Potentially pathogenic biocide tolerant heterotrophic bacteria from sewage & river water

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Dissertation submitted in fulfilment of the requirements for the degree *Master of Science in Microbiology* at the North-West University

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DECLARATION

I declare that the dissertation submitted by me for the degree Masters in Microbiology at the North-West University (Potchefstroom Campus), Potchefstroom, North-West, South Africa, is my own independent work and has not previously been submitted by me at another university.

Signed in Potchefstroom, South Africa

Signature:

Date:

Brendon Mann

ABSTRACT

A problem we are facing in South Africa is the release of antimicrobial compounds into our natural water bodies. There have been reports on the emergence of biocide resistance and the accompanying possibility of antibiotic cross-resistance. Bacteria that exhibit tolerance to an antimicrobial might be selected by means of recurring exposure to a low concentration or increasing concentration of an antimicrobial. The aim of the study was to identify and characterise potentially pathogenic Triclosan (TCS) - and/or Chloroxylenol (PCMX) tolerant, heterotrophic plate count bacteria isolates from the wastewater treatment plant (WWTP) in Potchefstroom and the Mooi River. Nutrient agar supplemented with the biocides was used to isolate and maintain tolerant bacteria. Isolates were identified by sequencing of the 16S rDNA region and where relevant clonal relationships between specific isolates were elucidated using ERIC-PCR. Selected isolates were characterised for their MICs against TCS and PCMX, as well as antibiotics resistance profiles. Synergistic and antagonistic interactions between the biocides and selected antibiotics were also evaluated. Isolates were also screened for the presence of extracellular enzymes associated with virulence. These results along with antibiotic resistance profiles were used to generate a pathogenic potential index to assess potential pathogenicity and the associated health risks. Of the isolates obtained *Pseudomonas*, *Bacillus*, *Klebsiella* and *Aeromonas* spp. are well described opportunistic pathogens. Based on current fingerprinting methods it is not yet clear if the WWTP is the source from which these organisms enter the environment and more study is required to obtain more conclusive results. Isolates exhibited various levels of resistance to antibiotics and the biocides in question as well as several occurrences of synergy and to antagonisms, but further study is required to determine the resistance mechanisms involved. HPLC revealed the presence of both biocides in the WWTP influent, bacteria are thus exposed to these biocides during their initial inflow into the WWTP and possibly during the wastewater treatment process, but both TCS and PCMX seem to be sufficiently removed by the WWTP as there were no traces found in the WWTP effluent. Extracellular enzyme production along with antibiotic resistance profiles was used to generate a pathogenic potential index. This revealed that several of the isolates had

very high potential for virulence, but further study is required to identify the specific virulence genes associated with the isolates in question.

Keywords: Triclosan (TCS), Antimicrobial resistance, Chloroxylenol (PCXM), WWTP

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ABBREVIATIONS

A	
AIDS	Acquired Immunodeficiency Syndrome
A10	Amoxicillin
B	
bp	Base pairs
C	
C	Chondroitinase
C30	Chloramphenicol
D	
D	DNase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
E	
EDTA	Ethylenediaminetetraacetic acid
ERIC	Enterobacterial repetitive intergenic consensus
G	
G	Gelatinase
g/L	Gram per litre
H	
H	Hyaluronidase
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPC	Heterotrophic plate count
HPLC	High performance liquid chromatography
I	
I	Intermediate
K	
KCl	Potassium chloride
K30	Kanamycin
L	
L	Litre
Le	Lecithinase
Li	Lipase
M	
M	Molar
mm	Millimetre
$MgCl_2$	Magnesium chloride

mg/L	Milligram per litre
mg/ml	Milligram per millilitre
MIC	Minimum inhibitory concentration
mM	Millimolar
N	
NaCl	Sodium chloride
NaI	Sodium iodide
ng/l	Nano gram per litre
ng/µl	Nano gram per microlitre
O	
O30	Oxytetracycline
P	
P	Proteinase
PCMX	Chloroxylenol
PCP's	Personal care products
PCR	Polymerase chain reaction
pmol	Picomole
R	
R	Resistant
S	
S	Susceptible
SPE	Solid phase extraction
T	
TCC	Triclocarban
TCS	Triclosan
T5	Trimethoprim
U	
UPGMA	Unweighted pair group method with arithmetic mean
V	
V30	Vancomycin
W	
WWTP	Wastewater treatment plant
µ	
µg/l	Microgram per litre
µl	Microliter
µm	Micrometre

CHAPTER 1 – GENERAL INTRODUCTION

1.1. Introduction

Multidrug resistant bacteria pose a major health concern and the development of multidrug-resistance, as well as co- and cross-resistance, is driven by continuous exposure of bacteria to metals, preservatives, antibiotics and biocides (Wales & Davies, 2015; Romero *et al*., 2017). In recent times, there has been an upsurge in the use of biocides in household products, such as cleansers, soap and dishwashing detergents (Fraise, 2002; Levy, 2001). Large amounts of personal care products (PCP's) frequently enter wastewater treatment plants, which in turn act as a point source of pollution from which these substances can enter the environment (Byrns, 2001; Oppenheimer *et al.,* 2007; Fatta-Kassinos *et al*., 2010).

Two common antimicrobials found in several personal care products include triclosan (TCS) and Chloroxylenol (PCMX) (McDonnel & Russel, 1999; Chen *et al.,* 2014a). Studies by both Tabak *et al.* (2009) and Tattawasart *et al.* (1999), have previously demonstrated the occurrence of bacterial resistance to biocides such as TCS. A review of literature indicates that bacterial resistance to TCS, as well as cross-resistance to antibiotics is a very realistic problem and, considering TCS's environmental accumulation and persistence, more research into reduced bacterial susceptibility to TCS and to antibiotics, is worth investigating (Russel, 2002; Carey *et al*., 2015; Yueh & Tukey, 2016). There is very little evidence that PCMX resistance or cross-resistance to antibiotics is occurring. However, PCMX has been detected in river systems and, even though its environmental persistence is limited, the possibility of long term exposure to this compound and the possible development of resistance, should still remain a concern (Russel, 1998; Russel, 2002).

A large portion of South Africa's population still utilize untreated water leading to an increased risk of infection due to water borne pathogens (Zamxaka *et al.*, 2004; Momba *et al.,* 2006). It is therefore important to further examine any direct connections between antibiotic and biocide resistance and whether the presence of these biocides

can possibly lead to selection for multidrug resistant potentially pathogenic bacteria (Wales & Davies, 2015; Romero *et al*., 2017).

1.2 Problem Statement

Large amounts of PCP's frequently enter wastewater treatment plants (WWTP) (Oppenheimer *et al.,* 2007; Fatta-Kassinos *et al*., 2010; Byrns, 2001). Two common antimicrobials found in several PCP's include triclosan (TCS) and Chloroxylenol (PCMX) (McDonnel & Russel, 1999; Chen *et al,* 2014a). Bacteria may be exposed to these antimicrobials during the wastewater treatment process and in the receiving river systems if the WWTP does not remove or degrade these compounds during the wastewater treatment process. Thus, it is important to investigate whether these biocides are present in the WWTP and the receiving river system, and to determine if continuous bacterial exposure to these biocides possibly selects for potentially pathogenic multidrug-resistant bacteria (Wales & Davies, 2015; Romero *et al*., 2017). If this is, in fact, the case there may be a potential health concern considering that a large portion of South Africa's population still utilize untreated water on a regular basis (Zamxaka *et al.*, 2004; Momba *et al.,* 2006).

1.3 Aim

The aim of the study was to identify and characterise potentially pathogenic triclosan and/or chloroxylenol tolerant, heterotrophic plate count bacterial isolates from sewage effluent and river water from the WWTP in Potchefstroom and the Mooi River.

1.4 Objectives

Objectives include the following

- To isolate, identify and characterise potentially pathogenic TCS and/or PCMX tolerant, heterotrophic plate count (HPC) bacteria
- Assay for cross-resistance to selected antibiotics
- To determine if antibiotic resistance patterns enhance in the presence of varying concentrations of TCS and/or PCMX
- To determine minimum inhibitory concentrations (MICs)

- Measurement of TCS and/or PCMX concentrations before and after the wastewater treatment process as well as in wetland, up- and downstream samples by using high performance liquid chromatography (HPLC) methods

CHAPTER 2 – LITERATURE REVIEW

2.1 The Water Situation in South Africa

South Africa has been experiencing criticism for some period due to the incompetence of its municipal departments and local governments to deliver rudimentary services such as water and sanitation to the public (Brettenny & Sharp, 2016). Water quality issues in South Africa include eutrophication, microbial contamination, turbidity, salinization, toxicants, metal contaminants and acid mine drainage. These issues are mostly driven by poor wastewater treatment, informal dense settlements, urbanisation and the mining and agricultural industries (Dhemba & van Veelen, 2011). A significant amount of the disease burden in South Africa is also due to unsafe water and lack of sanitation (Lewin *et al.*, 2007).

Investigations done by the District Water Affairs, confirm that South Africa's situation regarding wastewater treatment and compliance with water regulations need to be addressed immediately, due to wastewater services that are highly unacceptable when compared to those of national and international standards (Department of Water Affairs, 2009). Outdated and inadequate water treatment regimes, sewage treatment plant infrastructure and unskilled personnel exacerbate the problem, although improvement on this front is ongoing with the help of the development of the Blue and Green Drop assessments (Oelofse & Strydom, 2010; Brettenny & Sharp, 2016). When examining an annual Green Drop service audit, it is clear that there has been improvement in municipal waste management, but a large number of municipalities are still in a poor to critical state and a large number of wastewater service systems are not currently being assessed (Department of Water Affairs, 2009).

Many people in South Africa obtain water from springs and rivers, and although government has implemented many rural water supply schemes, drinking water frequently remains of poor quality. In South Africa approximately 17% of the population do not have access to potable water and around 54% lack basic sanitation. Nearly 80% of South Africa's population rely on surface water as their primary source of water; this indicates that many people still utilize untreated surface water for

domestic use. The fact that such a large portion of South Africa's population still utilizes untreated water leads to an increased risk of infection due to water borne pathogens (Zamxaka *et al.*, 2004; Momba *et al.,* 2006).

2.2 Human Health

Water-borne pathogen contamination and the associated diseases, are a major human health concern throughout the world. Illnesses caused by protozoa, bacteria and viruses have been the cause of many outbreaks and affect millions of people, especially in developing countries such as those in Africa (Pandey *et al.,* 2014). Organisms such as *Shigella, Vibrio* and *Salmonella* spp*.* are frequently the causative agents involved in water-borne disease outbreaks. However, in recent times certain emerging pathogens such as *Aeromonas* spp*., Helicobacter pylori,* and *Burkholderia pseudomallei* which have the potential to spread through aquatic ecosystems, have also become a cause for concern (Ashbolt, 2004; Cabral, 2010; Pandey *et al.,* 2014). Opportunistic pathogens have previously been responsible for several cases of infection, particularly nosocomial infection of immunocompromised patients, and the increase in antimicrobial resistance among these organisms may make them incredibly difficult to treat (Levin *et al.,* 1999; Gilbert & McBain, 2003; Ventola, 2015).

Human Immunodeficiency Virus (HIV) causes Acquired Immunodeficiency Syndrome (AIDS). The main immune defect in AIDS results from a decrease in CD4+ helper – inducer T lymphocytes in numbers and effectiveness. This occurs due to the killing of CD4+ T lymphocytes by the virus but might also include additional mechanisms. Effects on the CD4+ T lymphocytes subsequently lead to failure of cell-mediated immune responses; antibody production is also affected, overwhelming the capacity of infected individuals to respond to specific antigens. The end result is that AIDS patients become more susceptible to a range of fungal, viral and bacterial infections. By 2007, an estimated 33.2 million people were living with HIV; sub-Saharan Africa was said to have 67% of all HIV-1 infected people in the world, and Southern Africa shared the disproportionate global burden of HIV and AIDS related deaths (Ahmad *et al.*, 2010).

During the last decade South Africa has made significant progress in reducing the incidence of HIV among its population, while also increasing the number of people on antiretroviral treatment, thus reducing mortality and increasing life expectancy. Regardless of the progress made a very large portion of the South African population still suffers from the HIV and AIDS epidemic (South African National HIV Prevalence, Incidence and Behaviour Survey, 2012). Many of these infected people do not have access to an adequate supply of potable water, and there is also a lack of proper sanitation in many areas. Untreated water leads to an increased risk of infection due to water borne pathogens; this along with the HIV epidemic poses a serious health risk especially from opportunistic pathogens (Zamxaka *et al.*, 2004; Momba *et al.,* 2006).

2.3 Xenobiotics

A very realistic problem we are facing is the release of xenobiotic compounds into the aquatic environment. Xenobiotics can be defined as any substance that is foreign to an ecological system (Byrns, 2001). Environmental pollution by xenobiotics is a common occurrence worldwide primarily due to human activity, and the release of these pollutants can have several effects on the receiving natural environment (Embrandiri *et al.,* 2016). These compounds can be extremely persistent once they enter the natural environment and may lead to bioaccumulation or biomagnification among food chains (Godheja *et al.,* 2016).

The two most important sources from which xenobiotic chemicals enter WWTP's are urban drainage and industrial discharge (Byrns, 2001). A large portion of these compounds that enter wastewater treatment plants are from products such as fragrances, flame retardants, plasticizers, nonylphenol, nonylphenol ethoxilates and household antimicrobials (Fatta-Kassinos *et al.,* 2010). If these compounds are not removed during the wastewater treatment process, a fraction or by-products of these compounds may be released into the environment as part of effluent or a component of sludge. Although much of the environmental contamination of these chemicals comes from non-point sources, a large fraction does come from WWTPs. Thus, WWTPs act as a continuous point source from which PCP's enter the environment. These substances can have a wide range of physical and chemical characteristics and effects on the aquatic environment (Byrns, 2001; Oppenheimer *et al.,* 2007).

Depending on their fate in the natural environment these compounds may come into contact with microorganisms which, in several instances, play a large role in the transformation and degradation of these compounds (Godheja *et al.,* 2016).

2.4 Antimicrobials

Antimicrobials are defined as substances with such satisfactory antimicrobial action that it may be used for the treatment or prevention of infectious diseases and can either kill bacteria (bactericidal), or inhibit bacterial growth (bacteriostatic) (Ahmad *et al.*, 2010). Biocides have been in use for centuries for several applications and are generally found in disinfectants, preservatives and antiseptics (Fraise, 2002; Wales & Davies, 2015).

In recent times there has been an increase in the use of biocides in household products (Fraise, 2002). Cleansers, soap, toothbrushes, dishwashing detergents, and hand lotions are all examples of household products containing antimicrobial agents; some biocides have even been incorporated into chopping boards and knife handles (Levy, 2001; Fraise, 2002). Reviews have shown that certain products containing antimicrobials may not necessarily be more effective in stopping infectious disease symptoms and decreasing bacterial numbers than using plain soap (Aiello *&* Larson, 2003, Aiello *et al.,* 2007). Common antimicrobials include Chlorhexidine, Alcohols, Triclocarban, TCS and PCMX (McDonnel & Russel, 1999; Chen *et al.,* 2014a).

2.4.1 Chlorhexidine

Chlorhexidine is a hexamethylene biguanide cationic biocide with an influence on a variation of Gram-positive and Gram-negative bacteria (Munoz-Gallego *et al.*, 2015)**.** It is a safe, pH dependent and frequently used biocide in dental and oral antiseptic products, particularly in mouthwash with bactericidal and bacteriostatic mechanisms of action that depend on membrane disruption. Chlorhexidine primarily has an effect on Gram-positive bacteria, but also affects Gram-negative bacteria, and is also believed to be useful against fungi (Barah, 2013).

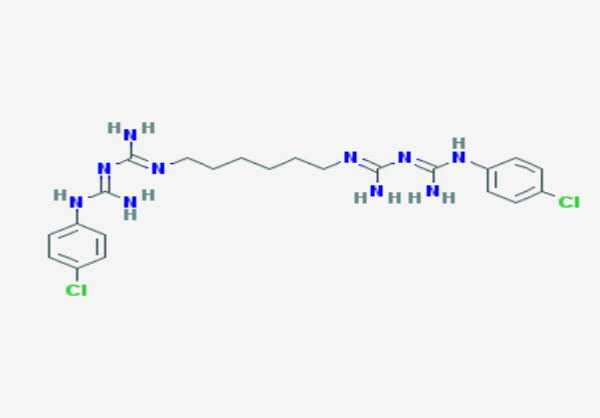

Figure 2.1: Chemical structure of chlorhexidine (National Centre for Biotechnology Information, 2018)

2.4.2 Alcohols

Alcohols are a broad-spectrum antimicrobial and display activity against bacteria, fungi and viruses, and although not considered to be sporicidal, they can inhibit spore germination and sporulation. Alcohols are not ideal for sterilization but remain effective for surface and hand disinfection and in some cases as a preservative in low concentrations. Little is known about alcohol's mechanism of action, but it is assumed to cause membrane damage and denaturation of proteins. Alcohols most commonly used as antimicrobials include ethyl alcohol, isopropyl alcohol and methyl alcohol (McDonnel & Russel, 1999; Barah, 2013).

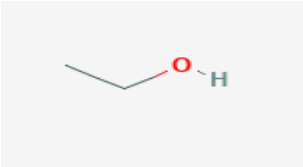

Figure 2.2: Chemical structure of ethyl alcohol (National Centre for Biotechnology Information, 2018)

2.4.3 Triclocarban

Triclocarban (TCC) is a polychlorinated, binuclear, aromatic antimicrobial used in bar soaps and detergents (Carey *et al.,* 2015). TCC primarily affects Gram-positive

bacteria and is not considered to be active against Gram-negative organisms (Walsh, 2003; Ahn *et al.,* 2008). Surveys have shown that TCC is one of the main antimicrobials used in liquid and bar soaps and is one of the most common compounds found in wastewater. It has shown a tendency to bio-accumulate in aquatic organisms and has been detected in some water ways at levels high enough to indicate widespread pollution of aquatic ecosystems (Ahn *et al.,* 2008; Brauch & Rand, 2011). Very little research has been done to establish a link between TCC and antibiotic resistance, but there has been increasing concerns that, as with TCS, resistance acquired by microbial contact to TCC could lead to cross-resistance to antibiotics (Carey *et al.,* 2015).

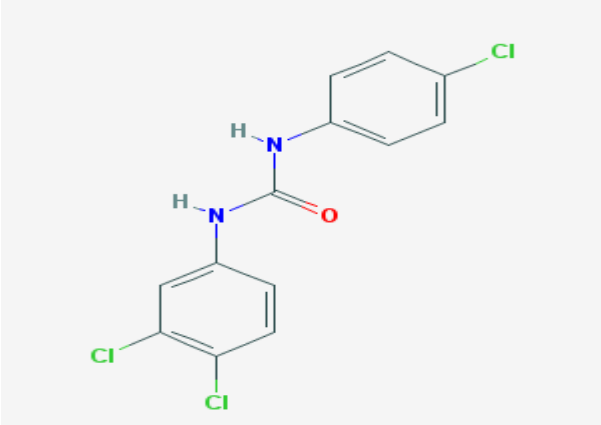

Figure 2.3: Chemical structure of triclocarban (National Centre for Biotechnology Information, 2018)

2.4.4 Triclosan

Triclosan (TCS) is a synthetic antimicrobial, introduced to the healthcare industry in the early 1970's and has been in use globally for over 40 years as a preservative, antiseptic and disinfectant in personal care products, as well as in clinical settings (Yueh & Tukey, 2016). TCS is a broad spectrum antimicrobial agent with an encouraging safety profile and is used in deodorants, shower gels and topical preparations, the majority of which end up in municipal wastewater (Bhargava & Leonard, 1996; Huang *et al*, 2016). TCS has been shown to be present in raw sewage, effluents from WWTP's and their receiving river systems (Ricart *et al.,* 2010; Dhillon *et al.,* 2015). In some instances, around 96% of TCS in consumer products is washed

down drains, leading to the accumulation of TCS in WWTP influent; TCS may also convert to other derivatives during the WWTP processes that may be more toxic or persistent than its parent compound in the environment. A wide range of TCS concentrations may be released into the environment, but this is highly dependent on the operation of the receiving WWTP. TCS tends to accumulate and has been found in surface water, estuarine sediment and fresh water. It has also been shown to bio-accumulate in aquatic biota (Yueh & Tukey, 2016).

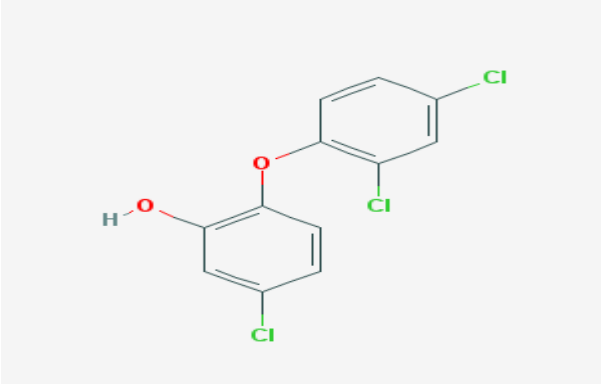

Figure 2.4: Chemical structure of triclosan (National Centre for Biotechnology Information, 2018)

At low doses TCS acts as a bacteriostatic agent inhibiting bacterial growth by inhibiting the enzyme enoyl-reductase required for lipid biosynthesis. This is essential for cellular division and leads to supressed growth of Gram-positive and Gram-negative bacteria. At high doses, TCS is believed to be bactericidal, inducing K^+ leakage and causing cell membrane damage (Adgent & Rogan, 2015; Yueh & Tukey, 2016). TCS exhibits specific activity against Gram-positive bacteria, but it is also effective against Gram-negative bacteria. Its efficiency against Gram-negative bacteria can also be enhanced by combining it with Ethylenediaminetetraacetic acid (EDTA) causing increased membrane permeability (McDonnel & Russel, 1999). TCS has been studied for its influence on antibiotic resistance and many TCS resistance mechanisms have been found in bacterial genera (Carey *et al.,* 2015). *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* have inherent resistance to TCS, believed to be due to non-susceptible enoyl-reductase, membrane impermeability or the expression of an efflux pump (Jo Yu *et al.*, 2010). TCS levels have been detected in urine, plasma and breastmilk in populations; this indicates that there is potential for long term to lifetime

exposure to TCS for humans in all age groups. TCS can possibly lead to many negative consequences such as impaired thyroid function and endocrine disruption (Yueh & Tukey, 2016).

2.4.5 Chloroxylenol

Chloroxylenol (PCMX) is an active ingredient of many therapeutic substances such as antiseptic agents and is also an ingredient of many other personal care products such as soap. Due to its vast production and its presence in many products and formulations, PCMX and its by products can be discharged into aquatic ecosystems either directly or by means of WWT effluent (Kasprzyk-Hordern *et al.*, 2009; Capkin *et al*., 2017). PCMX has been found in river systems, at a high concentration in raw sewage as well as in WWTP effluent but are mostly removed to a high degree during the WWTP process, depending on the operation of the WWTP (Daughton & Ternes, 1999; Kasprzyk-Hordern *et al.*, 2009).

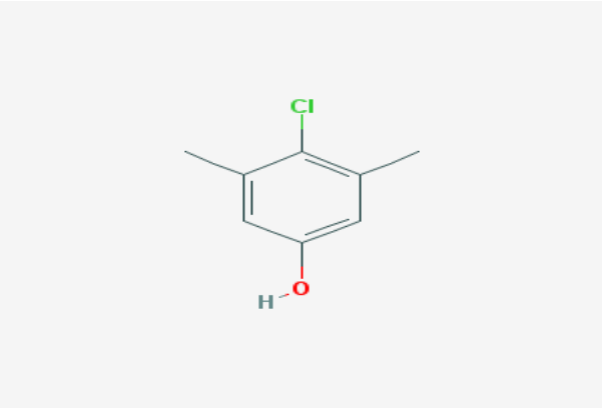

Figure 2.5: Chemical structure of chloroxylenol (National Centre for Biotechnology Information, 2018)

PCMX is a broad spectrum bactericidal agent with distinctive antiseptic properties and is described as being an efficient antimicrobial agent against common infectious bacteria. However, bacteria such as *Pseudomonas aeruginosa* and certain fungi/moulds have shown a high level of resistance. PCMX's mechanism of action has not been widely studied but due to its phenolytic nature, it is presumed to affect microbial membranes; phenol induces leakage of intracellular constituents, including

K^+ by causing cell membrane damage. It is also described as a proton gradient disruptor causing a deficiency in ATP, leading to death of bacteria from starvation (Latosinska *et al*., 2009; Barah, 2013). Studies have indicated that although PCMX can be absorbed through the skin it is excreted very quickly and cannot be detected in blood levels unless very high doses are used, and there is no evidence of carcinogenic and hormonal effects or reproductive toxicity (Food and Drug Administration, 2014).

2.5 Resistance to antimicrobials

Resistance can be described as the insusceptibility of a bacterium to a compound such as an antibiotic or antimicrobial (Willey *et al.,* 2011). For antibacterial substances, susceptibility is usually referred to as the minimum concentration required to have a notable effect such as the inhibition of growth. In instances where there is a change in susceptibility that result in an agent no longer being effective against an organism, that organism can be referred to as resistant (Gilbert & McBain, 2003). Different bacteria differ in their susceptibility to biocides with endospores being the most resistant and cocci generally the most sensitive (Russel, 1998). Resistance mechanisms to antimicrobials can be described as either intrinsic or acquired. Intrinsic meaning a natural ability of an organism and acquired implying resistance is the result of genetic changes due to mutation or acquisition of plasmids. Bacterial endospores, mycobacteria and Gram-negative bacteria such as *P. aeruginosa* exhibit intrinsic resistance while physiological adaptation can also modulate intrinsic resistance such as a biofilm containing cells (Russel, 1995; Russel, 1998).

Three classes of resistance driving chemicals have previously been described - these include antimicrobials, heavy metals and biocides - which are all known to select for resistance genes. The natural environment is frequently recognised for the role it may play in the spread of antimicrobial resistance. Municipal and industrial wastewater, agricultural runoff, aquaculture and mining activities are also known pollutant sources involved in the spread of resistance (Singer *et al.,* 2016). As previously mentioned biocides have been used for many years and common applications include their use as disinfectants on surfaces and equipment and as preservatives in PCP's (Wales & Davies, 2015).

