (sulphite-reducing *Clostridium* spp. and other indicator organisms) parameters were imported to Canoco for Windows 4.0 (Ter Braak and Smilauer, 1998) to create multivariate ordination diagrams, specifically Redundancy Analysis (RDA). These RDA depicts the correlation between the species and environmental data for different seasons in 2015 and 2016. Probability values lower than 0.05 (p-values ≤ 0.05) were considered statistically significant. MEGA 6 was used for the phylogenetic analyses (Tamura *et al*., 2013). A Neighbour-Joining tree, with a bootstrap of 1 000 replicates, was drawn using the Jukes-Cantor model to calculate evolutionary distance.

2.3. Results

2.3.1. Sulphite-reducing *Clostridium* (SRC) species relation to indicator organisms

Micro-organisms such as total coliforms, faecal coliforms, *E. coli* and faecal streptococci are used as an indicator of faecal contamination in water systems (Ashbolt *et al*., 2001). It has been suggested that sulphite-reducing *Clostridium* (SRC) species, like *Clostridium perfringens*, be used as a supplementary or even a surrogate indicator (Abia *et al*., 2015a; Mubazangi *et al*., 2012). These indicator organisms provide insight into the state and quality of the water systems (Ashbolt *et al*., 2001; Griffin *et al*., 2001). A Principal Component Analysis (PCA) was used to investigate if this proposed statement was plausible by determining any possible association between the levels of the different indicator organisms and the levels of SRC species. The microbiological parameters (Appendix A, Table A1 and A2) from the Schoonspruit, Crocodile and Groot Marico River for 2015 to 2016 was used in this analysis.

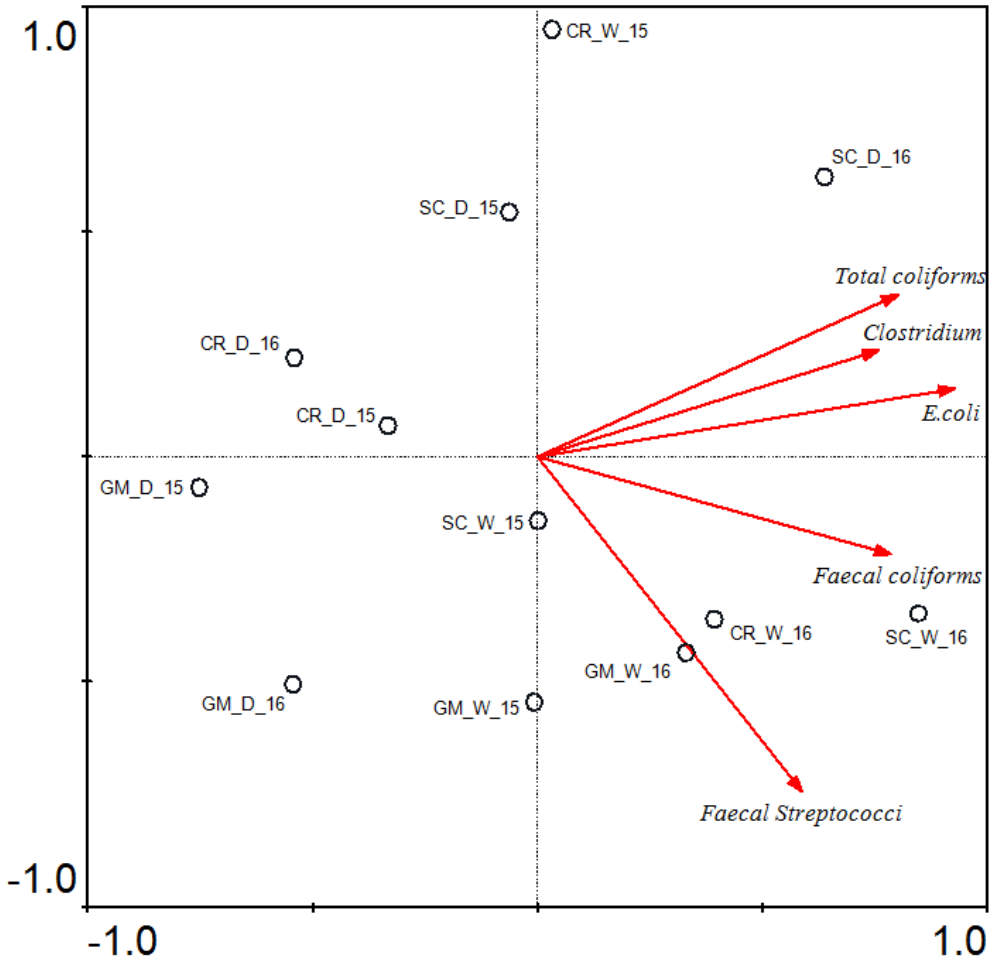

Figure 2.1: Principal Component Analysis (PCA) illustrating the association between the indicator organisms (total coliforms, faecal coliforms, *E. coli* and faecal streptococci) and the sulphite-reducing *Clostridium* species across the Schoonspruit (SC), Crocodile (CR) and Groot Marico (GM) Rivers during the warm-rainy (W), and cold-dry (D) seasons for 2015 and 2016. The microbiological parameters are indicated with red arrows and the surface water systems, along with the season and year are depicted in black circles.

A positive association can be observed between the levels of SRC (*Clostridium)* species and all the other indicator organisms, with total coliforms and *E. coli* being the most prominent and faecal coliforms and faecal streptococci being less so (Figure 2.1). The SRC species, along with *E. coli*, total and faecal coliforms had the greatest impact on the Groot Marico, Schoonspruit and Crocodile River during the warm-rainy season (2016), as well as the cold-dry season of the Schoonspruit in 2016. The cold-dry seasons of both the Crocodile and

Groot Marico in 2015 and 2016 were least impacted by the SRC species and the other indicator organisms.

2.3.2. Correlation between physico-chemical parameters and indicator organisms

To determine if the physico-chemical parameters of the surface water is directly related to the indicator organisms and sulphite-reducing *Clostridium* species, a redundancy analysis (RDA) ordination plot was constructed. The data compiled from the physico-chemical parameters over the four sampling runs for each river system was divided into two datasets, namely the warm-rainy season and the cold-dry season (Appendix A, Table A3 and A4).

The RDA results are presented in two segments, the one being for both sampling runs during the warm-rainy season and the other for the two runs during the cold-dry season. Both segments span over a duration of two years, 2015 to 2016, and was constructed by using the averages of the environmental and microbiological parameters of each river system for each season.

2.3.2.1. Warm-rainy season of 2015 and 2016

The RDA plot, shown in Figure 2.2, illustrates the correlation between the dominant environmental parameters and the indicator organisms and sulphite-reducing *Clostridium* species in the 3 surface water systems during the warm-rainy seasons of 2015 and 2016.

In Figure 2.2, a strong positive correlation can be observed between the SRC species and all the chemical parameters, such as phosphates, nitrates, sulphates and COD. These parameters had a great effect on the levels of SRC species in the Schoonspruit during the warm-rainy season in 2016, as it shows a noticeable positive correlation. The physical parameters also show a positive correlation to the SRC species, especially the temperature and, to a lesser extent, salinity, TDS and pH, respectively. When comparing the levels of the SRC species to the dissolved oxygen, a prominent negative correlation is noticed.

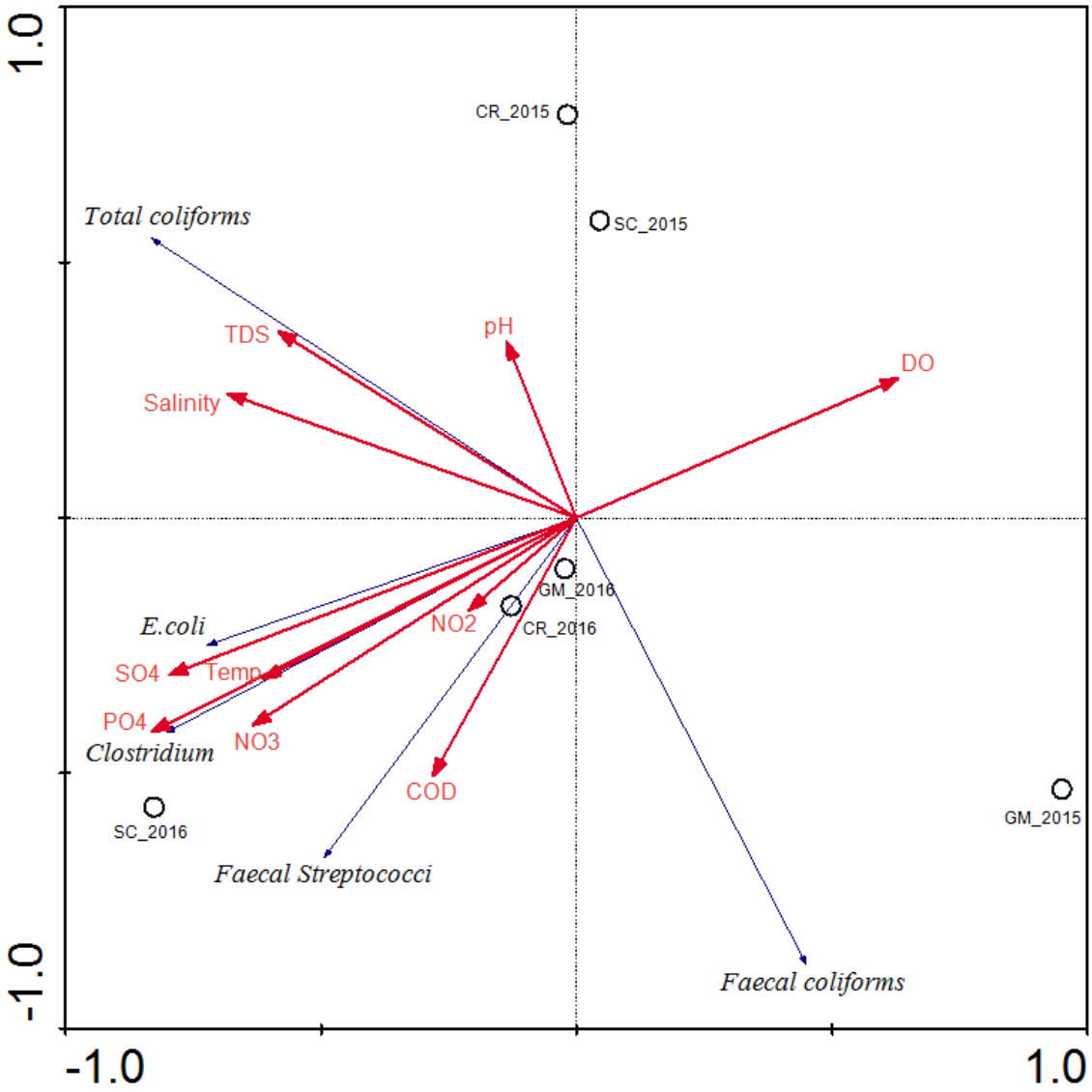

Figure 2.2: Redundancy analysis (RDA) triplot illustrating the correlation between the environmental parameters (pH, Temperature, TDS, Salinity, COD, DO, NO_2^-, NO_3^- and PO_4^{3}) and the microbiological indicators (*E. coli*, total coliforms, faecal coliforms, and faecal streptococci) and the sulphite-reducing *Clostridium* species levels, during the warm-rainy seasons of 2015 and 2016 from all 3 surface water systems. The red arrows represent the physico-chemical parameters, whereas the blue arrows represent the species.

The other indicator organisms, namely *E. coli* and faecal streptococci, show a similar trend to that of the sulphite-reducing *Clostridium* species, seeing that they indicate a positive correlation to all the chemical parameters and temperature, and a negative correlation to the dissolved oxygen. The total coliforms show a strong positive correlation to the TDS, salinity and pH, whereas the faecal coliforms indicate the exact opposite to these physical parameters.

2.3.2.2. Cold and dry season of 2015 and 2016

The RDA plot below (Figure 2.3) shows the correlation between the physico-chemical parameters and the levels of sulphite-reducing *Clostridium* species along with the indicator organisms in the Schoonspruit, Crocodile and Groot Marico Rivers for the duration of the cold-dry seasons in 2015 and 2016

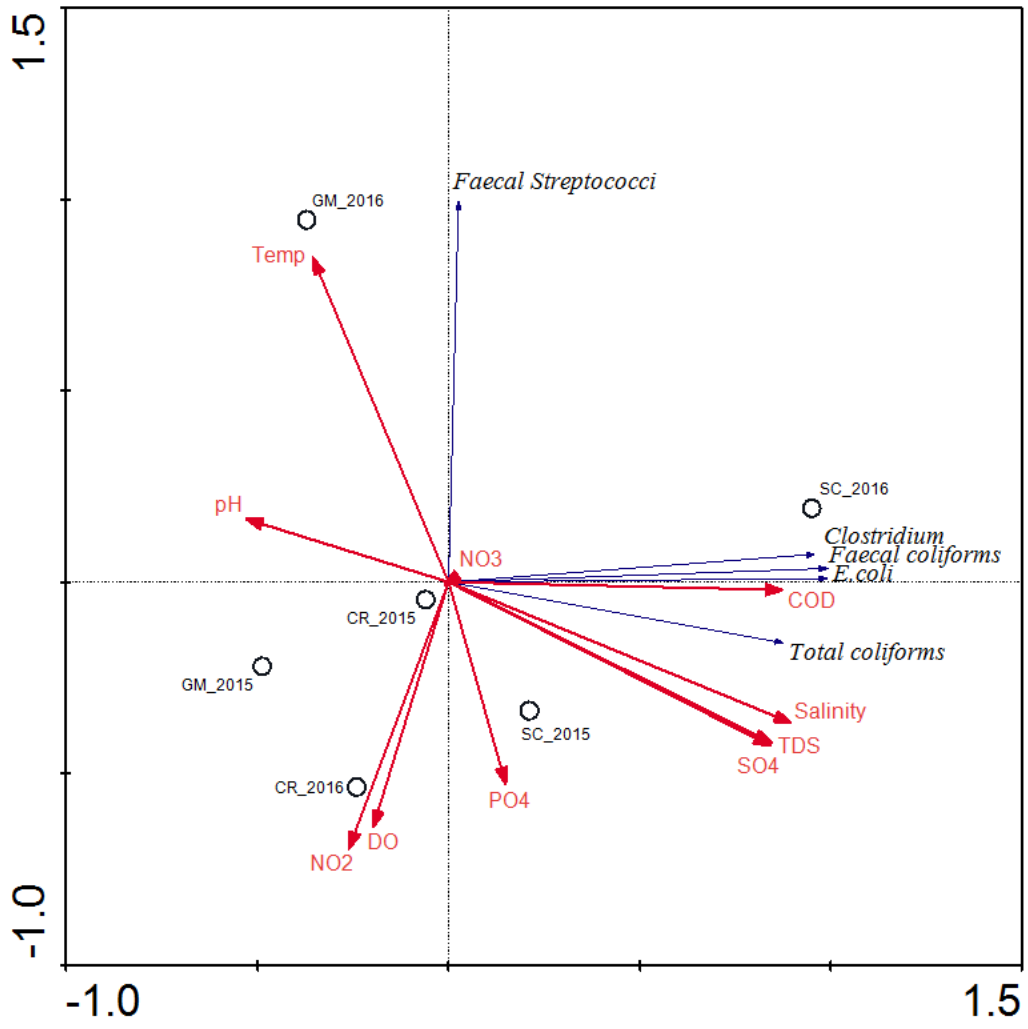

Figure 2.3: Correlation triplot showing the association between the environmental parameters (pH, Temperature, TDS, Salinity, COD, DO, NO_2^-, NO_3^- and PO_4^{3-}) and the microbiological indicators (*E. coli*, total coliforms, faecal coliforms, and faecal streptococci) along with the sulphite-reducing *Clostridium* species levels, for the cold-dry seasons (2015 and 2016) of all 3 surface water systems. The physico-chemical parameters are depicted in red, while the species are in blue.

The levels of sulphite-reducing *Clostridium* species display a positive correlation to the physical properties of the surface water, specifically salinity and TDS. A strong positive correlation can also be observed among the SRC species levels and the COD, but also sulphates and phosphates to a lesser extent. The SRC species showed a broad negative correlation to the temperature, pH, dissolved oxygen, nitrates and nitrites. All the coliforms followed a similar trend to the SRC species, whereas the faecal streptococci illustrated a positive correlation to temperature and pH and a negative correlation to the rest of the environmental parameters. The correlation triplot also demonstrated that the Schoonspruit River was mostly affected by the SRC species and all the coliforms in 2015 and 2016.

