Antibiotic resistance in triclosan heterotrophic plate count bacteria from sewage water

I Coetzee

10851585

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Supervisor: Dr JJ Bezuidenhout

Co-supervisor: Prof CC Bezuidenhout

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It all starts here ™

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DECLARATION

I declare that the dissertation submitted by me for the degree Magister Scientiae in Environmental studies 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:

Ilse Coetzee

ABSTRACT

The concentration of triclosan in antiseptics, disinfectants and preservatives in products exceeds the minimal lethal levels. Extensive use of triclosan and antibiotics results in bacterial resistance to their active ingredients. The precise relationship between use and resistance, however, has been challenging to define. The aim of the study was to identify and determine antibiotic resistance profiles of triclosan tolerant heterotrophic plate count bacteria isolates from sewage influent and effluent. R2 agar supplemented with triclosan was utilised to isolate the triclosan resistant bacteria. To determine the minimum inhibitory concentration (MIC), organisms were incubated for 24 hours at selected concentrations of triclosan. Polymerase chain reaction (PCR) amplification of the 16S rRNA region was done to identify isolates. An assay for cross resistance to various antibiotics was performed. Determination of enhanced resistance to antibiotics by adding antimicrobials to the medium will be performed by using three antibiotics. High performance liquid chromatography was conducted to quantified levels of triclosan persistent in sewage water. Forty-four isolates were resistant to levels of triclosan ranging from 0.25 mg/l to 0.5 mg/l. Minimum inhibitory concentration values of these isolates ranged from 0.125 mg/l to >1 mg/l of triclosan. 16S rDNA methods were used and five main genera namely, *Bacillus*, *Pseudomonas, Enterococcus, Brevibacillus* and *Paenibacillus* were identified. Cell wall targeting antibiotics showed more pronounced relation with the triclosan concentration. Relation to triclosan concentration is not as apparent with the antibiotic targeting protein synthesis. Combination of antimicrobials indicated that at certain triclosan concentrations synergism or antagonism is observed. The importance of applying the correct concentration and combination of antimicrobials is observed. Levels of triclosan were found throughout the sewage water. HPLC values indicated the presence of triclosan at post-grid removal and effluent of the WWTP. The triclosan concentrations decrease through the WWTP but small concentrations enter our water bodies. The presence of bacterial species that are resistant to high concentrations of triclosan and multiple antibiotics enter our natural water bodies and is cause for concern.

Keywords: Triclosan, Antibiotic resistance, HPLC, Synergy & antagonism, WWTP

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ABBREVIATIONS

µg/l	Microgram per litre
µl	Microlitre
bp	Base pair
DWAF	Department of water affairs and forestry
ENR	Enol-acyl carrier protein reductase
EPA	Environmental Protection Agency
HPC	Heterotrophic plate count
HPLC	High performance liquid chromatography
I	Induced
M	Molar
mg/l	Milligram per litre
mg/ml	Milligram/millilitre
MIC	Minimum inhibitory concentration
mm	Millimeter
MRSA	Methicillin resistant *Staphylococcus aureus*
ng/l	Nanogram per litre
PACD	Photo allergic contact dermatitis
PAT	Progress assessment tools
PCR	Polymerase chain reaction
ppb	Parts per billion
ppm	Parts per million
R	Resistant
RDA	Redundancy analysis
rpm	Rates per minute
S	Susceptible
SPE	Solid phase extraction
TCL	Triclosan
UHPLC	Ultra-high performance liquid chromatography
WWTP	Wastewater treatment plant
XOC	Xenobic organic components

CHAPTER 1: GENERAL INTRODUCTION AND RATIONALE

1.1 Introduction

Antimicrobials are defined as products that kill microorganisms or keep them from multiplying (reproducing) or growing and they are most commonly used to prevent or treat disease and infections due to microorganisms (May, 2012). For decades biocides or antimicrobials have been employed in the form of antiseptics, disinfectants and preservatives (Denyer & Maillard, 2002). Currently, antimicrobial usage is expanded to include consumer products such as toothpaste, mouthwashes, deodorants, hand soaps and personal care lotions. It is also incorporated in children's toys, cutting boards and plastic films to wrap meat products (Singh *et al.,* 2010). The benefit of adding antimicrobials to the last three products is, however, unclear (Baier-Anderson & Monosson, 2008). Recent studies have shown that the use of soap and water is just as effective in removing or reducing microorganisms as using products which contain antimicrobials (Chen *et al.*, 2008).

Typical antimicrobial compounds found in household products include triclosan, chloroxylenol, chlorhexidine, triclocarban and ethyl alcohol (Bessom, 2012). As the use of antimicrobial products continues to increase, the antimicrobial resistance in bacteria also increases. However the precise relationship between use and antimicrobial resistance has been challenging to define (Rubin & Samore, 2002).

It is generally believed that bacteria rarely acquire resistance to biocides due to their broad spectrum of activity and action at several target sites (Rodriquez *et al.,* 2007). However the careless use of these biocides or antimicrobials inside and outside of healthcare settings has become of concern. The more often bacteria are exposed to antimicrobials the more likely resistance will be acquired by them to these antimicrobials. The applied concentration at which these antimicrobials are applied is of importance as the sub-therapeutic use may result in antimicrobial resistance. Therefore, responsible use and education regarding these products is of importance (Rodriquez *et al.,* 2007). According to Brenwald & Fraise (2003), triclosan acts as a biocide with multiple cytoplasmic and membrane targets. At lower triclosan

concentrations, the mode of action appears bacteriostatic and targets bacteria mainly by inhibiting fatty acid synthesis. Brenwald & Fraise (2003) stated that triclosan is used as a broad spectrum biocide agent against *Staphylococcus aureus* and is recommended to eliminate carriage of methicillin resistant *S. aureus* (MRSA). Furthermore according to Tattawasart & Maillard (1999) evidence suggests that biocide insusceptibility can confer cross-resistance to other biocides as well as to a variety of antibiotics. A decreased susceptibility to triclosan correlates with decreased susceptibility to two classes of antibiotics, according to Tkachenko *et al.,* (2007).

Synergy between triclosan and antibiotics has long since been noted and is being reviewed in an article written by Schweizer (2001). In an article by Russel (1993) and cited by Schweizer (2001) it was stated that there is a link between antibiotics and biocide resistance and that specific biocides select for antibiotic resistance. Heath *et al.,* (1999) stated that the *FabI* (Enol-Acyl Carrier Protein Reductase) mutation selected by exposure to triclosan causes cross-resistance with other antimicrobial agents. Those authors also demonstrated that biocides share targets with antibiotics. Widespread use of biocides also selects for resistance to useful drugs (Heath *et al.,* 1999).

Triclosan ends up in the water system through domestic wastewater and is treated at the municipal wastewater treatment plants (WWTP). Studies done in the United States of America revealed that over 95% of the uses of triclosan are in consumer products and that triclosan was the most frequently found organic contaminant (Glaser, 2004). Incomplete removal of triclosan in wastewater treatment plants results in triclosan presence in soils and surface waters (SCCP/1251/09). Depending on the technical capabilities of the WWTP, between 58 – 99% of triclosan may be removed during wastewater treatment (McAvoy *et al*., 2002). Incomplete removal of triclosan results in bio-accumulation and production of toxic compounds in the aquatic environment (Aranami & Readman, 2007). Although triclosan is chemically stable, it can readily be degraded in the environment via photo degradation to compounds such as dibenzofurans and dioxins. These products have grater toxicities than the parent compound (Latch *et al.,* 2005).

As a result of antimicrobials being washed into the water supply the accumulation of antimicrobials is applying selective pressure on bacterial resistance to their active ingredients (Baier-Anderson, 2008). The fate of compounds like triclosan in the environment is of concern as it influences the activities of bacteria in eco-systems (SCCP/1251/09).

1.2 Problem statement

For decades biocides or antimicrobials have been employed in the form of antiseptics, disinfectants and preservatives (Denyer & Maillard, 2002). Currently, antimicrobial usage is expanded to include consumer products such as toothpaste, mouthwashes, deodorants, hand soaps, and lotions. It is also incorporated in children's toys, cutting boards and plastic films to wrap meat products (Singh *et al*., 2010). The benefit of adding antimicrobials to the last three products are, however, unclear (Baier-Anderson & Monosson, 2008). Recent studies have shown that the use of soap and water is just as effective as using products which contain antimicrobials (Chen *et al*., 2008). As the use of antimicrobial products continues to increase, the antimicrobial resistance in bacteria also increases. The precise relationship between use and resistance, however, has been challenging to define (Rubin & Samore, 2002).

It is generally believed that bacteria rarely acquire resistance to biocides due to their broad spectrum of activity and action at several target sites (Rodriquez *et al*., 2007). However the careless use of these biocides/ antimicrobials inside and outside healthcare settings has become of concern and the more often bacteria are exposed to antimicrobials the more likely resistance will be acquired. The applied concentration of these antimicrobials is of importance as the sub-therapeutic use may result in antimicrobial resistance development. Therefore, education regarding these products is of importance (Rodriquez *et al.,* 2007).

As a result of antimicrobials being washed into the water supply the accumulation of these products is causing the appearance of bacteria resistant to their active

ingredients (Baier-Anderson, 2008). It is important to evaluate the fate of triclosan in the environment. Bacterial activities change in the presence of triclosan and at different concentrations of triclosan it also affects these activities (SCCP/1251/09). Triclosan ends up in the water system through domestic wastewater and is treated at the wastewater treatment plant (WWTP). Only 58 – 99% of triclosan is removed from the water by the WWTP, but this depends on the technical capabilities of the WWTP (McAvoy *et al*., 2002).

Due to extended use of Triclosan in personal care products these substances are washed into our wastewater and inefficient removal of this antimicrobial leads to triclosan remaining in our water bodies. Exposure to triclosan is not only causing health concerns but may be causing bacterial resistance to antibiotics. Therefore the following aim and objectives were investigated to determine if triclosan is present in wastewater and what the antibiotic resistant profiles are.

1.3 Aim

The aim of the study was to identify and determine antibiotic resistance profiles of triclosan tolerant heterotrophic plate count bacterial isolates from sewage influent and effluent.

1.4 Objectives

Objectives included the following:

- Isolation and identification of triclosan tolerant bacteria by using phenotypical and molecular methods
- Determination of minimum inhibitory concentrations (MIC)
- Assay for cross-resistance to selected antibiotics
- Measurement of triclosan concentrations and reduction in sewage water by using HPLC methods
- To determine if antibiotic resistance patterns enhance in the presence of varying concentrations of triclosan.

CHAPTER 2 – LITERATURE REVIEW

2.1. Antimicrobials

Antimicrobials can be defined as products that kill microorganisms or keep them from multiplying (reproducing) or growing, thus effectively eliminating organisms over time. They are most commonly used to prevent or treat disease and infections resulting from, or aggravated by microorganisms (May, 2012).

For centuries biocides or antimicrobials have been employed in the form of antiseptics, disinfectants and preservatives (Denyer & Maillard, 2002). Currently, antimicrobial usage is expanded to include consumer products such as toothpaste, mouthwashes, deodorants, hand soaps, and lotions. It is also incorporated in children's toys, cutting boards and plastic films to wrap meat products (Singh *et al.,* 2010). The benefit of adding antimicrobials to the last three products are however unclear (Baier-Anderson & Monosson, 2008). Recent studies have shown that the use of standard soap and water is just as effective as using products which contain antimicrobials (Chen *et al.*, 2008). But contradicting findings have been published that explain the synergy between antimicrobial use and antibiotic resistance.

Typical compounds found in household products include triclosan, chloroxylenol, chlorohexidine, triclocarban and ethyl alcohol (Bessom, 2012). According to Downs (2008) triclocarban and triclosan were developed in the 1950s and 1960s and were first used mainly as antiseptic agents in hospitals. Sales of consumer antibacterial products took off in the early 1990s, backed by multimillion-dollar advertising campaigns for popular soap (Downs, 2008). By 2001, manufacturers were introducing hundreds of new antibacterial products every year (Levy, 2001). These products were introduced for their antimicrobial effect on bacteria.