Reduced susceptibility to biocides is increasing and may possibly be attributed to an ever increasing use of biocides in the community (Poole, 2002; Russel, 2002). There have been many debates regarding the increasing use of antimicrobial substances in consumer merchandises and the probability that, as with antibiotics, the overuse of these biocides may contribute to an increase in the overall resistance to biocides and antibiotics (Gilbert & McBain, 2003). Mechanisms of biocide resistance to date are poorly understood but are in some cases believed to be similar to that of antibiotic resistance mechanisms such as impermeability, efflux, drug degradation or modification of certain target sites (Russel, 2002). Resistance to antimicrobials varies between agents but typically involves alteration of the drug target, enzymatic destruction or modification of the drug or active drug efflux. The majority of resistance mechanisms are specific to the agent involved but there are many examples of multidrug efflux systems providing resistance to a range of unrelated antimicrobials (Poole, 2002). Biocides tend to act simultaneously on many targets, thus resistance is often mediated by non-specific means, such as cell wall changes, reducing permeability and efflux pumps that act on a broad range of chemically unrelated compounds (Fraise, 2002).

Studies by Tabak *et al.* (2009) and Tattawasart *et al.* (1999) both describe the occurrence of bacterial resistance to biocides. During a study by Tattawasart *et al.* (1999), it was shown that *Pseudomonas stutzeri* developed resistance to the biocide's chlorhexidine diacetate and cetylpyridinium chloride during exposure to progressively increasing concentrations of these antibacterial agents. Thus it is important to determine whether there is a direct connection between antibiotic and biocide resistance and whether there is a possibility that biocides can select for antibiotic resistance as there have been numerous reports of biocide-antibiotic cross-resistance (Poole, 2002; Russel, 2002).

2.6 Biocide resistance and cross-resistance to antibiotics

The occurrence of co- and cross-resistance between antimicrobials, including biocides and antibiotics, is not uncommon and has been highlighted in several previous studies (Wales & Davies, 2015; Singer *et al.*, 2016). Cross-resistance is the occurrence

whereby selection for one gene can lead to resistance to multiple harmful compounds. Efflux pumps can, in many cases, provide cross-resistance to multiple compounds (Singer *et al.,* 2016).

It is suggested that exposure to low concentrations of TCS are most likely to occur within the environment and, over a long period of time, could cause reduced susceptibility to TCS (Aiello *et al.*, 2003). Bacterial resistance to TCS occurs by several mechanisms. Known mechanisms of TCS resistance include efflux pumps, production of enoyl-reductase with low affinity for TCS and the expression of an enzyme that can degrade TCS (Yazdankhan *et al.,* 2006; Jo Yu *et al*., 2010). Variations in fatty acid profiles have been established in both *Escherichia coli* and *Staphylococcus aureus* strains for which MICs were raised. Studies with a divalent ion dependent *E. coli* TCS mutant, with a MIC ten times greater than a wild type strain, showed substantial differences in envelope fatty acids. Thus the suggestion was made that divalent ions and fatty acids may be limiting permeability of TCS to its primary site of action (McDonnel & Russel, 1999). *Acinetobacter baumannii* has been shown to exhibit intrinsic active efflux and acquired resistance by over-expression and mutation of the FabI gene (Chen *et al.,* 2009). *Pseudomonas aeruginosa* is known to express efflux pumps including MexAB-OprM, MexCDOprJ, and MexEF-OprN, *P. aeruginosa* isolates have also been found to express FabV leading to resistance of TCS (Zhu *et al.,* 2010). The production of enzymes that break down TCS has also been seen in two organisms, viz. *Pseudomonas putida TriRY* and *Alcaligenes xylosoxidans* subsp. *denitrificans TR1* (Meade *et al.,* 2001).

According to Birosova & Mikulasova (2008), the exposure of *Salmonella enterica serovar Typhimurium* to low concentrations of TCS has led to an increase in TCS-resistant strains. The presence of TCS in the environment may not necessarily lead to increased occurrence of strains with decreased susceptibility to antibiotics, but TCS does select for strains with elevated antibiotic MICs. Data indicates that TCS at sub inhibitory concentrations assists to preserve antibiotic-resistant cells in the population that have a shared mechanism of resistance to TCS. Therefore it is suggested that a decreased susceptibility to a biocide can confer cross-resistance to other biocides as well as to antibiotics (Tattawasart *et al.,* 1999). Multidrug-efflux systems that accommodate biocides such as TCS could mean that the strains expressing these are

both biocide-resistant and antibiotic-resistant. Thus there is a concern that agents such as TCS can select for strains resistant to several clinically important antibiotics (Poole, 2002). According to Carey *et al.* (2015) TCS has been studied for its effect on antibiotic resistance and that expression of an efflux pump that confers TCS resistance, can also lead to antibiotic resistance. There is a definitive need to determine if there is a link between antibiotic and biocide resistance and whether biocides can select for antibiotic resistance (Russel, 1995; Russel, 1998).

In studies, FabI mutations selected by exposure to TCS caused cross-resistance to other antimicrobial agents in *Escherichia coli*, leading to the fear that biocides may in fact share targets with antibiotics, and that antimicrobial resistance may very well lead to cross-resistance to antibiotics (Schweizer, 2001). According to Schweizer (2001), there is a clear link between TCS and antibiotics illustrated by two primary findings. Firstly TCS and antibiotics share multidrug efflux systems as a mechanism of resistance and they also cause expression of these efflux pumps by selecting related mutations in respective regulatory loci. Secondly, in *Mycobacterium tuberculosis* the up regulating mutation leading to isoniazid resistance in isolates was also obtained by selecting TCS resistance in the laboratory. A study by Chuanchuen *et al.* (2001), also demonstrates that exposure of bacteria to TCS can select for multidrug-resistance derivatives due to efflux. TCS and antibiotics associations have also been documented in laboratory settings, one described isoniazid resistance in *Mycobacterium smegmatis* selected by TCS by means of mutations in the InhA gene, the same target for TCS. TCS-resistant clones of *P. aeruginosa* have been associated with increased MICs of ciprofloxacin. *P. aeruginosa* is also known to confer high levels of intrinsic TCS resistance to antibiotics due to multidrug efflux pumps (Aiello & Larson, 2003). During a study by Beier *et al.* (2008), no connection between antibiotic resistance and antiseptic susceptibility was found, but it was found that the majority of vancomycin resistant *Enterococcus caesium* isolates examined had increased tolerance to TCS and were also resistant to many antibiotics. A review of literature shows that bacterial resistance to TCS is a very realistic problem and considering TCS's environmental accumulation and persistence, more research into reduced bacterial susceptibility to TCS and to antibiotics is worth investigating (Russel, 1998; Russel, 2002; Yueh & Tukey, 2016).

PCMX has not been as extensively studied as TCS in regard to resistance and cross-resistance to antibiotics. A study by Lear *et al.* (2006), found no evidence of antimicrobial resistance, cross-resistance to other antimicrobials or the development of antibiotic resistance among isolates exposed to PCMX. During an analysis of biocide and antibiotic susceptibility on isolates of methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *S. aureus* and *P. aeruginosa,* no correlation between PCMX use and antibiotic resistance was found (Lambert, 2004). Overall, according to existing literature there is very little evidence to suggest that PCMX resistance or cross-resistance to antibiotics is occurring, but PCMX has been shown to be present in river systems, and although its environmental persistence is limited the possibility of long term exposure to this compound and the possible development of resistance should remain a concern (Russel, 1998; Russel, 2002; Kasprzyk-Hordern *et al.*, 2009).

2.7 Virulence factors associated with potentially pathogenic bacteria

A pathogen can be referred to as a bacterial species capable of causing disease, while pathogenicity can be referred to as the ability of a bacterial species to cause a disease. Pathogenicity is associated with virulence and virulence factors that can be defined as microbial products or structures that contribute to pathogenicity. Typically, as the number of virulence factors associated with an organism increases, so does the degree of virulence and thus pathogenicity (Ahmad *et al.*, 2010; Willey *et al.,* 2011).

Virulence factors include chemicals that cause cell and tissue degradation, mechanisms to adhere to host cells and processes to overcome host defences. Adherence factors allow bacteria to adhere to, and colonise specific tissues by means of structures such as pili, fimbriae, capsule materials and specialized adhesion molecules. Besides serving as an adherence structure, the capsule may also play a role in protecting the pathogen from phagocytosis and opsonisation. Invasion factors are components that allow bacteria to invade host cells, such as lytic substances that alter the host tissue. An example of a virulence factor involved in bacterial pathogen invasion and dissemination is haemolysin that lyses erythrocytes. Exotoxins are proteins produced and/or secreted by pathogenic bacteria, and are grouped into 4 types: AB exotoxins, membrane active exotoxins, hydrolytic enzymes and super

antigen exotoxins. Endotoxins present in the outer membranes of some bacteria may be toxic to a specific host, causing fever, inflammation, shock, blood coagulation and several other effects. Virulence can be measured by determining the lethal dose 50 (LD50), this value refers to the number of pathogens or dosage required to cause the death of 50% of the hosts within a certain time period. Virulence can also be measured by cytopathology and by examining virulence factors (Peterson, 1996; Ahmad *et al.*, 2010; Willey *et al.,* 2011).

A number of determinants of bacterial pathogenicity are carried in plasmids, and the term virulence plasmid has been used to describe plasmids involved with pathogenicity and their gain or loss can lead to the modification of pathogenicity (Ramirez *et al*., 2014). Pathogenic islands are unique genomic segments associated with virulence, genes for adhesins, endotoxins, exotoxins and secretion systems which can all be included in a pathogenic island, and can be transferred to other bacterial strains by genetic exchange (Schmidt & Hensel, 2004).

Bacteria depend on various virulence factors associated with various genes. The identification of bacterial factors that promote virulence and persistence will always be a necessity. A study by Jorgensen *et al*. (2016) identified a gene cluster containing Clp ATPase, ClpK, from a strain of *Klebsiella pneumoniae* that enhances the ability of the organism to survive forms of heat treatment. The ClpK gene is part of a cluster containing a number of genes, all which are possibly involved in stress responses. The ClpK gene was common in *K. pneumoniae* isolates and found to be co-localized on transferable plasmids, possibly allowing for the co-dissemination of multidrug-resistance along with heat resistance to other bacteria. Similar ClpK gene clusters have also been shown to be present as genomic islands in other pathogenic bacteria (Jorgensen *et al*., 2016). *P. aeruginosa* has the ability to produce a variety of virulence factors and many classes of genes were identified encoding for these factors, and many additional factors involved with the pathogenicity of *P. aeruginosa* have yet to be discovered. It is evident from a review of literature that bacteria depend on several virulence factors associated with various genes (Choi *et al*., 2002; Filiatrault *et al*., 2006).

The presence of several extracellular enzymes may assist in determining the virulence potential of an organism. Some examples of these enzymes include: proteinase, gelatinase, lipase, lecithinase, DNase, hyaluronidase and chondroitinase (Pavlov *et al.,* 2004; Georgescu *et al.,* 2016). A study by Georgescu *et al.* (2016) highlight some of these enzymes associated with bacterial virulence during a study on *Pseudomonas aeruginosa* strains isolated from chronic leg ulcers. The enzymes tested for during the study included: proteinase, gelatinase, lipase, lecithinase and amylase.

2.7.1 Haemolysin

Many bacteria express haemolysis as a virulence factor but along with haemolysis they also express several other virulence factors. Haemolysis is caused by a subtype of exotoxins referred to as haemolysins which lyse erythrocytes and can further be divided into alpha haemolysin and beta haemolysin, in some cases to make iron available for bacterial growth. The majority of *Staphylococcus* strains show beta-hemolysis, but also express many other virulence factors. As an example *Streptococcus pyogenes* shows beta-haemolysis caused by either streptolysin O or streptolysin S, as well as other virulence factors such as M protein and pyrogenic exotoxins; the majority of *Enterococci* also show alpha haemolysis. The major virulence factors of *Listeria monocytogenes* are internalin and listeriolysin O, and also exhibit beta-haemolysis when grown on blood agar. Some species of *Clostridium (*e.g. *C. perfringens),* Enterobacteriacaea *(*e.g. *E. coli)* and *Pseudomonas (*e.g. *P. aeruginosa)* are also examples of bacteria exhibiting haemolysis as well as other virulence factors (Ahmad *et al.,* 2010; Willey *et al,* 2011).

Bacillus cereus also shows haemolysis due to the activity of the toxin Haemolysin BL, *Haemophilus haemolyticus* induces beta-haemolysis and *Helicobacter pylori* also exhibits haemolytic activity (Beecher *et al*., 1995; Bereswill *et al*., 1998; Anderson *et al*., 2012). Potentially pathogenic *Vibrio parahaemolyticus* has also been differentiated from non-pathogenic strains by testing for haemolysis (Twedt *et al*., 1970). From a review of literature, it is clear that haemolysis is a common virulence factor found in many potentially pathogenic bacteria and can be used for an initial screening process to determine possible pathogenicity.

2.7.2 Lecithinase

Lecithinase production by microorganisms has been investigated in an attempt to use it during the classification of bacteria and to associate its formation with virulence. Lecithinase/phospholipolytic activity has been identified by the formation of choline and phosphorous along with the precipitation of fat, after growth of bacteria on agar media containing egg yolk. The degradation of lecithin by lecithinase leads to the production of phosphorylcholin and diglyceride therefore causing toxicity. Lecithinase can also cause membrane disruption leading to cell lysis, cause haemolysis and damage tissue of the reproductive tract (Willey *et al.,* 2010; Sharaf *et al.,* 2014). Phospholipase involved in membrane disruption also plays an important role in host cell invasion (Istivan & Coloe, 2006).

2.7.3 Hyaluronidase & Chondroitinase

Glycosaminoglycans are important elements of the extracellular matrix, and have been implicated in a variety of diseases. Glycosaminoglycans include chondroitin sulfate and hyaluronan, each with unique disaccharide components and chemical links. Hyaluronidase and chondroitinase are glycosaminoglycan's degrading enzymes, and both are classified as virulence factors due to the fact that they make it possible for infecting microbes to penetrate tissue as they cause the depolymerisation of basic tissue constituents. For example, hyaluronidase hydrolyzes hyaluronic acid, which is a constituent of the extracellular matrix that binds cells together. Hyaluronic acid when intact prevents the passage of pathogens between intercellular spaces (de Assis *et al.,* 2003; Willey *et al.,* 2010; Jinno & Park, 2015).

2.7.4 DNase

DNase is DNA-specific and induces the degradation of nucleic acids, and has been shown to confer enhanced virulence. The innate immune response plays a critical role in host reaction to bacterial infection. Neutrophils migrate in large numbers to sites of infection and secrete neutrophil extracellular traps. Neutrophil extracellular

traps are composed of DNA and histones and the expression of DNase by bacteria confers resistance to the host's immune defence of extruded DNA/chromatin filaments (Pavlov *et al.,* 2004; Palmer *et al*., 2012). The potential role of DNase during bacterial infection has previously been studied in certain *Streptococcus* spp*.,* and it is stated that DNase likely contributes to the overall resistance of a pathogen to phagocytes in conjunction with other established virulence factors (Buchanan *et al.,* 2005).

2.7.5 Protease & Gelatinase

Also known as proteinases, proteases hydrolyse peptide bonds, and thus have the potential to degrade proteins and peptides that play a role in a range of biological functions, including the process of infection (Silva-Almeida *et al*., 2012). Bacterial pathogens rely on proteolysis for various purposes during infection, they degrade virulence regulators, provide tolerance to adverse conditions, they degrade host matrix components to allow for the spread of the infection, and interfere with host cell signalling. It can thus be said that proteolysis has been adopted by pathogens to ensure the success of the pathogen's contact with the host (Frees *et al*., 2013). Gelatinase is thought to contribute to virulence by degrading several substrates present in the infected host, some of which include collagen, fibrin and fibrinogen (Thurlow *et al.*, 2010). A previous study by Sifri *et al.* (2002) has highlighted the importance of extracellular proteases for the virulence of *Enterococcus* spp*.* such as *E. faecalis,* while a study by Thurlow *et al.* (2010), has described gelatinase as being the principle mediator of pathogenesis in endocarditis caused by *E. faecalis*.

2.7.6 Lipase

Many bacterial species produce lipases that hydrolyse esters of glycerol with preferably long-chain fatty acids (Jaeger *et al*., 1994; Boonmahome & Mongkolthanaruk, 2013). Bacterial lipases are important enzymes with applications in various industries, but it has become evident that extracellular lipases also play a role during microbial infections (Stehr *et al*., 2003). Lipase activity plays several roles during bacterial infection, and has been shown to interfere with immune responses, to hydrolyse host cell lipids during infection to supply an energy source in the form of

fatty acids and to assist with colonisation and persistence (Park *et al.,* 2013). Media containing Tween-80 or tributyrin as a substrate is generally used to determine the presence of lipase due to the formation of a turbid halo around colonies (Pavlov *et al.,* 2004; Mobarak-Qamsari *et al*., 2011).

2.8 Principles and applications: Methodologies available to study potentially pathogenic triclosan and/or chloroxylenol tolerant heterotrophic plate count bacteria

Methods are available to isolate and study antibiotic resistance of potentially pathogenic triclosan (TCS) and/or chloroxylenol (PCMX) tolerant heterotrophic plate count bacteria.

2.8.1 Isolation and characterisation methods for potentially pathogenic heterotrophic plate count bacteria (HPC)

Bacteria, yeast and fungi that use reduced, preformed organic molecules as a principle energy source are referred to as heterotrophs. The majority of pathogenic microorganisms are chemoorganoheterotrophs and form part of the HPC population (Burtscher *et al*., 2009; Willey *et al.,* 2011). Nutrient Agar has been used in several studies for the isolation and identification of heterotrophic plate count bacteria from several water sources (Panneerselvam & Arumugam, 2012; Mulamattathil *et al*., 2014). As previously stated haemolysis is a common virulence factor found in many potentially pathogenic bacteria and can be used for an initial screening process by plating isolates on blood agar (Ahmad *et al.*, 2010; Willey *et al.,* 2011).

Along with haemolysins, microorganisms also secrete various extracellular enzymes into their environment. Substances may be added to agar media to assay for the production of extracellular enzymes, for example media containing hyaluronic acid or chondroitin sulphate may be used to determine the presence of hyaluronidase and chrondroitinase, while media containing deoxyribonucleic acid may be used to screen for the presence of DNase (de Assis *et al.,* 2003; Pavlov *et al.,* 2004). Several extracellular enzymes are associated with virulence and screening for their presence

may therefore contribute to determine the pathogenic potential of an organism (Pavlov *et al.,* 2004; Georgescu *et al.,* 2016).

2.8.2 Identification of heterotrophic plate count bacteria

2.8.2.1 Phenotypical and molecular methods

Identification of bacteria can be conducted by two methods viz. phenotypical and molecular methods. Phenotypic methods are not always able to identify the microorganism to the species level, and much less to the strain level; thus in most cases molecular techniques are required (Graciela *et al.,* 2015). In addition, phenotypic identification is in some instances also difficult and extremely time-consuming (Tang *et al.,* 1998).

Molecular identification arose as an alternative or complement to existing phenotypic methods. Molecular identification of bacteria involves the amplification of certain conserved genetic targets by polymerase chain reaction (PCR), followed by sequencing and comparison to a known database (Tang *et al.,* 1998). One example of a gene used for the molecular identification of bacteria is the 16S rRNA gene which has emerged as a preferred genetic technique and is considered to be more accurate than phenotypic identification, although it requires more technological and cost considerations (Tang *et al.,* 1998; Clarridge, 2004). Use of the 16s rRNA gene for molecular identification has been highlighted in several articles (Stackerbrandt & Goebel, 1994; Lane *et al.*, 1985; Burtscher *et al*., 2009).

2.8.2.2 DNA Fingerprinting

There are several DNA-fingerprinting techniques available, all of which offer indirect access to DNA sequence polymorphism in order to assess species or clonal identity of bacterial organisms, or to examine bacterial genome composition. Several DNA fingerprinting techniques have been described in the past, namely: amplified restriction fragment polymorphism, restriction fragment length polymorphism, random amplified polymorphic DNA, and enterobacterial repetitive intergenic consensus (ERIC)

sequences PCR; these are but a few examples of DNA fingerprinting techniques (Lin *et al.,* 1996; Auda *et al.,* 2017). Clonally related organisms are those of the same species that share certain characteristics such as virulence factors and other genomic traits. It is thus possible to classify, differentiate and compare organisms isolated from various different sources due to sufficient diversity at the species level using fingerprinting techniques (Szczuka & Kaznowski, 2004; Auda *et al.,* 2017).

ERIC-PCR refers to enterobacterial repetitive intergenic consensus (ERIC) sequences, which are 127-bp elements that have a conserved central inverted repeat, occurring in several copies in the extragenic regions of the bacterial genome of enteric bacteria and vibrios. ERIC-sequences are highly conserved, implying that they may have in time acquired some form of function. The principle involves the use of primers specific for ERIC-sequences that bind to several loci to amplify sequences from an intricate DNA template. Amplified fragments varying in size yield a unique fingerprint that can be viewed by means of agarose gel electrophoresis (Vaneechoutte, 1996; Olive & Bean, 1999; Wilson & Sharp, 2006). The technique has been used for species typing and strain typing of several bacterial families other than Enterobacteriaceae as well, making the technique very suitable for the determination of clonal relationship and strain typing among varying bacterial species (Szczuka & Kaznowski, 2004; Asgarani *et al.*, 2015; Auda *et al.,* 2017).

2.8.3 Antimicrobial susceptibility

2.8.3.1 Kirby-Bauer disk diffusion susceptibility test

Developed in the 1960's by William Kirby and A. W. Bauer, the Kirby-Bauer disk diffusion susceptibility test is often used to estimate a pathogen's susceptibility to drugs in a timely manner. The principle of this method is to plate out bacteria onto the surface of a Mueller-Hinton agar plate; after the surface has dried for a few minutes, antibiotic test discs are placed on it with sterilized forceps or an applicator device. The plate is incubated at 35-37°C for 16-24 hours, after which the diameter of the zones of inhibition are measured. Results are usually interpreted using a table that relates zone

diameters to the degree of resistance (Boyle *et al.,* 1972; Willey *et al.,* 2011; Hudzicki, 2012).

2.8.3.2 Minimum Inhibitory Concentrations (MICs)

The minimum inhibitory concentration refers to the lowest concentration of a drug that inhibits the growth of an organism. The organisms are generally dispensed into a micro-well plate together with the different concentrations of the selected substance. The micro-well plate reader measures the optical density of the mixture hourly over a selected time period. A typical growth curve is used and lag time measured after 24 hours, giving a determination of the lowest concentration of a substance at which an organism will still grow. Biocide concentration is said to be one of the most important factors regarding its effectiveness, and several reports on the emergence of biocide resistance are based on the determination of MICs. Bacteria that exhibit tolerance to a biocide might be selected by a low concentration of said biocide. The level of resistance can increase through selection by means of recurring exposure to a low concentration or increasing concentration of a biocide, the correct use and thus the applied concentration of antimicrobials is very important as incorrect concentration use may result in antimicrobial resistance (Scientific Committee on Emerging and Newly Identified Health Risks, 2009).

During a study to determine the MICs of fungi in the presence of antifungal agents, a multi-detection micro-well plate reader was used. The optical density is measured over a period of 24 hours at 37°C. Growth curves were obtained for different concentrations of the antifungal agent during incubation. It was stated that the spectrophotometric MICs delivered a more objective result than visual MICs (Kaya & Ozbilge, 2012). A similar method was also used during a study to determine the antibacterial activity of aucubigenin and aucubin, where results demonstrated that the micro-well dilution assay was more accurate than the paper disc diffusion method (Li *et al.,* 2014).

2.8.3.4 Synergy and antagonism between antimicrobials (Checkerboard assay)

Antimicrobials used in combination may lead to synergistic and/or antagonistic effects. The interaction is said to be synergistic if the joint effect on cells by the antimicrobial combination is stronger than either antimicrobial by itself, and antagonistic if the joint effect is weaker (Bollenbach, 2015). An example of a synergistic combination can be seen with antibiotic pairs such as trimethoprim with sulphonamides; the combination can reduce side effects and increase the potency of drugs that might be ineffective alone (Pillai *et al.,* 2005). Drug combinations and the study of synergistic and antagonistic effects may also offer methods for controlling the evolution of drug resistance. These effects may also be used in research as a way of studying several perturbing cellular functions, to reveal relationships in cell physiology (Lehar *et al.,* 2007; Bollenbach, 2015).

Synergy may occur if for example one drug increases the permeability of a cell to another drug; it may also occur due to physical interaction between drugs at their target site. Synergistic effects may also occur if antimicrobials share a similar target or if both target a similar pathway involved in cell physiology (Bollenbach, 2015). Synergy may similarly occur if bacterial cells use the same mechanism to defend itself from both classes of antimicrobials and a double attack may overwhelm the function of the defensive mechanism (Tabak *et al*., 2009). Antagonistic effects may occur due to physical and/or chemical interaction between the drugs themselves, or due to the antimicrobial activity of the drugs involved. Interactions between bacteriostatic and bactericidal antimicrobials have been substantiated and these antagonistic effects are attributed to the fact that bactericidal antimicrobials generally require the occurrence of cell growth which is prevented by bacteriostatic drugs (Ocampo *et al*., 2014; Bollenbach, 2015). Antagonism may also occur due to genetic interactions, a study by Haaber *et al.* (2015) demonstrates that exposing *Staphylococcus aureus* to one antimicrobial (colistin), triggers global gene expression changes similar to those in vancomycin resistant mutants, thus protecting the cell from vancomycin. Some antimicrobials may share multidrug efflux systems and the combination of two drugs may trigger the overexpression of multiple efflux mechanisms also leading to antagonistic effects (Schweizer, 2003).

Synergy and antagonism between TCS and antimicrobials has previously been described. Tabak *et al*. (2009) demonstrated synergistic effects between TCS and antibiotics on *Salmonella typhimurium*, while a different study by Movahed *et al.* (2016), indicated that amphotericin B and fluconazole, along with TCS induces apoptosis-like cell death in *Cryptococcus neoformans*. Antagonism has also been observed between TCS and fluconazole during an investigation of its activity against *Candida albicans* (Higgins *et al*., 2012). Several occurrences of synergistic and/or antagonistic interactions between TCS and antibiotics have been observed, but there is still very little insight as to the precise mechanisms involved. PCMX has not previously been studied as extensively as TCS in relation to interaction with antibiotics and very little information is currently available in this regard.