2.3.3. Identification and conformation of potential *Clostridium* isolates

2.3.3.1. Primary phenotypical characterisation

All information regarding the Gram staining, Endospore staining and identity of all isolates used in this chapter is depicted in Table A5 and A6 in Appendix A. These tables also show the surface water system and the specific sampling site where each isolate was obtained from. A total of 42 isolates were obtained from the Schoonspruit, Crocodile and Groot Marico Rivers, collectively during both warm-rainy seasons of 2015 and 2016. Results showed Gram-positive rods for all these isolates. The majority of isolates were endospore formers with the exception of 2 isolates. These 2 isolates were included for further identification because there are some *Clostridium* strains that are known to be non-endospore formers. The 67 isolates from the cold-dry seasons of 2015 and 2016 were also subjected to the same process and showed that all isolates were pure, Gram-positive rods and endospore formers.

2.3.3.2. Identification by 16S rDNA

To confirm their identity, the DNA of each pure isolate was extracted and the 16S gene was amplified by means of polymerase chain reaction (PCR) and sequenced. Figure 2.4 below shows a 1.5% (w/v) agarose gel of 27 isolates that were successful in the 16S rDNA amplification, with a no template DNA control in lane 28 (NT). All amplicons were at an expected size of approximately 1 465 bp, with no non-specific bands and primer dimers present. No contamination was found as supported by the absence of a band in the no template DNA control. The 16S rDNA amplification of all 109 isolates were successful. All amplicons were then purified and sequenced as described in Section 2.2.10.

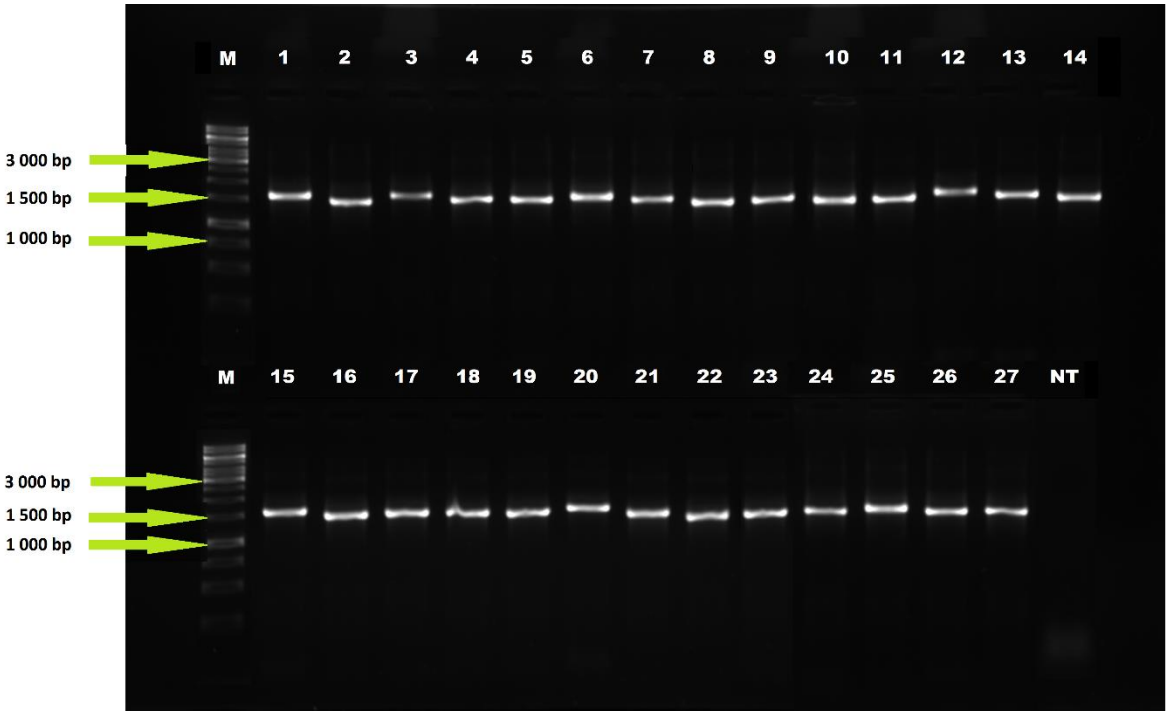

Figure 2.4: Gel electrophoresis of a 1.5% (w/v) agarose gel with 27 successful 16S rDNA amplifications with the expected size of 1 465 bp in lane 1-27. The lanes marked M and NT shows a 1 kb molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US) and the no template DNA control, respectively.

The chromatograms obtained through sequencing were viewed in Finch TV and analysed by BLAST to determine their identity. Identities of all isolates are summarised in Table A5 (warm-rainy seasons) and A6 (cold-dry seasons) in Appendix A. These tables also include additional information such as the isolate ID, accession number from GenBank, E-value, and the percentage (%) similarity. The closer the E-value is to zero, the greater the similarity is between the obtained sequence and that of the NCBI database match (http://www.ncbi.nlm.nih.gov/).

There was a total of 7 different *Clostridium* species identified during this study, namely *Clostridium bifermentans*, *C. perfringens*, *C. sordellii*, *C. ghonii*, *C. lituseburense*, *C. dakarense* and *C. baratii*. Table 2.1 below shows the abundance and distribution of each species during the warm-rainy (W/R) and cold-dry (C/D) seasons in the Crocodile, Groot Marico and Schoonspruit Rivers for 2015 and 2016, collectively. The most recurring species that were isolated during the warm-rainy season were *C. bifermentans* (76%), *C. perfringens* (7%) and *C. sordellii* (7%), *C. baratii* (5%) and *C. lituseburense* (2%). The cold-dry seasons showed a similar trend with *C. bifermentans* (57%) being the most dominant again, followed

by *C. perfringens* (13%), *C. sordellii* (12%), *C. ghonii* (3%), *C. lituseburense* (3%) and *C. baratii* (3%).

Table 2.1: Distribution of *Clostridium* species in the 3 surface water systems during the warm-rainy (W/R) and cold-dry (C/D) seasons for 2015 and 2016, collectively.

Surface water system	**Seasons**	***Clostridium* species**						
		C. bifermentans	*C. perfringens*	*C. sordellii*	*C. lituseburense*	*C. ghonii*	*C. dakarense*	*C. baratii*
Crocodile	W/R	9	2		1		2	
	C/D	26	4	2	2			1
Groot Marico	W/R	11						
	C/D	6						
Schoonspruit	W/R	12	1	3				
	C/D	6	5	5		2		1
Total		**70**	**12**	**10**	**3**	**2**	**2**	**2**

The Crocodile River had a greater diversity of *Clostridium* species with *C. bifermentans*, *C. perfringens*, *C. sordellii*, *C. lituseburense*, *C. dakarense* and *C. baratii*. It was also the only surface water system where *C. dakarense* was present. The Schoonspruit River was the second diverse with the absence of *C. lituseburense* and *C. dakarense*, and the addition of *C. ghonii*, which was solely isolated from this system. *Clostridium bifermentans* was the only species isolated in the Groot Marico River, showing the lowest diversity of all the surface water systems.

2.3.4. Phylogenetic analysis

A phylogenetic tree illustrates the evolutionary relation among different species and their common ancestors (Hall, 2013; Baum, 2008). Figure 2.5 shows a Neighbour-Joining phylogenetic tree of *Clostridium* species (CGON, CSOR, CBIF, CLIT, CBAR, CPER and CDAK) isolated from all 3 surface water systems for 2015 and 2016.

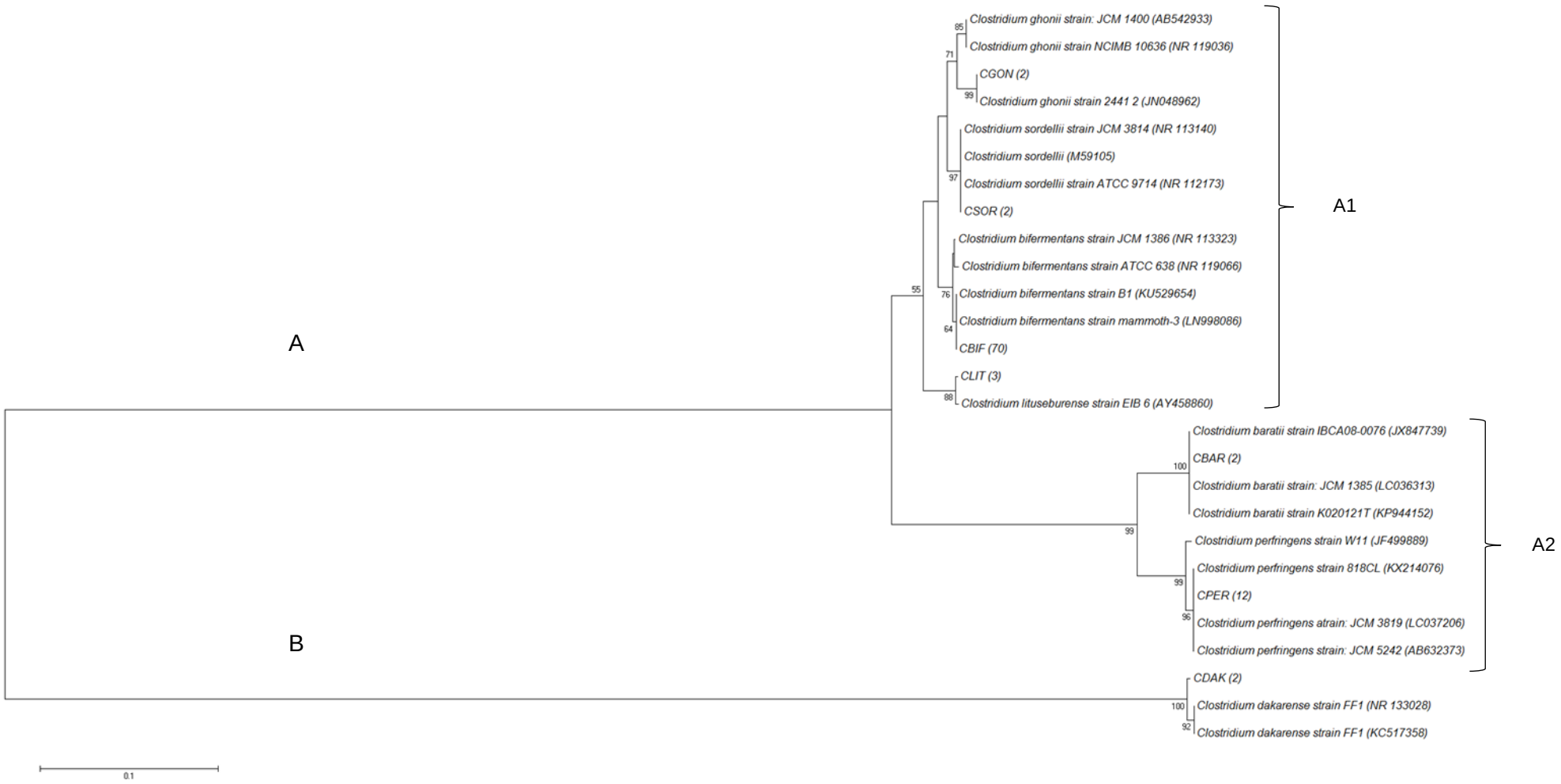

Figure 2.5: Neighbour-Joining tree showing the phylogenetic relationship of GenBank sequences and the sequences of *Clostridium* species isolated from 3 surface water systems in 2015 and 2016. The tree is constructed from partial 16S rRNA gene sequences. The Jukes Cantor model and 1000 bootstraps were used to generate this tree in MEGA 6.0. Percentages are indicated at the branching points of the dendrogram.

The number in brackets represents the number of isolates that were identified as the same *Clostridium* species in this study (Table 2.1). Reference 16S rRNA gene sequences of the main identified *Clostridium* species, along with their accession numbers, were exported from GenBank (NCBI) and are included in the analysis to show their phylogenetic relation with the isolated *Clostridium* species. Supporting bootstrap probability values are shown at the branch nodes.

The tree in Figure 2.5 is divided into 2 clusters (A and B), whereas cluster A is again divided into 2 sub-clusters, namely A1 and A2. When studying cluster A, the sub-cluster A1 comprises of *C. ghonii*, *C. sordelli, C. bifermentans* and *C. lituseburense* species. Sub-cluster A2 consists of *C. perfringens* and *C. baratii*. Cluster B solely consist of *C. dakarense*. The sub-cluster A1 showed a common ancestor to sub-cluster A2, while the length of the branch in cluster B indicates that it is more distantly related to those of sub-clusters A1 and A2.

The 70 isolates identified as *Clostridium bifermentans* (CBIF) in this study, showed relation to confirmed *Clostridium bifermentans* strains (LN998086 and KU529654). This is supported by the 64% bootstrap (Figure 2.5). The 96% bootstrap supports the phylogenetic similarity between *Clostridium perfringens* (CPER) isolates and the GenBank sequences of this species (LC037206 and AB632373). Both *Clostridium dakarense* (CDAK) and *Clostridium baratii* (CBAR) showed 100% bootstrap confidence amongst their affiliated species sequences. Furthermore, high bootstrap support (99-88%) was also observed for *Clostridium ghonii* (CGON), *Clostridium sordelli* (CSOR) and *Clostridium lituseburense* (CLIT) isolates when compared to the GenBank sequences.

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2.4. Discussion

2.4.1. The use of sulphite-reducing *Clostridium* (SRC) species as a faecal pollution indicator organism

To support the use of sulphite-reducing *Clostridium* (SRC) species as an indicator organism for faecal contamination in surface water, it was important to determine if these SRC species were present, along with the other indicator organisms (*E. coli*, total coliforms, faecal coliforms, and faecal streptococci), in the surface water systems. The levels of the SRC were then compared to the levels of the other indicator organisms acquired across all the sites in the Schoonspruit, Crocodile and Groot Marico from 2015 and 2016 (Table A1 and A2 in Appendix A).

During the course of this study, the Schoonspruit River had the highest recorded levels of sulphite-reducing *Clostridium* species, *E. coli*, total coliforms and faecal coliforms, all of which were most abundant at site 4. The Crocodile River also showed elevated levels of SRC species and indicator organisms, with an increase specifically at site 2. Both these sampling sites are situated downstream from local wastewater treatment plants (WWTP) in those areas.

Studies have found that WWTPs in South Africa contribute to the decline in microbial quality in rivers (Teklehaimanot *et al*., 2014; Gemmell and Schmidt, 2013). The Green Drop certification program was adopted by South Africa in 2008 as an incentive-based regulation model to recognise, reward and resolve noncompliance and encourage development in the wastewater sector. The 2014 Green Drop Report indicated an increase from 73% to 86% of the wastewater treatment plants in the North West Province which do not comply with the quality standard set for the final wastewater effluent (DWA, 2014). The province has 27 wastewater treatment plants that are deemed as high risk, which includes the 2 treatments plants near the Schoonspruit and Crocodile Rivers. Wastewater treatment plants that are not performing at optimal functionality can lead to partly treated waste or even raw waste being introduced into the surface water systems (Abia *et al*., 2015b; Teklehaimanot *et al*., 2014).

According to the State of Water Resources report from the Department of Water and Sanitation (DWS, 2014) the country is facing surface water quality challenges specifically due to faecal pollution. The data from this study for the Crocodile and Schoonspruit Rivers are proof of this. With the high levels of SRC species, alongside the other indicator organisms present at these sites where faecal contamination is clearly present, it can be suggested that SRC species are a viable option for indicating faecal pollution in surface waters.

High levels of sulphite-reducing *Clostridium* spp. and other indicator organisms were also recorded at sites further downstream in the Crocodile River. These sites in the Crocodile River are impacted by various anthropogenic factors such as agricultural activities. According to the Department of Water Affairs and Forestry (2008), the Crocodile River is surrounded by various citrus orchards and vegetable farms. A study by Paulse and colleagues (2009) found that agriculture is a contributor to faecal contamination in surface waters. Fertilisers are used to promote crop yield, but due to runoff from farms, it finds its way into the surrounding water systems. Interestingly, the second last site in the Crocodile River showed a decrease in the levels of all indicator organisms except for the SRC species, which showed an increased. Similar results were obtained by Abia and co-workers (2015b). The higher levels of *Clostridium* species in surface water can be attributed to its spore forming ability. This allows *Clostridium* species to survive for longer time periods than other indicator organisms, which furthermore makes it a better indicator for past faecal pollution (Robles *et al*., 2000). In these situations, solely relying on SRC species could indicate a false positive result of recent faecal pollutants.