2.1.1 Triclosan

Triclosan (polychloro phenoxy phenol, Figure 2.1) is an antimicrobial (antibacterial and antifungal) agent used in most household antiseptics, disinfectants and

preservatives (McAvoy *et al.*, 2002). Figure 2.1 represents the chemical structure of triclosan which is a chlorinated aromatic compound that has functional groups representative of both ethers and phenols. In appearance it is a white powder with a slight aromatic odour. Triclosan is only slightly soluble in water, but soluble in ethanol, methanol, diethyl ether and strong basic solutions such as 1 M sodium hydroxide (Tsai, 2008).

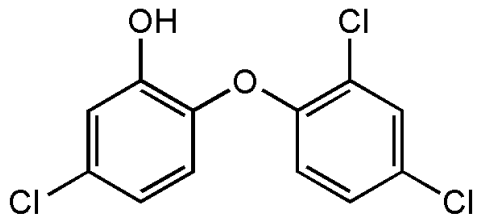

Figure 2.1: Chemical structure of triclosan

Triclosan (Figure 2.1) is used in household bars of soaps at 0.6% and cosmetics at lower concentrations. In deodorants it is used as a preservative at a concentration of 0.3% (Lanxess, 2010). Triclosan is an antimicrobial or germ-killing agent first used in hospital settings as a surgical scrub. Currently triclosan is found in hundreds of products because of its ability to stop the growth of bacteria, fungi and mildew. However, triclosan was first registered by the EPA as a pesticide in 1969 and introduced as a surgical scrub in 1972. The marketed product of triclosan in plastics and clothing is called Microban® and in acrylic fibers it is called Biofresh® (Glaser, 2004).

The main resistance mechanisms which a bacterium develops are efflux pumps and the *fabI* gene that encodes the enol reductase for fatty acid synthesis (McBain & Gilbert, 2011). These mechanisms are discussed later under triclosan resistance (section 2.3).

2.1.2 Triclocarban

Triclocarban (Figure 2.2) is also known as TCC or 3, 4, 4'-trichlorocarbanilide. It is a white powder and is used as an antibacterial agent in houshold bars of soaps at 0.6% and cosmetics at lower concentrations (Lim *et al*., 2012). This compound is

also included as a preservative in deodorants to deodorize, eliminate dermatophytosis and underarm odour at concentrations of 0.3% or even less. Approximately 30% of bar soaps have antibacterial agents incorporated into them. Triclocarban is insoluble in water but is soluble in a variety of alcohols (Lim *et al*., 2012).

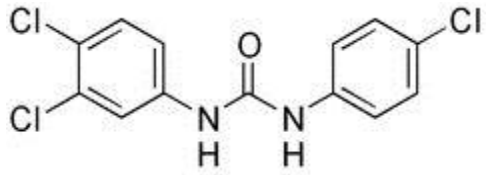

Figure 2.2: Chemical structure of triclocarban

Triclocarban is an antiseptic agent which is rarely used in clinics. This compound is active against Gram positive organisms, but less active against Gram negative organisms and fungi (Beaver *et al.,* 1957). Triclocarban acts by adsorbing to and destroying the semipermeable cytoplasmic membrane of a given bacterium, leading to cell death (Hamilton, 1971). It is metabolized by humans and excreted in the faeces and urine but does not bio-accumulate in humans when using the correct concentration of triclocarban. Studies do not indicate high levels of triclocarban in recycled water (Lanxess, 2010). This compound may thus not pose environmental concerns.

2.1.3 Chlorhexidine

Chlorhexidine (1:6di [4chlorophenyldiguanido] hexane) is characterized as being a strong base with cationic properties and is active against various bacteria, viruses, bacterial spores and fungi. Figure 2.3 illustrates the chemical structure of chlorhexidine. In appearance it is a colourless, odourless salt or solution and has an extremely bitter taste. In healthcare settings chlorhexidine it is used as a salt because of its ability to dissolve in water (Knuuttila *et al*., 1978).

This antimicrobial belongs to the biguanides group and is mostly used in antiseptics for example hand washing and oral products, disinfectants and preservatives. It is used because of its broad-spectrum efficacy and substantively for the skin and

causes low irritation (Gardner & Gray, 1991). It is pH dependent and reduces in the presence of organic matter (Russel & Day, 1993).

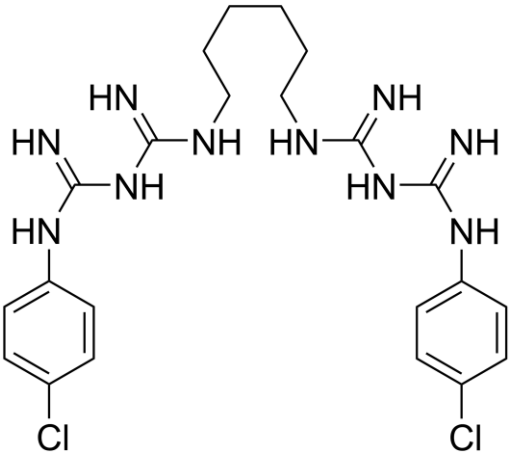

Figure 2.3: Chemical structure of chlorhexidine

The following mechanisms of antimicrobial action of chlorhexidine were presented by McDonald & Denver Russell (1999). Chlorhexidine is not sporocidal but it inhibits spore growth and development of spores. For Mycobacteria this agent is static but not cidal. Other nonsporulating bacteria the mode of action is on the membrane of the bacteria, causing lysis of the protoplast and spheroplast. High concentrations of this antimicrobial results in intracellular coagulation (McDonald & Denver Russell, 1999).

It was stated by El-Moug *et al*. (1985) that the uptake depends on the concentration and pH of the antimicrobial. The concentration and pH of the antimicrobial damages the outer cell layers, but is not sufficient enough to induce lysis or cell death (El-Moug *et al*., 1985). It can cross the cell wall by passive diffusion and then attacks the bacterial cytoplasmic membrane.

It kills the micro-organisms associated with various mouth and throat infections and other common conditions in the mouth (Ellen & Neuenfeldt, 2011). This includes *Candida albicans,* that causes thrush infection in the mouth and bacteria that may infect mouth ulcers or other sore areas in the mouth, for example following dental surgery. Infection of these areas increases discomfort and delays healing (Ellen & Neuenfeldt, 2011).

2.1.4 Chloroxylenol

Chloroxylenol (Figure 2.4) is a white to light beige crystal in appearance and is soluble in water. It belongs to the halophenol group and is described as 4-chloro-3,5-dimethylphenol. Chloroxylenol is an antiseptic/disinfectant agent and is bactericidal (Bruch, 1996). Although this antimicrobial is used in abundance little is known about the mechanism of action. It is presumed that because of the phenolic nature of chloroxylenol, it is hypothesized that the compound may affect the microbial membrane (Russell & Day, 1993). The bacteria which cause septicaemia and inflammation, *Pseudomonas aeruginosa* are resistant to chloroxylenol (Bruch, 1996).

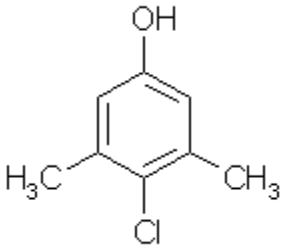

Figure 2.4: Chemical structure of chloroxylenol

2.1.5 Ethyl alcohol

Ethyl alcohol (Figure 2.5) is the most commonly used antimicrobial and is a broad-spectrum antimicrobial agent against vegetative bacteria, viruses and fungi (Morton, 1983. Because of its non-sporicidal effect (inhibits sporulation and spore germination) (Yasuda-Yasuki *et al*., 1978) it is not recommended for sterilization but can be used for surface disinfection and at lower concentration as a preservative (Bush *et al*., 1986)

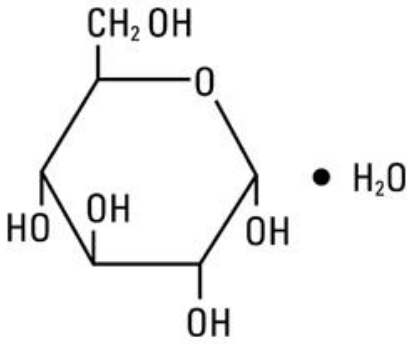

Figure 2.5. Chemical structure of ethyl alcohol

Ethyl alcohol is only one of the antimicrobials which are used in a hospital. Research by Bouzada *et al*. (2010) on the microbial resistance and persistence in a hospital environment, stated that the incorrect use of antimicrobials contributes to bacterial resistance to these antimicrobial products, (Bouzada *et al*., 2010). Little is known about the mode of action of ethyl alcohol but it results in membrane damage and rapid denaturation of the proteins which will lead to cell death (Larson & Morton, 1991).

Antimicrobials were developed to prevent diseases and have been effective the last 40 years, but because of the fact that they are widely used for long periods of time, the organisms have come to adapt to them. According to the Centres for Disease Control and Prevention (CDC), approximately two million people in the United States become infected with antimicrobial resistant bacteria every year and of these about 23 000 people die (Rubin & Samore, 2002).

2.2 Resistance to antimicrobials

As the use of antimicrobial products continues to increase, the antimicrobial resistance in bacteria also increases. The precise relationship between use and resistance, however, has been challenging to define (Rubin & Samore, 2002).

In a review Jones, (1999), argued that antimicrobial resistance has no correlation to antibiotic resistance in bacteria. The argument was that the appearances of low-level *in vitro* antiseptic resistance were the result of genetically acquired, efflux-mediated systems (Jones, 1999). No correlation exists because the plasmids

encoding for antimicrobial resistance may have evolutionarily preceded the introduction and use of antimicrobials. It was further argued that topical antimicrobial wash products have been used successfully to combat outbreaks of antibiotic-resistant bacteria. This author (Jones, 1999) also indicated that additional study may be warranted. The scientific literature at that stage did not illustrate a link between the use of topical antimicrobial formulations and the emergence of antiseptic or antibiotic resistance (Jones, 1999). It was concluded that further investigation was needed to determine whether a potential role exists for the use of topical antimicrobial products to reduce the incidence and emergence of bacterial resistance.

Jones, (1999) noted that there is no antibiotic/antimicrobial concern. Routine disinfection and household protocols presently used in hospitals need not be altered due to concerns about the potential for environmentally mediated transmission of antibiotic-resistant microorganisms. By using common disinfectant formulations at and below recommended concentrations with antibiotic-resistant/susceptible strains of *S. aureus, S. epidermidis, E. coli, K. pneumoniae, Salmonella choleraesius,* and *P. aeruginosa* demonstrated that antibiotic resistance does not correlate to increased disinfectant resistance (Jones, 1999). Thus using the correct concentration of antimicrobials does have a bactericidal effect and has no effect on antibiotic resistance in bacteria. This article is in contradiction to later studies done regarding antimicrobial and antibiotic resistance, such as Rodriques (2007). This author Rodriquez (2007), stated that it is generally believed that bacteria rarely acquire resistance to biocides due to their broad spectrum of activity and action at several target sites. However the careless use of these biocides/ antimicrobials both inside and outside of healthcare settings has become a matter of concern. The more often bacteria are exposed to antimicrobials the more likely resistance will be acquired by them against the antimicrobials in question. The applied concentration of these antimicrobials is of great importance as the incorrect applied concentration will also result in antimicrobial resistance. Therefore education regarding these products is of great importance (Rodriquez *et al*., 2007).

2.3 Triclosan resistance and synergy between triclosan and antibiotics

According to Brenwald & Fraise (2003), triclosan acts as a biocide with multiple cytoplasmic and membrane targets. According to McBain & Gilbert (2011) triclosan is a multi-target agent with two cellular targets. Firstly: a single mutation by blocking the lipid synthesis inducing the mutation in the *fabI* gene. The *fabI* gene encodes the enol reductase for fatty acid synthesis. McMurray *et al*. (1998) explained that sub-lethal concentrations of triclosan result in microbial resistance. Secondly: Efflux pumps but at lower concentrations of triclosan: the bacteria use efflux pumps to transfer substances out of the cell (Gomez *et al*., 2005). *Pseudomonas aeruginosa* is naturally resistant to triclosan because of intrinsically having an efflux pump but can increase resistance to triclosan by turning on additional efflux pumps (Schweizer, 2001).