The checkerboard method has previously been used during several studies for the purpose of examining synergistic and antagonistic effects between antimicrobials (Sopirala *et al.,* 2010; Spoorthi *et al.,* 2011). The method involves the use of microwell plates - one antimicrobial is serially diluted along the ordinate while the other is diluted along the abscissa. A study by Orhan *et al.* (2005), demonstrates the use of the checkerboard method to determine synergy and antagonism between antimicrobials against *Brucella melitensis,* the study further demonstrates that the checkerboard method is equally effective in comparison to E test methods, when studying synergistic and antagonistic interaction.

2.8.4 Chromatography

It is important to determine whether biocides are present in wastewater, wastewater effluents and the receiving river systems. Several chromatography techniques such as gas chromatography-mass spectrometry, liquid chromatography-mass spectrometry, ultra-high performance liquid chromatography–tandem mass spectrometry, and conventional high performance liquid chromatography (HPLC) with UV detection, have previously been employed to determine the presence of personal care products, antimicrobials and pharmaceuticals in WWTP's and their effluents (Hao *et al*., 2007; Zhou *et al.,* 2009; Gracia-Lor *et al*., 2012).

HPLC involves the use of pumps to pass a pressurized liquid and a sample mixture (mixture of solvents referred to as the mobile phase) through a column filled with absorbent, for example silica, leading to the separation of the components found in a sample. Components are separated due to varying degrees of interaction with the absorbent as they flow out of the column (Bird, 1989). According to Zhou *et al.* (2009), a method for the study of TCS using solid phase extraction (SPE) and HPLC with Ultra-Violet detection, has been developed and used to determine TCS concentrations in three WWTP's in Shanghai. Results indicated that the concentration of TCS ranged from 533 ng/L to 774 ng/L in raw wastewater samples and from 80.14 ng/L to 249.72 ng/L in the WWTP effluent samples. The removal efficiencies of the entire processes ranged from 62.59% to 67.74%. HPLC has been used during several other studies to determine the presence and concentration of TCS in varying water samples (Piccoli *et al.,* 2002; Ricart *et al.,* 2010; Madikizela *et al.,* 2014).

HPLC methods have not been applied for the detection of PCMX as frequently as for TCS. According to Gatti *et al.* (1997) HPLC methods have previously been proposed for the analysis of PCMX in commercial pharmaceutical dosage forms while a study by Thompson (2001) demonstrates the detection of PCMX in commercial formulations by HPLC methods. A technical guide on the analysis of cleaning and personal care products by gas and liquid chromatography by Restek, a leading developer and manufacturer of chromatography products, also demonstrates that PCMX can be detected by HPLC methods (Restek, 2008).

According to Dhillon *et al.* (2015) TCS has been detected in sewage treatment plant effluents, surface, ground and drinking water. The overuse of anti-microbial products such as TCS, might lead to increased tolerance and/or resistance among bacteria, and may also result in the development of cross-resistance to antibiotics. PCMX has not been as extensively studied as TCS, but it has been shown to be present in river systems and in wastewater treatment plant effluents, thus the possible development of resistance should remain a concern (Russel, 1998; Russel, 2002; Kasprzyk-Hordern *et al.,* 2009).

CHAPTER 3 – MATERIALS AND METHODS

3.1 Screening, isolation and maintenance of isolates

In total five sampling points were selected. Samples were collected from the pre- and post-chlorination points of the WWTP Potchefstroom North-West, after which three more samples were collected: one from the effluent after it has passed through the wetlands before entering the Mooi River, one upstream sample, and one downstream sample. Samples collected were plated onto nutrient agar containing different concentrations of TCS and PCMX. TCS and PCMX were dissolved in 99% methanol. The range of concentrations for TCS consisted of 0.25, 0.375, 0.5 and 1 mg/L, and the range of concentrations for PCMX consisted of 10, 20, 30 and 40 mg/L. Isolates were incubated at 37°C for 24 hours.

Isolates were spot inoculated onto blood agar to test for haemolysis, as a screening process for potential pathogens. Isolates testing positive for either alpha or beta-haemolysis were then streaked out onto nutrient agar containing the same concentration of TCS or PCMX that they were isolated from, to obtain pure cultures. Isolates not testing positive for either alpha or beta haemolysis, were discarded. Isolates were maintained on nutrient agar with TCS and PCMX concentrations corresponding with the concentration initially obtained at.

3.2 Molecular Identification and DNA Fingerprinting

3.2.1 Amplification of 16S rRNA region

For identification purposes, PCR amplification of the 16S rRNA region was done. To obtain DNA, broth cultures of the desired isolates were prepared and extracted according to the manufacturer's instruction using the Chemagic DNA Bacteria Kit (Perkin Elmer, Germany), followed by PCR amplification of the 16S rRNA region.

The mixture for the PCR consisted of the following: 12.5 µl Master Mix (Containing: Taq DNA polymerase, dNTPs, reaction buffer, $MgCl_2$, KCl and PCR stabilizer), 1 µl

(10 µM) forward primer (27F) targeting the 16S rRNA region (5'-AGAGTTTGATCMTGGCTCAG-3'), and 1 µl (10 µM) reverse Primer (1492R) targeting the 16S rRNA region (5'-TACGGYTACCTTGTTACGACTT-3'), 8.5 µl Nuclease free water and 1 µl DNA. The total volume per sample was 25 µl. The following thermal PCR cycle protocol was used: 95°C for 5 minutes (Initial denaturation), followed by 30 cycles of 95°C for 1 minute (Denaturation), 51°C for 45 seconds (Annealing), 72°C for 2 minutes (Elongation) and the last cycle was 72°C for 5 minutes (Final elongation). To determine if the PCR product is present, gel electrophoresis was performed. A 1.7% Agarose gel with the addition of 5 µl of ethidium bromide was loaded with 3 µl PCR product, along with 2 µl of a 30% Glycerol solution. The electrophoresis ran for 45 minutes at 80 Volts.

Sequencing: First clean-up was done using the in-house silica matrix method. 150 µl of NaI (6 M) was added to a 25 µl PCR reaction mixture after which 10 µl of silica matrix (100 mg/ml) was added. The mixture was left to incubate at room temperature for 5 minutes. After incubation, the mixture was centrifuged for 10 seconds and maximum speed and the supernatant was removed. After removal of the supernatant the remaining pellet was washed 3 times with 200 µl of washing buffer (consisting of 50 mM NaCl, 10 mM Tris pH 7.5, 2.5 mM EDTA, 50% v/v ethanol and ultra-pure water) removing the supernatant after each wash step. Once washing was complete, the pellet was left open to air dry for 5 minutes, after which the matrix was re-suspended in 20 µl of PCR water. After re-suspension, the mixture was again centrifuged for 5 minutes after which the DNA eluate was transferred to a new tube. Thereafter, sequencing PCR of the purified PCR product was done (Big Dye Terminator v3.1 cycle sequencing kit from Life Technologies) by making up a master mix of 4 µl Ready Reaction Premix (1:10 dilution), 2 µl 5x Big Dye Sequencing buffer, 3.2 pmol Forward primer(27F), 9.8 µl nuclease free water. One microliter (1 µl) of DNA was added. The following cycle conditions were used: 96°C for 1 minute (Initial denaturation), 96°C for 10 seconds (Denaturation), 50°C for 5 seconds (Annealing), 60°C for 4 minutes (Elongation), (step b were repeated for 25 cycles, (c) Hold at 4°C. Second clean-up and preparation for sequencing was done using the Zymo Research DNA sequencing clean-up kit according to the instructions of the manufacturer. After the second clean-up, 8 µl of each sample was loaded into a 96 well micro-well plate along with 5 µl of

HiDi Formamide in preparation for capillary electrophoresis (ABI 3130 genetic analyser, Applied Biosystems, USA).

3.2.2 DNA Fingerprinting (ERIC-PCR)

Polymerase chain reaction (PCR) amplification of the enterobacterial repetitive intergenic consensus (ERIC) sequences was done to determine the potential clonal relationship of same species isolates. Primers specific for ERIC-sequences bind to several loci yielding a unique DNA fingerprint. DNA extraction was done according to the following procedure. Broth cultures of the desired isolates were prepared and extracted according to manufacturer's instruction using the Chemagic DNA Bacteria Kit (Perkin Elmer, Germany). Next, PCR amplification of the ERIC sequences was done. The mixture for the PCR consisted of the following: 12.5 µl Master Mix (Containing: Dream Taq DNA polymerase, dNTPs, reaction buffer, $MgCl_2$, KCl and PCR stabilizer), 1 µl (2 µM) ERIC1 primer (5'-ATGTAAGCTCCTGGGGATTCAC-3'), 1 µl (2 µM) ERIC2 primer (5'-AAGTAAGTGACTGGGGTGAGCG-3'), 5 µl (20 ng/µl) DNA and 5.5 µl Nuclease free water (Katara *et al*., 2012). The total volume per sample was 25 µl.

For *Aeromonas* spp*.*, the following thermal cycling conditions were used: 95°C for 7 minutes (Initial denaturation), followed by 30 cycles of 90°C for 30 seconds (Denaturation), 52°C for 1 minute (Annealing), 65°C for 8 minutes (Elongation), and a final elongation step at 65°C for 16 minutes (Szczuka & Kaznowski, 2004). For *Bacillus* spp*.* the following thermal cycling conditions were used: 95°C for 5 minutes (Initial denaturation), 4 low stringency cycles of 94°C for 5 minutes (Denaturation), 40°C for 5 minutes (Annealing) and 72°C for 5 minutes (Elongation), followed by 30 high stringency cycles of 94°C for 1 minute (Denaturation), 55°C for 1 minute (Annealing) and 72°C for 2 minutes (Elongation), and final elongation step at 72°C for 10 minutes (Shangkuan *et al.,* 2000). A 2% Agarose gel was loaded with 10 µl PCR product along with 5 µl of a 30% Glycerol solution. The electrophoresis ran for 2-3 hours at 80 Volts.

The final gel products were analysed, and a clustering analysis was performed with the aid of Phoretix 1D pro (Total Labs: Version 1.0). Clustering analysis was done

based on the Dice similarity coefficient and the Unweighted Pair Group Method with Arithmetic mean (UPGMA)

3.3 Extracellular enzyme production

Media containing substrates specific for certain enzymes were used to assess extracellular enzyme production. The following enzymes were selected: proteinase, gelatinase, lipase, lecithinase, DNase, hyaluronidase and chondroitinase. These results provided information about the potential virulence of the organisms (Pavlov *et al.,* 2004; Beceiro *et al.,* 2013, Georgescu *et al.,* 2016).

3.3.1 Proteinase

Skimmed milk agar plates were prepared to screen for proteolytic enzyme production. Plates contained 3% (w/v) skimmed milk (Oxoid, England) and Mueller-Hinton agar at 35 g/L (Merck, Germany) (Patidar *et al.,* 2013). Ingredients were prepared and autoclaved separately after which ingredients were mixed and dispensed. Isolates were spot inoculated onto the plates and incubated at 37˚C for 24-48 hours. Development of a clear zone around the colonies indicated proteolytic activity (Pavlov *et al.,* 2004*;* Bhowmik *et al.,* 2015).

3.3.2 Gelatinase

Gelatinase production was detected by the inoculation of isolates onto agar plates containing 40 g/L tryptone soy agar, (Merck, Germany) and 3% (w/v) gelatine powder (Merck, Germany) (Patidar *et al.,* 2013). Isolates were spot inoculated onto the plates and incubated at 37˚C for 24-48 hours. After incubation, plates were cooled and the appearance of a turbid halo or clear zone around colonies was accepted as an indication of gelatinase activity (Pavlov *et al*., 2004*;* Patidar *et al.,* 2013).

3.3.3 Lipase

Media containing 40 g/L tryptone soy agar (Merck, Germany) was prepared and supplemented with 1% Tween-80 (Sigma, Germany) to serve as a substrate. Isolates were spot inoculated onto plates and incubated at 37˚C. The formation of a turbid halo after 24-72 hours of incubation was considered to be an indication of lipase activity (Pavlov *et al.,* 2004; Georgescu *et al.,* 2016).

3.3.4 Lecithinase

Lecithinase production was determined by the use of egg yolk agar. Media containing 40 g/L tryptone soy agar (Merck, Germany) was prepared and supplemented with 100 ml 50% egg yolk emulsion (Merck, Germany) per 1 L of media. Plates were spot inoculated and incubated at 37˚C. The formation of a distinct zone of opacity underneath or around the colony after incubation for 24-48 hours indicated lecithinase production (Pavlov *et al.,* 2004; Georgescu *et al.,* 2016).

3.3.5 DNase

To determine the presence of DNase, DNase agar (Oxoid, UK) was prepared according to the manufacturer's instruction. Plates were spot inoculated and incubated at 37˚C for 18-24 hours. After incubation plates were flooded with 1 M HCL, the formation of a clear zone around the colony was a positive indicator of DNase activity (Pavlov *et al*., 2004; Gundogan *et al.,* 2006).

3.3.6 Hyaluronidase

Hyaluronidase production was tested by preparing a media containing 10 g/L Noble agar (Conda, Spain), 37 g/L brain heart infusion broth. A second aqueous substrate containing 2 mg/ml hyaluronic acid (Merck, Germany) and 5% bovine albumin fraction V (Hyclone labs, USA), was prepared. The solution was filtered with a 0.22 µm filter (GVS, UK). The media was autoclaved and the second substrate was added after the

media was allowed to cool. Isolates were spot inoculated onto plates and incubated at 37˚C for 24-48 hours. The formation of a clear zone around the colonies indicated a positive result (de Assis *et al.,* 2003; Pavlov *et al*., 2004).

3.3.7 Chondroitinase

Chondroitinase production was tested by preparing a media containing 10 g/L Noble agar (Conda, Spain), 37 g/L brain heart infusion broth. A second aqueous substrate containing 4 mg/ml chondroitin sulphate (Roth, Germany) and 5% bovine albumin fraction V (Hyclone labs, USA), was prepared. The solution was filtered with a 0.22 µm filter (GVS, UK). The media was autoclaved and the second substrate was added after the media was allowed to cool. Isolates were spot inoculated onto plates and incubated at 37˚C for 24-48 hours. The formation of a clear zone around the colonies indicated a positive result (de Assis *et al.,* 2003; Pavlov *et al*., 2004).

3.4 Antimicrobial susceptibility

3.4.1 Assay for cross-resistance to antibiotics

Cross-resistance to antibiotics was done by using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. Six antibiotics were used to assay for resistance to antibiotics. Antibiotics selected included: Vancomycin (30 µg), Kanamycin (30 µg), Trimethoprim (5 µg), Oxytetracycline (30 µg), Amoxicillin (10 µg) and Chloramphenicol (30 µg). Amoxicillin and Kanamycin are described as being predominantly bactericidal while Trimethoprim, Tetracycline and Chloramphenicol are described as being predominantly bacteriostatic. Tetracycline and Kanamycin both inhibit protein synthesis by binding to the small ribosomal subunit, while Chloramphenicol inhibits protein synthesis by binding to the large ribosomal subunit. Amoxicillin inhibits cell wall synthesis and Trimethoprim blocks folic acid synthesis (Ahmad *et al.*, 2010; Willey *et al.,* 2011; Nemeth *et al*., 2015). Isolates were spread out using the spread plate method onto Mueller-Hinton agar (Merck, Germany). Antibiotic disks were placed on the agar containing isolates and incubated at 37ºC for 24 hours.

Table 3.1: Zone diameter interpretive standards recorded in NCCLS (2014).

Antibiotic	Abbreviation	Concentration	R	I	S
Vancomycin	VA30	30 µg	≤ 14	15-16	≥ 17
Amoxycillin	A10	10 µg	≤ 13	14-17	≥ 18
Chloramphenicol	C30	30 µg	< 12	13-17	≥ 18
Kanamycin	K30	30 µg	≤ 13	14-17	≥ 18
Trimethoprim	T5	5 µg	≤ 15	16-18	≥ 19
Oxytetracycline	O30	30 µg	≤ 14	15-18	≥ 19

R = resistant, I = Intermediate resistance, S = susceptible

Inhibition zones were measured after 24 hours and divided into three groups classifying the isolates as resistant, susceptible or inhibited by using the Performance Standards for Antimicrobial Disk Susceptibility Tests. Table 3.1 represents the NCCLS standards for the determination of antibiotic susceptibility for the selected antibiotics (NCCLS, 2014).

3.4.2 Minimum inhibitory concentrations

Minimum inhibitory concentrations were conducted to determine lag time measurement and TCS and PCMX minimum inhibitory concentrations. Isolates were incubated for 24 hours at 37ºC in Mueller-Hinton broth at different concentrations of TCS (0, 0.125, 0.25, 0.5, 0.75, 1 and 2 mg/L) and PCMX (0, 10, 20, 30, 50, 75, and 100 mg/L). The control treatment was set up by incubating the isolates in Mueller-Hinton broth without TCS or PCMX, and a solvent control was run by incubating the isolates in Mueller-Hinton broth containing varying concentrations of methanol. Bacterial growth was accessed by observing the turbidity of the medium by using a micro-well plate reader (BioTek Power Wave HT, Micro well plate reader). The results were noted as resistant or susceptible. These tests were performed in 96-well micro plates. The MIC was accepted as the lowest concentration of the substance being studied that inhibited visible growth after 24 hours of incubation at 37ºC (Kaya & Ozbilge, 2012). The importance and effectiveness of measuring lag time variations is

made evident in a study by Li *et al.,* (2016), where the effects of antimicrobials on the lag phase of bacterial communities were studied.

3.4.3 Checkerboard assay

A checkerboard assay was designed to determine if there were any synergistic or antagonistic effects between antibiotics at sub inhibitory concentrations of TCS or PCMX. Concentrations for the checkerboard assay were determined by examining the results of antibiotic resistance profiles, as well as MICs of the biocides in question. The antibiotic range was determined by comparing antibiotic resistance profiles, and susceptibility levels noted by the NCCLS standards (NCCLS, 2014). Isolates were thus incubated for 24 hours at 37ºC in Mueller Hinton broth (MHB), at different concentrations of TCS (0, 0.25, 0.5, 1 and 2 mg/L) and PCMX (0, 10, 20, 30 and 40 mg/L) with the addition of selected antibiotics at varying concentrations (0, 1, 2, 4, 8, 16, 32 and 64 µg/ml). Bacterial growth was assessed by the observation of visual growth after isolates were incubated for 24 hours at 37ºC. These tests were performed in 96-well micro plates and results were noted as the presence or absence of visible growth.

3.5 High performance liquid chromatography

HPLC methods were used to determine the presence of TCS and PCMX in the WWTP and up and downstream samples, as well as to quantify the levels present.

3.5.1 Preparation of water samples

Extractions were performed according to Horizon Technologies' method for the determination of pharmaceuticals and personal care products by solid phase extraction (SPE). The SPE-DEX 4790 Automated Extraction System run with the Envision® Platform Controller, was used to automatically extract aqueous samples. Atlantic™ HLB-L Disks were used during the extraction process. Influent and effluent water samples were collected in triplicate from the WWTP, as well as up and downstream samples. Glass bottles were cleaned with

methanol and double distilled water before sampling. All samples were pre-filtered to remove large particulate matter. One sample was treated with 500 ppm of TCS and PCMX. Sodium thiosulphate (80 mg) was added to each sample and acidified with HCL to a pH of 2, after which ethylenediaminetetraacetic acid (500 mg) was added. Samples were left for 1.5 hours to equilibrate before extraction.

3.5.2 SPE and HPLC

The Acid Purge method was carried out according to the Horizon Technologies 4790 method. Table 3.2 represents the acid-purge procedure.

Table 3.2: Acid Purge Method

Step	Solvent	Air Dry Time
Prewet 1	pH 2 water	0:15 min
Prewet 2	Reagent water	0:15 min
Prewet 3	Methanol	0:15 min
Wash 1	Reagent water	0:15 min
Rinse 1	Acetone/Methanol (1:1)	0:15 min
Rinse 2	Methanol	0:15 min

The Acid extraction method was then performed to the specifications of Horizon Technologies' 4790 method. Table 3.3 represents the Acid-extraction procedure.

Table 3.3: Acid Extraction Method

Step	Solvent	Soak time	Air Dry Time
Prewet 1	Methanol	1:30 min	0:30 min
Prewet 2	Methanol	1:00 min	0:05 min
Prewet 3	Reagent Water	1:30 min	0:02 min
Prewet 4	pH 2 Water	1:00 min	0:00 min
	Sample process		
Wash 1	Reagent water	1:00 min	0:30 min
Wash 2	Reagent water	1:00 min	0:30 min

Wash 3	Reagent water	1:00 min	0:30 min
	Air dry 15:00 min		
Rinse 1	Methanol	1:30 min	1:00 min
Rinse 2	Methanol	1:00 min	1:00 min
Rinse 3	Acetone/Methanol (1:1)	1:30 min	1:00 min
Rinse 4	Acetone/Methanol (1:1)	1:00 min	1:30 min

Following SPE, the eluted samples were concentrated using a TurboVap II (Caliper Lifesciences, USA). Concentrated samples were re-suspended in 75% acetonitrile to a final volume of 1 ml and syringe filtered before injection into the HPLC. An HPLC with a UV detector was used with a 250 x 4.6 Venusil XBP C18 (2) 5 u column. The mobile phase was made up of 75% acetonitrile, 10 µl of sample was injected into the column at 25°C and the wavelength was set to 280 nm (Xue-fei *et al.,* 2009).

CHAPTER 4 – RESULTS

4.1 Screening, isolation and maintenance of Isolates

Initially, 88 colonies were selected from agar plates supplemented with either TCS or PCMX, colonies were spot inoculated onto blood agar of which 34 tested positive for either alpha or beta-haemolysis. Impure isolates were streaked out to obtain pure cultures and were again spot inoculated onto blood agar to test for haemolysis. In total 37 isolates tested positive for haemolysis. Gram staining was performed on the isolates and the morphology of the isolates was noted according to the shape and colour of the colony. Isolates analysed were labelled as follows, T (Triclosan) or C (Chloroxylenol) which indicated the supplement added to nutrient agar, followed by the concentration. Sampling points are indicated as either Pre (Pre-chlorination), Post (Post-chlorination), U (Upstream), W (Wetland) or D (Downstream), and numbers were added to represent the colony obtained from the original plate. As an example, isolate T-1-Pre-2 was supplemented with TCS at 1 mg/L, obtained from the pre-chlorination sample and was isolate number 2, obtained from the original plate. Table 4.1 indicates bacterial morphology, colony descriptions, Gram staining characteristics and the results of haemolysis tests for final isolates obtained for identification purposes.

Table 4.1: Final isolates selected for identification

Isolate	Haemolysis	Gram	Morphology	Colony description
T-1-Pre-2	β	-	Bacilli	large, white, concave
T-1-Pre-3	β	-	Bacilli	large, white, concave
T-0.5-Pre-1A	β	-	Bacilli	transparent, green-discolouration, round
T-0.5-Pre-1B	β	-	Bacilli	white, round
T-0.25-Pre-1	β	-	Bacilli	small, dark green-discolouration, round
C-40-Pre-5	β	-	Bacilli	small, white, round
C-10-Pre-2D	β	+	Bacilli	large, white, concave with ruffled edges
T-1-Post-4	β	-	Bacilli	white, round
T-0.5-Post-4	β	+	Bacilli	white, round
T-0.375-Post-2	β	+	Bacilli	white, concave
T-0.25-Post-6	α	+	Bacilli	white, round with ruffled edges
T-0.25-Post-7	β	-	Bacilli	transparent, brown-discolouration, round

C-40-Post-1	β	+	Bacilli	white, round with ruffled edges
C-40-Post-2	β	-	Bacilli	small, transparent, round
C-20-Post-2	β	-	Bacilli	very small, transparent, round
C-20-Post-4	α	+	Bacilli/Capsule	gelatinous, white, round
C-10-Post-4	α	+	Bacilli	white, concave with ruffled edges
T-1-U-3	β	-	Cocci	white, green-discolouration, round
T-1-U-4A	α	-	Cocci	light-brown, round
C-40-U-1	β	+	Bacilli/Capsule	gelatinous, white, round
C-30-U-4	β	+	Bacilli	white, round with ruffled edges
C-10-U-1	β	+	Cocci	small, orange, round
T-1-W-3	β	-	Cocci	large, white, concave
T-1-W-4	β	-	Bacilli	white, dark-white centre, round
C-40-W-1	β	-	Bacilli	white, round
C-40-W-2	β	-	Cocci	white, round
C-30-W-2	β	-	Cocci	white, round with ruffled edges
T-1-D-5	β	-	Bacilli	small, white, round
T-1-D-6	β	+	Bacilli	white, concave
T-1-D-8	β	-	Bacilli	white, round with ruffled edges
T-0.375-D-1	β	+	Bacilli	white, round
C-40-D-5	β	+	Bacilli	white, round
C-30-D-3	β	-	Bacilli	light-brown, round
C-30-D-5	β	-	Bacilli	white, round
C-10-D-2A	β	-	Bacilli	transparent, green-discolouration, round
C-10-D-2B	β	+	Bacilli	white, brown-discoloration, round
C-10-D-3	β	+	Bacilli	large, white, round with ruffled edges

4.2 Molecular Identification and DNA Fingerprinting

4.2.1 16S Identification

Identification was done by sequencing of 16S rDNA amplicons, acquired by polymerase chain reaction (PCR). Figure 4.1 is an illustration of a 1.7 % agarose gel of the amplicons. A 1 kb ladder was used to indicate the size of the bands. All the amplicons were in the range of 1500 bp. These fragments were purified and sequenced. Chromatograms were viewed using Finch TV and were BLAST searched on NCBI to determine the identity of the isolates.