The surface water system with the lowest levels of SRC species was the Groot Marico River. In 2005, the Department of Environmental Affairs and Tourism reported that the Groot Marico was in a good, natural state (DEAT, 2005). The surface water was of decent quality, showing no organic pollutants. They also went on by describing the area surrounding the river. It is mainly covered with forest, thicket and bushland, with a low population density and recreational activities, like diving and camping taking place at the upper reaches of the river. The absence of anthropogenic activity such as agriculture or sanitation in the lower parts of the Groot Marico is depicted by the low, to no levels of SRC species in the surface water. These results are expected since there are no reasons for faecal pollution at these sites, except for minor contributions that the animal inhabitants present may have. The increase in both SRC species and the indicator organisms at the upper reaches of the Groot Marico River could be due to the interaction between humans and the river for recreational reasons, and runoff from the surrounding areas.

2.4.2. Seasonal variation on the levels of sulphite-reducing *Clostridium* species and other indicator organisms

An interesting observation concerning the levels of SRC species and indicator organisms was that they differed between seasons, with the lowest being during the cold-dry season. Both the Crocodile and Groot Marico Rivers showed this phenomenon. This phenomenon could be explained by the influence of seasonal rainfall.

Rainfall has for long been known to have an impact on water quality, both positively and negatively (Lucas *et al*., 2014; Biggs *et al*., 2013). The rain contributes to the volume of water present in the river, and with this addition of water, it dilutes any contaminants that may be present (Sibanda *et al*., 2013). In the present study the rainfall had a negative effect on the Crocodile and Groot Marico Rivers. Rainfall is a promoter for non-point pollution sources, such as surface runoff from storm water, feedlots, landfills and agriculture (Samie *et al*. 2009). Surface runoff is therefore a major cause of pollution in water (Li *et al*., 2016). During the rainy seasons, the storm water flushes out all the pollutants present on the surrounding surfaces into the nearest water system, either directly or through storm drains (Zhang *et al*., 2013). This could explain the higher levels of SRC species and indicator organisms during the warm-rainy seasons.

The levels of SRC species and other indicator organisms are relatively low throughout the Groot Marico River, especially during the cold-dry seasons. However, when comparing the levels of SRC species to that of the other indicator organisms during the warm-rainy season, they show an unbalanced distribution. The high rainfall during the warm-rainy season introduces faecal contaminants, which may have been present in the surrounding environments, into this surface water system. The lower parts of the Groot Marico River are surrounded by vegetation, ruminant animals and birds that utilize its water with little anthropogenic activities (DEAT, 2005). Several studies have found that faeces of herbivores, much like those present around this river system, do not harbour SRC species such as *Clostridium perfringens* (Vierheilig *et al*., 2013; Mueller-Spitz *et al*., 2010). The presence of these herbivores could possibly be the source of faecal pollutants in this river due to the surface runoff that occurs during warm-wet seasons (Vierheilig *et al*., 2013). This could also explain the deviation between the levels of the SRC species and other organisms. This absence could limit the use of SRC species as a sole faecal pollution indicator in surface water of the Groot Marico River.

However, the last sampling sites which are situated around the upper parts of the Groot Marico showed an increase in SRC species alongside the other indicator organisms. These sites are affected by various human and recreational activities which are usually associated with warmer seasons and with that comes more pollutants that may be introduced into the surface water system, resulting in a higher level of SRC species and indicator organisms (Zhang *et al*., 2013; DEAT, 2005).

According to Sibanda and co-workers (2013), higher levels of micro-organisms during the warm-rainy season is the norm and should be expected when compared to the cold-dry seasons. Any deviation from this trend is seen as unusual. The level of SRC species and indicator organisms are very comparable during these two seasons, which it is not supposed to be. Teklehaimanot and associates (2014) suggested that the levels of faecal pollutants in the surface water systems, due to the seasonal surface runoff, can be exasperated by the contamination from point source pollution such as wastewater or industrial effluent. This occurrence can, once again, be linked to the WWTP situated along the Schoonspruit River.

2.4.3. Influence of seasons on *Clostridium* species with regards to physico-chemical parameters

The physico-chemical parameters of surface water have a great impact on the micro-organisms that are found in it (Jordaan and Bezuidenhout, 2013). It is one of the controlling factors that determines the composition and distribution of micro-organisms (Basu *et al*., 2013), thus affecting the levels of species such as *Clostridium* in these water systems. These parameters are greatly influenced by seasonal variation throughout the year, along with anthropogenic factors such as agriculture and mining (Shabalala *et al*., 2013; Raibole and Singh, 2011).

According to Pachepsky and co-workers (2014), temperature is the most essential physical parameter that controls the survival and growth of micro-organisms in the aquatic environments. Temperature had a great impact on the levels of *Clostridium* species. During both warm-rainy seasons, and the higher temperatures, higher levels of *Clostridium* species were enumerated than during the lower temperatures of the cold-dry season. According to Chapman (1996), the temperature of surface water usually ranges between 0°C and 30°C, and is influenced by the seasons and time of day. Furthermore, temperature also has an impact on other environmental parameters such as the dissolved oxygen. With an increase in water temperature, the solubility of gasses such as oxygen decreases. This combination could potentially explain the higher levels of *Clostridium* species enumerated during the warm-rainy season, because they are able to grow at a broad spectrum of temperatures, but flourish in oxygen deprived environments (Hippe *et al*., 1992).

As shown Figure 2.2, nutrients such as nitrates (NO_3^-) and phosphates (PO_4^-), along with sulphate (SO_4^{2-}) salts had an effect on the levels of *Clostridium* species. All these compounds were more positively correlated with the levels of *Clostridium* species during the warm-rainy season than the cold-dry season (Figure 2.3). The strong positive correlation between the *Clostridium* and phosphate can possibly be due to point source pollution from

inadequately treated effluent of WWTP, since it has been reported to be a major source of both *Clostridium* and phosphate in surface water systems (Colvin and Burns, 2011). Elevated levels of phosphates, nitrates and sulphates in surface water can also be attributed to non-point pollution, such as runoff from agricultural, urban and mining areas (Ambujam and Sudha, 2016; Chen *et al*., 2015). There is an increase in urbanisation, mining and industrial development near these surface water systems, some of which are illegal and unmonitored (DWA, 2013). Rainfall during the warm-rainy season increases the runoff from these settlements into the aquatic environments (Sibanda *et al*., 2013). This rainfall runoff may contain high levels of these nutrients and salts, resulting in elevated levels of these compounds in river systems during this period. Previous work states the abundance of phosphates and nitrates in rivers, along with elevated surface water temperature, can result in eutrophication (Minaudo *et al*., 2015). This lowers the dissolved oxygen levels in the river, establishing microaerophilic conditions for *Clostridium* to grow (Marcheggiani *et al*., 2008). The combination of all these incidences are in agreement with the findings in this study, seeing that several parts of the Crocodile and Schoonspruit Rivers have been reported as eutrophic (DEAT, 2005; DWAF, 2009).

With the regards to the chemical oxygen demand (COD), Figure 2.2 and 2.3 shows a positive correlation between COD and the *Clostridium* species during both the warm-rainy and cold-dry seasons. According to Alam (2015), COD values reflect the values of organic pollutants in water. Thus, the higher the COD values are, the more organic pollutants are present in the water. Several studies have found *Clostridium* species to be able to decontaminate organic pollutants which are present in the aquatic environment (Chen *et al*. 2005; Wang *et al*., 2003). This is done through fermentative hydrogen production, where *Clostridium* spp. utilize the organic substrates to produce hydrogen (H_2) (Chen *et al*. 2005; Wang *et al*., 2003). Consequently, the positive association between the COD levels and *Clostridium* spp. in this study could be explained by this process.

2.4.4. *Clostridium* species diversity in surface water and associated diseases

A total of 7 different *Clostridium* species were identified in this study by 16S rDNA sequencing. These included both enterotoxic and histotoxic clostridia, such as *Clostridium bifermentans*, *C. perfringens*, *C. sordellii*, and neurotoxic Clostridia, like *C. baratii* (Popoff and Bouvet, 2013; Songer, 2010; Montecucco *et al*., 2006). Other species identified were *C. ghonii*, *C. lituseburense* and *C. dakarense*. Similar studies done have found these species in the aquatic environment, with exception of *C. lituseburense* and *C. dakarense* (Leja *et al*., 2014; Sartory *et al*., 2006).

Clostridium bifermentans was the only *Clostridium* species to be identified in all 3 surface water systems and was the most abundant of all the species. This species of *Clostridium* is known to inhabit the intestinal tract of animals and has been isolated from various sources such as soil and aquatic sediment (Saraswat *et al*., 2011). *Clostridium bifermentans* has long been considered as non-pathogenic, but recent studies have found the opposite (Sasi Jyothsna *et al*., 2016; Edagiz *et al*., 2015; Wong *et al*., 2014). A case study by Edagiz and colleagues (2015) presented *Clostridium bifermentans* as the causal agent of empyema, an infection where the pleural space is filled with purulent fluid (Yu, 2011). *Clostridium bifermentans* have also be linked to several human infections like bacteraemia, septic arthritis, osteomyelitis, necrotizing pneumonia and gas-gangrene in animals (Hale *et al*., 2016; Leja *et al*., 2014; Scanlan *et al*., 1994).

Clostridium perfringens is probably the most popular of all the *Clostridium* pathogens to date. It could be found in the intestinal tract of humans and animals as part of their normal gut microbiota, but have also been isolated from environmental samples (Mueller-Spitz *et al*., 2010; Timbermont *et al*., 2008). Its pathogenicity is vast, ranging from gastrointestinal diseases, such as gastroenteritis from food poisoning, to necrotising gas-gangrene (Irikura *et al*., 2015; Stevens and Bryant, 2002). *Clostridium perfringens* was only isolated from sampling sites 2 and 4 in the Crocodile and Schoonspruit Rivers, respectively. Interestingly, both of these sites are downstream from local wastewater treatment plants in these two surface water systems. The presence of *Clostridium perfringens* in water sources is used as an indicator for faecal contamination in various countries (Graziano *et al*., 2007; Bitton, 2005), but in a South African context, it has not been applied. Wastewater effluent is a major contributor to the introduction of this pathogen into the aquatic environment, since their endospores are highly resistant to the treatment processes and environmental stressors (Ajonina *et al*., 2015; Marcheggiani *et al*., 2008; Graziano *et al*., 2007). The presence of this organism in these surface water systems is of great health risk to humans and animals.

The Schoonspruit and Crocodile Rivers were the only hosts for *Clostridium sordellii*. Many *Clostridium sordellii* strains are non-pathogenic, however, most strains are virulent (Aldape *et al*., 2006). According to Winikoff (2006), this *Clostridium* species is an uncommon pathogen in humans, but more so in veterinary medicine. Nonetheless, several reports have shown a rapid increase of occurrence in human infections (Aldape *et al*., 2006; Winikoff, 2006). *Clostridium sordellii* is known to cause enterotoxaemia and enteritis in livestock and Clostridial septic shock in humans (Meites *et al*., 2010; Aldape *et al*., 2006). A study by de la Fe and collaborators (2006) found that *Clostridium sordellii*, that was present in drinking water, could be linked to the sudden death of a pride of lions and that the water was the

most probable source of exposure to this pathogen. Consequently, any livestock drinking the water from these sites in the Schoonspruit and Crocodile Rivers are at risk of infection.

C. baratii, *C. ghonii*, *C. lituseburense* and *C. dakarense* were all present in low levels. *Clostridium baratii* was only isolated at the two sampling sites downstream from the wastewater treatment plants in the Schoonspruit and Crocodile Rivers. This pathogen has been associated with infant botulism, bacteraemia, lung and liver abscesses (Huang *et al*., 2012; Barash *et al*., 2005). Although this *Clostridium* species was obtained downstream from the wastewater treatment plant, no studies have shown any association between the two. A study by Marcheggiani *et al.* (2008) isolated *Clostridium baratii* and *Clostridium ghonii* from river sediment. *Clostridium ghonii*, isolated at site 5 in the Schoonspruit River, is commonly found in soil and has also been isolated from human faeces. Its pathogenicity is not well studied but it has been linked to a variety of infections (Berger, 2015). *Clostridium lituseburens* and *Clostridium dakarense* were only isolated from site 5 and 3 in the Crocodile River, respectively. According to literature, *Clostridium lituseburens* was previously isolated from soil and swine faecal matter (Liu *et al*., 2010; Cotta *et al*., 2003), whereas *Clostridium dakarense* was first described in 2011, where it was also isolated from faecal matter from an infant suffering from gastroenteritis. Sequencing analyses of *Clostridium dakarense* showed 96.90% 16S rRNA nucleotide similarity with that of *Clostridium lituseburense* (Lo *et al*., 2013). The pathogenicity of both of these *Clostridium* species are unknown.

2.5. Chapter summary

In this study, the presence of sulphite-reducing *Clostridium* species and other indicator organisms were detected across various sampling sites in the Schoonspruit, Crocodile and Groot Marico Rivers. The occurrence of these micro-organisms in surface water during both the warm-rainy and cold-dry seasons indicates possible faecal pollution. Agriculture and wastewater treatment plants situated alongside the rivers are the foremost contributors of faecal contaminates in surface water systems. Therefore, the elevated levels of SRC species alongside other indicator organisms at these sites where faecal pollution are expected, supports the possibility of SRC species as a suitable alternative indicator for faecal pollution in surface water systems. However, limitations such as *Clostridium* species not being present in the faeces of herbivores and its ability to survive in surface water long after faecal pollution has occurred, suggests that it could be used in collaboration with other indicator organisms for the recent indication of faecal pollution.

This study also showed that levels of *Clostridium* species are impacted by various physico-chemical parameters, such as temperature, dissolved oxygen, chemical oxygen demand,

phosphates, sulphates and nitrates, depending on the season. Several pathogenic *Clostridium* species were identified in the surface water, *Clostridium perfringens*, *Clostridium bifermentans* and *Clostridium sordelli*, just to name a few. These species have been linked to severe enterotoxic, histotoxic and neurotoxic infections. The presence of these known pathogens, along with the faecal contaminant, in surface water systems are cause for concern, especially for many rural and informal settlements are directly dependent on these water sources.

Chapter 3

Antibiotic resistant *Clostridium* spp. isolated from selected surface water systems and aquatic sediment in the North West Province, South Africa

3.1. Research rationale

Antibiotic resistance is a worldwide problem (CDC, 2013). Studies done in South Africa have found various cases of antibiotic resistance in clinical isolates, particularly in anaerobic bacteria such as *Clostridium* (Meyer *et al*., 2006; Lubbe *et al*., 1999). With antibiotics being used in various fields like medicine, both human and veterinary, aquaculture, livestock and agriculture, it was bound to have an effect on the environment (Wright, 2010). Due to the anthropogenic factors such as agricultural and industrial runoff and poorly treated sewage effluent, antibiotics are introduced into surface water and sediment of rivers (Baquero *et al*., 2008; Kim and Carlson, 2007). This creates an ideal setting for the development of antibiotic resistance bacteria and genes.

Overuse and misuse of antibiotics in these areas have contributed to the development of antibiotic resistance genes which encode for mechanisms to oppose the effects of antibiotics (Gilchrist *et al*., 2007). These genes are the result of either vertical gene transfer (spontaneous mutation in the chromosome as a result of selective pressure of antibiotics) or horizontal gene transfer (the transfer of genes to or between organisms through mobile genetic elements) (Soucy *et al*., 2015). Several antibiotic resistance mechanisms exist which can be mediated by the expression of efflux pumps and penicillin-binding proteins (PBP's), modification of target enzymes, changes in membrane penetrability as well as the inactivation of target enzymes (Alekshun and Levy, 2007).

Antibiotic resistance, especially in *Escherichia coli*, have been thoroughly investigated in surface water, all across South Africa (Adefisoye and Okoh, 2016; Mulamattathil *et al*., 2014; Luyt *et al*., 2012; Olaniran *et al*., 2009). However, little information is available on antibiotic resistant bacteria in aquatic sediment (Abia *et al*., 2015b). Surprisingly, no studies have been conducted on antibiotic resistant *Clostridium* species in the environment. Thus, the study of these pathogens and their antibiotic resistance in South African aquatic environments, particularly the North West Province, is unknown and warrants investigation.

The aim of this study was to investigate antibiotic resistance in *Clostridium* spp. isolated from selected surface water and aquatic sediment in the North West Province, South Africa. The

specific objectives were: (I) to isolate and identify antibiotic resistant *Clostridium* species from selected surface water and aquatic sediment using Fung Double tube and 16S rDNA sequencing, (II) to determine antibiotic resistance profiles in *Clostridium* spp. obtained from surface water and aquatic sediment, (III) to determine the minimum inhibitory concentration (MIC) of antibiotics and (IV) determining the presence of specific antibiotic resistance genes in *Clostridium* spp. by PCR.