At sub-lethal concentrations, however, triclosan appears bacteriostatic and targets bacteria mainly by inhibiting fatty acid synthesis. Triclosan resistance in *S. aureus* can occur as a result of over-expression of *fabI* and amino-acid changes in *FabI*. Thus, if it targets a specific mechanism, resistant bacterial strains may be selected in its presence. In fact, *E.coli* with a missense mutation in the *fabI* gene has been shown to have a 64-fold higher minimum inhibitory concentration (MIC) for triclosan when compared to a wild type strain. Brenwald & Fraise (2003) stated that triclosan is used as a broad spectrum biocide agent against *Staphylococcus aureus* and is recommended to eliminate methicillin resistant *S. aureus* (MRSA). Isolates of MRSA with a low triclosan resistance had a MIC value between 2 mg/l to 4 mg/l isolated from a patient using soap that contained triclosan. A study by Tuffnell *et al*., (1987) illustrated that MRSA is sensitive to a concentration of triclosan between the values of 0.1-2 mg/l and may or may not have increased resistance to this antimicrobial (Tuffnell *et al*., 1987). These organisms were cross-resistant to the antibiotic mupirocin. The resistance was transferred via a plasmid (Aiello & Larson, 2003). A highly resistant strain of Staphylococcus aureus had mutations in *FabI* and overproduces the enzyme by three-to five-fold (Fan *et al.,* 2002).

Tkachenko *et al*. (2007), demonstrated a triclosan-ciprofloxacin cross-resistant mutant strain of *Staphylococcus aureus* displayed an alteration in the expression of several cell membrane structural and functional genes. Methods that were used included the growth of the bacteria in the presence and at different concentrations of triclosan. Triclosan-resistant bacteria were then plated on 1.0 mg/ml ciprofloxacin media. At this concentration ciprofloxacin killed wild-type cells. The aim of the study was to determine whether triclosan resistance contributes to ciprofloxacin resistance in *Staphylococcus aureus*. Results showed that the mutant sequesters the triclosan in the cell membrane which leads to triclosan resistance and increases the resistance to ciprofloxacin. Triclosan resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) was also determined by Brenwald & Fraise (2003). MRSA were cultivated in the presence of triclosan and tested for cross-resistance to mupirocin. Triclosan is used in hospitals to treat patients that were infected with MRSA. Results that were obtained indicated low mupirocin resistance even though previous studies have shown higher resistance to mupirocin when MRSA were treated with triclosan (Brenwald & Fraise, 2003).

Furthermore according to Tattawasart *et al.* (1999) evidence suggests that a decreased biocide susceptibility can confer cross-resistance to other biocides as well as to a variety of antibiotics. A decreased susceptibility to triclosan correlates with decreased susceptibility to two classes of antibiotics (Tkachenko *et al*., 2007). The antimycobacterial agent isoniazid targets the mycobacterial homolog of ACP (enoyl acyl carrier protein) reductase *InhA* (an enzyme involved in mycolic acid biosynthesis). The mutants are cross resistant to isoniazid and triclosan (Tkachenko *et al*., 2007). The second class is the quinolones. Bacterial resistance to triclosan also correlates with quinolone resistance in *E.coli* and *Pseudomonas aeruginosa*. This antibiotic inhibits DNA gyrase and topoisomerase IV. These enzymes are essential to bacterial DNA metabolism. Quinolone antibiotics and triclosan share the same resistance genes encoding multi-drug efflux pumps (Tkachenko *et al*., 2007).

Research has shown on a molecular level that bacteria with a low susceptibility to triclosan are resistant to a wide variety of antibiotics. Triclosan is shown to act as a substrate for multidrug efflux pumps and allows selection of pump mutations (Aiello, 2003). Because triclosan acts as a substrate for efflux pumps, the pump mutation

can be transferred to other susceptible species (Aiello, 2003). Mutations of ENR gene showed dramatic change in MIC value (McMurry *et al*., 1998; Chen *et al*., 2009) and capturing of triclosan and increasing the expression of important regulators or metabolic enzymes (Yu *et al*., 2010) were also reported as mechanisms of resistance to triclosan

The bacterial enoyl-acyl carrier protein reductase (ENR) encoded for by the *fabI* gene is an enzyme critical for cell-wall synthesis of bacteria. Subsequent studies show that triclosan is an inhibitor of ENR (Heath *et al.,* 1998, 1999; Levy *et al.,* 1999; Qiu *et al*., 1999). Because triclosan is broadly used, the resistance may cause serious global health problems (Levy, 2001; Aiello & Larson, 2003; Russell, 2004). ENR is now one of the most important drug targets (Fidock *et al*., 2004; Kuo *et al*., 2003; Wang *et al*., 2006) for methicillin-resistant *Staphylococcus aureus* (MRSA) (Priyadarshi *et al*., 2010), Tuberculosis (TB) (Gagneux *et al.,* 2006) and Malaria (Hall *et al.,* 2005). These are three major infectious problems worldwide.

Thus understanding the structural origin of triclosan resistance could help in solving other resistance problems and may lead to new generation antibiotics for global infection problems. Hence, it is vital to explore the structural changes of ENRs (enoyl-acyl carrier protein reductase) caused by mutation (Singh *et al.,* 2010). In the study of Singh *et al.* (2010) it was demonstrated that one-point mutation of ENR of the bacteria leads to resistance against a human-designed antimicrobial drug. It is believed that the molecular design which improves the binding strength around the flexible region, would improve the binding efficiency of the drug with ENR. The information provided could be utilized to design a new generation of antibiotics which will effectively act on mutant ENR, helping avoid serious health concerns due to the TCL resistance (Singh *et al*., 2010).

Synergy between triclosan and antibiotics has long since been observed and reviewed by Schweizer (2001). In an article by Russell (1999) and cited by Schweizer it was stated that there is a link between antibiotics and biocide resistance and those biocides select for antibiotic resistance. Tabak *et al.* (2009) observed synergy between triclosan and ciprofloxacin and a reduction of viable cells. Synergy was more pronounced at the lower triclosan concentrations. Thus the concentration

of the antimicrobial determines the synergistic effect of the efficacy of the antimicrobials. It was observed that exposure of *Salmonella typhimurium* to triclosan before exposure to antibiotics resulted in a reduction of viable cells and that this reduction was dependent on the concentrations of triclosan and the antibiotic. It was also noted that applying the drugs simultaneously an additive effect was observed at lower concentrations and synergy at higher concentrations of triclosan and the applied antibiotic (Tabak *et al*., 2009). Synergy between antibiotics and antimicrobials were also studied by Tong *et al*., (2011). These authors observed that the combination of these drug and the concentrations thereof will depend the efficacy of the treatment. It was also observed that different antimicrobial combinations will deliver different synergy results because of the antimicrobial target mechanisms (Tong *et al.,* 2009).

2.4. Triclosan in the aquatic environment

Baier-Anderson & Monosson (2008) found triclosan and triclocarban in streams across the United States of America. Similar concerns have been found in South Africa, but there is still a lack of studies done on our wastewater effluent system (Baier-Anderson, 2008). The presence of these antimicrobials has also been noted in sewerage sludge which is applied to farmland and according to a recent Centres for Disease Control and Prevention (CDC) study, triclosan was detected in the urine of nearly 75% of 2 517 people tested, indicating broad exposure (Baier-Anderson, 2008). As a result of antimicrobials being washed into the water supply the accumulation of these products is causing the appearance of bacterial resistance to their active ingredients (Baier-Anderson, 2008). According to Aiello (2003) triclosan was one of the most frequent substances found in water.

According to Bessom (2012) the use of triclosan and other antimicrobials contributes to bacterial resistance. Gram negative bacteria tends to thicken their capsule which makes the bacteria less susceptible to antimicrobials and antibiotics. The thick capsule also functions as a resistance mechanism to phagocytosis as enables the bacterium in adherence to surfaces (Willey *et al*., 2008). Safety of water supplies can be compromised as a result of bacteria having a capsule which is an extra-

cellular slime layer made of polysaccharides. The polysaccharides making up capsules are sticky: therefore capsules are one of the main reasons bacteria can form biofilms and the mucoid and sticky nature of the capsule makes biofilms hard to remove. These biofilms accumulate in the pipes of our water supplies and compromise the safety of water supplies (Bessom, 2012).The capsule of the bacteria stores excess food in the capsules and provide an osmotic buffer zone that allows bacteria to tolerate osmotic shock (Bessom, 2012). This makes these bacteria more pathogenic. These pathogenic bacteria become more resistant to antibiotic treatment (Bessom, 2012).

Discharge of triclosan through domestic use ends up in the wastewater. Studies done in the United States of America revealed that over 95% of the uses of triclosan are in consumer products and that triclosan was the most frequently found organic contaminant (Glaser, 2004). Incomplete removal of triclosan in the wastewater treatment plant results in triclosan presence in soils and surface waters (SCCP/1251/09). Studies done in Europe, USA, Canada, Australia, Japan and Hong Kong, indicated that high levels of triclosan were found in influents, effluents and biosolids of the WWTPs, lakes, rivers and sea water (Kanda *et al*., 2003; Xie *et al*., 2008; Kantiani *et al*., 2008; Dye *et al*., 2007; McAvoy *et al*., 2009; U.S. EPA 2009; Cha & Cupples, 2009; Hua *et al*., 2005; Fernandes *et al*., 2008; Okumura & Nishikawa, 1996; Chau *et al*., 2008, cited in 2005SCCP/1251/09, 2009).

In a study by Eriksson *et al*. (2003) grey wastewater was screened for xenobiotic organic compounds (XOCs). The purpose for this study was to determine if grey wastewater could be re-used for toilet flushing. Grey wastewater is water deriving from industrial and urban areas excluding water from toilets, bidets or heavily polluted process water. Seventy five present (75%) of wastewater is grey wastewaters (Hansen & Kjellerup, 1994). Chemical analysis by GC-MS identified a total of 191 compounds with their concentrations and only thermal stable compounds were processed. The results indicated the following preservatives: Ethyl, Methyl paraben, citric acid, phenoxy acetic acid and triclosan (Eriksson *et al*., 2003). Triclosan are used in shoes, toothpaste, etc. (Daughton & Ternes, 1999) and the levels of triclosan found (0.6 µg/l), correlate to observations in Sweden (0.56 to 5.9 µg/l) (Palmquist & Hanaeus, 2004; Eriksson *et al.,* 2003). The average triclosan

found in rivers ranged from 0.14-2.3 µg/l. They concluded that these chemical compounds found derived from pharmaceutical and personal care products (PPCPs) (Eriksson *et al*., 2003).

Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater were investigated by Novu *et al*., (2013). The aim of the study was to determine if there was an occurrence of antimicrobial residues and antibiotic resistant bacteria in sewage water. Triclosan was one of the antimicrobials tested for. The authors concluded that antibiotic resistance is not only contributed by antibiotic and antimicrobial residues in the water, but other factors such as temperature in the treatment plant may contribute to the observed resistance traits (Novu *et al.,* 2013). A major short-coming of the latter study is that the concentration of triclosan in the wastewater was not mentioned.

2.5. Impact of triclosan on the environment and related health concerns

It is important to evaluate the impact of triclosan in the environment. Bacterial activities change in the presence of triclosan and at different concentrations of triclosan it also affects these activities (SCCP/1251/09, 2010). Triclosan ends up in the water system through domestic wastewater and is treated at the WWTP. Depending on the technical capabilities of the WWTP, between 58 – 99% of triclosan may be removed during wastewater treatment (McAvoy *et al*., 2002).