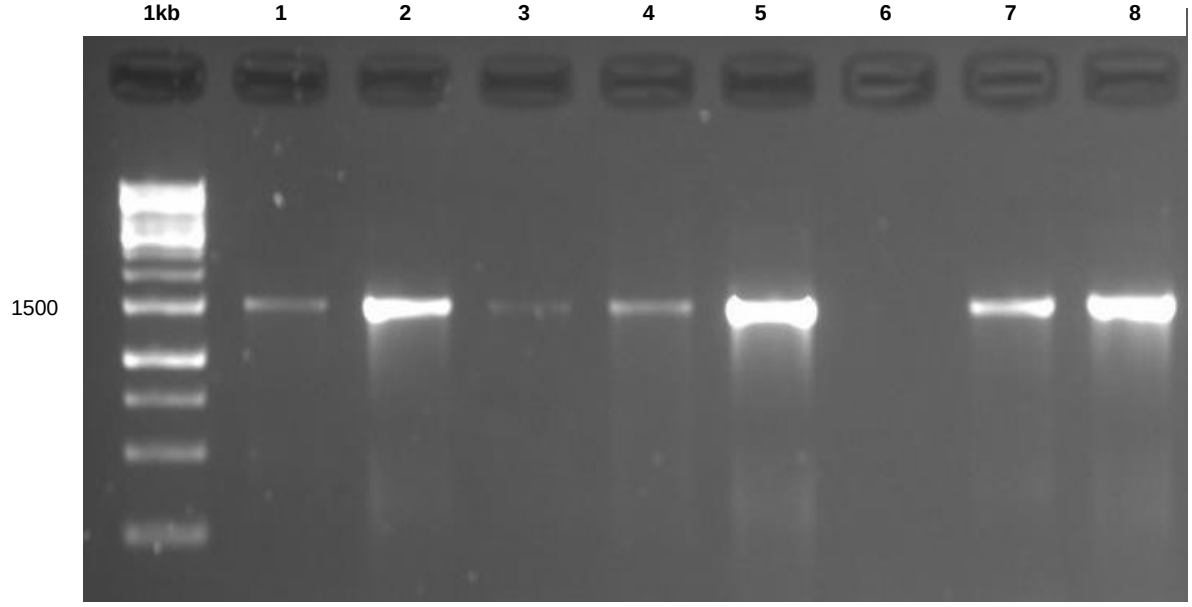

Figure 4.1: Photograph of the electrophoresis gel after amplification of the 16S rDNA

Table 4.2 indicates the species names with the isolate number obtained from the original nutrient agar plates supplemented with TCS or PCMX. Six genera were identified as *Klebsiella, Bacillus, Pseudomonas, Aeromonas, Exiguobacterium* and *Shewanella* spp.

Table 4.2: Identification of isolates

Species	**Isolate number (Accession number)**	**%ID**
***Bacillus* spp.**		
Bacillus toyonensis	C-10-Pre-2D(MG015920), T-0.375-Post-2(MG015921), T-0.25-Post-6(MG015922), C-40-Post-1(MG015923), C-30-U-4(MG015924), T-1-W-4(MG015925), C-40-D-5(MG015926), C-10-D-2A(MG015927)	100
Bacillus cereus	T-0.5-Post-4(MG015915), C-10-Post-4(MG015916), C-10-D-3(MG015917)	99.93
Bacillus subtilis	C-40-U-1(MG015919)	100
Bacillus stratosphericus	T-1-D-5(MG015918)	99.76
	13	
***Aeromonas* spp.**		
Aeromonas veronii	T-1-Pre-2(MG015904), T-1-Pre-3(MG015905), T-1-Post-4(MG015906), T-1-W-3(MG015907), C-40-W-1(MG015908), C-40-W-2(MG015909), T-1-D-6, (MG015910) T-1-D-8(MG015911), C-30-D-3(MG015912), C-30-D-5(MG015913), C-10-D-2B(MG015914)	99.82

Aeromonas caviae	T-0.5-Pre-1B (MG015902), T-1-U-3(MG015903)	100
	13	
***Pseudomonas* spp.**		
Pseudomonas monteilii	T-0.5-Pre-1A(MG015934)	100
Pseudomonas aeruginosa	T-0.25-Pre-1(MG015930)	100
Pseuduomonas alcaligenes	C-40-Post-2 (MG015931), C-20-Post-2 (MG015932), C-20-Post-4(MG015933)	100
Pseudomonas taiwanensis	T-1-U-4A(MG015935)	100
	6	
***Exiguobacterium* spp.**		
Exiguobacterium mexicanum	C-10-U-1(MG015928)	100
	1	
***Shewanella* spp.**		
Shewanella xiamenensis	T-0.25-Post-7(MG015936)	100
	1	
***Klebsiella* spp.**		
Klebsiella oxytoca	C-40-Pre-5(MG015929)	99.89
	1	

Similarity for identification varied between 99.73 – 100%, with *Aeromonas* (13) and *Bacillus* (13) being the most dominant isolates; there were also 6 *Pseudomonas* spp., *one Exiguobacterium* sp*., one Shewanella* sp*.,* and one *Klebsiella* sp*.* Isolates T-0.375-D-1 and C-30-W-2 could not be maintained under lab conditions and thus could not be identified.

4.2.2 DNA Fingerprinting (ERIC-PCR)

Aeromonas and *Bacillus* isolates were subjected to ERIC-PCR to determine clonal relation between isolates. PCR was followed by gel analysis using Phoretix 1D pro (Total Labs: Version 1.0). Variation among strains was assessed visually on the basis of the migration patterns of PCR products, and according to clustering analysis.

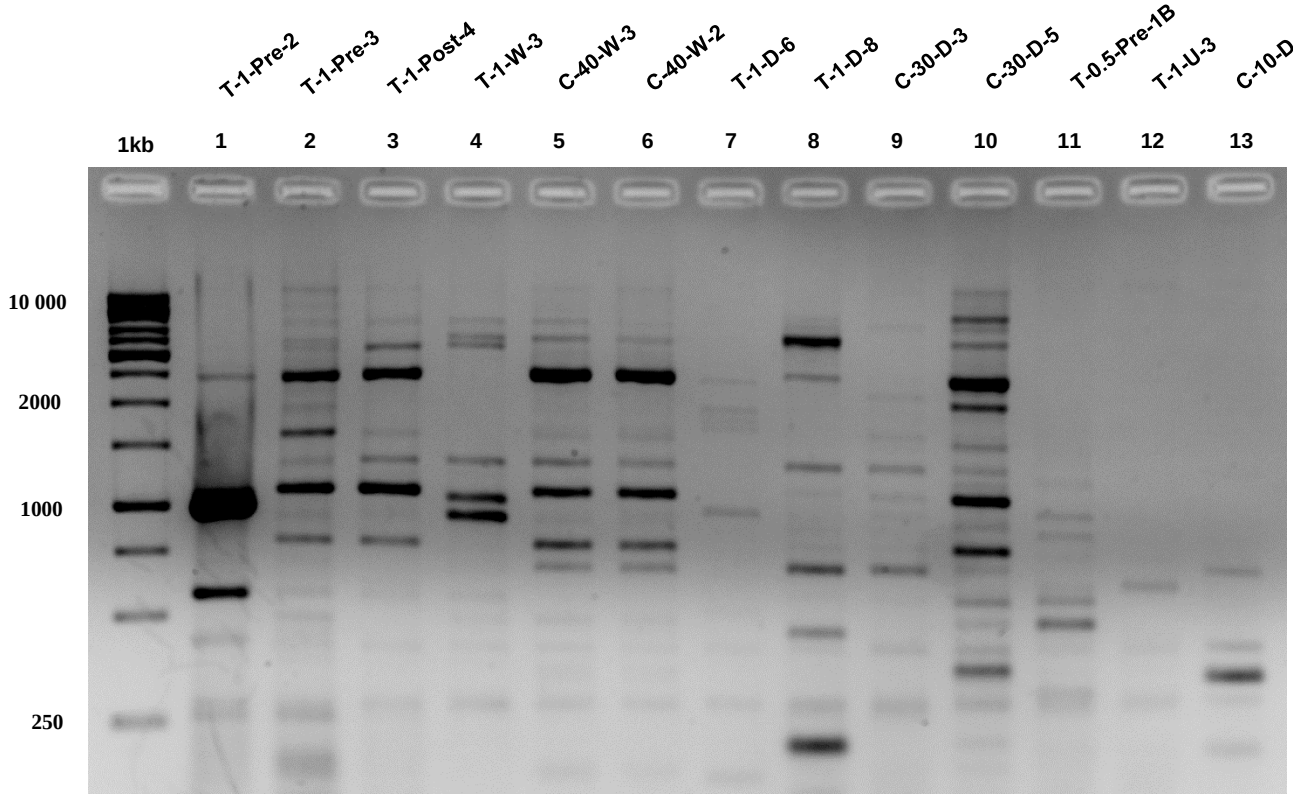

Figure 4.2: Agarose gel electrophoresis showing ERIC fingerprint of *A. veronii* isolates (Lanes 1-10 and 13), and *A. cavea* isolates (Lanes 11 and 12)

Figure 4.2 represents the ERIC fingerprint pattern obtained for *Aeromonas* isolates. ERIC-PCR yielded 2 to more than 18 PCR products, ranging in size from around 100 bp to over 10 000 bp. There were differences in the intensity of some bands, as well as the occurrence of several polymorphic bands. Isolates shared several similar bands, although differences in intensity for those bands were in some instances observed. With the exception of isolates T-1-Pre-2, T-1-D6, T-1-U-3 and C-10-D-2B, several similar banding patterns could be observed and several isolates shared bands of varying intensity at around 275, 800, 1100,1250, 1500, 2500 and 5000bp. Isolates C-40-W-3 and C-40-W-2 shared an almost identical fingerprint, while isolates T-1-Pre-3, T-1-Post-4, T-1-W-3, C-40-W-1, C-40-W-2, T-1-D-6, T-1-D-8, C-30-D-3 and C-30-D-5 all shared at least 2 or more identical bands. *A. cavea* isolates did not share any similar bands.

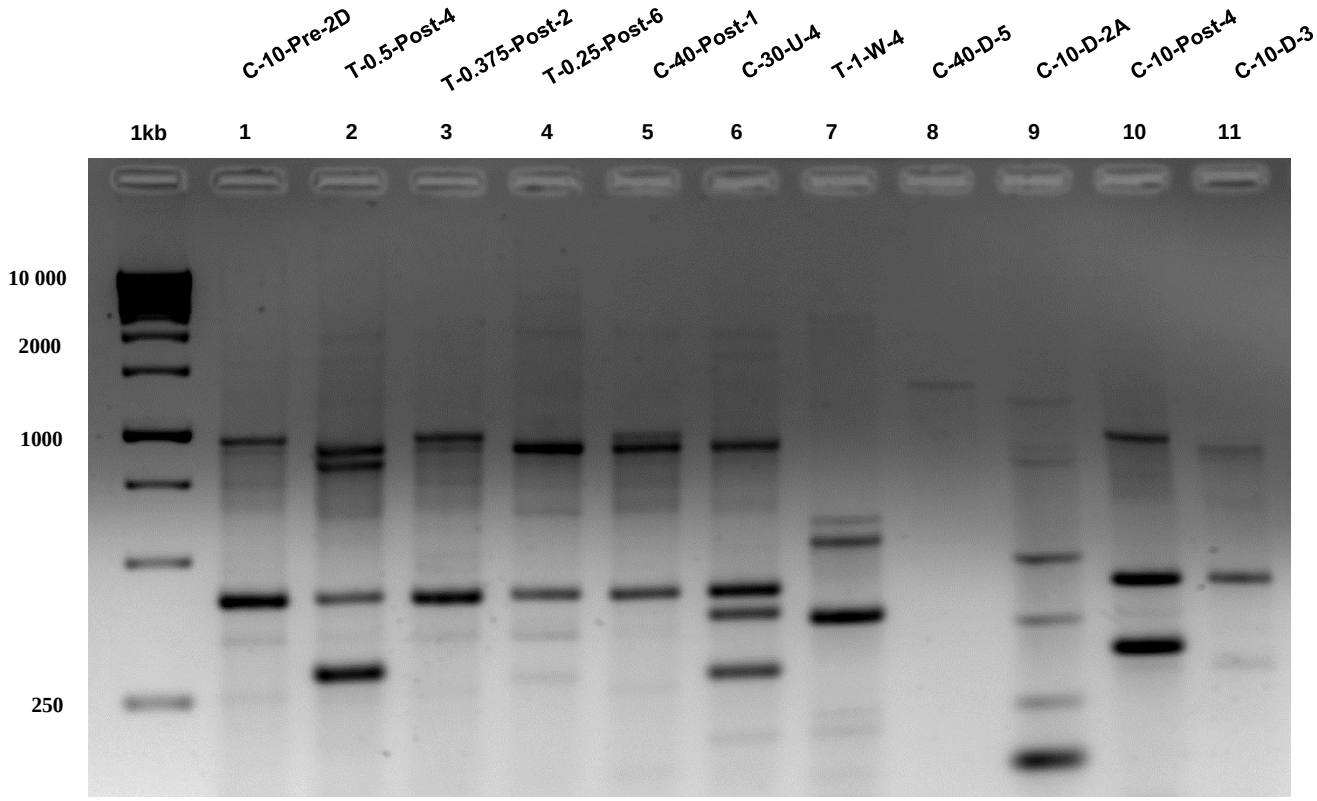

Figure 4.3: Agarose gel electrophoresis showing ERIC fingerprint of *B. toyonensis* isolates (Lanes 1 and 3-9), and *B. cereus* isolates (Lanes 2, 10 and 11)

Figure 4.3 represents the ERIC fingerprint pattern obtained for *Bacillus* isolates. ERIC-PCR yielded 1 to 7 PCR products, ranging in size from around 100bp to 1300bp. As with *Aeromonas* isolates, differences in concentration of certain bands, as well as polymorphic bands were visualised and several isolates shared similar bands. All isolates with the exception of isolates T-1-W-4, C-40-D-5 and C-10-D-2A shared a similar band at around 400 bp, the majority of *Bacillus* isolates regardless of the species identified as and with the exception of those previously mentioned, share 1 or more similar band with at least one of the other isolates. *B. toyonensis* isolates C-10-Pre-2D and T-0.375-Post-2 shared 2 similar bands at around 400 bp and 1000 bp, isolates T-0.25-Post-6, C-40-Post-1 and C-30-U-4 also shared 2 similar bands at around 400 bp and 950 bp in size. Among *B. cereus* isolates, T-0.5-Post-4 and C-10-D-3 share 2 similar bands at around 400 bp and 950 bp.

The final gel products were analysed and a clustering analysis was performed with the aid of Phoretix 1D pro (Total Labs: Version 1.0). Clustering analysis was done based on the Dice similarity coefficient and the Unweighted Pair Group Method with

Arithmetic mean (UPGMA). Figures 4.4 and 4.5 represent a dendrogram of the *Aeromonas* and *Bacillus* isolates, respectively.

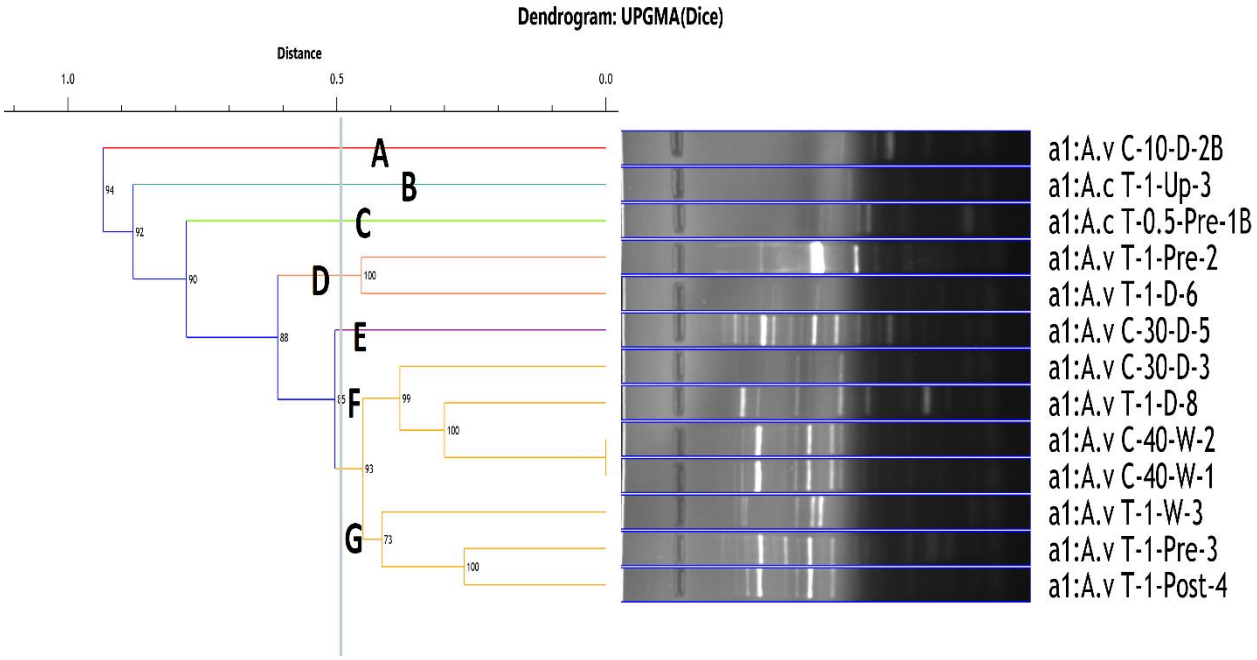

Figure 4.4: Cluster analysis by ERIC-PCR fingerprint of 11 *Aeromonas veronii* isolates and 2 *Aeromonas cavea* isolates. Clustering analysis performed with the aid of Phoretix 1D pro (Total Labs: Version 1.0) and based on Dice similarity coefficient and the Unweighted Pair Group Method with Arithmetic mean (UPGMA)

According to ERIC-PCR analysis, 7 major clusters labelled A-G were defined among *Aeromonas* (A.v) isolates. Cluster D consists of *A. veronii* isolates from the pre-chlorination sampling point (T-1-Pre-2), as well as the downstream sampling point (T-1-D-6). Clusters E, F and G all form part of a larger cluster which consists of *A. veronii* isolates from pre- and post-chlorination sampling points (T-1-Pre-3 and T-1-Post-4), as well as from the wetlands and downstream sampling points (C-30-D-5, C-30-D-5, T-1-D-8, C-40-W-1, C-40-W-2 and T-1-W-3).

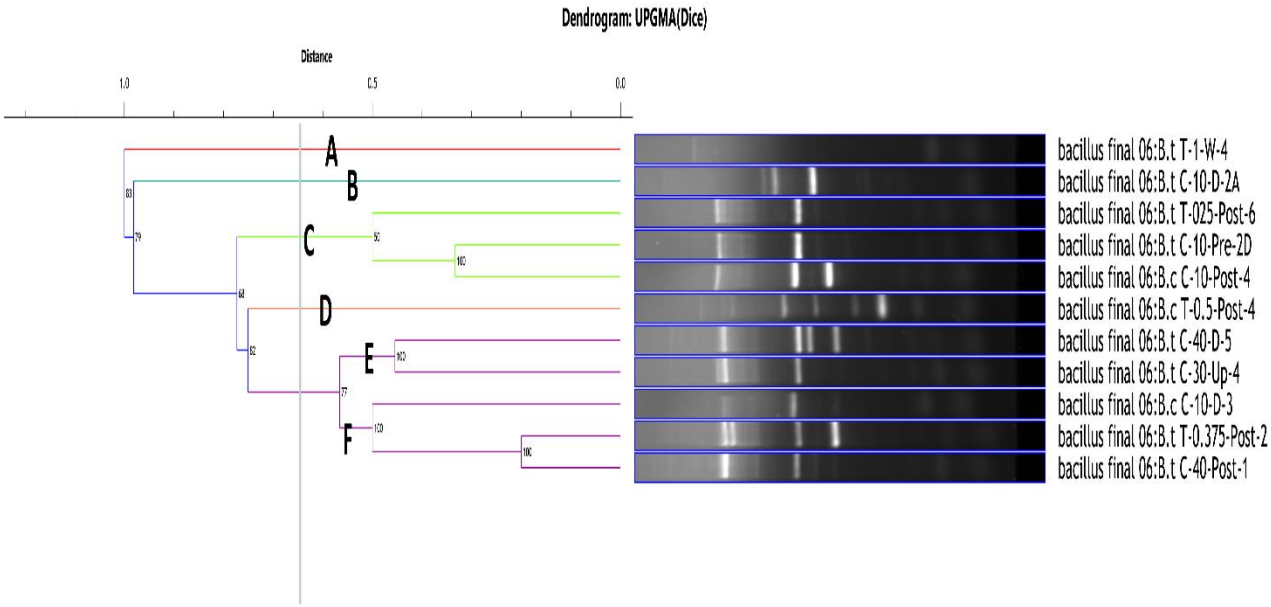

Figure 4.5: Cluster analysis by ERIC-PCR fingerprint of 8 *Bacillus toyonensis* isolates and 3 *Bacillus cereus* isolates. Clustering analysis performed with the aid of Phoretix 1D pro (Total Labs: Version 1.0) and based on Dice similarity coefficient and the Unweighted Pair Group Method with Arithmetic mean (UPGMA)

Among *Bacillus* isolates, 6 major clusters labelled A-F were defined. Cluster C consists of 1 *B. cereus* (B.c) isolate and 2 *B. toyonensis* (B.t) isolates, one from the pre (C-10-Pre-2D) and one from the post (T-0.25-Post-6) chlorination sampling points. Clusters D, E and F form part of a larger cluster that includes 2 *B. cereus* isolates, 1 one from the post-chlorination sampling point (T-0.5-Post-4) and one from the downstream sampling point (C-10-D-3). The cluster also consists of 4 *B. toyonensis* isolates from the post-chlorination sampling point (T-0.375-Post-2 and C-40-Post-1), the downstream sampling point (C-40-D-5) and the upstream sampling point (C-30-Up-4).

4.3 Extracellular enzyme production

All isolates were examined for haemolytic activity and the presence of seven selected extracellular enzymes associated with virulence. Table 4.3 represents results obtained after screening haemolytic positive isolates for the presence of extracellular enzymes. Enzymes were labelled as follows: Lecithinase (Le), hyaluronidase (H), chondroitinase (C), DNase (D), gelatinase (G), proteinase (P) and lipase (Li).

Table 4.3: Extracellular enzyme production

Isolate	Indentification	Haemolysis	Le	H	C	D	G	Pr	Li
T-0.5-Pre-1B	*A. caviae*	β		X	X	X	X	X	X
T-1-U-3	*A. caviae*	β	X	X	X	X	X	X	X
T-1-Pre-2	*A. veronii*	β			X	X	X	X	X
T-1-Pre-3	*A. veronii*	β	X		X	X	X	X	X
T-1-Post-4	*A. veronii*	β	X		X	X	X	X	X
T-1-W-3	*A. veronii*	β	X		X	X	X	X	X
T-1-D-6	*A. veronii*	β	X			X	X	X	X
T-1-D-8	*A. veronii*	β			X	X	X	X	
C-30-D-3	*A. veronii*	β	X	X	X	X	X	X	
C-30-D-5	*A. veronii*	β	X		X	X	X	X	X
C-10-D-2B	*A. veronii*	β	X		X	X	X	X	X
C-40-W-1	*A. veronii*	β	X	X	X	X	X	X	X
C-40-W-2	*A. veronii*	β	X			X	X	X	X
C-10-D-3	*B. cereus*	β	X	X	X		X		
T-0.5-Post-4	*B. cereus*	β	X	X		X	X	X	
C-10-Post-4	*B. cereus*	α	X		X	X			
C-40-U-1	*B. subtilis*	β	X		X		X	X	
C-10-Pre-2D	*B. toyonensis*	β	X			X	X		
T-0.375-Post-2	*B. toyonensis*	β	X	X	X	X	X		
T-0.25-Post-6	*B. toyonensis*	α	X		X	X	X		
C-30-U-4	*B. toyonensis*	β	X			X			
T-1-W-4	*B. toyonensis*	β			X		X		X
C-40-D-5	*B. toyonensis*	β	X	X		X		X	
C-10-D-2A	*B. toyonensis*	β			X				
C-40-Post-1	*B. toyonensis*	β	X		X	X	X		
T-1-D-5	*B.stratosphericus*	β		X	X	X	X		
C-10-U-1	*E. mexicanum*	β				X	X		
C-40-Pre-5	*K. oxytoca*	β				X			
C-20-Post-2	*P. alcaligenes*	β							X
C-40-Post-2	*P. alcaligenes*	β				X			X
C-20-Post-4	*P. alcaligenes*	α							X
T-0.25-Pre-1	*P. aeruginosa*	β	X	X	X	X	X	X	
T-0.5-Pre-1A	*P. monteilii*	β							
T-1-U-4A	*P. taiwanensis*	α							
T-0.25-Post-7	*S. xiamenensis*	β			X		X		

Lecithinase (Le), hyaluronidase (H), chondroitinase (C), DNase (D), gelatinase (G), proteinase (P) and lipase (Li).

The presence of extracellular enzymes was most prevalent among *Aeromonas* isolates followed by *P. aeruginosa* and *Bacillus* isolates. *P. monteilii*, and *P. taiwanensis* isolates exhibited no enzyme activity while *P. alcaligenes* exhibited very low enzyme activity. Figure 4.6 represents the final percentage of isolates testing positive for the presence of each extracellular enzyme.

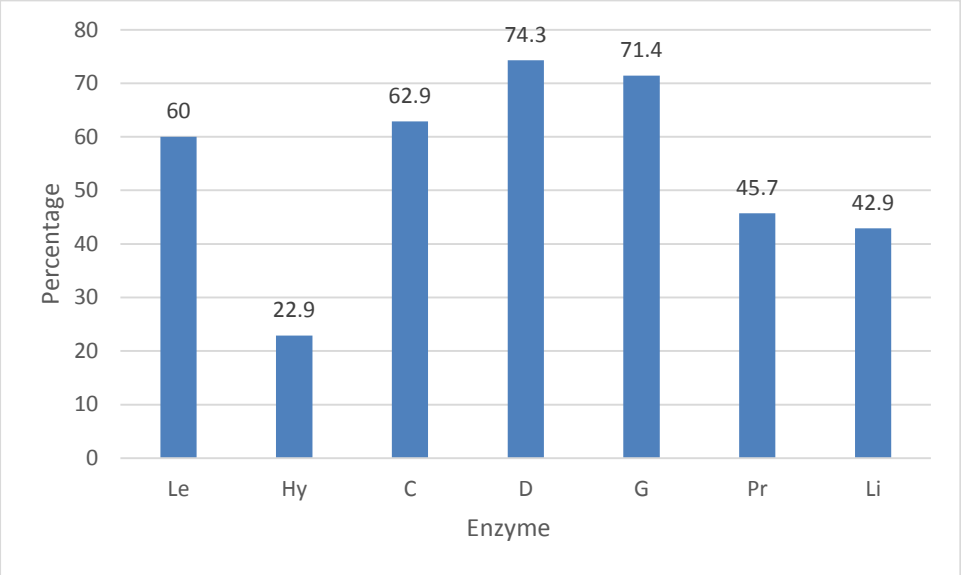

Figure 4.6: Percentage extracellular enzyme production

Of the isolates examined, 60% tested positive for the presence of lecithinase (Le), 22.9% for the presence of hyaluronidase (H), 62% for the presence of chondroitinase (C), 74.3% for the presence of DNase (D), 71.4% for the presence of gelatinase (G), 45.7% for the presence of proteinase (P) and 42.9% tested positive for the presence of lipase (Li). DNase and gelatinase were the most prevalent of the enzymes examined while hyaluronidase was the least prevalent.