3.2. Material and methods

3.2.1. Water and sediment collection

Water and sediment samples were collected from 3 different surface water systems (Crocodile River, Groot Marico and Schoonspruit) in the North West Province, South Africa in 2016 (Figures 1.1-1.3 in Section 1.9). Surface water samples were collected using the direct or dip technique as previously described by Molale (2012). The sampling of sediment was done as stated by Sibali and associates (2008). Sediment was only collected from sites that were accessible to the bank of the river. A spade was used to excavate sediment about 5 cm below the water, placed in zip lock bags and labelled. These bags were then placed on ice in a cooler box, along with the water samples and transported to the laboratory for further analyses.

3.2.2. Antibiotic resistance screening

Screening for resistance was done against three classes of antibiotics, specifically Lincosamides, β-lactam and Tetracyclines. The resistant *Clostridium* spp. were isolated with the use of the Fung double tube method (Barrios *et al*., 2013), in combination with the tryptose sulphite cycloserine (TSC) agar (Oxoid; UK).

According to the Clinical and Laboratory Standards Institute (CLSI) (2014), anaerobic bacteria (*Clostridium* spp. included) are classified as resistant to Clindamycin (DA) at a minimum inhibitory concentration (MIC) of 8 µg/ml, Ampicillin (AMP) at 2 µg/ml and Tetracycline (TE) at 16 µg/ml. Three test tubes containing TSC agar was each supplemented with an antibiotic matching its MIC. One millilitre (1 ml) of water sample was then mixed with content of the test tube. For the sediment, 1 g of the sample was suspended in 9 ml of autoclaved distilled water and a dilution series was made up to 10^{-2}. One millilitre of the 10^{-2} dilution was then added to the test tube and mixed together. All the test tubes were then incubated at 44°C for 12 hours or until there was growth present. Colonies were purified by streak plating on Reinforced Clostridia agar (Oxoid; UK) containing the same antibiotic concentration that the isolates were originally obtained from and incubated under anaerobic conditions.

3.2.3. Cross resistance of antibiotics and minimum Inhibitory concentration (MIC)

Resistance/susceptibility against 6 antibiotics were tested by using MIC gradient diffusion method (M.I.C.E. strips) and the agar dilution method (Schuetz, 2014), which, according to the CLSI, is the gold standard method for anaerobic antibiotic susceptibility testing. The antibiotics used were Tetracycline, Clindamycin, Ampicillin, Amoxicillin, Chloramphenicol and Metronidazole.

For the agar dilution, molten Reinforced Clostridia agar was supplemented with different concentrations of an antibiotic, ranging from 0.015 µg/ml up to 256 µg/ml. The mixture was then poured into sterile petri dishes and allowed to set. Isolates were spot inoculated on each plate containing a different concentration of antibiotic and incubated anaerobically at 37°C for 24-48 hours. All plates were then inspected and the lowest concentration where there was no visible growth was noted as the MIC for that isolate.

The M.I.C.E. strips (Oxoid; UK) was used for Tetracycline, Clindamycin and Ampicillin. An overnight culture in Reinforced Clostridia medium (Biolab Merck, Germany) of the isolate was prepared. One hundred microliter volume of this preparation was pipetted onto Mueller-Hinton agar and spread over the plate. The M.I.C.E. strip was then placed on the plate as per instruction of the manufacturer. Plates were then incubated under anaerobic conditions at 37°C for 24-48 hours. The MIC value was then read from the strip (point where the oval edge meets the scale on strip) and recorded in µg/ml.

3.2.4. Genomic DNA extraction

Genomic DNA was extracted from bacterial cells obtained from an overnight culture grown in Reinforced Clostridia medium (Biolab Merck, Germany) at 37°C (anaerobic). Genomic DNA (gDNA) was isolated using a NucleoSpin® Tissue kit (Macherey-Nagel, Germany). The kit was used according to the instructions of the manufacturer. Purity and integrity of the gDNA were determined by the A260 nm/A280 nm ratio using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, US) and by 1.5% (w/v) agarose gel electrophoresis.

3.2.5. Polymerase chain reaction (PCR) amplification

All PCR reactions with a final volume of 25 µl were performed containing 2X PCR Master Mix (Fermentas Life Science, US), Nuclease free water (Fermentas Life Sciences, US) and 1 µl gDNA (20-80 ng/µl). For the 16S, *bla*$_{TEM}$, *erm*B and *erm*F genes, 0.4 µM of both primers (Inqaba Biotec; SA) were added to the reaction mixture, with the exception for *tet*K and *tet*L

reactions. The primer concentration for these two reactions were 0.2 µM of each primer (Applied Biosystems, UK). All primer sequences are showed in Table 3.1 below.

3.2.5.1. 16S rDNA amplification

The 27F and 1492R primers were used to amplify the 1 465 bp 16S gene (Weisburg *et al*., 1991). The following thermal cycling conditions were used: initial denaturing at 94°C for 2 minutes, 35 cycles of denaturing at 94°C (30 seconds), annealing at 54°C (60 seconds) and extension at 72°C (60 seconds). The reaction was concluded by an additional extension at 72°C for 5 minutes.

3.2.5.2. *bla*$_{TEM}$

The *bla*$_{TEM}$ gene was amplified under the following PCR conditions: initial denaturing at 95°C for 5 minutes, where after 35 cycles began, consisted of denaturing at 94°C (60 seconds), annealing at 63°C (30 seconds) and extension at 72°C (60 seconds). The reaction was finished by an additional extension at 72°C for 10 minutes with an expected amplicon size of 1 150 bp (Costa *et al*., 2007).

3.2.5.3. *tet*L and *tet*K

For the amplification of the *tet*L and *tet*K genes, the same PCR conditions were employed. The reactions underwent 35 cycles which began with denaturing at 95°C for 1 minutes, annealing at 50°C (1 minute) and extension at 72°C (30 seconds). The reaction was completed by an additional extension at 72°C for 5 minutes. According to Duarte and co-workers (2005), this amplification will deliver amplicon sizes of 1 159 bp and 1 077 bp for *te*tK and *tet*L, respectively.

3.2.5.4. *erm*F and *erm*B

The successful amplification of *erm*F has previously been described by Chung and associates (1999). The PCR conditions used in this study were similar: denaturing at 94°C for 30 seconds, annealing at 50°C (30 seconds) and extension at 72°C for 2 minutes. Each cycle was repeated 35 times and deliver an expected amplicon size of 466 bp. For the amplification of the *erm*B gene, the same conditions were used, with the exception of annealing temperature at 48°C for 1 minute, to deliver a 639 bp amplicon.

Table 3.1: Oligonucleotide primers for PCR amplification of 16S rDNA, *bla*$_{TEM}$, *erm*F, *erm*B, *tet*K and *tet*L. F- Forward primer and R- Reverse primer.

Primer	Sequence (5'-3')	Amplicon size (bp)	Annealing Temp (C°)	Reference
16S rDNA				
27F	5'- AGA GTT TGA TCM TGG CTC AG- 3'	1 465	54	Jiang *et al*., 2006
1492R	5'- GG TTA CCT TGT TAC GAC TT- 3'			
***bla*$_{TEM}$**				
TEM-F	5'- ATT CTT GAA GAC GAA AGG GC- 3'	1 150	63	Costa *et al*., 2007
TEM-R	5'- ACG CTC AGT GGA ACG AAA AC- 3'			
***erm*F**				
ermF1	5' -CGG GTC AGC ACT TTA CTA TTG- 3'	466	50	Chung *et al*., 1999
ermF2	5' -GGA CCT ACC TCA TAG ACA AG- 3'			
***erm*B**				
ermB-F	5' -GAA AAG GTA CTC AAC CAA ATA- 3'	638	48	Tran *et al*., 2013
ermB-R	5' -AGT AAC GGT ACT TAA ATT GTT TAC- 3'			
***tet*K**				
TetK-F	5' -TAT TTT GGC TTT GTA TTC TTT CAT- 3'	1 159	50	Duarte *et al*., 2005
TetK-R	5' -GCT ATA CCT GTT CCC TCT GAT AA- 3'			
***tet*L**				
TetL-F	5' -ATA AAT TGT TTC GGG TCG GTA T- 3'	1 077	50	Gholamiandehkordi *et al*., 2009
TetL-R	5' -AAC CAG CCA ACT AAT GAC AAT GAT- 3'			

3.2.6. Sequencing and identification

A silica resin was used to purify all successful 16S PCR products and in doing so, removing all remaining primer (Li *et al*., 2010). The NanoDrop 1000 Spectrophotometer (Thermo Scientific; US) was utilized to determine the quantity and quality of the DNA. The Cycle Sequencing BigDye Terminator Kit (Zymo Research, US) was used to perform the sequencing PCR. A Zymo Research DNA-Sequencing Clean up kit™ (Zymo Research, US), was used to purify the sequencing PCR products following the manufacturer's protocol. An ABI 3130 Genetic Analyser (Applied Biosystems, UK) was utilized to sequence the amplicons. The isolates were identified by comparing their sequences to known sequences in GenBank (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Further details of the process are provided in Section 2.2.10.

3.2.7. Statistical analysis

Agglomerative hierarchical clustering (AHC) analysis was performed on all the multiple antibiotic resistance (MAR) data of *Clostridium* spp. based on Ward's method in XLSTAT (Version 2016.18.06.36087)

3.3. Results

Surface water and sediment samples were obtained from the Crocodile, Groot Marico and Schoonspruit River during March and July of 2016. The presence of antibiotic resistant *Clostridium* species was screened against several antibiotics, focusing on resistance against Tetracycline, Ampicillin and Clindamycin. Antibiotic resistant isolates were obtained from the Schoonspruit and Crocodile River, however, none were isolated in the Groot Marico River. The results obtained are presented in the following sections.

3.3.1. Identification of antibiotic resistant *Clostridium* species

A total of 67 antibiotic resistant *Clostridium* species were obtained from surface water and aquatic sediment of the Schoonspruit (62 isolates) and Crocodile (5 isolates) Rivers. DNA from isolates were extracted as described in Section 3.2.4. The concentration of the DNA ranged from 15.26 ng/µl to 73.7 ng/µl, with the A260 nm/A280 nm ratio values between 1.83 and 2.11. Ratio values between 1.7 - 1.9 are considered best for PCR. The concentration and purity of the DNA extracted were suitable for PCR. Figure 3.1 depicts the composition of all the antibiotic resistant *Clostridium* species identified through 16S rDNA sequencing. *Clostridium perfringens* was the dominant species (90%), being isolated from both surface water and sediment of the Schoonspruit and Crocodile Rivers. *Clostridium bifermentans*

(3%), *Clostridium sordelli* (4%) and *Clostridium baratii* (3%) were also being present in low quantities only in the Schoonspruit River.

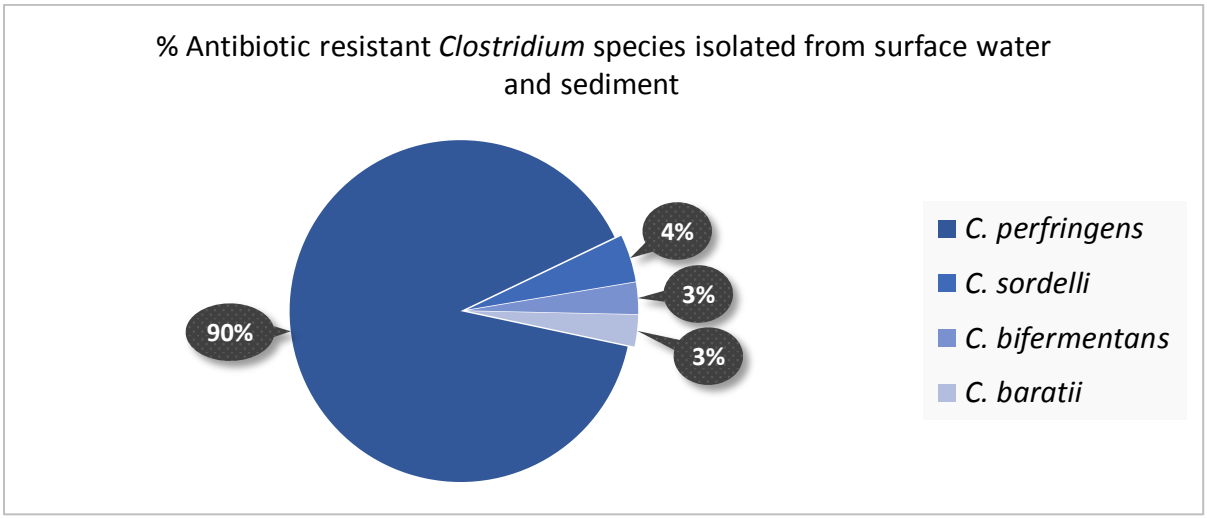

Figure 3.1: Circle graph illustrating the percentage of antibiotic resistant *Clostridium* species isolates from surface water and sediment of the Schoonspruit and Crocodile Rivers.

3.3.2. Minimum inhibitory concentration (MIC) and classification

Of the 67 antibiotic resistant *Clostridium* isolates, 17 were isolated through Tetracycline screening, 20 through Ampicillin and 30 through Clindamycin. The susceptibility profiles for all these isolates are listed in Table B1 in Appendix B. Table 3.2 below summarizes the MIC values of the 6 antibiotics used to screen for cross-resistance against all the antibiotic resistant *Clostridium* isolates. The classification of the isolates is also included in this table.

Among the 67 *Clostridium* isolates, 68.1% (46 isolates) were resistant to Ampicillin, 66.7% (45 isolates) to Tetracycline and 37.3% (25 isolates) to Clindamycin. Clindamycin also had the highest MIC concentration, with 33.3% of the isolates being able to grow at a concentration of 256 µg/ml or higher. Metronidazole showed a low MIC of 49.9% (33 isolates), whereas 72.7% (49 isolates) and 72.2% (48 isolates) were highly susceptible to Amoxicillin and Chloramphenicol, respectively. Although the majority of the isolates demonstrated resistance against Ampicillin, they also showed the most susceptibility to Amoxicillin. This indicates a variation in β-lactam resistance phenotype among the isolates.

Table 3.2: Percentage of antibiotic resistant *Clostridium* species isolated from the Crocodile and Schoonspruit Rivers. The numbers in the columns indicate percentage of the 67 clostridia that were able to grow at the particular antibiotic concentration. The classification percentage column is an indication of the percentage of the 67 that were sensitive, intermediate resistant or resistant to the particular antibiotic.

Antibiotics (µg/ml)	Distribution (%) of MICs											Classification (%)		
	0.25	0.5	1	2	4	8	16	32	64	128	>256	S	I	R
TE		5.0	5.0		6.7	16.7	40.7	10.3	7.8		7.8	16.7	16.7	66.7
DA	56.9	2.0		3.9		3.9					33.3	62.7		37.3
AMP	20.1	9.8	2.0	68.1								29.9	2.0	68.1
AMX	36.3	16.2	10.9	0.0	10.3	5.6	19.7					72.7	7.5	19.7
C	25.9	15.0	2.0	7.8	2.0	10.9	12.0	21.7				72.2	6.1	21.7
MTZ	21.8	2.2	3.6	6.7		15.6	22.0	25.9	2.2			49.9	22.0	28.1

* TE: Tetracycline; DA: Clindamycin; AMP: Ampicillin; AMX: Amoxicillin: C: Chloramphenicol; MTZ: Metronidazole; S: Susceptible; I: Intermediate resistance; R: Resistant. Red values indicate Resistant MIC according to the CLSI.

3.3.3. Multiple antibiotic resistance patterns of *Clostridium* species

Of the 67 antibiotic resistant *Clostridium* isolates, 28 were resistant to 3 or more antibiotics. All the multiple resistant (MAR) isolates were from either downstream from the wastewater treatment plant (WWTP) or near Orkney, in the Schoonspruit River, with the exception of one, which was obtained from the site in the Crocodile River just after Brits. The only antibiotic that was consistently present in all MAR phenotypes was Ampicillin, except for the one isolate from the Crocodile River. As shown in Table 3.3, the most dominant MAR phenotype was AMP-TE-DA-MTZ-C-AMX. A total of 9 isolates presented this phenotype. All these isolates were from the same site (SC4) in the Schoonspruit River and isolated from sediment. This could explain the similar MAR phenotype of these isolates. The phenotype AMP-TE-DA-MTZ-AMX was observed in 4 *Clostridium* species obtained from only surface water at the same site in the Schoonspruit River. Two isolates with the phenotype AMP-TE-DA-MTZ-C were isolated from surface water and sediment. The disturbance of sediment, resulting in resuspension of bacteria in surface water, could explain this occurrence. Several *Clostridium* species isolated downstream from the WWTP and the site near Orkney in the Schoonspruit River showed similar MAR phenotypes. These 2 sites are in close proximity to each other, so this could be the reason for the similarity in phenotypes.