Bio-accumulative and toxic compounds are produced when triclosan enters the aquatic environment (Aranami & Readman, 2007). Although triclosan is chemically stable, it can readily be degraded in the environment via photo degradation to compounds such as dibenzofurans and dioxins. These products have more toxic characteristics than the parent compound (Latch *et al.,* 2005). Studies done by Canosa *et al*., (2005a) showed that the main triclosan degradation pathways are the chlorination of the phenolic ring and cleavage of the ether bond in chlorinated water (Canosa *et al*., 2005a). Chloroform and other chlorinated organics are formed from free chlorine mediated oxidation of triclosan (Rule *et al*., 2005; Fiss *et al*., 2007).

However triclosan can effectively be removed from the wastewater system by ozone treatment but the degradation products were not identified (Suarez, 2007; Wert *et al*., 2009; Dodd *et al*., 2009).

Dioxins and dibenzofurans are produced not only by photo degradation, but also when triclosan reacts with chlorine in water. Dioxin is a chemical compound which is an environmental pollutant. It accumulates in the food chain, mainly in the fatty tissue of animals. More than 90% of human exposure is through food, mainly meat and dairy products, fish and shellfish. Dioxins are highly toxic and can cause reproductive and developmental problems, damage the immune system, interfere with hormones and also cause cancer. The concentration of dioxins becomes greater in the food chain as it bioaccumulate (Kogevinas, 2001). Dibenzofurans bind to other particles in water. Large amounts of this substance can be lethal to animals and humans. It can also cause reproductive problems and harm unborn infants. Kidney and liver problems are also associated with intake of dibenzofurans (Draggan, 2008).

The use of triclosan can cause skin irritation or contact dermatitis; this occurs when the skin comes in contact with products containing triclosan and causes photo allergic contact dermatitis (PACD) when exposed to sunlight (Schena *et al*., 2008). Eczematous rash ia caused by PACD on the face, neck, back of the hands or sun-exposed areas on the arms. Manufacturers of products containing triclosan claim that the ingredient lasts up to 12 hours. Thus continuing contact with triclosan occurs even if it only takes 20 seconds to wash your hands.

Triclosan is lipophilic and can thus bio-accumulate in fatty tissues and often in high quantities (Adolfsson-Erici, 2000).

2.6. Department of Water Affairs and Green Drop Certification

The Department of Water Affairs and Forestry (DWAF) introduced the water quality guidelines and the scope of this document is specifically designed to include safety of our water sources. For domestic use, specific requirements were documented

and its main purpose is to give information in order to make judgement of the quality of water for human consumption and household uses, (DWAF, 2009). The document is used by the department of water and forestry for information and to judge the fitness of water. Thus in this document they defined the term water quality as the physical, chemical, biological and aesthetic properties of water. This information is used to determine the quality and fitness of a variety of uses and thus in the end protect health and the integrity of aquatic ecosystems (DWAF, 2009).

According to the Department of Water Affairs in South Africa, Green Drop Progress Requirements are based on the following objectives; (a) It seeks to identify and develop core competencies to improve sustainably the level of wastewater management in South Africa, (b) It is a form of regulation to synergise current goodwill of water services, institutions and existing government support programmes to give focus, commitment, planning and resources to achieve excellence in wastewater management (DWAF, 2009). The Minister's undertaking, as stated in the Green Drop Progress Requirements, was, "...to provide the water sector and its stakeholders with on-going current, accurate, verified and relevant information on the status of wastewater services in South Africa..." (DWAF, 2009).

Green Drop certification and audits are done every second year. Progress assessments are done during the Green Drop gap year using the Progress assessment tools (PAT) to assess the cumulative risk status of the treatment systems (DWAF, 2009). This Green Drop Report and Progress Reports provide information on three levels. (1) System specific – Consists of data and information about the performance of the wastewater collector and treatment system, (2) Province specific – Consists of figures and information highlighting the strengths, weaknesses, historic trends within a province, (3) National overview – Summarize and combine the above findings for National view and performance (DWAF, 2009).

According to the Department of Water Affairs' Annual Report of 2009, South Africa's wastewater management industry comprises 850 municipal treatment plants and treating approximately 7589 Mega litres of wastewater on a daily basis. It has been reported that the municipal wastewater treatment is far from acceptable as stipulated by the National Water Act of 1998 (Act nr 36). The Green Drop Certification was put

into action to fill in the gaps where necessary and raising performance on a basis of incentives towards optimising the wastewater treatment processes (DWAF, 2009).

The Tlokwe Local Municipality in the North West Province was awarded the best provincial performer during this study (DWAF, 2009). Although Tlokwe Local Municipality was awarded with this prize, the Green Drop results indicated that municipal wastewater treatment in the North West Province is in an unsatisfactory state. Reports showed that most of the wastewater treatment plants in the North West are at high risk. 82.4% of the systems performed very poor to critical (DWAF, 2009). The 2013/2014 report will only be published during 2014, (DWAF, 2009).

When wastewater treatment plants' functioning is not satisfactory, the water that ends up in our water bodies leads to major health and ecological concerns.

2.7 Principles and applications: Methodologies available to study triclosan tolerant heterotrophic plate count bacteria

Appropriate methods are available to isolate and study the characteristics of antibiotic resistance of triclosan tolerant heterotrophic plate count bacteria.

2.7.1 Isolation methods for heterotrophic plate count bacteria (HPC)

Measurement of HPC or standard plate count of microorganisms is applied in order to determine the heterotrophic microorganism population in drinking water. Heterotrophs are organisms that include bacteria, yeasts and moulds which use organic carbon as a growth factor. Isolation of HPC bacteria from water was originally developed by Robert Koch in 1881. This method is since used to isolate HPC bacteria by using a nutrient rich media (Burtsher *et al*., 2009). However R2A media, could be used to isolate these bacteria from wastewater as this medium contains casein acid hydrolysate, yeast extract, proteose peptone, dextrose, starch, di-potassium phosphate, magnesium sulphate, sodium pyruvate and agar. R2A media were also used in a study by Garcia-Armisen *et al*., (2011) for the

determination of antibiotic resistance of heterotrophic bacteria in sewage-contaminated rivers (Garcia-Armisen *et al*., 2011).

2.7.2 Identification of heterotrophic plate count bacteria

Identification of bacteria can be conducted by two main methods, phenotypical and molecular methods. Both methods have their advantages and limitations. Lane *et al*., (1985) stated that because of evolutionary trends of bacteria, using only one method could limit reliable information. Therefore both methods should be used for correct identification of isolates.

By amplification of the 16S rDNA, identifications can be obtained and these methods were published in a variety of articles. With this method rapid expansion of the 16S rDNA could be used for the identification of bacteria (Lane *et al*., 1985; Stackerbrandt & Goebel, 1994; Simon *et al*., 1992; Smit *et al*., 1999; Fox *et al*., 2014).

2.7.3 Kirby-Bauer Disk Diffusion Susceptibility Test

Alexander Flemming developed the first antibiotic in 1928 which is regarded a milestone in the history of medicine (Willey *et al*., 2008). Thereafter more antimicrobials were developed for treatment of infectious diseases. But bacterial resistance to antimicrobials have become of concern and therefore laboratories developed ways to test different concentrations of antimicrobials on pathogens (Hudzicki, 2012). The Kirby-Bauer disk diffusion susceptibility test protocol was first published by Kirby and Bauer in the early 1960's and hence the name of this particular tests (Hudzicki 2012 & Boyle *et al*., 1972). The principle of this method is to plate out a bacterial lawn onto the agar plate. The antibiotic disks are placed onto the bacterial lawn and incubated at 37 ºC for 24 hours. This method is a fairly easy, rapid and accurate way of establishing susceptibility and resistance based on measuring the inhibition zone. The inhibition zone measurement can then be interpreted by using Antimicrobial Disk Susceptibility Test Standards (Hudzicki, 2012).

2.7.4. Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) method is used to determine the lowest concentration of a substance at which an organism can still grow. The organisms are dispensed into a 96 microwell plate together with the different concentrations of the selected substance (in this report the substance is triclosan). The microwell 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. Multidetection microwell plate reader was used by Kaya & Ozbilge (2012) to determine the MIC's of fungi in the presence of antifungal agents. The optical density was measured over a period of 24 hours at 37 °C. Growth curves were obtained in the presence of varying concentrations of the antifungal agent during the incubation period. It was reported that the spectrophotometric MIC's over a period of 24 hours delivered more objective results than the visual MIC's (Kaya & Ozbilge, 2012).

2.7.5 High Performance Liquid Chromatography (HPLC)

According to Piccoli *et al*., (2002) triclosan is hydrolytically stable, relatively non-volatile and hydrophobic, lipophilic with a water solubility of 10 mg/L (at 20 °C). Xue-fei *et al*., (2009) tested two methods to determine the presence of triclosan in wastewater. Solid Phase Extraction and High Performance Liquid Chromatography with ultra-violet methods were used. In previous studies liquid chromatography (Hua *et al.,* 2005; Ying *et al.,* 2007) and gas chromatography (Ying & Rai, 2007; Canosa *et al*., 2005; Darius *et al.,* 2003) were used but shown to have limitations. Thus the purpose of the study of Xue-fei *et al.*, (2009) was to establish a method to determine triclosan at trace level. High performance liquid chromatography (HPLC) and solid phase extraction method were used to determine trace levels of triclosan in wastewater. They concluded that the method for analysing triclosan in wastewater using these methods was established and that this method is suitable for the detection of limits up to 3.91 ng/L. It was also concluded that triclosan can be detected in wastewater using this method. The triclosan concentration in the raw wastewater was in the range between 533 ng/L to 774 ng/L and in the effluent from

80.14 ng/L to 249 ng/L. Thus the removal rates varied from 62.59% to 67.74% (Xue-fei *et al*., 2009).

Aufiero *et al*., (2012) tested methods for the detection of triclosan and the removal of triclosan in water using activated carbon and zeolites. The following methods were tested as possible methods of detection; Fluorescence spectroscopy, gas chromatography and high-pressure liquid chromatography. By using the fluorescence spectrometer the intensity of the samples with varying concentration appeared to have no clear trend. Thus the results were not viable because the concentration of triclosan were outside the detectable range (Aufiero *et al.,* 2012). Gas chromatography did show results but at lower concentrations the peaks were too small to read. HPLC method was the most successful method by determining triclosan concentrations at a variety of peaks (Aufiero *et al*., 2012). Thus by using the HPLC method the concentrations of Triclosan in wastewater can effectively be determined.

CHAPTER 3 – MATERIALS AND METHODS

3.1 Screening, isolation and maintenance of isolates

Effluent water samples were taken from the WWTP in Potchefstroom, North West, South Africa. Samples were taken in triplicate, designated as A, B and C. Initial screening was conducted to select the ranges for triclosan in the media. The range started at 10 mg/l triclosan and decreased until the first growth was observed.

Water samples were plated onto R2A media (Merck KGaA, Germany), with different concentrations of Triclosan (Irgasan, Sigma, Germany). Triclosan was dissolved in 95% ethanol. The concentration range consisted of 0.25 mg/L, 0.375 mg/L, 0.5 mg/L and 1 mg/L. Cultures were then streaked out onto the same concentrations to obtain pure cultures. For culture maintenance isolates were also streaked unto R2A agar with a triclosan concentration corresponding with the concentration the isolates were initially obtained from.

3.2. Primary phenotypical characterisation

Gram staining was performed on all the cultures to determine if they were Gram negative or Gram positive and the morphology of the colonies was noted (Willey *et al.,* 2008). Isolates were plated onto Cetrimide agar (Biolab Merck, Germany) to screen for candidate *Pseudomonas* species.

3.3. Phenotypically and molecular identification

Isolates can be identified by phenotypical and molecular methods. Both of these methods has proven to have reliable results. The methods of identification are described below.

3.3.1 Phenotypical identification

The following methods were performed to determine the identification of the heterotrophic plate count bacteria and included the following tests; starch hydrolysis,

Methyl Red & Voges Proskauer, indole, lactose, hydrogen sulphide, motility and urease.