4.4 Antimicrobial susceptibility

4.4.1 Assay for cross-resistance to antibiotics

To determine cross-resistance to antibiotics, the Kirby-Bauer disc diffusion method was used. Antibiotics used included Vancomycin (30 µg), Kanamycin (30 µg), Trimethoprim (5 µg), Oxytetracycline (30 µg), Amoxicillin (10 µg) and Chloramphenicol (30 µg).

Table 4.4: Antibiotic resistance profiles

Isolate	Identification	V30	A10	K30	C30	T5	O30
T-0.5-Pre-1B	*A. caviae*	NA	R	S	S	R	S
T-1-U-3	*A. caviae*	NA	R	S	S	I	S
T-1-Pre-2	*A. veronii*	NA	R	S	S	S	R
T-1-Pre-3	*A. veronii*	NA	R	S	S	S	R
T-1-Post-4	*A. veronii*	NA	R	S	S	S	S
T-1-W-3	*A. veronii*	NA	R	S	S	I	S
T-1-D-6	*A. veronii*	NA	R	S	S	R	R
T-1-D-8	*A. veronii*	NA	R	S	S	S	S
C-30-D-3	*A. veronii*	NA	R	S	S	R	I
C-30-D-5	*A. veronii*	NA	R	S	S	I	S
C-10-D-2B	*A. veronii*	NA	R	S	S	S	S
C-40-W-1	*A. veronii*	NA	R	S	S	R	S
C-40-W-2	*A. veronii*	NA	S	S	S	S	R
C-10-D-3	*B. cereus*	R	R	I	S	R	S
T-0.5-Post-4	*B. cereus*	S	R	I	S	R	I
C-10-Post-4	*B. cereus*	I	R	S	S	R	R
C-40-U-1	*B. subtilis*	S	S	S	S	S	S
C-10-Pre-2D	*B. toyonensis*	R	R	I	I	R	R
T-0.375-Post-2	*B. toyonensis*	I	R	S	S	R	I
T-0.25-Post-6	*B. toyonensis*	I	R	S	S	R	R
C-30-U-4	*B. toyonensis*	S	R	S	S	R	I
T-1-W-4	*B. toyonensis*	I	S	S	S	S	S
C-40-D-5	*B. toyonensis*	R	R	S	S	R	S
C-10-D-2A	*B. toyonensis*	I	S	S	S	R	S
C-40-Post-1	*B. toyonensis*	I	R	R	S	R	R
T-1-D-5	*B.stratosphericus*	I	R	I	I	R	R
C-10-U-1	*E. mexicanum*	NA	R	S	S	R	R
C-40-Pre-5	*K. oxytoca*	NA	S	S	S	S	R
C-20-Post-2	*P. alcaligenes*	NA	I	S	S	I	R
C-40-Post-2	*P. alcaligenes*	NA	R	S	S	R	R
C-20-Post-4	*P. alcaligenes*	NA	S	S	S	S	S
T-0.25-Pre-1	*P. aeruginosa*	NA	R	R	R	R	R
T-0.5-Pre-1A	*P. monteilii*	NA	R	S	I	R	I
T-1-U-4A	*P. taiwanensis*	NA	R	S	I	R	R
T-0.25-Post-7	*S. xiamenensis*	NA	I	S	S	S	I
	Resistance percentage:	**23.1%**	**77.1%**	**11.4%**	**2.9%**	**57.1%**	**45.7%**

R = resistant, I = Intermediate resistance, S = susceptible, NA = Not Applicable

Table 4.4 represents results obtained after antibiotic susceptibility testing using the Kirby-Bauer disc diffusion protocol; the following antibiotic resistance patterns could be observed. Among Gram positive isolates, 23.1% were resistant to Vancomycin (30 µg), among all isolates 77.1% were resistant to Amoxicillin (10 µg), 11.4% were resistant to Kanamycin (30 µg), 2.9 % were resistant to Chloramphenicol (30 µg), 57.1% were resistant to Trimethoprim (5 µg) and 45.7% were resistant to Oxytetracycline (30 µg).

Among organisms of the same genus, several antibiotic resistance patterns could be observed. *Aeromonas* spp. exhibited resistance primarily to Amoxicillin while Trimethoprim and Oxytetracycline resistance was also observed. *Bacillus* spp. exhibited resistance to a variety of antibiotics, Amoxicillin and Trimethoprim resistance being most prevalent, but varying levels of resistance to all other antibiotics tested was also found. *P. aeruginosa, P. monteilii* and *P. taiwanensis* isolates exhibited various levels of resistance to all antibiotics except Kanamycin, while *P. alcaligenes* isolates primarily exhibited resistance to Amoxicillin, Trimethoprim and Oxytetracycline. No variation could be seen on the type of biocide supplemented media the isolates were found on or based on sampling location, except among *B. toyonensis* isolates. *B. toyonensis* isolates found at the pre- and post-chlorination sampling points exhibited resistance to more antibiotics than isolates found at the wetlands, and up and downstream sampling points.

4.4.2 Minimum inhibitory concentration

Isolates showing similar patterns of antibiotic susceptibility were selected to determine MICs. Isolates were grouped according to antibiotic susceptibility patterns and one isolate from each group was selected for MICs. Thus, in total 14 isolates were selected for MICs. Figures 4.7 and 4.8 indicate a typical growth curve obtained after isolates were supplemented with varying concentrations of TCS or PCMX. The zero concentration was used as a control for each isolate.

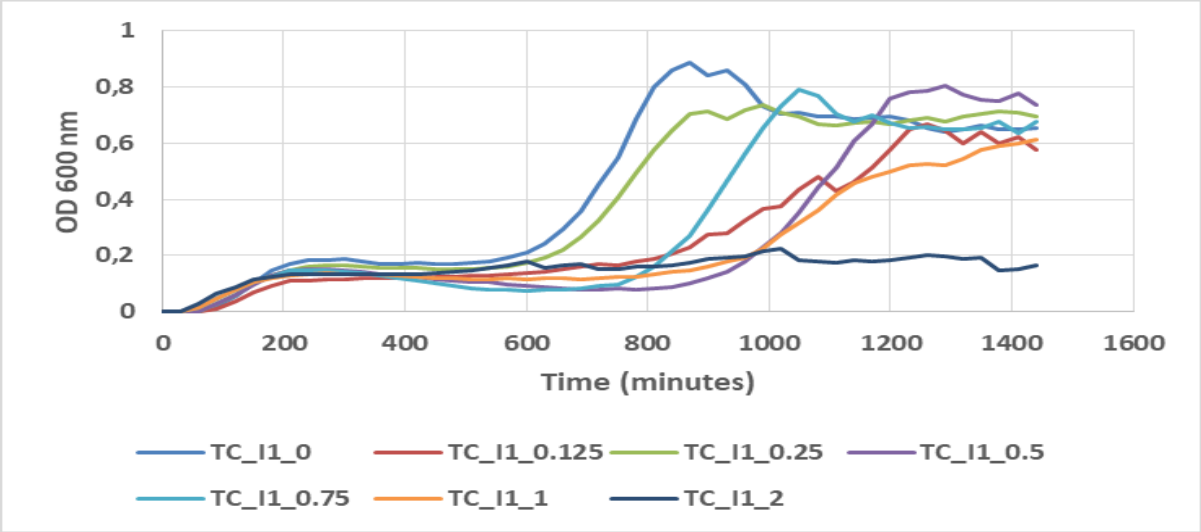

Figure 4.7: Typical growth curve data obtained after 24 hours (TCS, *A. veronii* (T-1-Pre-2))

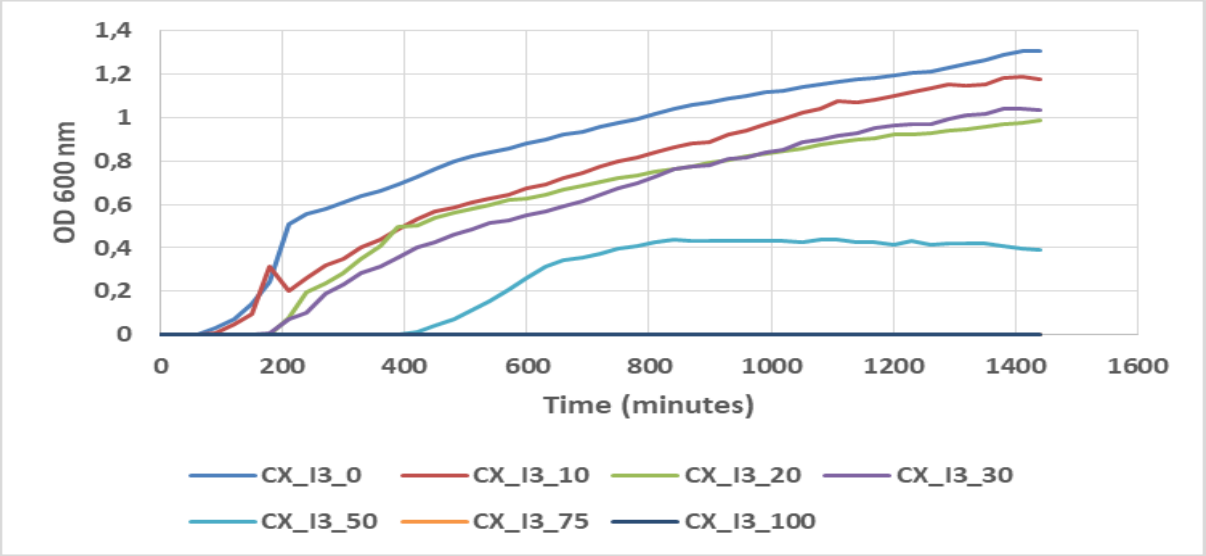

Figure 4.8: Typical growth curve data obtained after 24 hours (PCMX, *P. monteilii* (T-0.5-Pre-1A))

In Figures 4.7 and 4.8, the optical density was measured at 600 nm for a period of 24 hours. Blue indicates the growth of the isolate that was grown in media without TCS or PCMX. The fourteen isolates were grown in Mueller-Hinton broth supplemented with different concentrations of TCS or PCMX and incubated for 24 hours. After 24 hours in the micro-well plate reader, the lag times of the isolates was measured to indicate if these isolates were susceptible or inhibited by the different concentrations of TCS or PCMX. Tables 4.5 and 4.6 indicate the measurements of the lag time of the isolates supplemented with varying concentrations of TCS or PCMX. The optical density of the medium containing the isolates was measured over a period of 24 hours.

Table 4.5: Table containing lag time measured from growth curves and minimum inhibitory concentration value for TCS (mg/L)

Isolate Group	ID	0	0.125	0.25	0.5	0.75	1	2
T-1-Pre-2; T-1-Pre-3; T-1-Post-4	*A. veronii*	0.5	0.5	0.5	0.5	0.5	0.5	0.5
T-1-W-3; C-40-W-1; C-40-W-2	*A. veronii*	0.5	0.5	0.5	0.5	0.5	0.5	0.5
T-1-D-6	*A. veronii*	8	11.5	14	11	8	8	8
T-1-D-8; C-30-D-3; C-30-D-5, C-10-D-2B	*A. veronii*	0.5	0.5	0.5	0.5	0.5	0.5	0.5
T-0.5-Pre-1B; T-1-U-3	*A. caviae*	3	1.5	1.5	1.5	6.5	6.5	6.5
T-0.5-Pre-1A	*P. monteleii*	1.5	1.5	1.5	1.5	0.5	1	1.5
T-0.25-Pre-1	*P. aeruginosa*	1.5	1.5	3	1.5	2.5	2.5	4
C-40-Post-2; C-20-Post-2, C-20-Post-4	*P. alcaligenes*	5	0.5	5	6	8	8	11.5
T-1-U-4A	*P. taiwanensis*	2	2	2	2	0.5	0.5	2.5
C-10-Pre-2D; C-40-Post-1	*B. toyonensis*	0.5	0.5	0.5	0.5	0.5	0.5	1.5
T-0.375-Post-2; T-0.25-Post-6; T-1-W-4; C-10-D-2A C-30-U-4; C-40-D-5	*B. toyonensis*	8	13	13	10	10	10	8
T-0.5-Post-4; C-10-Post-4; C-10-D-3	*B. cereus*	0.5	2.5	2.5	2.5	3	0.5	1.5
T-0.25-Post-7	*S. xiamenensis*	0.5	0.5	0.5	0.5	0.5	0.5	0.5
C-10-U-1	*E. mexicanum*	0.5	0.5	0.5	6.5	8	8.5	14

Isolates incubated with TCS indicated lag times for the control group between 0.5 and 8 hours; thereafter lag times varied or remained the same. It can thus be assumed that all isolates had a MIC value above 2 mg/L TCS, but further investigation is required to obtain a precise value.

Table 4.6: Table containing lag time measured from growth curves and minimum inhibitory concentration value for PCMX (mg/L)

Isolates	ID	0	10	20	30	50	75	100
T-1-Pre-2; T-1-Pre-3; T-1-Post-4	*A. veronii*	16.5	6.5	16.5	10	24	24	24
T-1-W-3; C-40-W-1; C-40-W-2	*A. veronii*	3	4	4	4	24	24	24
T-1-D-6	*A. veronii*	6	7.5	7.5	16	24	24	24
T-1-D-8; C-30-D-3; C-30-D-5, C-10-D-2B	*A. veronii*	7.5	7.5	7.5	7.5	24	24	24
T-0.5-Pre-1B; T-1-U-3	*A. caviae*	9	7.5	6.5	8	24	24	24
T-0.5-Pre-1A	*P. monteleii*	1	1.5	3	3	6.5	24	24
T-0.25-Pre-1	*P. aeruginosa*	2.5	1.5	4	3	24	24	24
C-40-Post-2; C-20-Post-2, C-20-Post-4	*P. alcaligenes*	0.5	5	8	4	24	24	24
T-1-U-4A	*P. taiwanensis*	0.5	0.5	0.5	3	24	24	24
C-10-Pre-2D; C-40-Post-1	*B. toyonensis*	0.5	0.5	0.5	0.5	24	24	24
T-0.375-Post-2; T-0.25-Post-6; T-1-W-4; C-10-D-2A C-30-U-4; C-40-D-5; T	*B. toyonensis*	0.5	0.5	0.5	0.5	24	24	24
T-0.5-Post-4; C-10-Post-4; C-10-D-3	*B. cereus*	0.5	0.5	0.5	0.5	24	24	24
T-0.25-Post-7	*S. xiamenensis*	0.5	1.5	1.5	0.5	24	24	24
C-10-U-1	*E. mexicanum*	0.5	0.5	0.5	0.5	1.5	24	24

The isolates incubated with PCMX indicated lag times for the control group between 0.5 and 16.5 hours, after which lag times varied or remained the same. The majority of isolates indicated no change in optical density over 24 hours when exposed to 50, 75 and 100 mg/L of PCMX. This indicates that the organisms remained in lag phase

and thus the MIC for these isolates are regarded as being between 30 and 50 mg/L. The *P. monteleii* and *E. mexicanum* isolates indicated no change from 75 mg/L and upwards, thus the MIC for these isolates is regarded as being more than 50 mg/L.

When observing the results in Figure 4.7, the following can be concluded. There is a marked decrease in growth between the control (0 mg/L) and the 0.125 mg/L TCS. However, between 0.125 mg/L to 0.25 mg/L an increase in the growth of the isolate can be observed. A decrease in growth can be observed when the TCS increases to 0.25 mg/L, followed by an increase in growth at 0.75 mg/L, similar to that observed between 0.125 mg/L and 0.25 mg/L TCS. At the remainder of the exposure concentrations (0.75 mg/L through 2 mg/L), a dose related decrease in growth of the isolate can be observed. This suggests possible induction of an efflux related system to expel the biocide from the cell, which appears to display two induction levels, contributing to the observed resistance. These types of responses were observed with either TCS or PCMX among the *Aeromonas* (T-1-Pre-2, T-1-D-6, T-1-Pre-2 and T-0.5-Pre-1B)*, Pseudomonas* (C-40-Post-2, T-1-U-4A and T-0.25-Pre-1) and *Bacilus* (C-40-Post-1, T-0.375-Post-2 and T-0.5-Post-4) isolates.

For the remainder of the isolates, for example Figure 4.8 (T-1-W-3, T-1-D-8, T-1-D-6, T-0.5-Pre-1A, C-40-Post-2, T-0.5-Pre-1B, T-1-U-4A, T-0.375-D-1, C-10-U-1, T-0.25-Post-7), a typical dose-dependent effect was observed for either TCS or PCMX as there was a consistent decrease in observed growth as the concentration of the biocide (PCMX and TCS) increased. More examples of these observations can be found in appendix B.

4.4.3 Checkerboard assay

Synergy and antagonism between TCS and PCMX, and selected antibiotics was done by a checkerboard method as described in Chapter 3. Based on previous antibiotic resistance profiles (Table 4.4), the following isolates were selected: T-0.5-Pre-1B (*A. cavea*), T-1-D-6 (*A. veronii*), T-0.25-Pre-1 (*P. aeruginosa*), C-40-Post-2 (*P. alcaligenes*), C-10-Pre-2D (*B. toyonensis*), and T-0.5-Post-4 (*B. cereus*). Antibiotics included the following: Kanamycin, Amoxicillin, Chloramphenicol, Trimethoprim and

Tetracycline. Vancomycin was not included during the assay due the fact that Vancomycin is a very narrow spectrum antibiotic. The presence or absence of visible growth was observed after isolates were incubated for 24 hours at 37ºC. Micro-wells were loaded in triplicate.

	A0	A1	A2	A4	A8	A16	A32	A64
T0	T.0_A0	T.0_A1	T0_A2	T0_A4	T0_A8	T0_A16	T0_A32	T0_A64
T.25	T.25_A0	T.25_A1	T.25_A2	T.25_A4	T.25_A8	T.25_A16	T.25_A32	T.25_A64
T.5	T.5_A0	T.5_A1	T.5_A2	T.5_A4	T.5_A8	T.5_A16	T.5_A32	T.5_A64
T1	T1_A0	T1_A1	T1_A2	T1_A4	T1_A8	T1_A16	T1_A32	T1_A64
T2	T2_A0	T2_A1	T2_A2	T2_A4	T2_A8	T2_A16	T2_A32	T2_A64

	A0	A1	A2	A4	A8	A16	A32	A64
C0	C0_A0	C0_A1	C0_A2	C0_A4	C0_A8	C0_A16	C0_A32	C0_A64
C10	C10_A0	C10_A1	C10_A2	C10_A4	C10_A8	C10_A16	C10_A32	C10_A64
C20	C20_A0	C20_A1	C20_A2	C20_A4	C20_A8	C20_A16	C20_A32	C20_A64
C30	C30_A0	C30_A1	C30_A2	C30_A4	C30_A8	C30_A16	C30_A32	C30_A64
C40	C40_A0	C40_A1	C40_A2	C40_A4	C40_A8	C40_A16	C40_A32	C40_A64

Figure 4.9: Checkerboard (Trimethoprim + TCS)

An example of the micro-well plate design containing the raw data used to determine the final checkerboard can be found in Appendix A. Figure 4.9 represents an example of the final checkerboard obtained for one antibiotic (Trimethoprim), one biocide (TCS) and one isolate T-0.5-Pre-1B (*A.cavea*). Table 4.7 A-F represents the final results obtained for the checkerboard assay to determine the occurrence of synergy and/or antagonism between the biocides in question and selected antibiotics.

The occurrence of synergy was labelled as S, antagonism as A, and the occurrence of both was labelled as A/S, while the concentrations at which no effects were observed was labelled N/A. Among the *Aeromonas* isolates selected, synergy and antagonism was observed to varying degrees in relation to all antibiotics tested except for Trimethoprim, which showed no interaction. Among *Pseudomonas* isolates, interaction was observed only with Tetracycline for *P.aeruginosa,* and only with Amoxicillin and Trimethoprim for *P.alcaligenes*. Both *Bacillus* spp. isolates showed interaction with Kanamycin, Tetracycline and Chloramphenicol.

Table 4.7 A-F: Synergy and/or Antagonism between antimicrobials

A

T-0.5-Pre-1B (*A.cavea*)

Antibiotic	TCS				PCMX			
Biocide concentration(mg/L):	0.25	0.5	1	2	10	20	30	40
Kanamycin	A	N/A	N/A	A	S	S	S	S
Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline	S	N/A	N/A	S	A	N/A	S	S
Chloramphenicol	A	A	A	A	S	S	S	S
Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

B

T-1-D-6 (*A.veronii*)

Antibiotic	TCS				PCMX			
Biocide concentration(mg/L):	0.25	0.5	1	2	10	20	30	40
Kanamycin	N/A	A	A	A/S	S	S	S	S
Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline	N/A	N/A	S	S	N/A	N/A	S	S
Chloramphenicol	N/A	N/A	N/A	S	N/A	N/A	N/A	N/A
Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	S

C

T-0.25-Pre-1 (*P.aeruginosa*)

Antibiotic	TCS				PCMX			
Biocide concentration(mg/L):	0.25	0.5	1	2	10	20	30	40
Kanamycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline	S	S	S	S	N/A	N/A	S	S
Chloramphenicol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

D

C-40-Post-2 (*P.alcaligenes*)

Antibiotic	TCS				PCMX			
Biocide concentration(mg/L):	0.25	0.5	1	2	10	20	30	40
Kanamycin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Trimethoprim	N/A	S	S	S	A	A	A	A
Tetracycline	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Chloramphenicol	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Amoxicillin	N/A	S	S	S	N/A	N/A	N/A	N/A

E

C-10-Pre-2D (*B.toyonensis*)

Antibiotic	TCS				PCMX			
Biocide concentration(mg/L):	0.25	0.5	1	2	10	20	30	40
Kanamycin	S	N/A	N/A	N/A	S	S	S	S
Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	N/A	N/A	N/A	N/A	N/A	N/A
Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

F

T-0.5-Post-4 (*B.cereus*)

Antibiotic	TCS				PCMX			
Biocide concentration(mg/L):	0.25	0.5	1	2	10	20	30	40
Kanamycin	S	N/A	A	A	N/A	S	S	S
Trimethoprim	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Tetracycline	S	S	N/A	N/A	N/A	N/A	N/A	S
Chloramphenicol	A	A	A	N/A	A	A	A	A
Amoxicillin	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

In total, 80 occurrences of synergy and/or antagonism were observed with different antibiotics and various concentrations of TCS or PCMX. Synergistic and/or antagonistic effects were the least prevalent with Amoxicillin (4 occurrences) and Trimethoprim (7 occurrences), while synergistic and/or antagonistic effects were most prevalent with Tetracycline (26 occurrences). Synergistic and/or antagonistic effects overall were equally prevalent in antibiotic/TCS combinations and antibiotic/PCMX combinations (39 occurrences each). Synergy was more prevalent between antibiotic/PCMX combinations (31 versus 26 occurrences), while antagonism was most prevalent between antibiotic/TCS combinations (14 versus 9 occurrences).

4.5 High Performance Liquid Chromatography

Water samples were collected from the influent and effluent points of the wastewater treatment plant, as well as at upstream and downstream sampling sites. Two sampling runs were performed: with one representative of the end of the wet season and one of the end of the dry season. Samples were treated according to previously mentioned

methods in Chapter 3. Four 1 L samples were collected from each site, the method was performed in triplicate, and one water sample was spiked with 500 ppm of TCS and PCMX to determine efficiency. The method was optimised to ensure a specific retention time for TCS and PCMX by injecting various concentrations of TCS or PCMX in the HPLC. Retention times observed were 6.079 for PCMX and 14.586 min for TCS as represented by Figure 4.10.

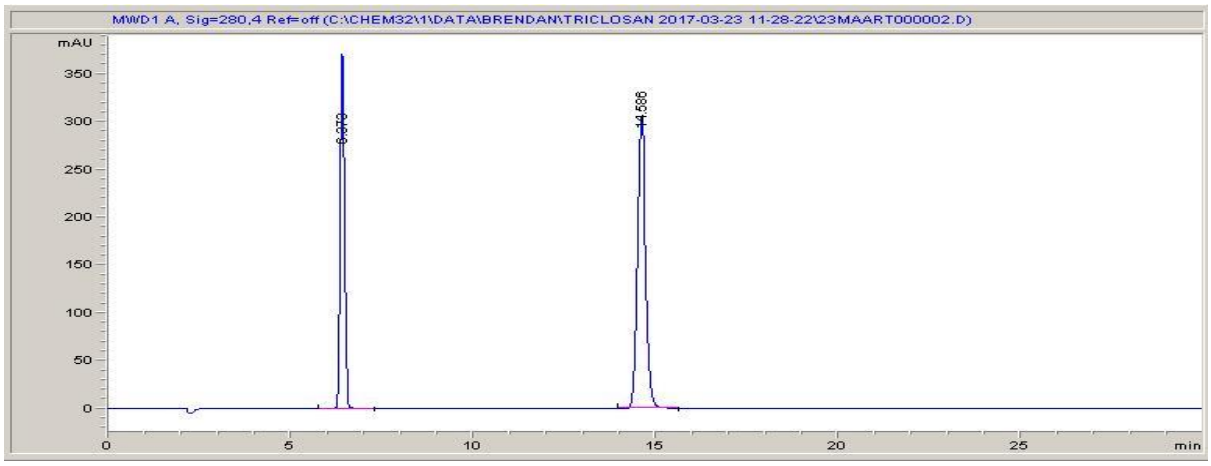

Figure 4.10: Chromatogram showing the retention time peaks at 6.079 for PCMX and 14.586 min for TCS

A standard curve was constructed using the following standard concentrations: 0, 25, 50, 100, 250 and 500 ppm. The area under the curve obtained was plotted as indicated by figure 4.11 for TCS and figure 4.12 for PCMX, respectively.