Table 3.3: Multiple antibiotic resistance (MAR) phenotypes of 28 *Clostridium* species.

No. of isolates	Antibiotic pattern	MAR average	Isolate ID
9	AMP, TE, DA, MTZ, C, AMX	1	SC4-Se-C6, SC4-Se-C9, SC4-Se-C11, SC4-Se-C12, SC4-Se-C15, SC4-Se-C16, SC4-Se-C18, SC4-Se-C21, SC4-Se-C30
2	AMP, TE, DA, MTZ, C	0.833	SC4-Su-C24, SC4-Se-C8
4	AMP, TE, DA, MTZ, AMX	0.833	SC4-Su-C26, SC4-Su-C27, SC4-Su-C28, SC4-Su-C29
1	AMP, TE, MTZ, C, AMX	0.833	SC4-Se-T15
4	AMP, TE, DA, MTZ	0.667	SC4-Su-C25, SC5-Se-C17, SC4-Su-C3, SC4-Su-C10
1	AMP, DA, MTZ, C	0.667	SC4-Se-C5
2	AMP, DA, MTZ, AMX	0.677	SC4-Su-C2, SC4-Su-C3
3	AMP, TE, DA	0.5	SC5-Su-C19, SC5-Su-C20, SC4-Se-T5
1	AMP, DA, AMX	0.5	SC4-Su-C14
1	TE, DA, C	0.5	CR2-Se-C4

* TE: Tetracycline; DA: Clindamycin; AMP: Ampicillin; AMX: Amoxicillin: C: Chloramphenicol; MTZ: Metronidazole; SC: Schoonspruit River; CR: Crocodile River; Se: Sediment; Su: Surface water.

3.3.4. Cluster analysis of antibiotic resistant patterns observed in *Clostridium* species isolated from surface water and sediment across the Schoonspruit and Crocodile Rivers

The minimum inhibitory concentration (MIC) data from the susceptibility testing done on all 67 *Clostridium* species were subjected to a cluster analysis. This illustrates if there were any similarities in the antibiotic resistant patterns of the isolates. The dendrogram depicted in Figure 3.2 below show three clusters, A, B and C, where all three clusters are each subdivided into a further two minor clusters, A1 and A2, B1 and B2, C1 and C2, respectively.

Cluster A is mainly comprised out of isolates screened for by using Clindamycin. The same could be seen in Cluster B, but with the isolates that were screened for by using Ampicillin. Isolates screened for by using Tetracycline generally clustered together in cluster C, however some of the isolates were found in cluster A and B. Cluster C also included a few isolated from the Clindamycin containing media. When focusing on each cluster individually, the 2 minor clusters are clearly divided by sediment and surface water sample, with the isolates obtained from the sediment showing more antibiotic resistance than that of the surface water. This trend can be observed in each minor cluster throughout. The surface water and sediment from Schoonspruit River was the main source of the isolates

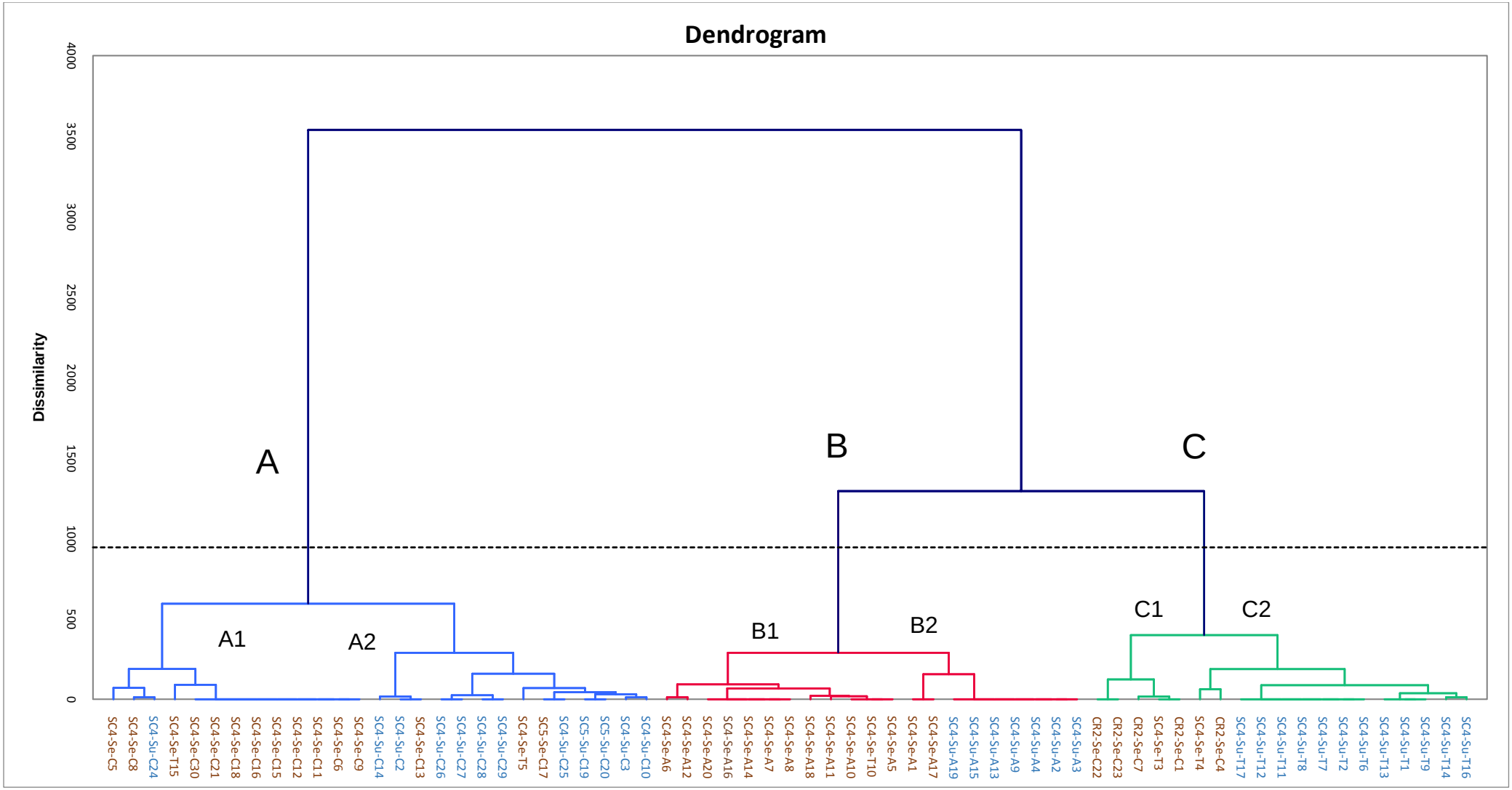

Figure 3.2: Dendrogram showing the relationship of 67 *Clostridium* spp. isolates obtained from surface water and aquatic sediment from the Schoonspruit and Crocodile Rivers in 2016, cluster formation based on their resistance profiles. SC: Site in Schoonspruit River; CR: Site in Crocodile River; Se: sediment (also depicted in Brown); Su: Surface water (also depicted in Blue); C: Clindamycin screened isolated; A: Ampicillin screened isolate; T: Tetracycline screened isolate.

in cluster A, B and minor cluster C2, whereas cluster C1 isolates were obtained from only sediment of the Crocodile and Schoonspruit Rivers.

3.3.5. Presence of antibiotic resistance genes in *Clostridium* species

The presence of several antibiotic resistance genes was screened for in *Clostridium* species isolated throughout this study. As shown in Table 3.4 below, three of these genes were found to be present in antibiotic resistant *Clostridium* isolates obtained from both surface water and sediment. Figure 3.3 shows representatives of the three antibiotic resistance genes, *erm*B (lane 1), *tet*L (lane 2) and *tet*K (lane 3), that were successfully amplified through PCR.

Table 3.4: Destitution of antibiotic resistance genes (*erm*B, *tet*K and *tet*L) in *Clostridium* species isolated from surface water and sediment in the Schoonspruit and Crocodile Rivers.

Antibiotic resistant gene	**Source**	**Isolate ID**	**Clostridium spp. ID**
*erm*B (n= 9)	Surface water	SC4-Su-C24	*Clostridium perfringens*
		SC4-Su-C26	*Clostridium perfringens*
	Sediment	SC4-Se-C13	*Clostridium perfringens*
		SC4-Se-C15	*Clostridium perfringens*
		SC4-Se-C16	*Clostridium perfringens*
		SC5-Se-C17	*Clostridium perfringens*
		SC4-Se-C18	*Clostridium perfringens*
		SC4-Se-C21	*Clostridium perfringens*
		CR2-Se-C22	*Clostridium perfringens*
tet L (n= 4)	Surface water	SC4-Su-T6	*Clostridium perfringens*
		SC4-Su-T7	*Clostridium baratii*
		SC4-Su-T9	*Clostridium perfringens*
	Sediment	SC4-Se-T10	*Clostridium baratii*
*tet*K (n= 1)	Surface water	SC4-Su-T1	*Clostridium bifermentans*

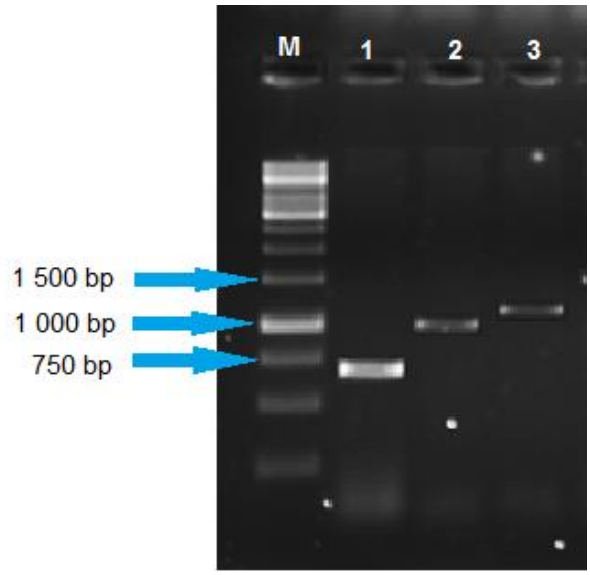

Figure 3.3: Gel electrophoresis of a 1.5% (w/v) agarose gel with representatives of the 3 antibiotic resistant genes that were successfully amplified. Lane 1 represent the *erm*B gene (639 bp) that was identified in 9 *Clostridium* isolates, Lane 2: *tet*L gene (1 077 bp) identified in 4 *Clostridium* isolates and Lane 3: *tet*K gene (1 159 bp) identified in 1 *Clostridium* isolate. The lane marked M shows a 1 kb molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US).

Of all the *Clostridium* species isolates that were resistant to Tetracycline, 4 isolates harbour the *tet*L gene and only 1 the *tet*K gene. All *tet* genes were found in isolates obtained from the site downstream from the WWTP in the Schoonspruit River. Furthermore, these *tet* genes were found in a number of *Clostridium* species such as *Clostridium perfringens*, *Clostridium baratii* and *Clostridium bifermentans*. Of the 2 macrolide-lincosamide-streptogramin (MLS) resistance genes (*erm*B and *erm*F), encoding for resistance against Clindamycin, only the *erm*B gene was found in 9 of the isolates. The isolates that possessed this gene was exclusively identified as *Clostridium perfringens* that were isolated from both the Schoonspruit and Crocodile Rivers. The β-lactam resistance gene, bla_{TEM}, was not carried by any of the *Clostridium* isolates that showed resistance against Ampicillin.

3.4. Discussion

The presence of antibiotic resistant *Clostridium* species was investigated in the surface water and the sediment of selected river systems, specifically Schoonspruit, Crocodile and Groot Marico, in the North West Province. These river systems serve as a vital source of water for the surrounding communities. Subsequently, the water quality of the river systems is continually affected by various anthropogenic pollutants, such as antibiotics. The extensive and unnecessary use of antibiotics are hazardous to both the aquatic environment and human health (Rahman *et al*., 2014). As a result, bacteria which are present in the aquatic environment, like *Clostridium*, are exposed to selective pressure imposed by these antibiotic pollutants (Coutinho *et al*., 2014). Under these conditions, the bacteria can develop antibiotic resistance by horizontal gene transfers (Rahman *et al*., 2014; Kolář *et al*., 2001).

3.4.1. Prevalence of antibiotic resistant *Clostridium* species in surface water and sediment

Of the 67 *Clostridium* species isolated during this study, more than half (53.7%) were obtained from aquatic sediment. Although the distribution of the antibiotic resistant *Clostridium* species varies between surface water and sediment, their resistance profiles are very contrasting. Studies focusing on the concentration of antibiotics in the environment have proven that aquatic sediment contains higher levels of antibiotics than that of the adjoined surface water (Mims, 2015; Kümmerer, 2009). Thus, bacteria present in sediment are more likely to show resistance to antibiotics.

The occurrence of antibiotic resistant *Clostridium* species in both surface water and sediment is consistent with the Centre for Disease Dynamics, Economics & Policy report (2015) on antibiotic-resistance in the environment. It states that antibiotic residue present in the environment can lead to antibiotic resistance in micro-organisms, which could create reservoirs for resistance gene transfer (CDDEP, 2015). There are limited studies on antibiotic resistant *Clostridium* species in the aquatic environments. However, Xiong and colleagues (2015b) identified several genera, including *Clostridium*, from sediment and freshwater sources that showed resistance to various classes of antibiotics. These isolates also had various resistance genes present in their genomes. The prevalence of antibiotic resistant *Clostridium* species in the Schoonspruit and Crocodile Rivers suggest that these river systems are exposed to various pollutants. The Schoonspruit and Crocodile Rivers are known to receive wastewater from wastewater treatment plants which are situated alongside the river banks. Several studies revealed that the effluent from wastewater treatment plants are primarily responsible for the introduction of antibiotics and antibiotic resistant bacteria into aquatic environments (Xu *et al*., 2015; Gatica and Cytryn, 2013; Börjesson, 2009). This

could explain the presence of antibiotic resistant *Clostridium* species in the river systems. The majority of the isolates were obtained from the sites downstream from the wastewater treatment plants in both the Schoonspruit and Crocodile Rivers.

As showed in Table 3.2, most of the *Clostridium* species were susceptible to Amoxicillin and Chloramphenicol, 72.7% and 72.2%, respectively. There were, however, some resistance exhibited against these two antibiotics. A study by Roberts and associates (2006) found similar susceptibility profiles against Amoxicillin, whereas Brook (2014) also reported Chloramphenicol susceptibility amongst various *Clostridium* species. Both Amoxicillin and Chloramphenicol are broad-spectrum antibiotics and are normally, but not always, effective against *Clostridium* species (Brook, 2014; Roberts *et al*., 2006; Schwartzman *et al*., 1977). This statement is coherent with the findings in this study, since these two antibiotics were the most effective in inhibiting the growth of *Clostridium* species, though some expressed resistance.

In this study, *Clostridium* species showed the highest resistance to Ampicillin and Tetracycline, 68.1% and 66.7%, respectively. These isolates showed high minimum inhibitory concentrations (MIC) of greater than 256 µg/ml for Tetracycline and 2 µg/ml for Ampicillin. Both antibiotics are broad-spectrum which are widely utilized against Gram-positive and Gram-negative infections (Romanis, 2013). However, there have been numerous reports of *Clostridium* species showing resistance to Ampicillin and Tetracycline in South Africa (Meyer *et al*., 2006; Montso and Ateba, 2014) and around the world (Xiong *et al*., 2015a; Soge *et al*., 2008). The *Clostridium* species from the Crocodile River showed little to no resistance to Ampicillin, whereas numerous isolates from the Schoonspruit River were Ampicillin resistant. This could suggest that the Crocodile River is unthreatened by Ampicillin, while the Schoonspruit River is very much exposed to it. A similar study by Coutinho and co-workers (2014) isolated *Clostridium* species from urban aquatic environments that expressed resistance to Ampicillin. Studies have found that resistance to β-lactams, such as Ampicillin, can be mediated by one of three mechanisms: (i) through a decrease in permeability, (ii) by the expression of low-affinity penicillin-binding proteins, or (iii) via the inactivation through enzymes such as β-lactamases (Rice, 2012; Hecht, 2004). *Clostridium* species expresses their resistance through the latter.