3.3.2 Molecular identification

DNA extraction was isolated according to the following procedure. Isolates were grown in nutrient broth for 24 hours at 37 °C. Ten microliters (µl) of culture and 20 µl of nuclease free water (Fermentas Life Science, US) were centrifuged (Boeco Centrifuge U-32R) for one minute at 13400 rpm to obtain a pellet. This mixture was then microwaved for 2 min and centrifuged for 90 sec at 13400 rpm. The supernatant (1 µl) was then used for the next step.

Polymerase chain reaction (PCR) amplification of the 16S rRNA region was done. The mixture for the PCR consisted of the following: (a) 12.5 µl Master Mix (contains Taq DNA polymerase, dNTPs, reaction buffer, $MgCl_2$, KCL and PCR stabilizer), (Fermentas Life Science, US), (b) 0.15 µl (10 mM) forward (27F) targeting the 16S rRNA region (5'-AGAGTTTGATCMTGGCTCAG-3'), amplification length of 1465 bp, (Lane, 1991) and 0.15 µl (10mM) reverse Primer (1492R) targeting the 16S rRNA region (5'-TACGGYTACCTTGTTACGACTT-3'), amplification length of 1465 bp, (Lane, 1991), (c) 11.2 µl Nuclease free water (d) (Fermentas Life Sciences, US) and 1 µl DNA. The total volume per sample was 25 µl. The following thermal PCR cycle protocol was use: 95 °C for 300 seconds, (the next step was repeated 40 times), 95 °C for 60 seconds, 51 °C for 45 seconds, 72 °C for 110 seconds and the last cycle was 72 °C for 300 seconds by using a ICycler termal cycler (BioRad, UK).

Determination if the PCR product is present in the sample was done by Gel Electrophoresis. 1.5% Agarose gel was made up for the electrophoresis run (45 minutes) and the gel loaded with 5 µl PCR product together with 5 µl GelRed (to stain PCR product). The electrophoresis run was set up for 45 minutes and 80 Volts. Sequencing: First cleanup was done by using the Macherey-Nagel Gel and PCR cleanup kit. Thereafter PCR-Sequencing was done by making up a master mix of 4 µl Ready Reaction Premix, 2 µl BigDye Sequencing buffer, 3.2 µl Forward primer(27F), 9.8 µl nuclease free water. One microliter (1 µl) of DNA was added. The following cycle conditions were used; (a) 96 °C for 1 min (Initial denaturation), (b) 96 °C for 10 seconds (Denaturation), 50 °C for 5 seconds (Annealing), 60 °C for 4

min (Extension), (step b were repeated for 25 cycles, (c) Hold at 4 °C. Second cleanup (Zymo Research Corp) was done and samples were prepared for sequencing using the instructions of the manufacturer.

3.4. Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations were conducted to determine lag time measurement and triclosan inhibitory concentrations. Isolates were incubated for 24 hours at 37 ºC in Mueller Hinton broth (MHB), (Biolab Merck, Germany) at different concentrations of triclosan (0, 0.125, 0.25, 0.375, 0.5, 0.75, 1 µg/L). The control treatment was set up by incubating the isolates in MHB without triclosan. Bacterial growth was accessed by observing the turbidity of the medium by using a microwell plate reader (Powerwave X, Micro well plate reader). The results were noted as resistant or susceptible. After 24 hour incubation isolates were plated out on to Mueller Hinton Agar. The results were noted again as resistant, susceptible or inhibited. These tests were performed in the 96-well micro plates. The MIC was defined as the lowest concentration of drug that inhibited visible growth after 24 h of incubation at 37 ºC (Kaya & Ozbilge, 2012).

3.5. Assay for cross resistance to antibiotics

Antibiotics were tested by using the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. Seven antibiotics (Mast Group Ltd.) were used to assay for cross resistance to antibiotics. Antibiotics applied were; Penicillin G (10 µg), Vancomycin (30 µg), Streptomycin (25 µg), Erythromycin (15 µg), Trimethoprim (2.5 µg), Tetracycline (30 µg) and Amoxycillin (10 µg). Isolates were spread out by using the spread plate method onto Mueller Hinton agar (Merck, Germany). Antibiotic disks were placed on the Mueller Hinton agar containing isolates. Isolates were incubated at 37 ºC for 24 hours. 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 (NCCLS, 1999).

Table 3.1: Zone diameter interpretive standards recorded in NCCLS vol. 19 (1999).

Antibiotic	Abbreviation	Concentration	R	I	S
Vancomycin	VA30	30 µg	≤ 14	15-16	≤ 17
Erythromycin	E15	15 µg	≤ 13	14-22	≤ 23
Penicillin G	PG10	10 µg	≤ 19	20-27	≤ 28
Streptomycin	S25	25 µg	≤ 6	7-9	≤ 10
Trimethoprim	TM2,5	2,5 µg	≤ 10	11-15	≤ 16
Tetracycline	T30	30 µg	≤ 15	19-22	≤ 23
Amoxycillin	A10	10 µg	≤ 13	14-17	≤ 18

3.6. Assay for cross resistance to antibiotics in the presence of triclosan

Three antibiotics (Mast Group Ltd.) were used to assay for cross resistance to antibiotics in the presence of a variety of triclosan concentrations. The following antibiotics were used according to their resistance patterns. Antibiotics used were; Penicillin G (10 µg), Vancomycin (30 µg), Erythromycin (15 µg). Mueller Hinton agar plates were prepared with different concentrations of triclosan (0, 0.125, 0.25, 0.375, 0.5, 0.75, 1 mg/l). Isolates were spread out by using the spread plate method. Antibiotics were placed on the Mueller Hinton agar (Merck, Germany) containing the isolates. Isolates were incubated at 37 ºC for 24 hours. 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 (NCCLS, 1999).

3.7. High Performance Liquid Chromatography (HPLC)

HPLC methods were used to determine not only the presence of triclosan in the WWTP but also to quantify the levels present.

3.7.1 Preparation of water samples

Horizon technology provided a method for determining pharmaceuticals and personal care products in water by automated solid phase extraction. The method

used was SPE-DEX 4790 to automatically extract aqueous samples using SPE techniques. The system used for the solid phase extraction was the Horizon Technology SPE-DEX® 4790 Automated Extraction System, and the Envision® Platform Controller. The Atlantic™ HLB-L Disk was used for extraction purposes.

Water was sampled at the influent and effluent in triplicate from the water sewage system. Glass bottles were cleaned with methanol and water before sampling. Water sample (1 Liter) was centrifuged and filtered. One sample was treated with 500 ppb of triclosan. Eighty milligrams (80 mg) of sodium thiosulfate were added to each sample and acidified with HCl to a pH of 2-3 to reduce biological activity. 500 mg of $Na_4EDTA.2H_2O$ were added to each sample. The samples were left for a time period of 1.5 hours to equilibrate.

The Acid Purge method was then performed according to the Horizon Technologies method 4790. Table 3.2 and 3.3 illiterates the purge procedures.

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

Acid extraction method was then performed to the specifications of Horizon Technologies method 4790. The following table indicates the Acid extraction method:

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

The eluted samples were damped off by using Horizon technologies DryVap at a temperature of 37 °C. The sample was damped off to a volume of 2 ml and syringe filtered before the samples were injected into the HPLC.

HPLC equipped with a UV detector was used with a 250 x 4.6 Venusil XBP C18 (2) 5u column. The mobile phase was made up of 75 acetonitrile: 25 Milli-Q, water. The HPLC conditions were set at a flow rate of 1 ml/min. Twenty microliters (20 µl) of sample were injected into the column at 25 °C and wavelength of 280 nm (Xue-fei *et al.*, 2009).

3.8. Statistics

The inhibition zones were then used to determine the average and standard error by means of breakdown and one-way ANOVA, using STATISTICA 12 (StatSoftInc ©, 2013). Multivariate analysis, specifically Redundancy Analysis (RDA), was performed on the data using Canoco for Windows 4.5 (Ter Braak & Smilauer, 1998).

CHAPTER 4 – RESULTS

4.1 Screening and isolation of heterotrophic plate count bacteria

4.1.1 Screening and isolation

Effluent samples were taken from the wastewater treatment plant in Potchefstroom, North-West Province in South Africa. Water was specifically sampled from the effluent, before the water returns to the river after purification. Isolation of heterotrophic plate count bacteria was done on R2A media supplemented with varying concentrations (0.25, 0.375, 0.5, 1 mg/l) of triclosan. Isolates were incubated at 37 °C for 24 hours. A total of forty-four isolates were obtained. Figure 4.1 is an illustration of isolates obtained from R2A media, supplemented with 0.25 mg/l triclosan.

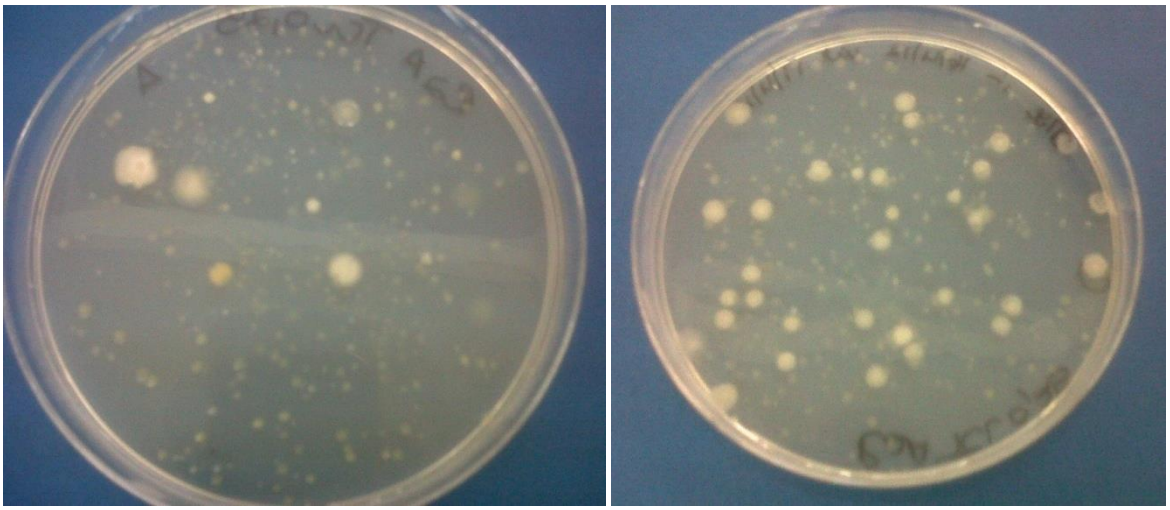

Figure 4.1: Isolates obtained from R2A supplemented with 0.25 mg/l triclosan

Fifteen isolates were obtained from 0.25 mg/l concentration of triclosan, twelve isolates obtained from 0.375 mg/l and seventeen isolates from the 0.5 mg/l triclosan concentration. No isolates were obtained from the R2A plate supplemented with 1 mg/l of triclosan.

4.1.2 Gram staining and morphology

Gram staining was performed on the isolates. The morphology of the isolates was noted according to the shape and colour of the colony. The results are recorded in Table 4.1. Samples were analysed and are indicated as samples A, B & C. The

numbering of the isolates also reflects concentration of triclosan in the original plate. The number 2 in isolate 2A1 represents the 0.25 mg/l triclosan, “A” represents the water sample and “1” represents the colony obtained from the original plate.

Table 4.1 contains the results of bacterial as well as colony morphology. Within the 44 isolates tested twenty seven were Gram positive and seventeen were Gram negative. The morphology of the isolates indicated that twenty one of the isolates were bacilli and had endospores. Twenty isolates were bacillus shaped with absence of endospores. Two isolates were bacilli with an oval shape and a thick cell wall and the cells of one isolate were coccus shaped and grouped into clusters.