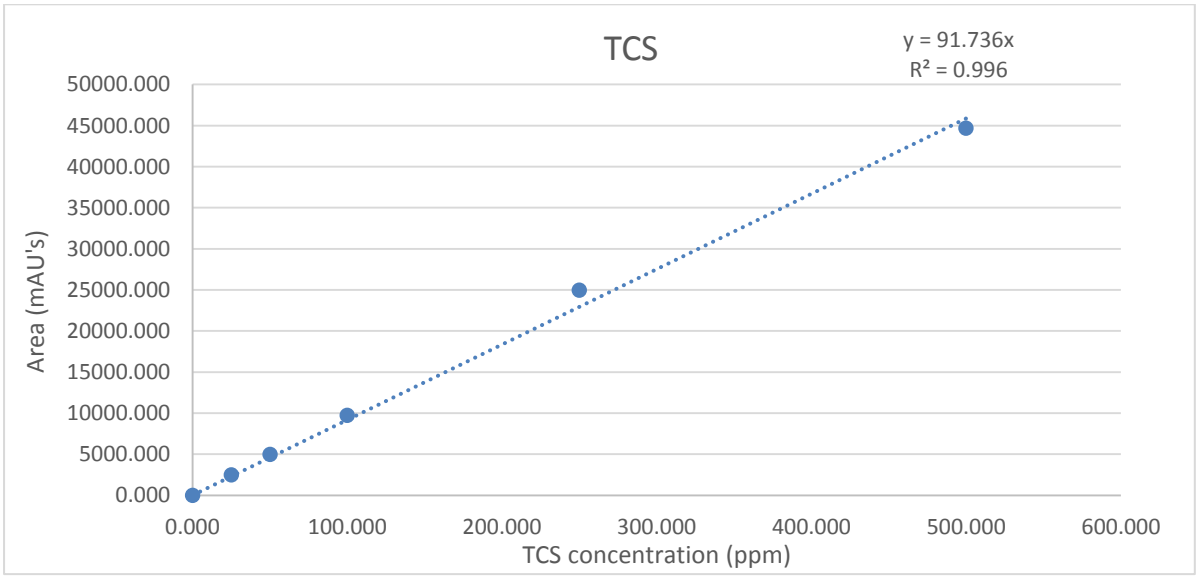

Figure 4.11: TCS concentration gradient (0, 25, 50, 100, 250, 500 ppm)

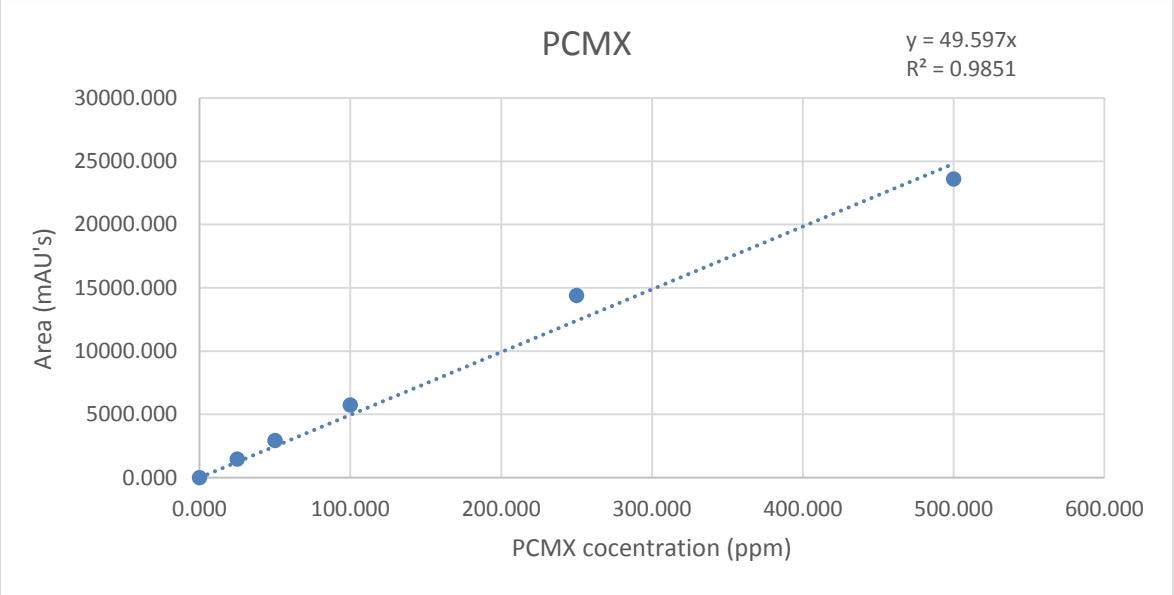

Figure 4.12: PCMX concentration gradient (0, 25, 50, 100, 250, 500 ppm)

The influent and effluent points of the WWTP were selected, as the influent site is representative of wastewater entering the plant before the occurrence of any treatment, while the effluent site represents the final product before entering the river systems. From the comparison of these two sites, it can be determined whether TCS and/or PCMX enter the WWTP, and whether their levels are reduced during the treatment process before entering the environment as effluent. The upstream sampling point was selected to determine if TCS and/or PCMX are present in the river prior to the outflow from the WWTP, and the downstream sampling point was selected, as it would be indicative of the presence of the biocides downstream from the WWTP.

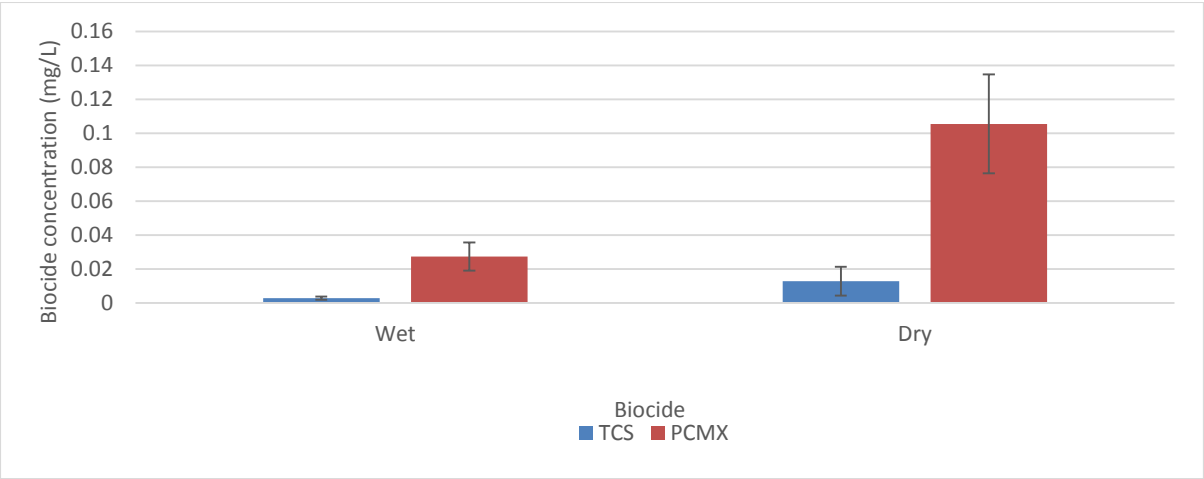

Figure 4.13: Concentration of TCS and PCMX measured in the WWTP influent during the wet and dry seasons

The levels of TCS and/or PCMX were determined by plotting the area obtained after HPLC onto the previously determined standard curves. Effluent, up- and downstream samples did not indicate the presence of either TCS or PCMX. As indicated by Figure 4.13, influent samples had concentrations of 0.00267 mg/L (End of wet season) and 0.01274 mg/L (End of dry season) for TCS and 0.0272 mg/L (End of wet season) and 0.10544 mg/L (End of dry season) for PCMX, respectively. At the end of the dry season, TCS and PCMX levels were much higher in comparison to the end of the wet season.

4.6 Pathogenic potential

The bacterial isolates obtained from the WWTP North-West Potchefstroom and the associated river system had various virulence characteristics. Thus, the outcomes of tests to determine antibiotic resistance profiles (Table 4.4) and extracellular enzyme production (Table 4.3) were combined to create a pathogenic potential index. This index was used to compare the level of potential risk posed by the isolates identified that are also known to be potential human pathogens (Horn *et al*., 2016).

Isolates exhibiting haemolysis were allocated scores of 1 for alpha and 1.2 for beta-haemolysis. Beta-haemolysis causes the complete lysis of red blood cells and is thus attributed a more significant score, as it may be regarded to have more severe effects on the host (Pakshir *et al.*, 2013). A score of 1 was allocated for a positive test for the presence of each extracellular enzyme. Scores were also assigned based on antibiotic resistance profiles: if an isolate tested resistant, a score of 1 was assigned, 0.5 if the isolate was inhibited and 0.2 for those considered to be not applicable (N/A), in which instance the organism is known to already be intrinsically resistant to the antibiotic tested.

Table 4.8: Pathogen score

	Virulence characteristics								Antibiotic resistance						Pathogen score
Isolates	**Haemolysis**	**Le**	**H**	**C**	**D**	**G**	**P**	**Li**	**V30**	**A10**	**K30**	**C30**	**T5**	**O30**	
Pathogen weight	β = 1.2; α = 1			X = 1					R = 1; I = 0.5, NA = 0.2						
A. caviae	β			X	X	X	X	X	NA	R	S	S	R	S	8,2
A. caviae	β	X		X	X	X	X	X	NA	R	S	S	I	S	8,9
A. veronii	β			X	X	X		X	NA	R	S	S	S	R	7,2
A. veronii	β	X		X	X	X	X		NA	R	S	S	S	R	8,4
A. veronii	β	X		X	X	X	X		NA	R	S	S	S	S	7,4
A. veronii	β	X		X	X	X	X	X	NA	R	S	S	I	S	8,9
A. veronii	β	X			X	X	X		NA	R	S	S	R	R	8,4
A. veronii	β			X	X	X	X		NA	R	S	S	S	S	6,4
A. veronii	β	X		X	X	X	X		NA	R	S	S	R	I	8,9
A. veronii	β	X		X	X	X	X	X	NA	R	S	S	I	S	8,9
A. veronii	β	X		X	X	X	X	X	NA	R	S	S	S	S	8,4
A. veronii	β	X		X	X	X	X		NA	R	S	S	R	S	8,4
A. veronii	β	X			X	X	X	X	NA	S	S	S	S	R	7,4
B. cereus	β	X		X		X			R	R	I	S	R	S	7,7
B. cereus	β	X			X	X	X		S	R	I	S	R	I	8
B. cereus	α	X		X	X				I	R	S	S	R	R	7,5
B. subtilis	β	X		X		X	X		S	S	S	S	S	S	5,2
K. oxytoca	β				X				NA	S	S	S	S	R	3,4
P. alcaligenes	β								NA	I	S	S	I	R	3,2
P. alcaligenes	β				X				NA	R	S	S	R	R	5,4
P. alcaligenes	α								NA	S	S	S	S	S	1,2
P. aeruginosa	β	X		X	X	X	X		NA	R	R	R	R	R	10,4
P. monteilii	β								NA	R	S	I	R	I	4,4

Lecithinase (Le), hyaluronidase (H), chondroitinase (C), DNase (D), gelatinase (G), proteinase (P) and lipase (Li). R = resistant, I = Intermediate resistance, S = susceptible, NA = Not Applicable

Table 4.8 represents the virulence scores assigned to each potentially pathogenic isolate based on haemolytic activity, antibiotic resistance profiles and the presence of extracellular enzymes. Scores for *Aeromonas* spp. ranged from 6.4-8.9, while scores for *Bacillus* spp. ranged from 5.2-8. *Pseudomonas* spp. had both the highest and lowest scores *P. aeruginosa* scored 10.4, *P. monteilii* scored 4.4 and scores for *P. alcaligenes* ranged from 1.2-5.4 while *K. oxytoca* had a relatively low score of 3.4.

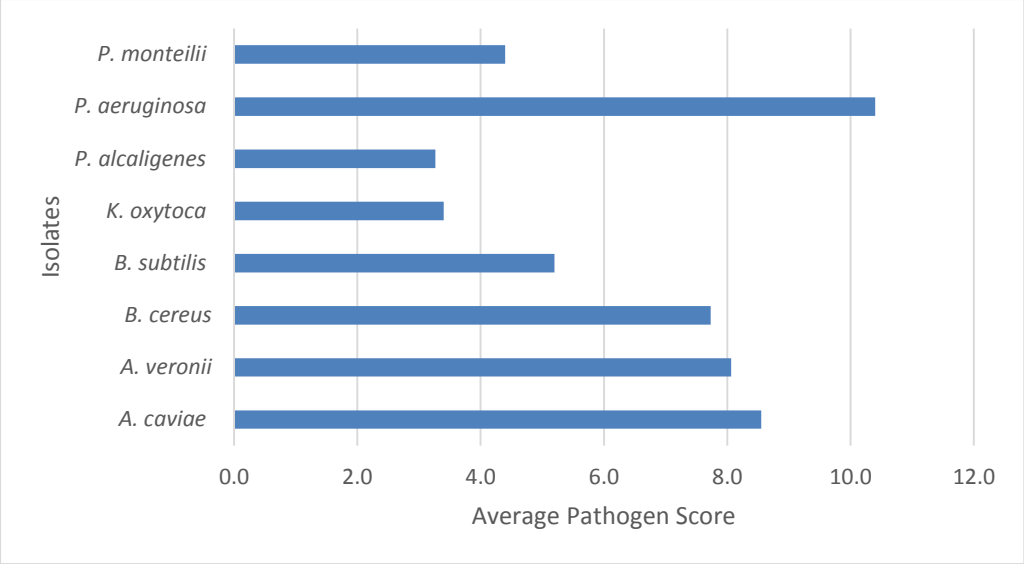

Figure 4.14: Pathogenic potential based on average pathogen scores

Figure 4.14 demonstrates the pathogenic potential of each species known to be a potential pathogen. The *P. aeruginosa* isolate scored the highest, followed by *A. veronii* and *A. cavea.* The next highest scores were among *Bacillus* spp. while the lowest scores were attributed to *P. monteilii, K. oxytoca* and *P. alcaligenes.*

4.7 Summary of results

In total, 5 samples were taken. A pre- and post-chlorination effluent water sample was taken from the WWTP in Potchefstroom, North-West, South Africa, after which 3 more samples were taken, one from the effluent after it had passed through the wetlands before entering the Mooi River, one upstream sample, and one downstream sample. Heterotrophic plate count bacteria were grown in the presence of varying concentrations of triclosan or chloroxylenol. In total 37 isolates tested positive for haemolysis and underwent identification. Six genera were identified as *Bacillus, Pseudomonas, Aeromonas, Exiguobacterium*, *Klebsiella* and *Shewanella* spp. Of the isolates identified, *Bacillus, Pseudomonas, Klebsiella* and *Aeromonas* spp. are known to be opportunistic pathogens. Isolates were tested for the presence of extracellular enzymes- 60% tested positive for the presence of Lecithinase (Le), 22.9% for the presence of Hyaluronidase (H), 62% for the presence of Chondroitinase (C), 74.3% for the presence of DNase (D), 71.4% for the presence of Gelatinase (G), 45.7% for

the presence of Proteinase (P) and 42.9% tested positive for the presence of Lipase (Li).

To determine cross-resistance to antibiotics the Kirby-Bauer disc diffusion method was used. Among gram positive isolates 23.1% were resistant to Vancomycin (30 µg), among all isolates 77.1% were resistant to Amoxicillin (10 µg), 11.4% were resistant to Kanamycin (30 µg), 2.9 % were resistant to Chloramphenicol (30 µg), 57.1% were resistant to Trimethoprim (5 µg) and 45.7% were resistant to Oxytetracycline (30 µg). In total, 14 isolates were selected for MICs. All of the isolates incubated with TCS remained in lag phase over a 24-hour time period. It can thus be assumed that all isolates have an MIC value above 2 mg/L TCS. However further investigation is required to obtain a precise value. Of the isolates incubated with PCMX, the majority indicated no change in optical density over 24 hours when exposed to 50, 75 and 100 mg/L of PCMX. This indicates the isolates remained in lag phase and thus the MIC for these isolates are regarded as being more than 30 mg/L. *Pseudomonas monteilii* indicated no change from 75 mg/L and upwards, thus the MIC for this isolate is regarded as being more than 50 mg/L. Six isolates were selected for the checkerboard assay; all showing various degrees of synergy and/or antagonism to certain antibiotics. Tetracycline showed the highest prevalence and variation of effects in combination with the biocides, while amoxicillin showed the least.

Antibiotic resistance profiles and extracellular enzyme production was used to generate a pathogenic potential index to represent the virulence potential of the Isolates obtained, *P. aeruginosa* scored the highest and *P. alcaligenes* scored the lowest. HPLC was used to determine the presence and quantity of TCS and/or PCMX at various sampling points. Effluent, up- and downstream samples did not indicate the presence of either TCS or PCMX, but both were present in the influent samples, which had concentrations of 0.00267 mg/L (End of wet season) and 0.01274 mg/L (End of dry season) for TCS and 0.0272 mg/L (End of wet season) and 0.10544 mg/L (End of dry season) for PCMX, respectively.

CHAPTER 5 – DISCUSSION

5.1 Screening and isolation

TCS and PCMX tolerant heterotrophic plate count bacteria was found in the pre- and post-chlorination samples of the wastewater treatment plant, as well as in samples taken at up- and downstream sites and the wetland outflow. Isolates managed to grow in the presence of TCS up to a concentration of 1 mg/L, and in the presence of PCMX up to a concentration of 40 mg/L. To screen for potential pathogens isolates were spot inoculated on blood agar, as haemolysis is considered to be a commonly found virulence factor (Ahmad *et al.,* 2010; Willey *et al.,* 2011). Initially, eighty-seven isolates were obtained from nutrient agar plates supplemented with various concentrations of TCS and PCMX. Of these isolates, thirty-seven tested positive for haemolysis.

5.2 Molecular Identification and DNA fingerprinting

5.2.1 16S identification of potential pathogens

Of the 35 isolates identified by 16S sequencing, *Pseudomonas*, *Aeromonas, Bacillus* and *Klebsiella* spp. are well described as opportunistic pathogens. Thus, the discussion focuses primarily on the results related to these species. *Pseudomonas* spp. were identified at pre- and post-chlorination and in the upstream sampling points, and the one *Klebsiella* sp. was found at the pre-chlorination sampling point. *Aeromonas* and *Bacillus* spp. on the other hand were found at the pre, post, wetlands, as well as the downstream sampling points that may be an indication that the wastewater treatment plant is a possible source of these species entering the environment.

5.2.1.1 *Pseudomonas* spp.

Pseudomonas is a genus of Gram-negative, Gammaproteobacteria, that belong to the family Pseudomonadaceae. *Pseudomonas* demonstrates a great range of metabolic diversity and can thus colonize a wide range of niches. There is a large number of

Pseudomonas species, some of which are capable of opportunistic infections. Some of the lesser known species include *Pseudomonas monteleii, Pseudomonas alcaligenes, and Pseudomonas taiwanensis* (Ahmad *et al.,* 2010).

The clinical significance of *P. monteilii* is still unknown, but the bacterium has been isolated from clinical specimens. The current hypothesis is that *P. monteilii* is a rare opportunistic pathogen (Elomari *et al.,* 1997). *P. alcaligenes* has been studied for bioremediation purposes of certain chemical substances, as it can degrade polycyclic aromatic hydrocarbons (Yi *et al.,* 2015; O'Mahony *et al*., 2006). It may be a human pathogen, but their occurrence as such is said to be extremely rare. To date, only two fatal cases of *P. alcaligenes* infection has been documented: namely a case of endocarditis and a case of blood stream infection (Suzuki *et al.,* 2013; Valenstein *et al.,* 1983). There is no evidence to suggest that *P. taiwanensis* may be a human pathogen but it has been studied extensively for its insecticidal activity towards certain agricultural pests, due to the activity of the TccC protein (Chen, 2014b).

Pseudomonas aeruginosa is considered to be the most important species as it is difficult to frequently attach pathogenic significance to any of the other *Pseudomonas* species. *P. aeruginosa* exhibits the most consistent resistance to antimicrobials of all medically important bacteria. This species is extremely versatile and can survive and thrive over a wide range of temperatures and in almost any environment and has also been shown to be frequently present in recreational waters (Lister, 2009; Ahmad *et al.,* 2010; Guida *et al*., 2016). The clinical manifestations of the infections depend on the organ system involved and once established, infections are extremely virulent and difficult to treat. It is also common that the majority of infected patients usually have some form of compromised immunity. Infections have resulted from growth of *P. aeruginosa* in contact lens solutions, medication, and even disinfectants (Lister, 2009; Ahmad *et al.,* 2010).

Although considered to be an opportunistic pathogen, *P. aeruginosa* possesses several virulence factors, making this organism particularly virulent. Most *P. aeruginosa* strains produce exotoxins, as well as endotoxins, considered to be a main virulence factor in septic shock and bacteraemia. Infection following surgery or burns may result in fatal bacteraemia and the introduction of *P. aeruginosa* on catheters can

lead to urinary tract infections. *P. aeruginosa* has also been found to be a frequent coloniser of patients with cystic fibrosis. Necrotizing *P. aeruginosa* pneumonia may ensue in patients after the use of respirators that have been contaminated. Occasionally, *P. aeruginosa* has been identified as the causative agent of meningitis following lumbar puncture and endocarditis after cardiac surgery, and has also been associated with some diarrheal disease episodes and skin infections (Iglewski, 1996; Ahmad *et al.,* 2010; Guida *et al*., 2016).

5.2.1.2 *Bacillus* spp.

Bacillus is a genus of Gram-positive, rod-shaped bacteria belonging to the family Bacillaceae. *Bacillus* spp. are widespread and occupy several niches including air, soil, water, and animal by-products. Endospores associated with *Bacillus* spp. aid with their survival contributes to resistance to disinfection (Ahmad *et al.,* 2010). The ability of *Bacillus* spp. to form biofilms, allowing them to stay attached to biomedical devices, has also previously been described as problematic (Veysseyre *et al.*, 2015). Both *Bacillus cereus* and *B*. *subtilis,* are known to produce infection especially in immunocompromised individuals, with *B. cereus* being said to be the most likely to cause opportunistic infection (Ahmad *et al.,* 2010; Jeon *et al*., 2012). *B. cereus* pathogenicity is said to be associated with tissue destructive extracellular enzymes and the organism is particularly notorious for its association with food poisoning and eye infections. Furthermore, it has also been a causative agent in several other clinical conditions such as bacteraemia, wound infections, pneumonia, meningitis and endocarditis (Bottone, 2010; Veysseyre *et al.*, 2015). *Bacillus subtilis* is not well known as a potential pathogen, but the organism has been identified in clinical samples taken from deep and superficial body sites (Celandroni *et al*., 2016). One rare and unusual case has previously been reported by Jeon *et al.* (2012) in which bacteraemia and mediastinitis was reported due to a co-infection involving *Bacillus subtilis* and *Bacillus licheniformis* in a patient with oesophageal perforation.

5.2.1.3 *Klebsiella* spp.

Klebsiella is a genus of Gram-negative, nonmotile, rod shaped bacteria with a polysaccharide-based capsule. *Klebsiella* forms part of the family Enterobacteriaceae,

many of which survive readily in nature. These organisms are commonly found in nature and are often present in surface waters used for recreational purposes (Duncan, 1998; Ahmad *et al.,* 2010). According to Hagiwara *et al.* (2013), *Klebsiella* are opportunistic pathogens involved in a variety of diseases such as bacteraemia. *K. oxytoca* has been isolated to a much lesser degree than various other *Klebsiella* spp. from human clinical specimens, but there have been several cases of infection where *K. oxytoca* has been identified as the causative agent (Hagiwara *et al.,* 2013). A study by Zárate *et al.* (2008), described an outbreak in a renal transplant unit, while another study by Watson *et al.* (2005) has described the occurrence of catheter-associated bloodstream infections in a chemotherapy centre. An outbreak of multiple resistant *K. oxytoca* has also previously been documented (Decré *et al.,* 2004).

5.2.1.4 *Aeromonas* spp.

Aeromonas is a genus of Gram-negative, Gammaproteobacteria that belong to the family Aeromonadaceae. These organisms are an uncommon, but highly virulent cause of wound infections acquired in fresh or salt water. Onset can be extremely rapid and original cellulitis may progress to bacteraemia, myonecrosis or fasciitis in less than a day. *Aeromonas* is also the most frequent cause of infections associated with the use of leeches. Resistance to penicillins and cephalosporins are not unusual and most strains show susceptibility to Tetracycline with variable susceptibility to aminoglycosides. Also considered to be an opportunistic pathogen, most of the described *Aeromonas* species have been associated with human disease; two of the most important pathogens include *A. caviae*, and *A. veronii* (Graf, 2015; Ahmad *et al.,* 2010).

Human infections caused by *Aeromonas* most often occur in community settings but can occur in healthcare settings. Characteristically, susceptible individuals acquire the infection from oral consumption of, or direct contact with contaminated water or seafood (Igbinosa *et al.*, 2012). In humans, *A. veronii* can cause diseases ranging from wound infections and diarrhoea to septicaemia in immunocompromised patients. *Aeromonas* septicaemia has been documented and *A. caviae* and *A. veronii* account for a large number of blood borne infections (Janda & Abbot, 2010). *A. caviae* and *A. veronii* have also been implicated as a cause of traveller's diarrhoea that is one of the

main infectious diseases reported in persons traveling abroad (Vila *et al.,* 2003). The majority of clinical infections are associated with species *A. hydrophila*, *A. veronii*, *A. caviae*, and *A. dhakensis.* These species have been associated with gastrointestinal infections, biliary tract infections, soft tissue infections, pneumonia and certain specific infections such as endocarditis, meningitis and eye infections (Chen, 2014c; Figueras, 2009).