Resistance to Tetracycline was observed in both the Schoonspruit and Crocodile Rivers. Tetracycline resistance is mediated through numerous mechanisms, ranging from protecting the Tetracycline binding site to modification of Tetracycline or its binding site. However, *Clostridium* species counter attack the effects of Tetracycline through efflux pumps (Speer *et*

al., 1992). These resistance mechanisms can be the result of a genetic mutation within the bacteria itself, however, several studies have reported that Ampicillin and Tetracycline resistance can be transferred between *Clostridium* species and other bacteria in the aquatic environment (Rahman *et al*., 2014; Hecht, 2004; Courvalin, 1994). This phenomenon greatly reduces the effectiveness of antibiotics to combat diseases, thus leaving human and animal health exposed.

3.4.2. Prevalence of multiple antibiotic resistant (MAR) *Clostridium* species in surface water and sediment

Of the 67 antibiotic resistant *Clostridium* species, 28 isolates showed resistance against three or more antibiotics (MAR). The presence of multiple antibiotic resistant *Clostridium* species in the surface water systems suggests that these bacteria were once exposed to numerous antibiotic compounds. It is evident with the high levels of MAR *Clostridium* species in the Schoonspruit River, particularly at the site downstream from the wastewater treatment plant, that there are various antibiotics being introduced into this river system. This exposure to antibiotics could have occurred in humans or animals, or directly in the environment.

Interestingly, the majority of the MAR *Clostridium* species were isolated from the sediment of both the Schoonspruit and Crocodile Rivers. Nine of the isolates were resistant to all six of the antibiotics used in this study (AMP-TE-DA-MTZ-C-AMX). All these isolates were obtained from the sediment at the site downstream from the wastewater treatment plant in the Schoonspruit River. Abia and colleagues (2015a) found that sediment in rivers which are exposed to antibiotic pollutants (via effluent from WWTP) harbours large quantities of MAR bacteria. This is due to sorption-desorption of antibiotics in aquatic environments (Kümmerer, 2009). Antibiotics in the rivers systems can be absorbed into the underlying sediment, decelerating its degradation, thus becoming more stable and active for a longer period (Devarajan *et al*., 2016; Baquero *et al*., 2008). This results in an increase of bacteria, such as *Clostridium* species, in the sediment showing resistance against various antibiotics. The broad range of resistance found in the isolates in this study is an ideal example of this manifestation.

In Table 3.3, several MAR *Clostridium* species showed similar phenotype patterns. For instance, 2 isolates displayed identical patterns, specifically AMP-TE-DA-MTZ-C. Both isolates were obtained from the same site in the Schoonspruit River, but were isolated from different sources (surface water and sediment). Devarajan and associates (2016) attributed this to the disturbance of the aquatic sediment, resulting in the resuspension of antibiotics and MAR *Clostridium* species present in the sediment, into the adjoining surface water. This

disturbance could also clarify the number of isolates that were obtained from sediment and/or surface water, which displayed similar MAR phenotypes. However, these were isolated from different sites in the river. Isolates with the phenotype AMP-TE-DA-MTZ and AMP-TE-DA were present at both the site downstream from the wastewater treatment plant in the Schoonspruit River and the sampling point at Orkney (SC5). This shows that these MAR bacteria are being distributed throughout this river system.

The incidences of MAR *Clostridium* species in these river systems represent a great threat to the health of both humans and animals that utilize these water sources. *Clostridium* species are known to cause severe diseases and for them to be presenting MAR traits could lead to unsuccessful antibiotic therapy, therefore reducing treatment options.

3.4.3. Presence of antibiotic resistance genes (ARG) in *Clostridium* species isolated from surface water systems

The presence of several antibiotic resistance genes was screened for in the antibiotic resistant *Clostridium* species that were obtained from surface water and sediment in the Schoonspruit and Crocodile Rivers. These genes encode for mechanisms which are the basis of antibiotic resistance (Davies and Davies, 2010). Thus, they create unstoppable pathogens that threaten the health of all living beings. The *ermF* and *bla*$_{TEM}$ genes, encoding for macrolide-lincosamide-streptogramin (MLS) and β-lactam resistance, respectively, were not identified in any of the Clindamycin and Ampicillin-resistant *Clostridium* species in this study. Even though the *ermF* gene has previously been confirmed in several *Clostridium* species (Chung *et al*., 1999), the absence of this gene is concurrent with findings of Mayorga and co-workers (2015). Very little information is available in regards to β-lactam resistance amongst *Clostridium* species, (Kristiansen, 2007; Hecht, 2004). The *bla*$_{TEM}$ gene has previously been identified in *Clostridium perfringens*, but only in a single clinical isolate (Fatima *et al*., 2013). Manaia and colleagues (2016) did, however, report high prevalence of the *bla*$_{TEM}$ gene in the aquatic environments, including in a South African context (Adefisoye and Okoh, 2016; Igbinosa and Okoh, 2012). Yet, to our knowledge, the *bla*$_{TEM}$ gene has not been identified in any environmental *Clostridium* species. This may possibly suggest that Ampicillin resistance is facilitated by a different gene encoding for β-lactamases in these sources.

The Tetracycline resistance genes, *tet*(L) and *tet*(K), were used to confirm the presence of antibiotic efflux mechanisms (Roberts, 2005). These two genes were identified in the *Clostridium* isolates obtained from the surface water and sediment of only the Schoonspruit River. Incidentally, Molale and Bezuidenhout (2016) also reported the presence of

Tetracycline resistance genes, mainly *tet*(L), in several surface water systems in the North West Province, including the Schoonspruit River.

As shown in Table 3.4, it is evident that *tet*(L) was the most predominant of the 2 *tet* genes in this study and occurred in species such as *Clostridium perfringen*s and *Clostridium baratii*. Gholamiandehkordi and colleagues (2009) have previously detected the *tet*(L) gene in *Clostridium perfringens*, however, the presence of this gene in *Clostridium baratii* has not been reported. Throughout this study, only a single isolate, *Clostridium bifermentans*, carried the *tet*(K) gene. The low presence of *tet*(K) has been documented in *Clostridium* species which are allied with these findings (Gholamiandehkordi *et al*., 2009; Martel *et al*., 2004). *Clostridium bifermentans* have shown resistance against Tetracycline in previous works, but no studies have focused on the possible presence of *tet* genes in this *Clostridium* species (Leja *et al*., 2014).

Only a few of the Tetracycline-resistant *Clostridium* species isolated harboured the *tet*(L) or *tet*(K) gene. Roberts (2005) stated that there are over 40 different *tet* genes that encode for resistance against Tetracycline. The low prevalence of these two *tet* genes amongst all the Tetracycline resistant *Clostridium* species in this study suggest that other *tet* genes, such as *tet*(M), *tet*(W), *tet*(36) or *tet*(40) might be responsible for the observed resistance (Park *et al*., 2010; Roberts, 2005).

The majority of the *Clostridium* isolates that expressed resistance to Clindamycin, revealed high minimum inhibitory concentrations (MIC) of above 256 µg/ml. These elevated MIC values suggests the presence Clindamycin resistance genes. Resistance to Clindamycin in *Clostridium* species is encoded by *erm* genes and are usually mediated by the ribosomal methylation mechanism (Spigaglia, 2016; Stoll *et al*., 2012). However, not all the Clindamycin resistant isolates in this study possessed the *erm*B or *erm*F genes, which may indicate alternative resistance mechanisms in those isolates (Hecht, 2004).

Of the two macrolide-lincosamide-streptogramin (MLS) resistance genes, *erm*F and *erm*B, screened for in this study, the *erm*B gene is known to be more widely distributed in the environment (Berglund, 2015; Zhang *et al*., 2009). This is evident by the dominant presence of the *erm*B gene and the absence of *erm*F in various Clindamycin resistant isolates. In this work, the *erm*B gene was solely detected in *Clostridium perfringens* (Table 3.4). These findings are parallel with those of Perreten and colleagues (2005), who also reported the presence of this gene in *Clostridium perfringens* that were isolated from aquatic environments.

The detection of the *erm*B gene was more common in *Clostridium* isolates from the Schoonspruit than those from the Crocodile River. Also, when comparing the presence of the *erm*B gene in isolates obtained from surface water to those from aquatic sediment, the gene was more recurrent in isolates from the latter source. Several studies describe the aquatic environment, especially the sediment, as a reservoir for antibiotic resistant bacteria and genes (Marti *et al*., 2013; Mims, 2015; Kümmerer, 2009). In previous works, the *erm*B gene has been associated with mobile genetic elements, such as plasmids and non-conjugative transposons (Berglund, 2015; Spigaglia *et al*., 2005). Based on this knowledge, studies have proven that the *erm*B gene can be transferred into and from *Clostridium* species, such as *Clostridium perfringens*, through horizontal gene transfer (Spigaglia *et al*., 2005; Shoemaker *et al*., 2001). This could possibly explain the high occurrence of this gene in the various Clindamycin resistant isolates obtained from sediment, as well as surface water.

3.5. Chapter summary

The results of the study have shown that there are large numbers of antibiotic resistant and MAR *Clostridium* species present in the Schoonspruit and Crocodile Rivers, North West, South Africa. Majority of these isolates showed resistance to Ampicillin and Tetracycline. Studies have listed various contributors to the presence of antibiotic resistant and MAR bacteria in the aquatic environments. However, the elevated occurrence of these resistant bacteria observed in this study, could be due to pollution from WWTPs. The source of these antibiotic resistant *Clostridium* species varied between the surface water and aquatic sediment. The majority of the MAR *Clostridium* isolates were obtained from the sediment, which could suggest that it is a possible reservoir for these antibiotic resistant *Clostridium* species. Disruption of the sediment can resuspend these antibiotic resistant isolates into the adjoined surface water, as evident in the similar phenotypes observed in both sources, and dispersing them throughout the river system.

Several antibiotic resistant *Clostridium* spp. isolates tested positive for the presence of antibiotic resistance genes. The presence of these genes is worrisome, since they all have been found in the aquatic environment and linked to mobile genetic elements, such as plasmids. The expression of these genes could also contribute to MAR in potential pathogenic micro-organisms. Consequently, the presence of these antibiotic resistant *Clostridium* spp. and the associated genes responsible for it, contribute to the deterioration of surface water quality in these rivers. Therefore, negatively impacting the health of its users, especially the immune-compromised individuals and livestock. These results highlight

the necessity to screen for other antibiotic resistant pathogens in the aquatic environment and to further investigate the potential sources.

CHAPTER 4

Conclusions and recommendations

4.1. Conclusion

The main aim of this study was to characterize *Clostridium* species that were present in surface water and aquatic sediment of selected river systems across the North West Province, South Africa. To achieve this aim, two main objectives were set. The first was to determine the prevalence of *Clostridium* species in surface water of the Schoonspruit, Crocodile and Groot Marico Rivers and evaluate its potential as an indicator of faecal pollution, along with the possible health risks associated with these species. The second was to investigate antibiotic resistance in *Clostridium* species isolated from surface water and aquatic sediment obtained from these three surface water systems and the presence of antibiotic resistance gene in these isolates. The findings of these were presented in Chapter 2 and 3, as two separate studies with each having separate objectives to achieve the main aim of this study. The conclusions of each objective are briefly discussed below.

4.1.1. Prevalence of *Clostridium* species, the potential health risks and its use as a faecal pollution indicator in selected surface water systems in the North West Province

I. The use of sulphite-reducing *Clostridium* (SRC) species as a faecal pollution indicator

The presence of sulphite-reducing *Clostridium* species along with the faecal indicator organisms (FIO) were confirmed in the three surface water systems of interest. The high levels of SRC were correlated with those of FIO at sites in the river systems where faecal pollution was most likely. Wastewater treatment plants alongside the rivers were one of the major contributors of these SRC species and FIO in the surface water systems. These findings substantiate the prospect of SRC species being a suitable surrogate indicator for faecal pollution in surface water systems in the North West Province. However, a limitation of SRC species as a faecal pollution indicator was recognised. This included the absence of *Clostridium* spp. in the faeces of herbivores and its prolonged survival in surface water following faecal pollution (Vierheilig *et al*., 2013; Robles *et al*., 2000). For this reason, it is recommended that SRC species be used in combination with other FIO for a more recent and reliable indication of faecal contamination in surface water.

II. Correlation between the levels of *Clostridium* species and the physico-chemical parameters influenced by seasonal variation

In this study, it was demonstrated that the physico-chemical parameters of surface water are largely influenced by seasonal variation and anthropogenic factors. Physical parameters such as temperature and dissolved oxygen, along with chemical parameters such as chemical oxygen demand, nitrates, phosphates and sulphate salts in the water had effects on the *Clostridium* spp. levels. This was mainly due to non-point source pollution such as surface runoff from the surrounding environments which promoted eutrophication in parts of these river systems, creating ideal environments for *Clostridium* species to accumulate. This occurrence was more noticeable throughout the warm-rainy months of the year than that of the cold-dry months, therefore demonstrating the effect of seasonality on the physico-chemical parameters and levels of *Clostridium* species.

III. Identification of *Clostridium* species

All isolates were successfully identified as *Clostridium* species through the use of staining (Gram and endospore) and DNA sequencing. The 7 *Clostridium* species that were identified in this study included *Clostridium bifermentans*, *C. perfringens*, *C. sordellii*, *C. baratii*, *C. ghonii*, *C. lituseburense* and *C. dakarense*. *Clostridium bifermentans* was the most frequently isolated across all three surface water systems. This was followed by *Clostridium perfringens* and *Clostridium sordellii*, which were present in both the Schoonspruit and Crocodile Rivers. These 2 river systems also harboured *Clostridium baratii.* Furthermore, species such as *C. lituseburense* and *C. dakarense* only occurred in the Crocodile River, whereas *C. ghonii* was identified in solely the Schoonspruit River. Most of these *Clostridium* species have been linked to aquatic environments, except for *C. lituseburense* and *C. dakarense*. The identification of these species in surface water was unique to this study.

IV. The potential associated health risks *Clostridium* species present in surface water

Several of the *Clostridium* species identified throughout this study were known pathogens, with *Clostridium perfringens* being the most noteworthy. This pathogen, along with others such as *Clostridium sordellii* and *Clostridium bifermentans* produce enterotoxins and histotoxins, which cause severe gastrointestinal diseases and necrotising gas-gangrene in both humans and animals (Irikura *et al*., 2015; Stevens and Bryant, 2002). This is alarming since these pathogens were the 3 most abundant of all the *Clostridium* species identified across these surface water systems. Although the occurrence of *Clostridium baratii* was scarce, its presence may lead to neurotoxic diseases such as botulism. The pathogenicity of the other *Clostridium* species has not been well studied, however, all of them have been associated with some kind of medical illness. The presence of these *Clostridium* pathogens

in surface water are cause for concern. Some communities use these surface water systems as a direct source for domestic water, recreational activities and livestock watering. Exposure to these pathogens can be a major health risk to both humans and animals.

4.1.2. Antibiotic resistant *Clostridium* spp. isolated from selected surface water systems and aquatic sediment in the North West Province, South Africa

I. Isolation and identification of antibiotic resistant *Clostridium* species from selected surface water and aquatic sediment

Clostridium species that showed resistance against Ampicillin, Tetracycline or Clindamycin were successfully isolated from surface water and aquatic sediment. All antibiotic resistant *Clostridium* isolates were obtained from the Schoonspruit and Crocodile Rivers. The results of this study showed wastewater treatment plants to be the potential source of antibiotic resistant *Clostridium* species to these two surface water systems. No incidence of antibiotic resistant *Clostridium* species was observed in the Groot Marico River, which could indicate that this river system was minimally impacted by antibiotic pollutants. Using DNA sequencing, all the antibiotic resistant *Clostridium* isolates were successfully identified. The majority of the isolates were confirmed as *Clostridium perfringens*, with a couple of the isolates being identified as *Clostridium sordelli*, *Clostridium baratii* or *Clostridium bifermentans*. All these *Clostridium* species have been connected to serious diseases and infection.

II. Antibiotic resistance profiles in *Clostridium* spp. obtained from surface water and aquatic sediment.

All the antibiotic resistant *Clostridium* spp. were screened for cross-resistance against 6 antibiotics. Antibiotics such as Amoxicillin and Chloramphenicol were found to be the most effective in inhibiting the growth of antibiotic resistant *Clostridium* species, whereas the majority of the isolates showed resistance against Ampicillin and Tetracycline. Both of these are broad-spectrum antibiotics that are prescribed for Gram-positive and Gram-negative infections (Romanis, 2013). Surprisingly, none of the antibiotics tested for in this study was found to be a 100% effective against the *Clostridium* isolates. Furthermore, ten different multi-antibiotic resistant (MAR) phenotypes were also observed across these isolates. The most prevalent one being AMP-TE-DA-MTZ-C-AMX. All isolates that presented this phenotype were obtained from aquatic sediment, suggesting that aquatic sediment may be a reservoir for antibiotic resistance and MAR *Clostridium* species and should be closely monitored.