Isolates 2A1 and 2A3 were slimy white colonies with a concave shape. Isolate 2A2 had big white round colonies with a concave shape. Isolates 2A4 and 3A1, had small white granular colonies. Fifteen isolates (2A7, 2A9, 2A10, 2C2, 2C3, 3A4, 3B2, 3B3, 3B4, 5A1b, 5A2, 5A5, 5B2, 5C1 and 5C2) had small white round colonies. Six of the isolates (2A6, 2A11, 3A3, 3C4, 5C3 and 5C5); no colonies could be observed and were overgrown over the petridish. Seven isolates (2B1, 2B3, 2B4, 2C1, 3A2, 3B1 and 3C2) were round clear colonies with ruffled edges. Five isolates (3C1, 3C5, 5A1a, 5A3 and 5A7) were white round flat colonies. Four isolates (5A4, 5B1, 5B3 and 5B4) were small white colonies with granular edges. One isolate (5B5) had light white small round colonies and one isolate (5C4) had clear round small colonies.

Table 4.1: Gram staining and morphology results obtained from R2A supplemented with varying concentrations of triclosan (0.25 mg/l, 0.375 mg/l, 0.5 mg/l,).

Sample nr	Gram	Cetrimide	Morphology	Colony description
2A1	+	-	Bacilli + Spore	Slimy white colonies, concave
2A2	+	+	Bacilli + Spore	Big white colonies, round, concave
2A3	+	-	Bacilli + Spore	Slimy white colonies, concave
2A4	+	-	Bacilli + Spore	Small white colonies, granular
2A6	+	-	Bacilli + Spore	Overgrown
2A7	-	-	Bacilli	Small white round colonies
2A9	-	+	Bacilli	Small white round colonies
2A10	-	-	Bacilli	Small white round colonies
2A11	-	+	Bacilli	Overgrown
2B1	+	-	Bacilli + Spore	Round clear colonies with ruffled edge
2B3	+	-	Bacilli + Spore	Round clear colonies with ruffled edge
2B4	+	-	Bacilli + Spore	Round clear colonies with ruffled edge
2C1	+	-	Bacilli oval, thick cell wall	Round clear colonies with ruffled edge
2C2	+	-	Bacilli + Spore	Small white round colonies
2C3	-	+	Bacilli	Small white round colonies
3A1	+	+	Bacilli + Spore	Small white colonies, granular
3A2	+	-	Bacilli	Round clear colonies with ruffled edge
3A3	-	-	Bacilli	Overgrown
3A4	-	-	Bacilli	Small light white round colonies
3B1	-	-	Bacilli	Round clear colonies with ruffled edge
3B2	-	+	Bacilli	Small light white round colonies
3B3	-	+	Bacilli	Small light white round colonies
3B4	-	-	Bacilli	Small light white round colonies
3C1	+	-	Bacilli + Spore	White round flat colonies
3C2	+	-	Bacilli + Spore	Round clear colonies with ruffled edge
3C4	-	-	Bacilli oval/thick cell wall	Overgrown
3C5	+	-	Bacilli + spore	White round flat colonies
5A1a	+	+	Bacilli + Spore	White round flat colonies
5A1b	-	-	Bacilli	Small round white colonies
5A2	+	+	Bacilli + Spore	Small round white colonies
5A3	-	+	Bacilli	White round flat colonies
5A4	-	+	Bacilli	Small light white round colonies
5A5	+	-	Cocci/clusters	Small white round colonies
5A7	+	-	Bacilli	White round flat colonies
5B1	+	-	Bacilli + Spore	Small white colonies with granular edges
5B2	+	-	Bacilli + Spore	Small white colonies round
5B3	+	-	Bacilli + Spore	Small white colonies with granular edges
5B4	+	-	Bacilli + Spore	Small white colonies with granular edges
5B5	+	-	Bacilli	Light white small round colonies
5C1	+	-	Bacilli + Spore	Small white colonies round
5C2	+	-	Bacilli + Spore	Small white colonies round
5C3	-	+	Bacilli	Overgrown
5C4	-	-	Bacilli	Clear round small colonies
5C5	+	-	Bacilli	Overgrown

4.1.3 Testing for possible *Pseudomonas* candidates

Previous studies indicated that the species *Pseudomonas* are resistant to triclosan. These pathogens are normally present in sewage water derived from domestic wastewater. Therefore cetrimide studies were conducted to indicate possible *Pseudomonas* species.

Isolates were plated onto cetrimide agar to screen for the presence of *Pseudomonas* species. Table 4.1 indicates the isolates obtained from the R2A plate supplemented with varying concentrations (0.25 mg/l, 0.375 mg/l, 0.5 g/l) of triclosan plated onto cetrimide agar. Twelve heterotrophic plate count bacteria isolated from the WWTP indicated the possibility of *Pseudomonas* species.

4.2 Identification of isolates obtained

4.2.1 Identification by phenotypical methods

An attempt was made to identify the heterotrophic plate count bacteria by using phenotypical tests as described in Chapter 3. Results were inconclusive and were thus excluded from this report.

4.2.2 Identification by 16S rDNA

Identification of the heterotrophic plate count bacteria was done by sequencing of the 16S rDNA amplicons by polymerase chain reaction (PCR). Figure 4.2 is an illustration of a 1.5% agarose gel of the amplicons. Gel red was added to the loading dye before the gel was loaded. 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 as described in section 3.3.2.

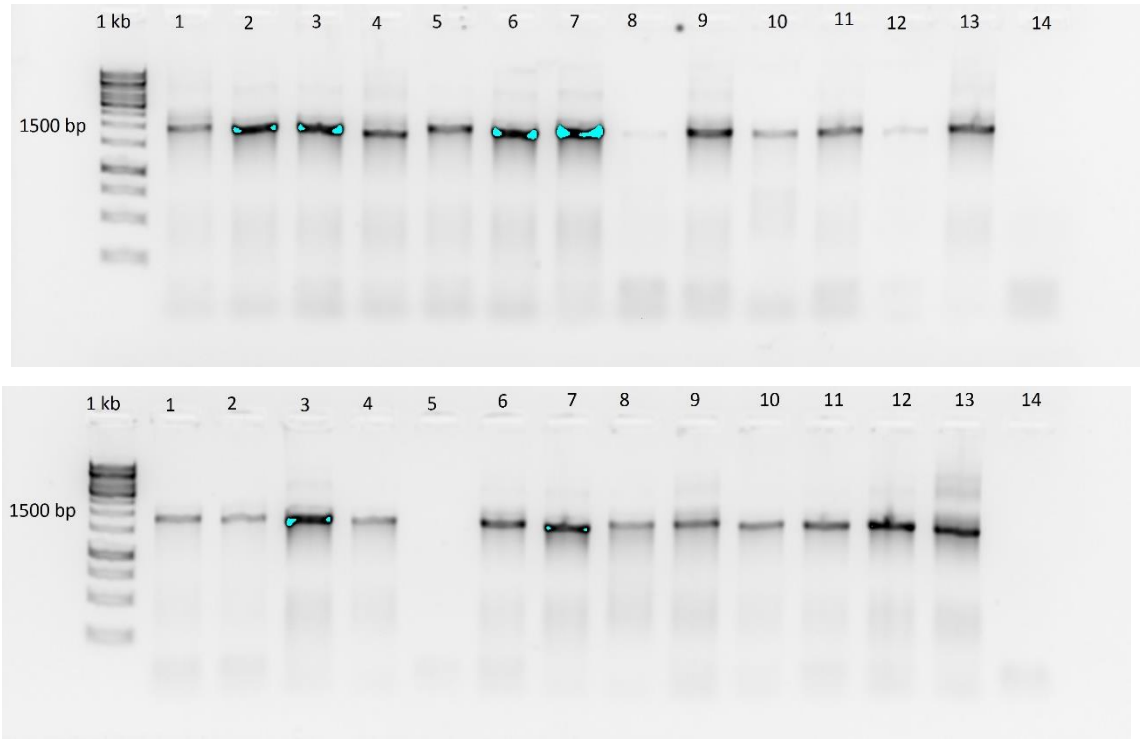

Figure 4.2: Photograph of the electrophoresis gel after amplification of the 16S rDNA

The chromatograms were viewed in Finsch TV and BlastN searched to determine the identity of the isolates. Table 4.2 indicates the species names with the isolate number obtained from the original R2A plate supplemented with triclosan. Five main genera of bacteria were identified as *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Brevibacillus* and *Enterococcus* spp.

Similarity to GenBank for the HPC isolated varied between 81 – 99 %. *Bacillus* (29) was the most dominant HPC isolated from the sewage water. Amongst these, *Bacillus thuringiensis* (7) was the most common followed by *Bacillus aerius* (5), *Bacillus cereus* (4), *Bacillus tequilensis* (4), and *Bacillus licheniformis* (4). There were also eight *Pseudomonas* spp. and four *Paenibacillus* spp.

Table 4.2: Identification of isolates by 16S rDNA amplification

Species	Isolate number	% ID
***Bacillus* spp**		
Bacillus subtilis	2A1	97
Bacillus cereus	2A2,2A423B1,3C5	89-99
Bacillus siamensis	2A3	99
Bacillus tequilensis	2A6,3C1,5A2,5A5	99
Bacillus aerius	2B1,3A2,3A4,3B2,3C2	96-99
Bacillus thuringiensis	2B3,2B4,2C2,3A1,5C2,5C3,5C4	81-99
Bacillus safensis	3B3	99
Bacillus licheniformis	3B4,3C4,5B1,5B3	99
Bacillus weihenstephanensis	5A1a	99
Bacillus stratosphericus	5C1	99
	29	
***Paenibacillus* spp**		
Paenibacillus jamilae	2C1	99
Paenibacillus cookii	2A10,5B4,5C5	88
	4	
***Pseudomonas* spp**		
Pseudomonas mosselii	2A9,2A11.2C3,5A4,5B2	94-99
Pseudomonas entomophila	5A1b	92
Pseudomonas xanthomarina	5A3,5B5	99
	8	
***Brevibacillus* spp**		
Brevibacillus parabrevis	2A7	91
	1	
***Enterococcus* spp**		
Enterococcus mundtii	3A3	99
	1	
Unknown	5A7	
	1	

4.3 Minimum inhibitory concentration (MIC)

Figure 4.3 indicates a typical growth curve obtained after isolates were supplemented with varying concentrations of triclosan. Each isolate was incubated for 24 hours at 37 ºC in Mueller Hinton broth supplemented with 0, 0.125, 0.25, 0.375, 0.5, 0.75 and 1 mg/l triclosan. The zero concentration was used as a control for each of the HPC isolates.

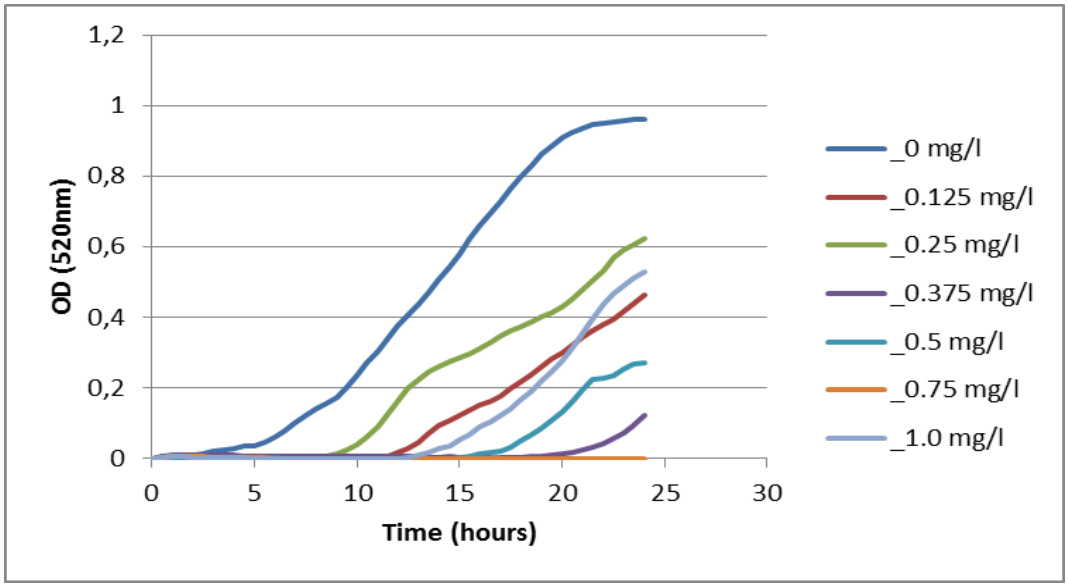

Figure 4.3: Typical growth curve data obtained after 24 hours

In Figure 4.3 the optical density was measured at 520 nm over a 24 hour period. Blue indicates the growth of the isolate that was grown in media without triclosan and had the shortest lag time (control). Forty four isolates were grown in Mueller Hinton broth supplemented with different concentrations of triclosan and incubated for 24 hours. After 24 hours in the microwell plate reader, the lag times of the isolates were measured to indicate if these isolates were susceptible or inhibited by the different concentrations of triclosan. This data determine what the minimum inhibitory concentration is for the isolates.