5.2.2 DNA fingerprinting (ERIC-PCR)

As previously stated a large portion of South Africa's population still rely on surface water as their primary source of water, and many still utilize untreated water (Zamxaka *et al.*, 2004; Momba *et al.,* 2006). Several studies have indicated the possibility that *Aeromonas* and *Bacillus* spp. may be the cause of infection in humans (Graf, 2015; Bottone, 2010). It was thus important during the course of this study to determine if the WWTP North-West Potchefstroom is the source of the potentially pathogenic bacterial isolates obtained at the pre- and post-chlorination sampling points as well as the wetlands and downstream sampling points. DNA fingerprinting by means of ERIC-PCR has been described as an effective method to determine clonal relationship and genetic similarity between isolates of the same species and has been previously used to type both *Aeromonas* and *Bacillus* spp. (Shangkuan *et al.*, 2000; Szczuka & Kaznowski, 2004; Katara *et al*., 2012).

B. toyonensis is not known to be pathogenic and is generally used as part of an animal feed additive (Casanovas-Massana *et al.,* 2014). *B. toyonensis* was included in the fingerprinting analysis as it may still act as a good indicator of the WWTP being the source off bacterial dissemination into the receiving river waters, since it was found at several sampling points. Based on visual and clustering analysis of the final ERIC-PCR gel product it is clear that the isolates found during the course of this study do not share any clonal relation, but there is a level of genetic homology present between isolates found at the pre- and post-chlorination sampling points as well as at the wetlands and downstream sampling points. The capability of aquatic bacteria to participate in horizontal gene transfer and the occurrence of mutation must also be considered. The visual and clustering analysis performed on selected isolates may

indicate various strains but that does not rule out the possibility that isolates of the same species may share common ancestry (Bryant *et al.,* 2012; Berglund, 2015). Although the visual and clustering analysis did not yield any conclusive results, the fact that similar species were found at pre- and post-chlorination sampling points as well as downstream from the WWTP, indicated that the possibility remains that the WWTP is the source from which these organisms enter the environment.

Several studies have previously indicated that many organisms harbouring antibiotic resistance genes (ARGs) are constantly released with wastewater into the aquatic environment. These genes inserted into mobile genetic elements (integrons, transposons and plasmids) are able to readily disseminate among aquatic bacterial communities (Baquero *et al.,* 2008; Bouki *et al.,* 2013) A previous study by Goñi-Urizza *et al.* (2000) has found that resistance was significantly increased among bacteria (*Enterobacteriacea* and *Aeromonas* spp.) collected downstream of a wastewater discharge point. As mentioned current results cannot confirm nor deny the WWTP North-West Potchefstroom as the source of the isolates obtained during this study, but these isolates did demonstrate various levels of antimicrobial resistance (*Aeromonas* and *Bacillus* spp.); thus the WWTP may still potentially be a source of ARG's entering the aquatic environment.

5.3 Extracellular enzyme production

As previously stated microorganisms synthesise and secrete exotoxins, some of which are enzymes, into their environment. Exotoxins released into the environment are toxic to their hosts, and thus testing for the presence of extracellular enzymes was done to contribute to determine potential pathogenicity (Pavlov *et al*., 2004; Georgescu *et al*., 2016). All *Aeromonas* isolates tested positive for the presence of at least 5 or more extracellular enzymes. *Aeromonas* spp. have been described to produce a broad range of extracellular enzymes, some of which may contribute to pathogenicity. Despite the presence of several virulence factors the virulence mechanisms of *Aeromonas* spp. still remain vague (Igbinosa *et al.,* 2012). All *Bacillus cereus* isolates tested positive for the presence of 3 or more extracellular enzymes, while the *B. subtilis* isolate tested positive for 4. The pathogenicity of *B. cereus* is said to be intimately

related to the production of tissue-destructive extracellular enzymes, such as proteases, phospholipases and haemolysins (Bottone, 2010).

Pseudomonas spp. tested positive for the presence of both the most (*P. aeruginosa*) and least (*P. alcaligenes*) amount of extracellular enzymes. Pathogenicity of *Pseudomonas* spp. has previously been stated to be dependent on the secretion of various virulence factors such as proteases and elastases, especially during the early stages of infection, and as with *Aeromonas* spp. the specific role of these virulence factors is still incompletely understood (van 't Wout *et al.,* 2015). The 1 *Klebsiella* sp. found tested positive for only the presence of 1 extracellular enzyme. According to Pereira & Vanetti (2015), the primary factors contributing to *Klebsiella* spp. pathogenicity include the production of siderophores, fimbrial adhesins, serum resistance properties, and particular capsular types. Other factors such as haemolysins and phospholipase production have also been said to play a role as intensifiers of virulence (Podschun *et al.,* 2001; Pereira & Vanetti, 2015).

Results for the isolates obtained indicate the presence of several extracellular enzymes that may all potentially contribute to virulence (Pavlov *et al*., 2004; Georgescu *et al*., 2016). The presence of extracellular enzymes was factored into a pathogenic potential index along with antibiotic resistance profiles to assess the pathogenic potential of each isolate that will be discussed later in Section 5.6 (Horn *et al*., 2016).

5.4 Antimicrobial susceptibility

5.4.1 Antibiotic resistance profiles

Bacteria may repel antibiotic action by means of various mechanisms. Some bacterial species exhibit intrinsic resistance to certain antibiotics, but the ongoing concern is the bacteria that become resistant to antibiotics, and consequently distribute under the selective pressure of use of these antibiotics. Numerous mechanisms of resistance to antibiotics are freely spread to a range of bacteria genera. Several bacteria have also become resistant to multiple classes of antibiotics. Resistance is not necessarily limited to a single antibiotic, but can apply to several unrelated compounds, and the

emergence of multiple drug resistant bacteria is a major concern (Scientific Committee on Emerging and Newly Identified Health Risks, 2009).

Of the five *Pseudomonas* isolates obtained all showed resistance or intermediate resistance to Amoxicillin, Trimethoprim and Oxytetracycline. Three isolates showed resistance or intermediate resistance to Chloramphenicol and only one viz. *P. aeruginosa* showed resistance to Kanamycin. The *P. monteleii* isolate obtained showed resistance or intermediate resistance to all antibiotics tested except Kanamycin. According to Bogaerts *et al.* (2011), a multidrug-resistant strain of *P. monteleii* has previously been isolated and after antibiotic susceptibility testing by agar disc diffusion according to CLSI guidelines, it was revealed that the isolate only remained susceptible to Colistin and intermediately susceptible to Aztreonam. Resistance in this case was attributed to the presence of a $bla_{\text{IMP-13}}$ gene, encoding for IMP-13 that is a class B metallo-β-lactamase. Resistance to various antibiotics exhibited by *P.* monteilii has also previously been attributed to the presence of a $bla_{\text{VIM-2}}$ gene encoding for VIM-2 β-lactamase (Ocampo-Sosa *et al*., 2015).

The *P. aeruginosa* isolate showed resistance to all antibiotics tested and it has been shown that *P. aeruginosa* strains tend to be multidrug-resistant and resistance to Amoxicillin, Tetracycline, Chloramphenicol, Trimethoprim and aminoglycosides has been well documented (Kohler *et al.,* 1996; Ahmad *et al.,* 2010). Multidrug-resistance among *P. aeruginosa* strains has previously been attributed to Mex drug efflux pumps, AmpC beta-lactamase and the porin OprD (Dumas *et al*., 2006). Efflux systems have been said to contribute significantly to multidrug-resistance among *P. aeruginosa* isolates; several efflux systems of the resistance nodulation division (RND) family have been well categorized (Henrichfreise *et al.*, 2007). According to Livermore (2002) *P. aeruginosa* is also known to carry plasmids that also contribute to multidrug-resistance.

It has been documented that *P. alcaligenes* has shown resistance to broad-spectrum cephalosporins and monobactams (Suzuki *et al.*, 2013). During a clinical case it has also been found that *P. alcaligenes* shows resistance to ß-lactam/ß-lactamase inhibitor combinations (Aztreonam, and Gentamicin) but it remained susceptible to carbapenems, fluoroquinolones, Trimethoprim and Colistin (Flores-Carrero *et al*.,

2016). According to Arslan *et al.* (2011) several *Pseudomonas* spp. show resistance to Trimethoprim but there is no direct reference to *P. alcaligenes.* The *P. alcaligenes* isolate obtained showed resistance to Amoxicillin, and intermediate resistance to both Trimethoprim and Oxytetracycline. *P. aeruginosa* has been well studied, and resistance to several antibiotics is not uncommon; *P. monteleii* and *P. alcaligenes* have not been as extensively studied as *P. aeruginosa* but resistance patterns observed during this study have previously been documented (Ahmad *et al.,* 2010; Bogaerts *et al.,* 2011).

The *P. alcaligenes* isolate obtained during the course of this study exhibited resistance to Trimethoprim, and according to Arslan *et al*. (2011) *Pseudomonas* species tend to exhibit resistance to Trimethoprim but there is no direct reference to *P. alcaligenes* exhibiting resistance. *B. cereus* isolates showed resistance to Amoxicillin and Trimethoprim, and varying levels of resistance to Vancomycin, Kanamycin and Oxytetracycline, while the *B. subtilis* isolate found showed resistance to none of the antibiotics tested. *B. cereus* is known to express marked resistance to penicillin and other beta-lactam antibiotics (Bottone, 2010; Malik-Tabassum *et al.,* 2017). Resistance of *B. cereus* to several other antibiotics such as cephalosporins, Trimethoprim, Chloramphenicol, Vancomycin, aminoglycosides and Tetracycline has also previously been reported (Turnbull *et al.,* 2004). Efflux mechanisms have also been said to be present in certain *Bacillus* spp. and may thus also contribute to multiple drug resistance (Li & Nikaido, 2009). The *K. oxytoca* isolate found during this study exhibited resistance only to Oxytetracycline. It has previously been documented that the majority of *K. oxytoca* strains produce K1 extended spectrum beta-lactamase (Arakawa *et al.,* 1989). According to Fenosa *et al.* (2009) *K. oxytoca* is often resistant to multiple antibiotics including Tetracycline, and goes on to state that these effects are most likely due to efflux mechanisms such as the AcrAB efflux mechanisms and a Tolc-like protein. *K. oxytoca* isolates have also been documented to harbour plasmids carrying ARG's (Shaikh *et al*., 2015).

All *Aeromonas* isolates except one showed resistance to Amoxicillin, and varying degrees of resistance could be seen for Oxytetracycline and Trimethoprim. According to Ahmad *et al.* (2010), resistance to penicillins and cephalosporins is not unusual and most strains show susceptibility to Tetracycline with variable susceptibility to

aminoglycosides. Trimethoprim resistance has been documented among *Aeromonas* spp. due to the action of cassette borne resistance genes such as drfA and drfB located in class 1 integrons (Kadlec *et al.*, 2011). Multidrug non-susceptible patterns among *Aeromonas* spp. including *A. veronii* and *A. caviae* are not uncommon and studies have indicated varying resistance patterns to several antibiotics (Goñi-Urizza *et al*., 2000; Aravena–Roman *et al*., 2012). A study by Odeyemi & Ahmad (2017) also found that *Aeromonas* spp. exhibit resistance to multiple antibiotics including Kanamycin, Oxytetracycline, Trimethoprim and Chloramphenicol. Efflux mechanisms responsible for resistance have also been documented in *Aeromonas* spp. and may also contribute to resistance to multiple antibiotics. Resistance profiles in general seem to depend largely on the particular aquatic environment but it is evident that *Aeromonas* spp. may exhibit resistance to multiple antibiotics. Resistance may also be conferred by plasmids carrying a number of different determinants contributing to antimicrobial resistance (Piotrowska & Popowska, 2015).

Overall the majority of potentially pathogenic isolates found during this study all show resistance to multiple antibiotics, the results of which were factored into a pathogenic potential index along with extracellular enzyme production to determine the pathogenic potential and health risks associated with these isolates, which will be discussed later in Section 5.6 (Horn *et al*., 2016).

5.4.2 Minimum inhibitory concentration of obtained *Pseudomonas, Aeromonas* and *Bacillus* spp. isolates

The minimum inhibitory concentrations of the organisms indicate the ability to survive in the presence of varying concentrations of the biocide. As previously stated biocide concentration is said to be one of the most important factors regarding its effectiveness, and several reports on the emergence of biocide resistance are based on the determination of MICs. Bacteria that exhibit tolerance to a biocide might be selected by a low concentration of said biocide. The level of resistance can increase through selection by means of recurring exposure to a low concentration or increasing concentration of a biocide, therefore the correct use and thus the applied concentration of antimicrobials is very important as incorrect concentration use may result in antimicrobial resistance (Scientific Committee on Emerging and Newly Identified

Health Risks, 2009). The importance and effectiveness of measuring lag time variations is made evident in a study by Li *et al.* (2016), where the effects of antimicrobials on the lag phase of bacterial communities were studied.

Several studies have indicated the occurrence of TCS resistance in microorganisms including some of clinical concern, leading to the concern that TCS resistance may contribute to a decrease in susceptibility of clinically important antimicrobials possibly due to co-resistance or cross-resistance mechanisms. TCS is said to be effective against the most susceptible organisms at a range of 0.1 to 2 mg/L and has been used as an antimicrobial against *Staphylococcus aureus* and is recommended to eliminate methicillin resistant *S. aureus*, but methicillin resistant *S. aureus* isolates with MIC values of up to 4 mg/L have been documented (Tuffnell *et al*., 1987; Brenwald & Fraise, 2003; Yazdankhan *et al*., 2006). *P. aeruginosa* is described to be highly resistant and MICs to TCS have been observed at being larger than 1000 mg/L (Chuanchuen, 2001). A study by Lear *et al.* (2006), also indicated that some bacterial strains from industrial sources had tolerance levels to TCS larger than 100 mg/L, while PCMX isolates showed tolerance to PCMX levels larger than 500 mg/L. PCMX tolerance found during the study may thus not be very high in comparison to this study but the occurrence of tolerance should still remain a concern.

The enoyl-acyl carrier protein reductase enzyme is the target for a series of antimicrobials including TCS, thus mutations of the fabI gene and its variants can be seen as one of the primary foundations of resistance to TCS (Yu *et al.,* 2010; Grandgirard *et al.*, 2015). Another mechanism by which organisms may gain resistance to antimicrobials is by means of efflux mechanisms. These efflux mechanisms may confer resistance and cross-resistance to several types of antimicrobials (Li & Nikaido, 2009). Efflux pumps have previously been identified in several studies to play a role in TCS resistance and in some cases antibiotic cross-resistance (Chuanchuen *et al.,* 2001; Levy, 2002; Schweizer, 2003). PCMX has not been as extensively studied as TCS as far as resistance to the biocide is concerned, but there is the potential that as with many biocides, PCMX resistance may also be due to efflux mechanisms or mutations altering the target of the biocide. *Aeromonas, Pseudomonas, Bacillus* and *Klebsiella* spp. found during this study have all previously been described as possessing efflux mechanisms. AcrAB homologues (*K. oxytoca*),

RND transporters such as AheB (*Aeromonas* spp.), MexAB-OprM (*P. aeruginosa*) and Bmr3 (*B. subtilis*) are but a few examples of efflux mechanisms involved in drug or multidrug efflux (Li & Nikaido, 2009).

Current results indicate that the MIC for PCMX averages are between 30 and 50 mg/L, for the majority of isolates, except for the *P. monteilii* isolate for which the MIC lies between 50 and 75 mg/L. All isolates showed MIC values for TCS of more than 2 mg/L of TCS, thus more study is required to determine exact TCS MICs. Results also indicated that certain concentrations induced an increased level of resistance to either TCS or PCMX. This observation as seen among *Aeromonas, Pseudomonas* and *Bacillus* isolates may be due to the fact that exposure to certain antimicrobials may trigger changes in gene expression (Haaber *et al*., 2015). As previously stated the antimicrobial resistance observed among the isolates in this study may be due to efflux mechanisms and it may be possible that certain levels of these antimicrobials trigger expression or overexpression of these efflux mechanisms (Schweizer, 2003; Khanam *et al*., 2017; Yılmaz & Özcengiz, 2017).

5.4.3 Checkerboard Assay

As previously stated the use of antimicrobials in combination may lead to synergistic and/or antagonistic effects. Interactions are said to be synergistic if the joint effect on cells by the antimicrobial combination is stronger than either antimicrobial by itself, and antagonistic if the joint effect is weaker (Bollenbach, 2015). The checkerboard method has previously been used during several studies for the purpose of examining synergistic and antagonistic effects between antimicrobials (Sopirala *et al.,* 2010; Spoorthi *et al.,* 2011).

5.4.3.1 TCS

Antagonism was observed during interaction between TCS, Kanamycin and Amoxicillin, which may be attributed to the fact that bactericidal antimicrobials generally require the occurrence of cell growth which is prevented by bacteriostatic agents; in this case TCS, two instances of synergy were observed at lower

concentrations of TCS that may also be attributed to the previously stated fact. At higher concentrations the growth of certain cells may be inhibited to a point where the bactericidal agents are no longer effective (antagonism), but at low concentrations growth is only slightly inhibited contributing to the bactericidal action (synergy) (Ocampo *et al*., 2014; Bollenbach, 2015). This occurrence may also be due to efflux mechanisms, which may be triggered at varying concentrations of the antimicrobials (Schweizer, 2003; Li & Nikaido, 2009). Antagonism was also observed between Chloramphenicol and TCS, which may be due to the presence of efflux mechanisms or changes in gene expression owing to simultaneous exposure to both antimicrobials (Schweizer, 2003; Li & Nikaido, 2009; Haaber *et al.* 2015).

Other than Kanamycin, synergy was also observed between TCS alongside Tetracycline, Trimethoprim, Chloramphenicol and Amoxicillin. Amoxicillin and TCS both inhibit cell wall synthesis, thus synergy may occur due to the fact that both antimicrobials share a similar target (Bollenbach, 2015). Tetracycline and Chloramphenicol are both protein synthesis inhibitors while Trimethoprim blocks folic acid synthesis; all three these antibiotics as well as TCS are described as bacteriostatic antimicrobials and thus synergy may be attributed to a joint effect on the inhibition of bacterial cell growth. It is also possible that resistance to bacteriostatic agents may be attributed to a similar mechanism and the combination of two such santimicrobials may overwhelm the cells' defences (Tabak *et al*., 2009; Bollenbach, 2015).

5.4.3.2 PCMX

Antagonism observed during interactions between PCMX, Tetracycline, Trimethoprim and Chloramphenicol; this can be attributed to the fact that both Trimethoprim and Chloramphenicol are primarily bacteriostatic agents while PCMX is a bactericidal agent. As previously stated, at higher antimicrobial concentrations the growth of certain cells may be inhibited to a point where the bactericidal agents are no longer effective (Ocampo *et al*., 2014; Bollenbach, 2015). Antagonistic effects may also be present due to the presence of efflux mechanisms or changes in gene expression owing to simultaneous exposure to both antimicrobials (Schweizer, 2001; Li & Nikaido, 2009; Haaber *et al.* 2015).

Synergy was observed between PCMX, Kanamycin, Chloramphenicol, Tetracycline and Amoxicillin. Kanamycin and Amoxicillin are both bactericidal agents the same as PCMX, thus synergy may be attributed to a joint bactericidal activity (Tabak *et al*., 2009; Bollenbach, 2015). According to Bollenbach (2015) resistance to antimicrobials may be due to similar mechanisms; thus synergy observed between PCMX, chloramphenicol and tetracycline may be due to the fact that in these cases a similar resistance mechanism is shared that becomes overwhelmed when exposed to both antimicrobials simultaneously. PCMX is known to cause cell membrane damage, thus increasing permeability for protein synthesis inhibitors such as Kanamycin, Chloramphenicol and Tetracycline, which may also lead to the observed synergistic effects (Tabak *et al*., 2009; Bollenbach, 2015).

Results obtained while determining MICs indicated that certain concentrations of antimicrobials induced an increased level of resistance; these observations may also contribute to the antagonisms seen between TCS and PCMX, and various antibiotics. Efflux mechanisms are known to confer resistance to various antimicrobials, for example the RND (Resistance-Nodulation-Division), SMR (Small multidrug resistance) and MFS (Major facilitator superfamily) efflux pump mechanisms are all known to confer resistance to biocides and several of the antibiotics used during the course of this study (Yılmaz & Özcengiz, 2017; Slipski et al., 2017). According to Yılmaz & Özcengiz (2017) the above-mentioned efflux systems confer resistance to tetracyclines, Trimethoprim, Chloramphenicol, β-lactams and aminoglycosides, while several studies have also demonstrated the role RND and MFS efflux mechanisms play in resistance to biocides such as TCS (Chuanchuen *et al*., 2002; Srinivasan *et al*., 2014). Thus, current results obtained during the determination of antibiotic resistance profiles, MICs and synergistic and antagonistic interactions all indicate the possibility that efflux mechanisms may be the primary means of resistance to both antibiotics and the antimicrobials in question (Schweizer, 2003; Khanam et al., 2017; Yılmaz & Özcengiz, 2017).

5.5 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) was used to determine the presence and quantity of TCS and/or PCMX present in the WWTP of the North-West Potchefstroom, and at up and down river sampling points. TCS is used in several personal care products, the majority of which end up in municipal wastewater, and has previously been shown to be present in raw sewage, effluents from WWTP's and their receiving river systems (Dhillon *et al.,* 2015; Huang *et al.,* 2016). PCMX is also an active ingredient of many personal care products, and has been found in river systems, at high concentrations in raw sewage as well as in WWTP effluent (Kasprzyk-Hordern *et al.,* 2009; Capkin *et al*., 2017). A wide range of antimicrobial concentrations may be released into the environment, but this is highly dependent on the operation of the WWTP (Yueh & Tukey, 2016; Kasprzyk-Hordern *et al.,* 2009; Daughton & Ternes, 1999).

Based on current methods there was no indication of the presence of TCS or PCMX at up or down stream sampling points or the effluent site of the WWTP, but both TCS and PCMX were present at the influent site of the WWTP. Levels measured indicated TCS concentrations in the influent at the wastewater treatment plant up to 0.01274 mg/L, and PCMX concentrations of up to 0.10544 mg/L (Figure 4.14). Biocide concentrations found in influent samples taken at the end of the dry season were higher than those taken at the end of the wet season. During the wet season rainfall may dilute personal care products. The higher temperatures occurring during the wet season may also play an important role in improved removal of personal care products and thus lower concentrations in the influent may be due to improved rates of biodegradation (Sui *et al.,* 2011).

TCS and PCMX have both previously been described as being extensively biodegraded and removed during the wastewater treatment process (Food and Drug Administration, 2014; Federle *et al.,* 2002). Current results suggest that TCS and PCMX are effectively removed by the WWTP North-West Potchefstroom. As previously stated, antimicrobial concentrations may be released into the environment, but this is highly dependent on the operation of the WWTP (Yueh & Tukey, 2016; Kasprzyk-Hordern *et al.,* 2009; Daughton & Ternes, 1999). Due to the presence of

these antimicrobials in the influent of the WWTP it should thus still remain a concern that these antimicrobials may enter the aquatic environment at other points with less efficient WWTP's.

The presence of these biocides in the WWTP influent means bacteria entering the WWTP are still exposed to these antimicrobials during the process of entering the WWTP, and possibly during certain stages of the wastewater treatment process. As previously indicated bacteria that exhibit tolerance to a biocide might be selected by means of recurring exposure to a biocide; there is also the fear that biocide resistance could lead to possible cross-resistance to antibiotics (Chuanchuen *et al*., 2001; Poole, 2002; Russel, 2002).

5.6 Pathogenic Potential

The isolates obtained during the course of this study had various characteristics that may contribute to their pathogenic potential. Thus the results of the antibiotic resistance tests and tests for extracellular enzyme production were used to generate a pathogenic potential index. The goal of this index was to determine and compare the degree to which these organisms may potentially cause disease. *Pseudomonas*, *Aeromonas, Bacillus* and *Klebsiella* spp. are well described as opportunistic pathogens capable of causing various diseases, and each isolate has its own set of virulence characteristics (Table 4.8). Figure 4.14 demonstrates the pathogenic potential of each isolate known to be a potential pathogen. The *P. aeruginosa* isolate scored the highest, followed by *A. veronii* and *A. cavea.* The next highest scores were among *Bacillus* spp. while the lowest scores were attributed to *P. monteilii, K. oxytoca* and *P. alcaligenes.*

As previously stated microorganisms are known to synthesise and secrete exotoxins, some of which are enzymes, into their environment, that play a role in pathogenicity. The presence of extracellular enzymes thus contributes to the pathogenic potential of a bacterial isolates (Pavlov *et al*., 2004; Georgescu *et al*., 2016). Antibiotic resistance in itself is not considered to be a virulence factor, but it may still perform an important role during the development of infection, contributing to the overall virulence of a pathogen (Beceiro *et al.,* 2013). The majority of isolates found during the course of

this study exhibited resistance to several antibiotics (Table 4.8) and various levels of resistance to the antimicrobials examined during this study (Table 4.5 and 4.6).

The majority of South Africa's population still rely on surface water as their primary source of water, and many still utilize untreated water. Unsafe water leads to an increased risk of infection due to water borne pathogens; this along with the HIV epidemic poses a serious health risk particularly from opportunistic pathogens (Zamxaka *et al.*, 2004; Momba *et al.,* 2006). These facts along with the results obtained showing the potential for virulence among the isolates, indicate that these organisms may pose a serious health risk (Igbinosa *et al*., 2012; Horn *et al*., 2016; Vouga & Greub, 2016).

5.6 Summary of discussion

Eighty-seven isolates were obtained from nutrient agar plates supplemented with various concentrations of TCS or PCMX. Of these isolates thirty-seven tested positive for haemolysis. *Pseudomonas*, *Aeromonas, Bacillus* and *Klebsiella* spp. were found at various sampling points and are all well described as opportunistic pathogens capable of causing various diseases and were thus the main focus of the discussion. ERIC PCR was used to determine clonal relationship between *Aeromonas* and *Bacillus* isolates found at various sampling points to determine if the WWTP North-West Potchefstroom may be the source from which these biocide tolerant potential pathogens enter the environment, but whether this is the case is not yet clear.