III. Minimum inhibitory concentration (MIC) of antibiotics

Of all the antibiotic resistant *Clostridium* isolates, 68.1% displayed a MIC of 2 µg/ml Ampicillin, which according to the CLSI, were classified as resistant isolates. Furthermore, 66.7% of isolates showed a MIC ranging from 16-256 µg/ml Tetracycline. A MIC of 256 µg/ml for Clindamycin was found among 33.3% of all *Clostridium* isolates, whereas the MIC of 28.1% of the isolates was between 16-32 µg/ml Metronidazole. Almost 22% of the *Clostridium* isolates had a MIC of 16 µg/ml Chloramphenicol and 19.7% a MIC of 16 µg/ml Amoxicillin. It has been found that elevated MIC values make treating an infection more difficult. Thus, the MIC values reported in this study are alarming.

IV. Presence of antibiotic resistance genes in antibiotic resistant *Clostridium* species

The presence of several antibiotic resistance genes was screened for in this study. One of the genes encoding for macrolide-lincosamide-streptogramin (*erm*F), and β-lactam (bla_{TEM}) resistance were not found in any Clindamycin and Ampicillin resistant isolates, respectively. This suggest that resistance against these antibiotics may be encoded by other genes present in *Clostridium* species. However, several Clindamycin-resistant *Clostridium* isolates were found to harbour the *erm*B gene, which also encodes for macrolide-lincosamide-streptogramin resistance. Two genes encoding for efflux mechanisms against Tetracycline (*tet*K and *tet*L) were found to be present in a couple of Tetracycline resistant isolates. The presence of these antibiotic resistant genes in environmental *Clostridium* species are worrisome. This could add to the decline of an already deteriorating surface water quality and subsequently affect the health of its users, particularly the immune-compromised individuals and livestock.

4.1.3. Concluding remarks

The data collected throughout this study period have shown the presence of potentially pathogenic *Clostridium* species in both surface water and the adjoined sediment. The presence of antibiotic resistant genes in *Clostridium* species isolated from these sources are also worrisome. The expression of these genes could contribute to MAR in these potential pathogenic micro-organisms. Also, these results emphasised the necessity to screen for other antibiotic resistant pathogens in the aquatic environment and to further investigate the possible sources. Finally, outcomes of these studies indicate that the surface water systems in the North West Province are exposed to various pollutants such as antibiotics and faecal contaminants from surface runoff and WWTP. This is cause for concern, considering that a large number of rural and informal settlements directly rely on these water as their primary water source. Health of the users, especially the immune-compromised individuals and livestock could be affected by these organisms

4.2. Recommendations

The following are recommendations for future studies:

A. The results from this study suggested that SRC could be used as a possible faecal indicator organism (FIO). This statement is supported by various studies (Abia *et al*., 2015b; Mubazangi *et al*., 2012; Vijayavel *et al*., 2009). The European Union, as well as the State of Hawaii, have implemented SRC species as indicator organisms in drinking, marine and fresh waters (Ashbolt *et al*., 2001). Therefore, it is recommended to develop standard operational protocols and techniques to determine the presence of SRC species in surface water and implement these for additional surveillance of water quality in South Africa.

B. Various *Clostridium* pathogens were identified, however, their pathogenicity was not determined in this study. It is therefore recommended to investigate the epidemiology and virulence characteristics associated with these *Clostridium* pathogens in the aquatic environments. This will indicate the probability of humans and animals being infected with *Clostridium* from these surface water systems and also support its use as a FIO.

C. Although the Fung double tube method used in this study was effective in isolating *Clostridium* species from surface water and sediment, additional culturing techniques and equipment was needed to obtain useable isolates for all the analyses. Thus, it is suggested to make use of culture-independent techniques such as metagenomics in future studies. According to Zhou and associates (2016), this approach could also asses the microbial community composition and the presence of other potential pathogens.

D. This study reveals the presence of various antibiotic resistance genes in environmental *Clostridium* species. However, not all the isolates harboured the genes screened for in this study. There are numerous genes which encode for antibiotic resistance mechanisms. With the use of whole genome sequencing, the possible presence of all these genes could be investigated.

E. Prior to the adoption of molecular techniques such as the use of 16S rRNA gene sequences, *Clostridium* species were classified by being Gram-positive, endospore forming anaerobic bacteria and non-sulphate reducers (Hippe et al., 1992). This led to the misclassifications of numerous *Clostridium* species and problems with the taxonomic structure (Wiegel *et al*., 2006). The molecular phylogenetics reported in this study illustrated this fact (Figure 2.5). It is therefore recommended that future studies should focus on the reclassification of some of these species by using molecular techniques like DNA-DNA hybridization and whole genome sequencing to resolve this problem.

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Appendix A

Table A1: The microbiological water quality analysis of each sampling site within the 3 respective surface water systems sampled during the warm-rainy season (2015-2016)

Surface water system	Site	Total coliforms (CFU/100ml)	Faecal coliforms (CFU/100ml)	*E. coli* (CFU/100ml)	Faecal streptococci (CFU/100ml)	*Clostridium* spp. (CFU/1ml)
Crocodile River	CR1	207.83	174.00	155.83	156.67	17.92
	CR2	300.00	254.00	204.67	147.67	20.67
	CR3	210.33	185.00	164.17	288.00	26.83
	CR4	222.67	164.33	144.50	200.83	26.42
	CR5	198.67	214.00	164.00	106.00	9.84
	CR6	11.33	7.33	10.00	2.33	12.17
	CR7	36.83	18.83	13.00	22.33	13.83
	Mean	169.67±97.44	145.36±88.10	122.31±72.16	131.98±92.15	18.24±6.75
Groot Marico River	GM1	121.83	61.50	70.33	78.17	0.50
	GM2	105.67	85.67	73.17	141.33	0.83
	GM3	144.00	152.50	113.33	145.00	0.92
	GM4	129.83	224.67	68.50	284.00	1.83
	GM5	300.00	300.00	300.00	300.00	1.17
	GM6	98.83	90.83	52.33	54.00	3.00
	GM7	219.80	236.67	222.17	288.00	11.33
	Mean	160.0±73.59	164.55±90.91	128.55±95.1	184.36±104.65	2.8±3.86
Schoonspruit River	SC1	300.00	38.67	22.67	53.67	0.00
	SC2	300.00	62.33	71.67	169.33	1.00
	SC3	300.00	194.00	88.33	276.00	2.67

SC4	300.00	300.00	300.00	300.00	300.00
SC5	300.00	300.00	211.67	300.00	48.00
Mean	300.0±0.0	179.0±125.31	138.87±113.84	219.8±107.44	70.33±129.98

Table A2: The microbiological water quality analysis of each sampling site within the 3 respective surface water systems sampled during the cold-dry season (2015-2016).

Surface water system	Site	Total coliforms (CFU/100ml)	Faecal coliforms (CFU/100ml)	*E. coli* (CFU/100ml)	Faecal streptococci (CFU/100ml)	*Clostridium* spp. (CFU/1ml)
Crocodile River	CR1	83.17	43.17	28.33	58.17	1.50
	CR2	211.67	128.67	119.83	25.83	14.34
	CR3	81.67	35.00	60.83	151.50	14.33
	CR4	117.67	141.67	96.67	145.67	12.50
	CR5	144.83	109.83	120.67	86.00	10.83
	CR6	29.33	13.00	23.83	20.83	9.50
	CR7	47.33	14.33	41.67	20.00	14.17
	Mean	102.24±62.11	69.38±55.46	70.26±41.86	72.57±57.13	11.02±4.6
Groot Marico River	GM1	8.50	7.00	6.67	14.00	0.00
	GM2	29.17	22.67	15.00	80.33	0.00
	GM3	17.17	13.17	11.33	178.17	0.17
	GM4	15.83	9.67	9.67	217.83	1.50
	GM5	17.33	19.00	3.00	300.00	2.34
	GM6	35.50	30.50	18.17	30.67	0.00
	GM7	83.83	47.00	67.50	140.33	0.17
	Mean	29.62±25.55	21.29±13.89	18.76±22.07	137.33±103.65	0.60±0.94
Schoonspruit River	SC1	300.00	11.67	12.00	11.00	0.00
	SC2	300.00	7.33	3.67	17.33	2.00

	SC3	275.67	159.67	62.33	24.00	0.00
	SC4	300.00	300.00	300.00	300.00	300.00
	SC5	300.00	300.00	300.00	157.17	96.00
	Mean	295.13±10.88	155.73±145.27	135.6±151.74	101.9±126.27	79.60±116.22

Table A3: The physico-chemical parameters of each sampling site within the 3 respective surface water systems sampled during the warm-rainy season (2015-2016).

Surface water system	Site	Temp	pH	TDS (ppm)	Salinity	DO (mg/l)	Nitrate (mg/l)	Nitrite (mg/l)	Phosphate (mg/l)	Sulphate (mg/l)	COD
Crocodile River	CR1	14.72	8.61	431.33	243.83	8.77	4.1	9	3.59	58.25	23.25
	CR2	14.98	8.59	483.5	274.67	8.02	4.25	9.25	3.56	61	31.75
	CR3	12.48	9.42	487.5	275	9.1	0.65	5.75	4.66	52	34.75
	CR4	13.13	9.05	537.17	308.83	8.83	1.3	8.25	3.03	64.25	18.75
	CR5	16.38	8.75	661.5	363.67	8.85	1.93	10.25	3.68	76	21.25
	CR6	14.57	8.95	611.33	350.67	8.48	1.13	13.75	5.57	72.25	27
	CR7	15.15	8.95	582	332.67	7.55	0.18	45.25	9.8	72.75	14.25
Mean		14.49	8.9	542.05	307.05	8.51	1.93	14.5	4.84	65.21	24.43
Standard deviation		1.3	0.29	80.98	44.42	0.55	1.63	13.77	2.35	8.8	7.24
Groot Marico River	GM1	19.13	7.99	214.83	123.17	4.58	0.85	9	0.21	1.5	8.5
	GM2	14.53	8.28	231.17	130	6.42	1.05	1.5	0.5	1	0
	GM3	13.48	8.14	103.17	58.83	6.02	0.53	0.75	0.3	19.75	0
	GM4	14.9	8.36	234.83	131	5.55	0.9	4	0.4	3.75	0
	GM5	14.23	8.46	505.33	267	8.87	0	6	0.15	76	20.5
	GM6	18.77	8.33	209.5	132.17	5.07	0.45	4	0.3	5.75	3.5
	GM7	15.63	8.48	278	155.33	5.73	2.05	2.75	0.09	14.25	18.5
Mean		15.81	8.29	253.83	142.5	6.03	0.83	4	0.28	17.43	7.29

Stander deviation		2.24	0.18	123.08	62.5	1.39	0.64	2.81	0.14	26.75	8.9
Schoonspruit River	SC1	13.07	8.19	574	311.67	10.83	0.2	21.5	0.04	5	14.5
	SC2	15.23	8.53	510.67	278.67	12.37	0.4	12	0.24	9	7
	SC3	14.2	8.04	591.67	337.67	6.42	1.3	7.75	0.28	103	5.5
	SC4	17.1	8.04	886.67	550.67	3.87	0.9	0.06	3.78	86	205.5
	SC5	12.73	7.7	922.33	532.67	3.43	1.43	7	4.78	119.75	29
Mean		14.47	8.1	697.07	402.27	7.38	0.85	9.66	1.82	64.55	52.3
Standard deviation		1.77	0.3	192.15	129.12	4.05	0.54	7.88	2.27	53.89	86.15

Table A4: The physico-chemical parameters of each sampling site within the 3 respective surface water systems sampled during the cold-dry season (2015-2016).

Surface water system	Site	Temp	pH	TDS (ppm)	Salinity	DO (mg/l)	Nitrate (mg/l)	Nitrite (mg/l)	Phosphate (mg/l)	Sulphate (mg/l)	COD
Crocodile River	CR1	20.18	8.34	437	250.33	4.55	2.03	7.5	2.96	69.75	27
	CR2	20.22	8.44	498.17	286.5	5.12	1.85	6.25	2.16	65.5	24.5
	CR3	19.32	9.2	495.33	279.33	5.1	0.5	3.5	2.49	51	46.25
	CR4	19.92	8.93	526.83	305.83	5.32	0.7	4	1.1	64	20.25
	CR5	17.93	8.53	745.67	376.33	1.9	0.65	6.5	1.51	78.5	32
	CR6	14.4	8.63	688	377	1.53	0.9	11	1.97	74	20
	CR7	21.35	8.6	499	282.83	4.02	0.13	7.75	1.22	59.25	23.25
Mean		19.05	8.67	555.71	308.31	3.93	0.96	6.64	1.92	66	27.61
Standard deviation		2.13	0.28	106.02	45.79	1.46	0.65	2.33	0.63	8.53	8.52
Groot Marico River	GM1	22.75	7.8	212	123.17	8.97	0.8	12	1.89	10.75	143.5
	GM2	21.12	8.08	224.5	129.5	7.58	1.6	5	0.53	5	53
	GM3	18.1	7.51	90.28	54	6.45	0.55	0	0.55	2.75	60.5
	GM4	23.03	7.94	227.17	131.5	5.97	0.3	4.5	0.53	1.25	47
	GM5	14.23	7.9	513.33	279	6.77	0	3	0.13	80	38
	GM6	23.68	8.08	209.67	121.5	4.97	0.45	8.5	0.38	5	10
	GM7	21.07	8	293.17	167.33	3.85	0.65	10	0.46	18.25	43
Mean		20.57	7.9	252.87	143.71	6.36	0.62	6.14	0.64	17.57	56.43

Standard deviation		3.35	0.2	129.68	68.53	1.68	0.5	4.21	0.57	28.13	41.58
Schoonspruit River	SC1	14.23	8.29	624	340.33	8.93	0.45	7	0.5	1	0
	SC2	15.53	8.14	648.67	356.67	6.63	0.05	4.5	0.61	28.5	9
	SC3	20.82	7.93	537.5	314.5	6.15	0.48	5.5	0.32	154	60.75
	SC4	26.63	7.81	812.67	507.33	2.2	6.2	17.5	8.5	210	200
	SC5	20.1	7.45	868.33	500.83	2.65	0.85	6.5	7.63	167.75	56.25
Mean		19.46	7.92	698.23	403.93	5.31	1.61	8.2	3.51	112.25	65.2
Standard deviation		4.91	0.32	137.69	92.68	2.84	2.58	5.29	4.17	91.88	80.13

Table A5: GenBank identification of the amplified *Clostridium* species isolates from the various surface water systems during the warm-rainy seasons (2015-2016).

Year	River system	Site	Isolate ID	Identity	Accession no.	E-value	Identity	Gram stain	Endospore
2015	Crocodile	2	CR1.1	*Clostridium bifermentans*	KT624613.1	0	100%	+	+
		2	CR1.2	*Clostridium perfringens*	AP017630.1	0	100%	+	+
		3	CR1.3	*Clostridium dakarense*	NR_133028.1	0	100%	+	+
		3	CR1.4	*Clostridium dakarense*	NR_133028.1	0	99%	+	+
		2	CR1.5	*Clostridium perfringens*	KU601406.1	0	99%	+	+
		5	CR1.6	*Clostridium bifermentans*	LN998086.1	0	100%	+	+
		5	CR1.7	*Clostridium bifermentans*	LN998086.1	0	100%	+	+
		6	CR1.8	*Clostridium sp.*	KF620499.1	0	99%	+	-
		6	CR1.9	*Clostridium bifermentans*	KT633855.1	5.00E-177	99%	+	+
		7	CR1.10	*Clostridium bifermentans*	KT887961.1	0	100%	+	+
	Groot Marico	3	GM1.1	*Clostridium bifermentans*	KP944171.1	0	99%	+	+
		6	GM1.2	*Clostridium bifermentans*	KT367517.1	0	100%	+	+
	Scoonspruit	4	SC1.1	*Clostridium sordellii*	KU531492.1	0	99%	+	+
		4	SC1.2	*Clostridium sordellii*	KU531492.1	0	99%	+	+
		4	SC1.3	*Clostridium bifermentans*	KT633855.1	5.00E-172	99%	+	+
		4	SC1.4	*Clostridium sordellii*	KR364762.1	0	99%	+	+
		4	SC1.5	*Clostridium bifermentans*	KT633855.1	3.00E-65	97%	+	+
		4	SC1.6	*Clostridium bifermentans*	JQ271547.1	0	99%	+	+
		4	SC1.7	*Clostridium bifermentans*	LN998086.1	0	94%	+	+

		4	SC1.8	*Clostridium bifermentans*	KT367517.1	0	100%	+	+
		4	SC1.9	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		2	SC1.10	*Clostridium bifermentans*	LN998086.1	0	100%	+	+
		4	SC1.11	*Clostridium bifermentans*	KT624614.1	0	99%	+	+
2016	Crocodile	1	CR3.1	*Clostridium bifermentans*	LN998086.1	0	100%	+	+
		4	CR3.2	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
		4	CR3.3	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
		4	CR3.4	*Clostridium lituseburense*	EU887828.1	0	98%	+	+
		4	CR3.5	*Clostridium bifermentans*	KT887961.1	0	99%	+	+
	Groot Marico	2	GM3.1	*Clostridium bifermentans*	KP944171.1	0	99%	+	+
		3	GM3.2	*Clostridium bifermentans*	KP944171.1	0	99%	+	+
		7	GM3.3	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
		7	GM3.4	*Clostridium bifermentans*	LN998086.1	0	100%	+	+
		7	GM3.5	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
		1	GM3.6	*Clostridium bifermentans*	LN998086.1	0	98%	+	+
		1	GM3.7	*Clostridium bifermentans*	KT887961.1	0	99%	+	+
		8	GM3.8	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
		1	GM3.9	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
	Scoonspruit	4	SC3.1	*Clostridium bifermentans*	KT367517.1	0	100%	+	+
		4	SC3.2	*Clostridium bifermentans*	KT624613.1	0	99%	+	+
		4	SC3.3	*Clostridium perfringens*	CP000312.1	0	99%	+	+
		4	SC3.4	*Clostridium bifermentans*	LN998086.1	0	99%	+	+
		4	SC3.5	*Clostridium bifermentans*	LN998086.1	0	100%	+	+

Table A6: GenBank identification of the amplified *Clostridium* species isolates from the various surface water systems during the cold-dry seasons (2015-2016).