Table 4.3 indicates the measurements of the lag time of the isolates originally obtained from wastewater effluent supplemented with varying concentrations of triclosan. The optical density of the medium containing the isolates were measured over a period of 24 hours.

Table 4.3: Table containing lag time measured from growth curves and minimum inhibitory concentration value (concentrations are in mg/l).

Isolate	NR	[0]	[0,125]	[0,25]	[0,375]	[0,5]	[0,75]	[1]
Bacillus aerius	2B1	2,5	17	8	7,5	8	8	7,5
Bacillus aerius	3A2	1	24	10	11	11	14	24
Bacillus aerius	3A4	2,5	15	4	4	2,5	3	15
Bacillus aerius	3B2	6,5	17,5	11,5	24	24	24	24
Bacillus aerius	3C2	4	24	2	10	10	15	20
Bacillus cereus	2A2	0,5	5	0,5	0,5	0,5	0,5	0,5
Bacillus cereus	2A4	1	1	1	1	1	1	1
Bacillus cereus	3B1	2,5	2,5	2,5	2,5	2,5	2,5	2,5
Bacillus cereus	3C5	1	24	1	1	1	1	1
Bacillus licheniformis	3B4	4	14	5	12	14	15	9
Bacillus licheniformis	3C4	1	3	1	0,5	1	1	1
Bacillus licheniformis	5B1	2,5	24	6	16	24	16	24
Bacillus licheniformis	5B3	2	16	12	13	15	15	20
Bacillus safensis	3B3	1	12	5	8	10	12	9
Bacillus siamensis	2A3	2	12,5	4,5	10	11,5	12,5	22
Bacillus stratosphericus	5C1	1	3	1	1	1	1	1
Bacillus subtilis	2A1	1,5	15	24	15	11	15	15
Bacillus tequilensis	2A6	0,5	1,5	0,5	0,5	1,5	0,5	1,5
Bacillus tequilensis	3C1	2	24	15	24	24	5	7
Bacillus tequilensis	5A2	4	24	24	10	1	4	15
Bacillus tequilensis	5A5	2,5	16	9	16	24	20	24
Bacillus thuringiensis	2B3	1,5	1,5	1,5	1,5	1,5	1,5	1,5
Bacillus thuringiensis	2B4	1	1	1	1	1	1	1
Bacillus thuringiensis	2C2	1,5	14	1,5	1,5	1,5	1,5	1,5
Bacillus thuringiensis	3A1	0,5	24	1	1	1	1	1
Bacillus thuringiensis	5C2	1,5	1,5	1,5	1,5	1,5	1,5	1,5
Bacillus thuringiensis	5C3	3	24	3	24	24	24	24
Bacillus thuringiensis	5C4	4	15	4	11	8	11	13
Bacillus weihenstephanensis	5A1a	2,5	8	2,5	8	6	6	8
Brevibacillus parabrevis	2A7	3	15	5	8	15	12,5	10
Enterococcus mundtii	3A3	1,5	1,5	1,5	1,5	1,5	1,5	1,5
Paenibacillus cookii	2A10	4	0	8	11	15	0	0
Paenibacillus cookii	5B4	2,5	24	24	24	24	24	24
Paenibacillus cookii	5C5	2,5	24	2,5	2,5	2,5	2,5	2,5
Paenibacillus jamilae	2C1	2	3	2	2	2	2	2
Pseudomonas entomophila	5A1b	4	15	12	15	16	16	14
Pseudomonas mosselii	2A9	3	18	5	10	17	15	10
Pseudomonas mosselii	2A11	4	12	5	13	7	15	13
Pseudomonas mosselii	2C3	0,5	15	5,5	15	11,5	14,5	19,5
Pseudomonas mosselii	5A4	2	15	15	15	24	24	15
Pseudomonas mosselii	5B2	1	1	1	1	1	1	1
Pseudomonas xanthomarina	5A3	7	17	12	24	24	24	24
Pseudomonas xanthomarina	5B5	2,5	18	5	16	24	24	18
Unknown	5A7	2,5	2,5	2,5	2,5	2,5	2,5	2,5

The HPC isolates tested indicated lag times for the control group (0 mg/l triclosan) between 0.5 hours – 7 hours. Thereafter the lag times at 0.125 mg/l increased or

remained the same as the control group. *Bacillus aerius* (3A2), *Bacillus licheniformis* (5B1) and *Bacillus tequilensis* (5A5) indicated no change in optical density over a period of 24 hours at 1 mg/l triclosan. In other words isolates remained in a lag phase and therefor the minimum inhibitory concentration (MIC) for these isolates are regarded as 1 mg/l. *Bacillus aerius* (3B2), *Bacillus thuringiensis* (5C3) and *Pseudomonas xanthomarina* (5A3) indicated no change in the lag times measured from 0.375 mg/l up to 1 mg/l over 24 hours. This is an indication that the MIC value for these isolates are 0.375 mg/l triclosan. *Peanibacillus cookii* (5B4) indicated the lowest MIC value of 0.125 mg/l. It can be speculated that the remaining isolates had a MIC value above 1 mg/l triclosan, but further investigation must be done for the precise value.

Clear MICs were observed for isolates 3A2, 5B1 and 5A5 (<1 mg/l), 3B2, 5C3 and 5A3 (<0.375 mg/l), 5B4 (<0.125 mg/l) and presented in Table 4.3. Conflicting results were observed for 3B2, which showed no growth at triclosan concentrations of 0.125 mg/l, 0.375 mg/l and 0.5 mg/l. However growth was observed at 0.25 mg/l, 0.75 mg/l and 1 mg/l. There were also other examples (Table 4.3). The identities of these are provided in Table 4.3.

4.4 Antibiotic resistance

Isolates obtained from the original R2A supplemented with triclosan were tested against seven antibiotics using the Kirby-Bauer disc diffusion method. Antibiotics used were penicillin G (PG10), vancomycin (VA30), streptomycin (S25), erythromycin (E15), trimethoprim (TM2.5), tetracycline (T30) and amoxycillin (A10). All the numbers in brackets indicate the concentrations (µg/ml) of the antibiotics. Antibiotic discs were placed on the Mueller Hinton agar containing a lawn of the isolates. Isolates were incubated at 37 ºC for 24 hours. Inhibition zones were measured after 24 hours and placed into three different groups indicating if the isolates are resistant, susceptible or intermediate resistant to the applied antibiotics. Classification of the inhibition zone measurements can be observed in Table 3.1. Penicillin G and vancomycin were only applied to the Gram positive isolates. Streptomycin, erythromycin, trimethoprim, tetracycline and amoxycillin were applied to all the isolates.

Table 4.4 represents results obtained, and the following antibiotic resistance could be observed. Eighty percent of Gram positive isolates were resistant to penicillin G and 68% of all isolates were resistant to amoxicillin. The lowest percentage of isolates were resistant to streptomycin (9%). Thirty four percent of the isolates tested indicated intermediate resistance to tetracycline and 30% indicated intermediate resistance to erythromycin. Two percent of the isolates were intermediate resistant to streptomycin, which were also the lowest percentage (Table 4.4).

To obtain a better understanding of the antibiotic profiles multivariate analysis was conducted (Figure 4.4, 4.5, 4.6). The following variables were used: phenotypical characteristics, triclosan concentration of original plates with which isolates were obtained, growth kinetics (measurement of the lag time) and antibiotic resistance. Separate RDA were compiled to indicate the antibiotic profiles of three antibiotics (penicillin G, vancomycin and erythromycin). The RDA legend nr in Table 4.4 indicates the numbering of the isolates represented in Figure 4.4, 4.5 and 4.6. The isolates were plotted into a redundancy analysis triplot (RDA) and each RDA highlights the resistance patterns of the three main antibiotics applied.

Table 4.4: Various antibiotics were tested on the isolates originally obtained from R2A supplemented with varying concentrations of triclosan (0.25 mg/l, 0.375 mg/l, and 0.5 mg/l). Measurements were noted as I (Inhibited), S (Susceptible) or R (Resistant). The RDA number was used in Figure 4.4, 4.5 and 4.6.

Species	NR	PG10	T30	VA30	E15	S25	TM2,5	A10	RDA LEGEND NR
Bacillus subtilis	2A1	-	I	-	S	I	S	R	C1
Bacillus cereus	2A2	-	R	-	I	S	R	R	C2
Bacillus siamensis	2A3	-	R	-	S	S	S	R	C3
Bacillus cereus	2A4	R	I	S	I	S	R	R	C4
Bacillus tequilensis	2A6	-	R	-	S	S	S	S	C5
Brevibacillus parabrevis	2A7	-	I	-	I	S	S	R	C6
Pseudomonas mosselii	2A9	R	R	R	I	S	I	R	C44
Paenibacillus cookii	2A10	S	R	S	S	R	R	S	C7
Pseudomonas mosselii	2A11	R	I	R	R	S	I	R	C45
Bacillus aerius	2B1	-	S	-	S	S	S	S	C8
Bacillus thuringiensis	2B3	-	R	-	S	S	R	R	C9
Bacillus thuringiensis	2B4	-	R	-	I	S	R	R	C10
Paenibacillus jamilae	2C1	I	R	S	R	R	R	R	C11
Bacillus thuringiensis	2C2	-	R	-	I	S	S	R	C12
Pseudomonas mosselii	2C3	R	R	R	I	S	R	R	C13
Bacillus thuringiensis	3A1	R	I	S	I	S	R	R	C14
Bacillus aerius	3A2	-	R	-	R	S	S	R	C15
Enterococcus mundtii	3A3	R	R	S	S	S	R	R	C16
Bacillus aerius	3A4	R	I	R	S	S	R	R	C17
Bacillus cereus	3B1	R	R	S	S	S	R	R	C19
Bacillus aerius	3B2	R	I	I	R	S	I	R	C20
Bacillus safensis	3B3	R	S	R	S	S	I	R	C46
Bacillus licheniformis	3B4	R	S	S	S	S	R	S	C47
Bacillus tequilensis	3C1	R	-	S	S	R	-	-	C21
Bacillus aerius	3C2	-	S	-	S	S	S	R	C22
Bacillus licheniformis	3C4	I	R	S	S	S	R	R	C23
Bacillus cereus	3C5	-	I	-	I	S	R	R	C24
Bacillus weihenstephanensis	5A1a	R	R	R	R	S	R	R	C25
Pseudomonas entomophila	5A1b	R	I	R	I	S	I	R	C26
Bacillus tequilensis	5A2	R	R	S	S	S	R	R	C27
Pseudomonas xanthomarina	5A3	R	I	R	I	S	I	R	C31
Pseudomonas mosselii	5A4	R	I	R	R	S	I	I	C32
Bacillus tequilensis	5A5	-	I	-	R	R	R	S	C38
Unknown	5A7	-	I	-	I	S	R	R	C39
Bacillus licheniformis	5B1	-	S	-	S	S	S	R	C28
Pseudomonas mosselii	5B2	-	S	-	S	S	S	S	C29
Bacillus licheniformis	5B3	-	S	-	S	S	S	S	C30
Paenibacillus cookii	5B4	R	S	S	S	S	S	R	C33
Pseudomonas xanthomarina	5B5	R	R	S	R	S	I	I	C40
Bacillus stratosphericus	5C1	-	I	-	S	S	S	S	C34
Bacillus thuringiensis	5C2	R	R	I	S	S	R	R	C35
Bacillus thuringiensis	5C3	R	R	R	I	S	R	I	C41
Bacillus thuringiensis	5C4	S	I	S	S	S	S	S	C42
Total resistant to antibiotic		80%	43%	38%	18%	9%	45%	68%	
Total intermediate resistance		8%	34%	8%	30%	2%	18%	7%	