All isolates underwent antibiotic susceptibility testing using various antibiotics, followed by MICs and testing for synergy and antagonisms between antibiotics and the biocides in question. Isolates exhibited various antibiotic resistance patterns which may be due to specific drug resistance mechanisms or multidrug-resistance mechanisms such as efflux pumps. The majority of isolates had TCS MICs of more than 2 mg/L while the majority of isolates had MICs for PCMX at 30 to 50 mg/L; resistance to these biocides may be due to efflux mechanisms or mutations of the target sites. Further analyses to determine the presence of synergy and antagonism revealed several different observations of both synergy and antagonism. Synergistic effects may be due to complementary effects of the drugs such as one increasing the

permeability of a cell for the other to then take effect, due to physical interaction between drugs at target sites, the targeting of similar pathways or due to the antimicrobials overwhelming a similar resistance mechanism. Antagonism on the other hand can be due to the antimicrobial activity such as one being bactericidal and one being bacteriostatic; it may also be possible that the antimicrobials share multidrug efflux systems and the combination of two drugs may trigger the overexpression of multiple efflux mechanisms.

HPLC results indicate that TCS and PCMX are effectively removed during the wastewater treatment process but were found to be present in the influent of the WWTP. Thus, bacteria entering the WWTP are still exposed to these antimicrobials during the process of entering the WWTP, and possibly during certain stages of the wastewater treatment process. Antibiotic resistance profiles and the assay for extracellular enzyme production were used to generate a pathogenic potential index. The *P. aeruginosa* isolate scored the highest, followed by *A. veronii* and *A. cavea.* The next highest scores were among *Bacillus* spp. while the lowest scores were attributed to *P. monteilii, K. oxytoca* and *P. alcaligenes.* Isolates exhibited a high potential for virulence that may pose a serious health risk.

CHAPTER 6 –CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions/Recommendations

The aim of the study was to identify and characterise potentially pathogenic Triclosan - and/or Chloroxylenol tolerant, heterotrophic plate count bacteria isolates from sewage effluent and river water from the wastewater treatment plant (WWTP) in Potchefstroom and the Mooi River.

6.1.1 Isolation, identification and DNA fingerprinting of potentially pathogenic Triclosan and/or Chloroxylenol tolerant, heterotrophic plate count bacteria

Potentially pathogenic TCS and PCMX tolerant heterotrophic plate count bacteria were isolated from pre- and post-chlorination sampling points of the WWTP in Potchefstroom, South Africa and the surrounding Mooi River. The bacterial species obtained are highly tolerant of TCS above concentrations of 2 mg/L. Eighty-seven isolates tolerant to TCS or PCMX were found of which thirty-seven tested positive for haemolysis.

Six genera were identified as *Klebsiella, Bacillus, Pseudomonas, Aeromonas, Exiguobacterium* and *Shewanella* spp.; of these genera *Bacillus, Klebsiella, Pseudomonas* and *Aeromonas* are well described opportunistic pathogens. It can be concluded that TCS and PCMX tolerant heterotrophic plate count bacteria can grow in the presence of varying concentrations of TCS and PCMX. A cause for concern should be that these organisms enter our natural water bodies; it should also be a concern that the WWTP may be the source of these organisms entering natural water bodies as *Aeromonas* and *Bacillus* spp. were found at the pre- and post-chlorination sampling points, and at the wetlands and downstream sampling points.

As previously stated visual and clustering analysis did not yield any conclusive results, but similar species were found at pre- and post-chlorination sampling points as well as

downstream from the WWTP, thus the possibility remains that the WWTP is the source from which these organisms enter the environment. The current study was carried out with isolates obtained during an initial sampling period, and among those isolates obtained only a small number were of the same species. Thus, to get a more conclusive result in the future a larger sampling pool is recommended using only one of the more prevalent species such as *Aeromonas* spp. as an indicator to gain more accurate insight into the possibility of these organisms surviving chlorination and entering the aquatic environment, after their pre-exposure to these antimicrobials. The application of other fingerprinting techniques may also contribute to the determination of a more conclusive result. Techniques such as whole genome sequencing may also provide clearer insight as to whether the isolates share common ancestry (Bentley & Parkhill, 2015).

6.1.2 Antimicrobial susceptibility

Antimicrobial susceptibility testing included a test for cross resistance to antibiotics, the determination of MICs for the biocides in question and the determination of synergistic and/ or antagonistic effects. Current results obtained strongly suggest that similar mechanisms of resistance are potentially responsible for the observed resistance to both antibiotics and biocides. Efflux mechanisms are known to confer resistance to multiple antimicrobials and thus seem to be the main reason behind the tolerance to these biocides and resistance to antibiotics but further in depth molecular analysis is required to identify and confirm this observation (Schweizer, 2003; Khanam *et al*., 2017; Yılmaz & Özcengiz, 2017).

6.1.3 High performance liquid chromatography

Levels of both TCS and PCMX were found at the WWTP and are a cause for concern. The antimicrobials are effectively removed during the wastewater treatment process but are present in the influent and may thus still select for potentially pathogenic antibiotic resistant bacteria that may end up in the aquatic environment. It is recommended that a method such as UHPLC–MS/MS be applied in future as this will

assist in determining whether the current HPLC method is sufficient in identifying the presence of and quantifying these antimicrobials (Gracia-Lor *et al*., 2012).

6.1.4 Pathogenic potential

Results from antibiotic resistance profiles and the assay for extracellular enzyme production were used to generate a pathogenic potential index. A large portion of South Africa's population still rely on surface water as their primary source of water, and many still utilize untreated water, this along with a very high prevalence of HIV makes the presence of organisms exhibiting multiple virulence characteristics and resistance to antibiotics a serious health concern (Zamxaka et al., 2004; Momba et al., 2006; South African National HIV Prevalence, Incidence and Behaviour Survey, 2012). Further study is required to determine which genes are associated with the production of these extracellular enzymes and to determine if they are in fact directly related to virulence. Future study is also recommended on the epidemiology of some of these organisms for example the emerging pathogens among *Aeromonas* spp. which were very prevalent, to determine if any of these opportunistic pathogens can be linked to actual clinical cases to get a clearer picture of potential health risks involved (Igbinosa *et al*., 2012).

6.1.5 Conclusion

In conclusion TCS and PCMX tolerant potentially pathogenic HPC bacteria were isolated from the WWTP North-West Potchefstroom; these isolates exhibit resistance and/or tolerance to various antibiotics and to the biocides in question. Isolates also tested positive for the presence of various extracellular enzymes associated with virulence. Both PCMX and TCS were found in the WWTP but based on current results are not released into the environment. The presence of these biocides in the WWTP may drive the process of selection for multidrug-resistant pathogens that may be entering the environment, posing a serious health concern due to downstream usage (e.g. recreational purposes) of the receiving waters.

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APPENDIX A

Table A: Example of the micro well plate design containing the raw data used to determine the final checkerboard for all isolates using one antibiotic (Tetracycline).

Isolate	4	5	7	9	14	29	4	5	7	9	14	29
TMP 5ug	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0	T0_A0
	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1	T0_A1
	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2	T0_A2
	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4	T0_A4
	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8	T0_A8
	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16	T0_A16
	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32	T0_A32
	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64	T0_A64
	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0	T0.25_A0
	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1	T0.25_A1
	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2	T0.25_A2
	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4	T0.25_A4
	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8	T0.25_A8
	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16	T0.25_A16
	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32	T0.25_A32
	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64	T0.25_A64
	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0	T0.5_A0
	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1	T0.5_A1
	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2	T0.5_A2
	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4	T0.5_A4
	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8	T0.5_A8
	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16	T0.5_A16
	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32	T0.5_A32
	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64	T0.5_A64
	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0	T1_A0
	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1	T1_A1
	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2	T1_A2
	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4	T1_A4
	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8	T1_A8
	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16	T1_A16
	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32	T1_A32
	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64	T1_A64
	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0	T2_A0
	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1	T2_A1
	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2	T2_A2
	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4	T2_A4
	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8	T2_A8
	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16	T2_A16
	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32	T2_A32
	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64	T2_A64

APPENDIX B

Figure A-J: Examples of possible induced increased resistance or classic dose response to either TCS or PCMX at certain biocide concentrations.

Induction

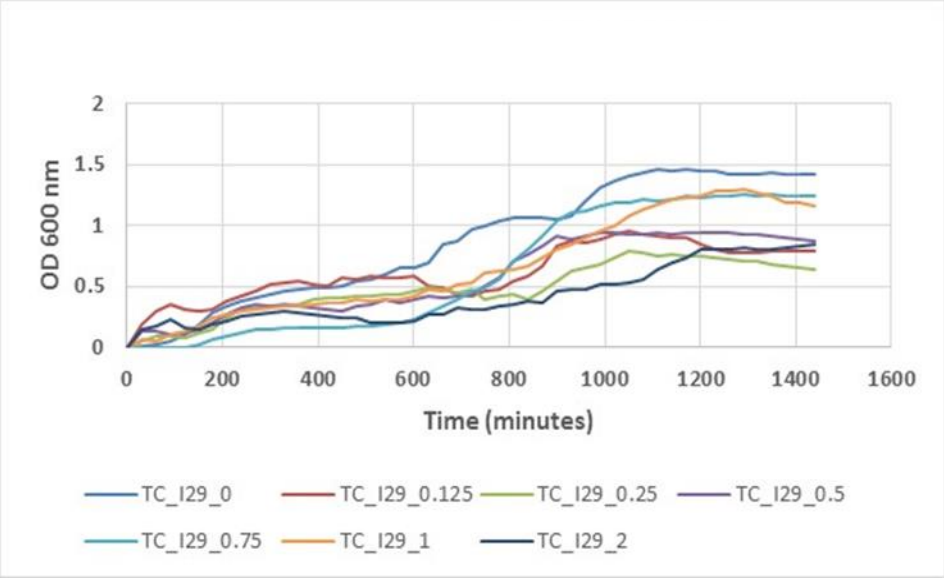

Figure A: Typical growth curve data obtained after 24 hours (TCS, *A. veronii* (T-1-D-6))

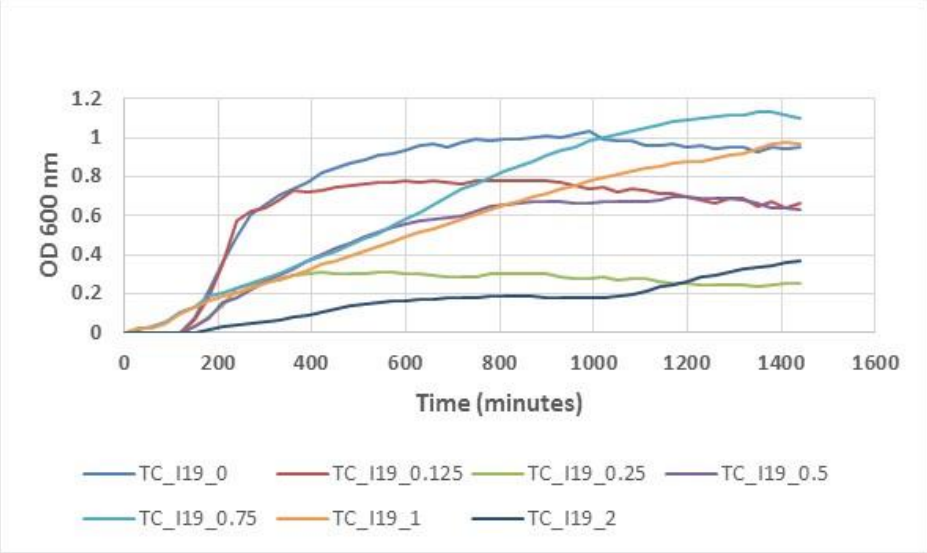

Figure B: Typical growth curve data obtained after 24 hours (TCS, *P. taiwanensis* (T-1-U-4A))

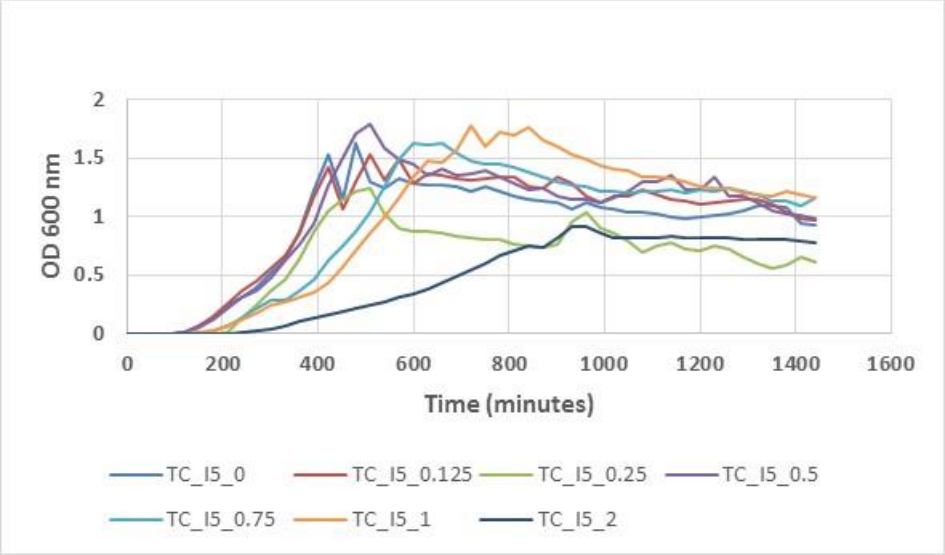

Figure C: Typical growth curve data obtained after 24 hours (TCS, *P. aeruginosa* (T-0.25-Pre-1))

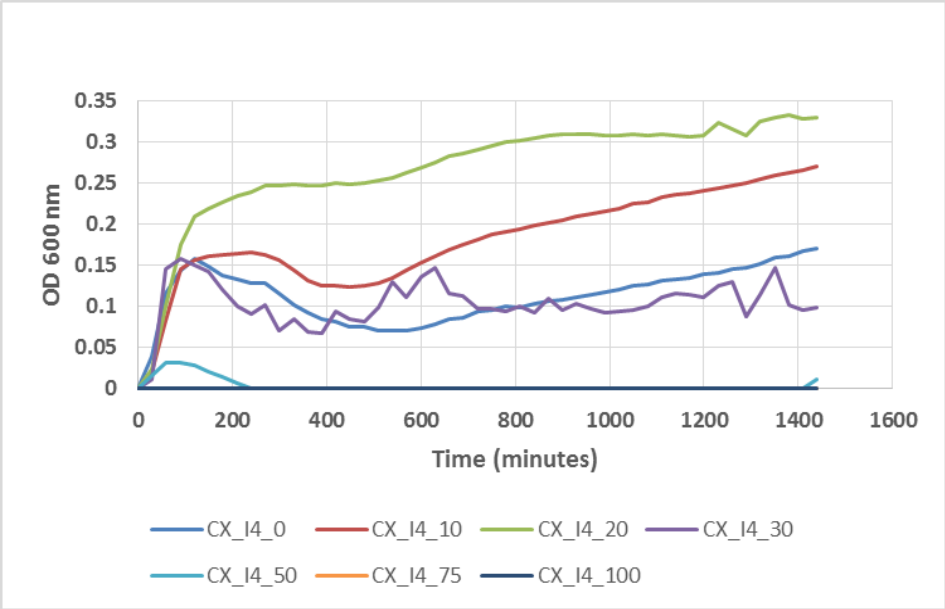

Figure D: Typical growth curve data obtained after 24 hours (PCMX, *A.cavea* (T-0.5-Pre-1B))

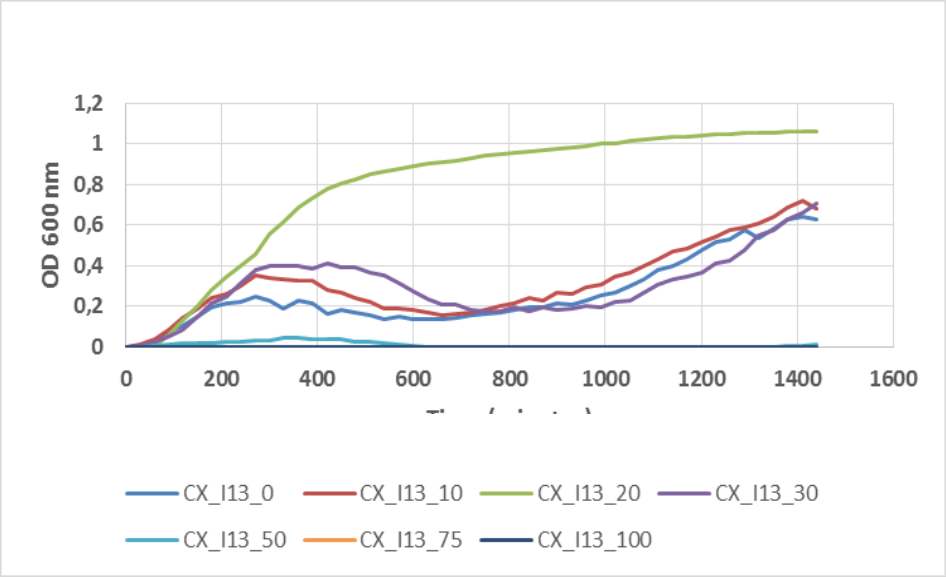

Figure E: Typical growth curve data obtained after 24 hours (PCMX, *B. toyonensis* (C-40-Post-1))

Dose response

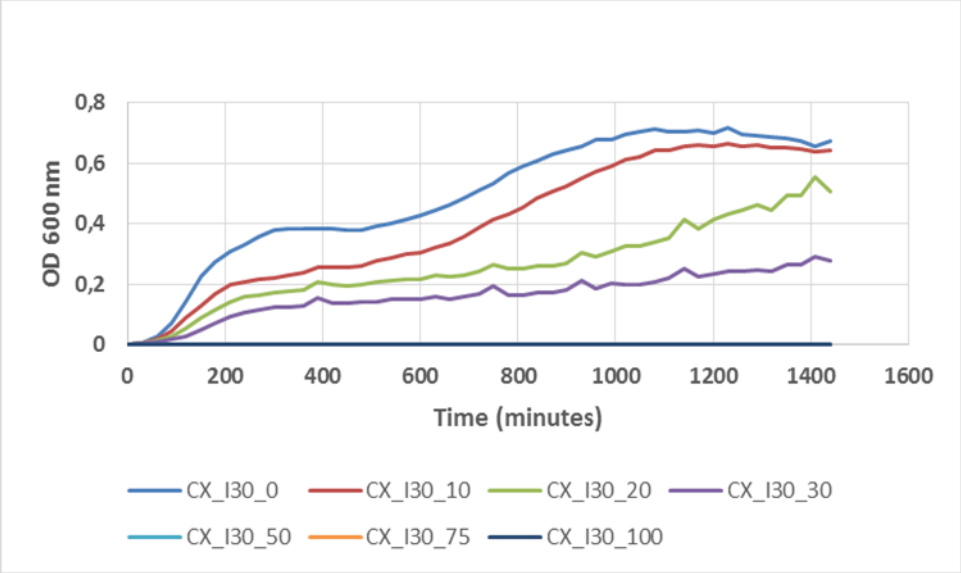

Figure F: Typical growth curve data obtained after 24 hours (PCMX, *A. veronii* (T-1-W-3))

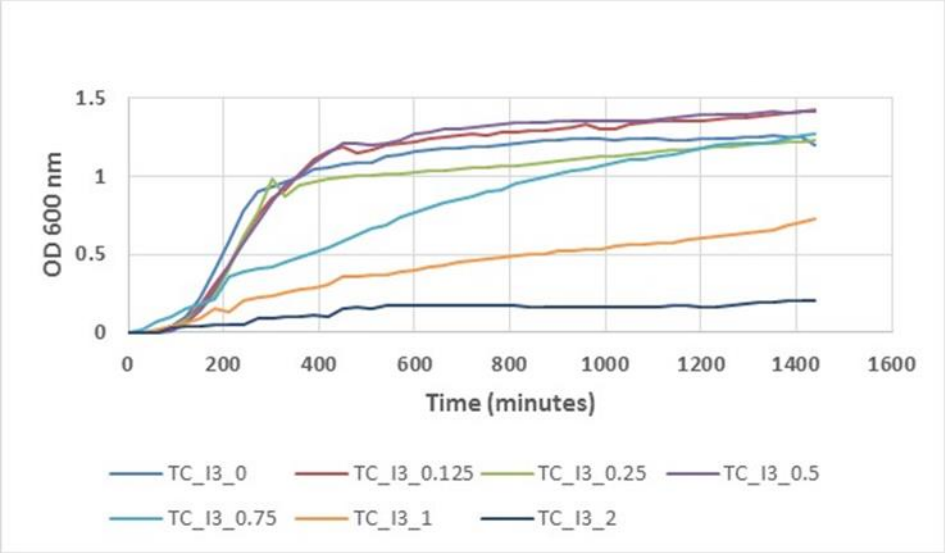

Figure G: Typical growth curve data obtained after 24 hours (TCS, *P. monteilii* (T-0.5-Pre-1A))

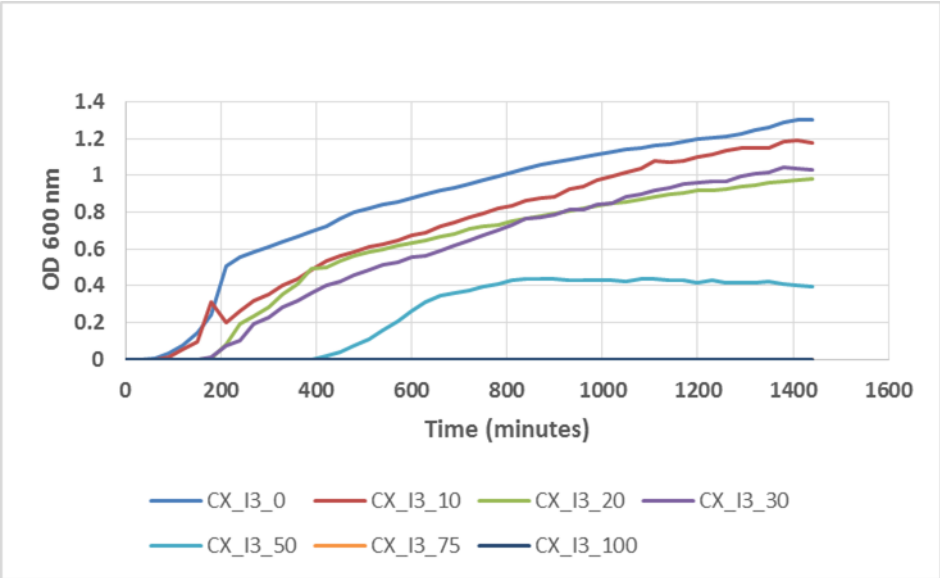

Figure H: Typical growth curve data obtained after 24 hours (PCMX, *P. monteilii* (T-0.5-Pre-1A))

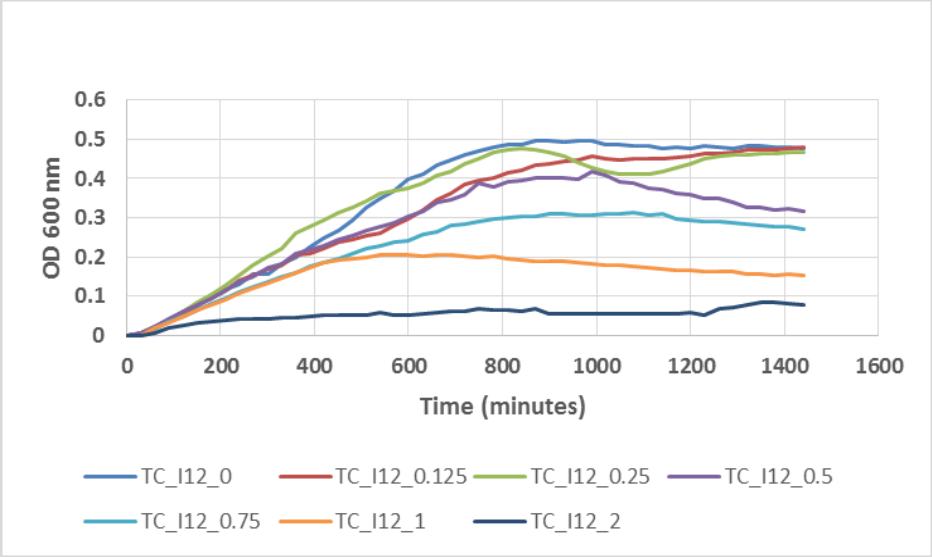

Figure I: Typical growth curve data obtained after 24 hours (TCS, *S. xiaminensis* (T-0.25-Post-7))

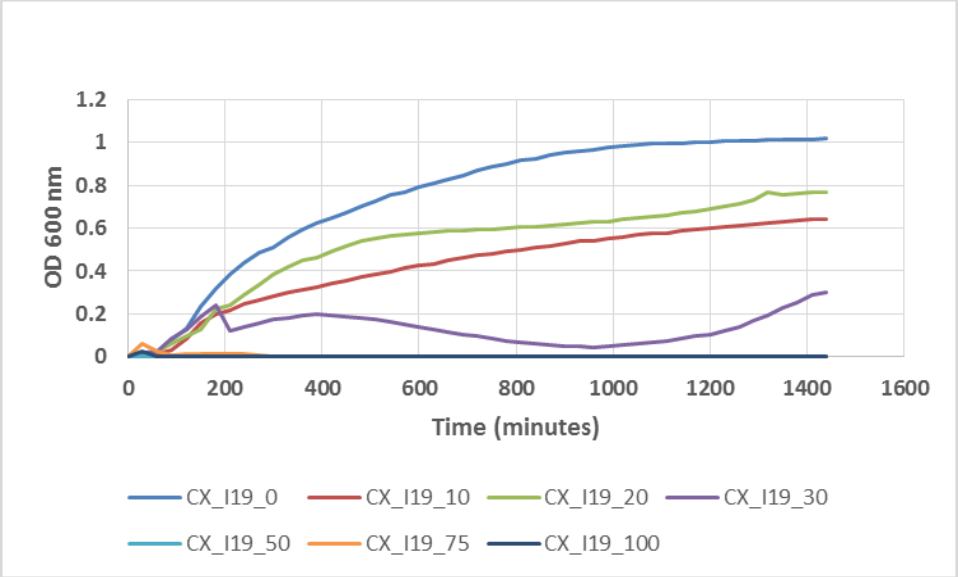

Figure J: Typical growth curve data obtained after 24 hours (PCMX, *P. taiwanensis* (T-1-U-4A))