Year	River system	Site	Isolate ID	Identity	Accession no.	E-value	Identity	Gram stain	Endospore
2015	Crocodile	1	CR2.1	*Clostridium bifermentans*	KP944171.1	0	100%	+	+
		1	CR2.2	*Clostridium bifermentans*	KT633855.1	1.00E-90	100%	+	+
		1	CR2.3	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		2	CR2.4	*Clostridium bifermentans*	KT633855.1	3.00E-138	100%	+	+
		3	CR2.5	*Clostridium bifermentans*	KT633855.1	2.00E-78	100%	+	+
		3	CR2.6	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		3	CR2.7	*Clostridium bifermentans*	KT624613.1	0	100%	+	+
		3	CR2.8	*Clostridium sp.*	KR364757.1	3.00E-97	97%	+	-
		4	CR2.9	*Clostridium bifermentans*	KT624614.1	0	100%	+	+
		5	CR2.10	*Clostridium bifermentans*	KT367517.1	0	100%	+	+
		5	CR2.11	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		5	CR2.12	*Clostridium bifermentans*	KT624614.1	0	100%	+	+
		6	CR2.13	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		6	CR2.14	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		6	CR2.15	*Clostridium bifermentans*	KT633855.1	3.00E-158	100%	+	+
		6	CR2.16	*Clostridium bifermentans*	KT633855.1	2.00E-149	100%	+	+
		7	CR2.17	*Clostridium sordellii*	KU531492.1	0	100%	+	+
		7	CR2.18	*Clostridium bifermentans*	KT887961.1	0	100%	+	+

	Groot Marico	4	GM2.1	*Clostridium bifermentans*	KT624614.1	0	100%	+	+
		6	GM2.2	*Clostridium bifermentans*	KT624614.1	4.00E-152	100%	+	+
		6	GM2.3	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		6	GM2.4	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
	Schoonspruit	5	SC2.1	*Clostridium ghonii*	JN048962.1	0	100%	+	+
		5	SC2.2	*Clostridium ghonii*	JN048962.1	0	100%	+	+
		5	SC2.3	*Clostridium sordellii*	KU531492.1	2.00E-50	100%	+	+
		5	SC2.4	*Clostridium bifermentans*	KT624614.1	0	98%	+	+
		5	SC2.5	*Clostridium sordellii*	KR364762.1	0	99%	+	+
		5	SC2.6	*Clostridium bifermentans*	KT633855.1	0	100%	+	+
		5	SC2.7	*Clostridium sp.*	KT002346.1	0	100%	+	+
		5	SC2.8	*Clostridium bifermentans*	KT633855.1	7.00E-78	100%	+	+
		5	SC2.9	*Clostridium sordelli*	KR364762.1	0	100%	+	+
		5	SC2.10	*Clostridium sordelli*	KR364762.1	0	100%	+	+
		5	SC2.11	*Clostridium sp.*	KT002346.1	0	100%	+	+
		5	SC2.12	*Clostridium sp.*	KT002334.1	0	100%	+	+
		5	SC2.13	*Clostridium sordelli*	KR364762.1	0	98%	+	+
		3	SC2.14	*Clostridium sp.*	JF312719.1	6.00E-94	100%	+	+
		5	SC2.15	*Clostridium bifermentans*	KT633855.1	3.00E-138	100%	+	+
2016	Crocodile	1	CR4.1	*Clostridium bifermentans*	KT633855.1	7.00E-155	100%	+	+
		7	CR4.2	*Clostridium bifermentans*	KT624614.1	0	100%	+	+
		3	CR4.3	*Clostridium sordellii*	KU531492.1	5.00E-177	100%	+	+
		7	CR4.4	*Clostridium bifermentans*	KT633855.1	5.00E-177	100%	+	+

		2	CR4.5	*Clostridium bifermentans*	KT633855.1	9.00E-118	100%	+	+
		7	CR4.6	*Clostridium bifermentans*	KT633855.1	7.00E-93	100%	+	+
		2	CR4.7	*Clostridium perfringens*	KX214076.1	0	100%	+	+
		2	CR4.8	*Clostridium baratii*	KP944152.1	0	100%	+	+
		2	CR4.9	*Clostridium perfringens*	CP010993.1	0	100%	+	+
		2	CR4.10	*Clostridium perfringens*	KX214076.1	0	99%	+	+
		2	CR4.11	*Clostridium perfringens*	KX214076.1	0	100%	+	+
		2	CR4.12	*Clostridium perfringens*	CP010993.1	0	100%	+	+
		5	CR4.13	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		5	CR4.14	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		5	CR4.15	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		5	CR4.16	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		5	CR4.17	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		5	CR4.18	*Clostridium lituseburense*	JN792340.1	0	99%	+	+
		5	CR4.19	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		5	CR4.20	*Clostridium lituseburense*	FM875817.1	0	99%	+	+
Groot Marico		3	GM4.1	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
		4	GM4.2	*Clostridium sp.*	JF312673.1	0	99%	+	+
		8	GM4.3	*Clostridium bifermentans*	KU529654.1	0	100%	+	+
Schoonspruit		4	SC4.1	*Clostridium bifermentans*	KT624613.1	0	100%	+	+
		4	SC4.2	*Clostridium bifermentans*	KT624613.1	0	100%	+	+
		4	SC4.3	*Clostridium perfringens*	KX214076.1	0	100%	+	+
		4	SC4.4	*Clostridium baratii*	KP944152.1	0	100%	+	+

	4	SC4.5	*Clostridium perfringens*	KX214076.1	2.00E-160	100%	+	+
	4	SC4.6	*Clostridium perfringens*	KU601406.1	0	100%	+	+
	4	SC4.7	*Clostridium perfringens*	KX214076.1	0	100%	+	+

Appendix B

Table B1: Antibiotic susceptibility profiles of all antibiotic resistant *Clostridium* isolates obtained from surface water and sediment from the the Schoonspruit and Crocodile Rivers against Tetracycline (TE), Ampicillin (AMP), Amoxicillin (AMX), Clindamycin (DA), Chloramphenicol (C), and Metronidazole (MTZ).

River system	Site	Source	Isolate ID	Species ID	Antibiotics					
					TE	AMP	AMX	DA	C	MTZ
Tetracycline Screened Isolates										
Crocodile	CR2	Surface water	SC4-Su-T1	*C. bifermentans*	R	S	S	S	S	I
	CR2	Surface water	SC4-Su-T2	*C. bifermentans*	R	S	S	S	S	S
	CR2	Sediment	SC4-Se-T3	*C. sordelli*	R	S	S	R	S	S
	CR2	Sediment	SC4-Se-T4	*C. perfringens*	R	S	S	S	R	S
	CR2	Sediment	SC4-Se-T5	*C. sordelli*	R	R	S	R	S	S
Schoonspruit	SC5	Surface water	SC4-Su-T6	*C. perfringens*	R	S	S	S	S	S
	SC5	Surface water	SC4-Su-T7	*C. baratii*	R	S	S	S	S	S
	SC5	Surface water	SC4-Su-T8	*C. perfringens*	R	S	S	S	S	S
	SC5	Surface water	SC4-Su-T9	*C. perfringens*	R	S	S	S	S	I
	SC5	Sediment	SC4-Se-T10	*C. baratii*	R	R	S	S	S	S
	SC5	Surface water	SC4-Su-T11	*C. perfringens*	R	S	S	S	S	S
	SC5	Surface water	SC4-Su-T12	*C. perfringens*	R	S	S	S	S	S
	SC5	Surface water	SC4-Su-T13	*C. perfringens*	R	S	S	S	S	I
	SC5	Surface water	SC4-Su-T14	*C. perfringens*	R	I	S	S	I	I
	SC5	Sediment	SC4-Se-T15	*C. perfringens*	R	R	R	S	R	R
	SC5	Surface water	SC4-Su-T16	*C. perfringens*	R	S	S	S	I	I
	SC5	Surface water	SC4-Su-T17	*C. perfringens*	R	S	S	S	S	S

Ampicillin Screened Isolates											
Schoonspruit		SC2	Sediment	SC4-Se-A1	*C. perfringens*	S	R	S	S	R	S
		SC2	Surface water	SC4-Su-A2	*C. perfringens*	S	R	S	S	S	S
		SC2	Surface water	SC4-Su-A3	*C. perfringens*	S	R	S	S	S	S
		SC5	Surface water	SC4-Su-A4	*C. perfringens*	S	R	S	S	S	S
		SC5	Sediment	SC4-Se-A5	*C. perfringens*	R	R	S	S	S	S
		SC5	Sediment	SC4-SeA6	*C. perfringens*	I	R	S	S	S	R
		SC5	Sediment	SC4-Se-A7	*C. perfringens*	I	R	S	S	S	S
		SC5	Sediment	SC4-Se-A8	*C. perfringens*	I	R	S	S	S	S
		SC5	Surface water	SC4-Su-A9	*C. perfringens*	S	R	S	S	S	S
		SC5	Sediment	SC4-Se-A10	*C. perfringens*	R	R	S	S	S	S
		SC5	Sediment	SC4-Se-A11	*C. perfringens*	R	R	S	S	S	I
		SC5	Sediment	SC4-Se-A12	*C. perfringens*	I	R	S	S	S	I
		SC5	Surface water	SC4-Su-A13	*C. perfringens*	S	R	S	S	S	S
		SC5	Sediment	SC4-Se-A14	*C. perfringens*	I	R	S	S	S	S
		SC5	Surface water	SC4-Su-A15	*C. perfringens*	S	R	S	S	S	S
		SC5	Sediment	SC4-Se-A16	*C. sordelli*	I	R	S	S	S	S
		SC5	Sediment	SC4-Se-A17	*C. perfringens*	S	R	S	S	R	S
		SC5	Sediment	SC4-Se-A18	*C. perfringens*	R	R	S	S	I	S
		SC5	Surface water	SC4-Su-A19	*C. perfringens*	S	R	S	S	S	S
		SC5	Sediment	SC4-Se-A20	*C. perfringens*	I	R	S	S	S	S
Clindamycin Screened Isolates											
Crocodile		CR2	Surface water	CR2-Se-C1	*C. perfringens*	R	S	S	R	S	S

	CR2	Surface water	SC4-Su-C2	*C. perfringens*	S	R	R	R	S	R
	CR2	Surface water	SC4-Su-C3	*C. perfringens*	R	R	I	R	I	R
	CR2	Surface water	CR2-Se-C4	*C. perfringens*	R	S	S	R	R	I
	CR2	Sediment	SC4-Se-C5	*C. perfringens*	S	R	S	R	R	R
	CR2	Sediment	SC4-Se-C6	*C. perfringens*	R	R	R	R	R	R
	CR2	Surface water	CR2-Se-C7	*C. perfringens*	R	S	S	R	S	I
	CR2	Sediment	SC4-Se-C8	*C. perfringens*	R	R	S	R	R	R
	CR2	Sediment	SC4-Se-C9	*C. perfringens*	R	R	R	R	R	R
	CR2	Surface water	SC4-Su-C10	*C. perfringens*	R	R	I	R	S	R
Schoonspruit	SC2	Sediment	SC4-Se-C11	*C. perfringens*	R	R	R	R	R	R
	SC2	Sediment	SC4-Se-C12	*C. perfringens*	R	R	R	R	R	R
	SC2	Surface water	SC4-Se-C13	*C. perfringens*	S	R	R	R	S	R
	SC5	Surface water	SC4-Su-C14	*C. perfringens*	S	R	R	R	S	I
	SC5	Sediment	SC4-Se-C15	*C. perfringens*	R	R	R	R	R	R
	SC5	Sediment	SC4-Se-C16	*C. perfringens*	R	R	R	R	R	R
	SC5	Surface water	SC5-Se-C17	*C. perfringens*	R	R	S	R	S	R
	SC5	Sediment	SC4-Se-C18	*C. perfringens*	R	R	R	R	R	R
	SC5	Surface water	SC5-Su-C19,	*C. perfringens*	R	R	I	R	S	I
	SC5	Surface water	SC5-Su-C20	*C. perfringens*	R	R	I	R	S	I
	SC5	Sediment	SC4-Se-C21	*C. perfringens*	R	R	R	R	R	R
	SC5	Surface water	CR2-Se-C22	*C. perfringens*	S	S	S	R	S	S
	SC5	Surface water	CR2-Se-C23	*C. perfringens*	S	S	S	R	S	S
	SC5	Sediment	SC4-Su-C24	*C. perfringens*	R	R	I	R	R	R

SC5	Surface water	SC4-Su-C25	*C. perfringens*	R	R	S	R	S	R
SC5	Surface water	SC4-Su-C26	*C. perfringens*	R	R	R	R	I	R
SC5	Surface water	SC4-Su-C27	*C. perfringens*	R	R	R	R	I	R
SC5	Surface water	SC4-Su-C28	*C. perfringens*	R	R	R	R	S	R
SC5	Surface water	SC4-Su-C29	*C. perfringens*	R	R	R	R	S	R
SC5	Sediment	SC4-Se-C30	*C. perfringens*	R	R	R	R	R	R

Appendix C

Table C1: GPS coordinates of different sample sites across the Crocodile, Groot Marico and Schoonspruit Rivers.

Surface water system	Site		Longitude (S)	Latitude (E)
Crocodile River	CR1	Before Brits	25°40'9.71"	27°47'33.66"
	CR2	After Brits	25°32'58.05"	27°42'50.84"
	CR3	Pienaars River	25°06'24.7"	27°33'55.2"
	CR4	Koedoeskop Bridge	25°03'59.6"	27°31'06.2"
	CR5	Thabazimbi	24°39'53.63"	27°22'40.39"
	CR6	After Thabazimbi	24°24'5.35"	27°05'51.73"
	CR7	Bend in Crocodile River	24°12'57.30"	26°53'54.28"
Groot Marico River	GM1	Marico Eye, source of Marico River.	25°47’22.16"	26°21'59.85”
	GM2	Marico River before its confluence with Sterkstroom, 20 km downstream of Eye.	25°39’34.64”	26°26’01.0”
	GM3	Sterkstroom 5 km before its confluence with Marico River.	25°38’50.82”	26°29’20.47”
	GM4	Marico River 10 km above Marico Bosveld Dam, after its confluence with Sterkstroom.	25°35’19.28”	26°24’38.85”
	GM5	Klein Marico River 1km below Klein-Maricopoort (Bospoort) Dam.	25°30’49.27”	26°09’30.78”
	GM6	Marico River immediately below Marico Bosveld Dam.	25°27’42.51”	26°23’30.92”
	GM7	Marico River at Derdepoort.	24°50’42.62”	26°29’11.08”
Schoonspruit River	SC1	Bodenstein	26°25'16.4"	26°43'42.8"
	SC2	Confluence Brakspruit	26°40'46.0"	26°34'58.7"
	SC3	Voortrekker	26°51'54.4"	26°39'30.3"
	SC4	Downstream WWTP	26°53'53.5"	26°38'30.4"
	SC5	Orkney	26°59'09.1"	26°37'54.7"