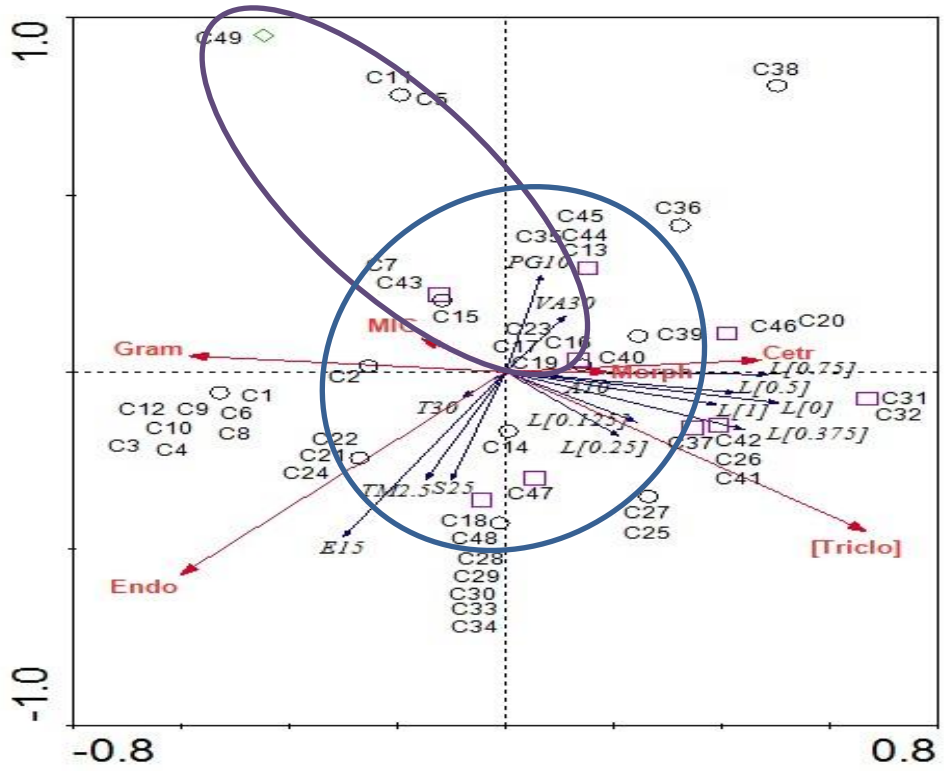

Figure 4.4: Redundancy analysis triplot- Penicillin G and triclosan effects on bacterial isolates (Black arrows indicate antibiotics used, e.g. PG10 – Penicillin G, VA30 – Vancomycin, A10 – Amoxycillin, E15 – Erythromycin, S25 – Streptomycin, TM2,5 – Trimethoprim, T30 – Tetracycline. Red arrows indicate other variables, e.g. Gram – Gram staining, MIC – Minimum inhibitory concentration, Cetr – Cetrimide agar, Morph – cell morphology, Endo – endospore. C[nr] – HPC bacteria)

Figure 4.4 represents the redundancy analysis triplot highlighting Penicillin G and triclosan concentration. Triclosan concentrations enhance as indicated by the arrow in the second quadrant. The purple circle indicates the grouping of the isolates inhibited by Penicillin G. The blue circle indicates the grouping of the isolates susceptible to Penicillin G. Grouping of the resistant isolates resistant to Penicillin G is not indicated in this figure. The reason being that the isolates which are resistant to Penicillin G are wide spread over the figure. The increase in triclosan concentration moves from the left to the right hand side of the figure.

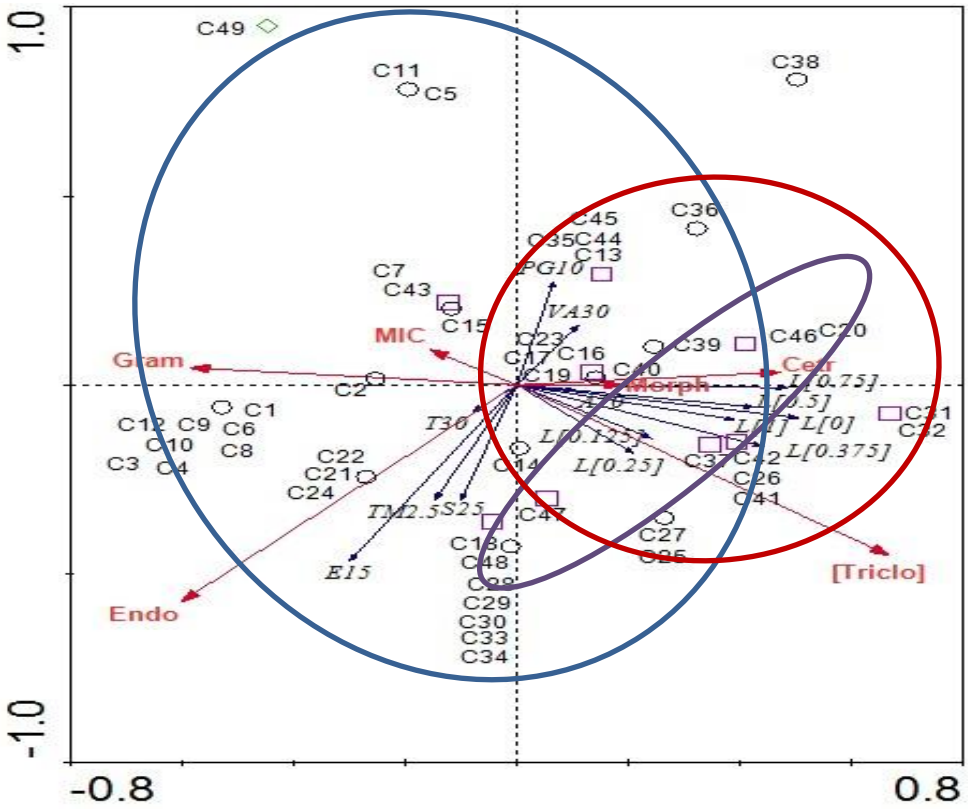

Figure 4.5: Redundancy analysis triplot – Vancomycin and triclosan effects on bacterial isolates. (Black arrows indicate antibiotics used, e.g. PG10 – Penicillin G, VA30 – Vancomycin, A10 – Amoxycillin, E15 – Erythromycin, S25 – Streptomycin, TM2,5 – Trimethoprim, T30 – Tetracycline. Red arrows indicate other variables, e.g. Gram – Gram staining, MIC – Minimum inhibitory concentration, Cetr – Cetrimide agar, Morph – cell morphology, Endo – endospore. C[nr] – HPC bacteria)

The effect of vancomycin and triclosan on the bacteria can be observed in Figure 4.5. The purple circle indicates the grouping of the isolates inhibited by Vancomycin. The blue circle indicates the grouping of the isolates susceptible to Vancomycin. The grouping of the isolates that are resistant to Vancomycin is indicated with a red circle. The triclosan concentrations enhance, as indicated by the [triclo] arrow, into the second quadrant. Vancomycin is a cell wall synthesis inhibitor and has a cidal effect on bacteria. Vancomycin only targets Gram positive organisms. Thus Vancomycin is only applied on these organisms.

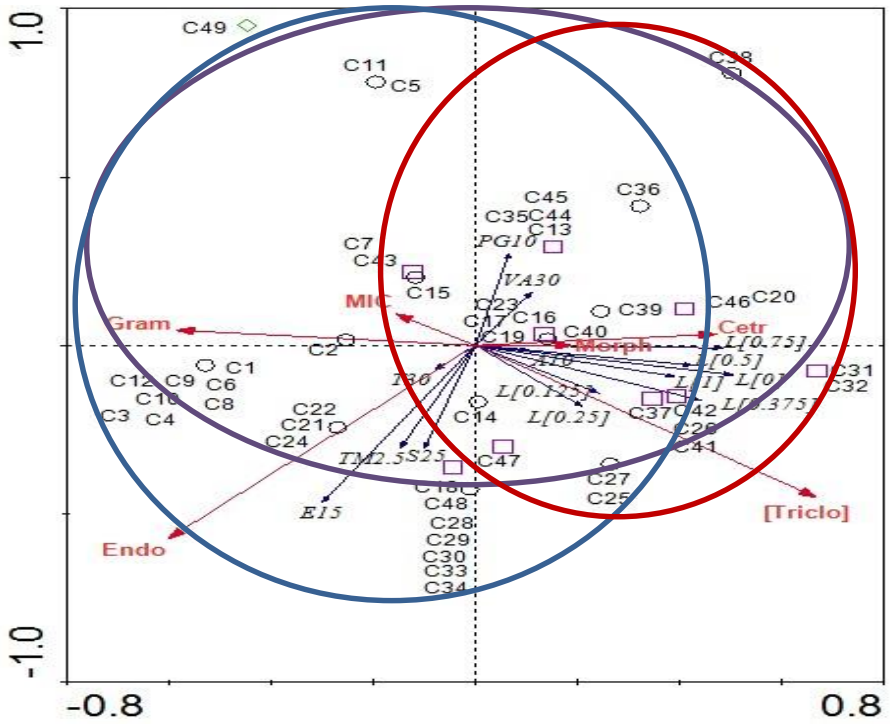

Figure 4.6: Redundancy analysis triplot – Erythromycin and triclosan effects on bacterial isolates. (Black arrows indicate antibiotics used, e.g. PG10 – Penicillin G, VA30 – Vancomycin, A10 – Amoxycillin, E15 – Erythromycin, S25 – Streptomycin, TM2,5 – Trimethoprim, T30 – Tetracycline. Red arrows indicate other variables, e.g. Gram – Gram staining, MIC – Minimum inhibitory concentration, Cetr – Cetrimide agar, Morph – cell morphology, Endo – endospore. C[nr] – HPC bacteria)

Figure 4.6 is the redundancy analysis triplot highlighting Erythromycin and Triclosan concentration. The purple circle indicates the grouping of the isolates inhibited by Erythromycin. The blue circle indicates the grouping of the isolates susceptible to Erythromycin. The grouping of the isolates that are resistant to Erythromycin is indicated with a red circle. The triclosan concentration, as indicated by the red arrow ([Trilco]) enhances into the second quadrant of the RDA. Erythromycin is a protein synthesis inhibitor and has a static effect on bacteria. Erythromycin targets Gram positive and Gram negative organisms. Erythromycin was applied to all the isolates.

4.5 Antibiotic resistance profiles in the presence of varying concentrations of triclosan

Antibiotic resistance profiles were performed in combination with varying concentrations of triclosan (0, 0.125, 0.25, 0.375, 0.5, 0.75, 1 mg/l). The antibiotics used were penicillin G, vancomycin and erythromycin. These antibiotics were chosen according to the resistance patterns observed in Figure 4.4, 4.5 and 4.6. Inhibition zones were measured after incubation of 24 hours at 37 °C.

Multivariate analysis was conducted using the measurements of the inhibition zones, phenotypical characteristics and triclosan concentration. Zone diameter interpretive standards recorded in NCCLS (1999) (Table 3.1) were used to indicate if the isolates were resistant, susceptible or induced.

4.5.1 *Bacillus* species

Redundancy analysis triplot (RDA) highlighting *Bacillus* spp*,* triclosan concentration and antibiotics resistance patterns are indicated in Figure 4.7. Antibiotics are indicated by E_Diam (erythromycin), V_Diam (vancomycin) and P_Diam (penicillin G). Triclosan is indicated by [Tricl] and the concentrations thereof are indicated in the figure legend under samples. Furthermore the following variables are also present: minimum inhibitory concentration (MIC), Gram staining (Gram) and Endospore presence (Endos).

Enterococcus mundtii is also included in this RDA plot because of the pathogenity of these bacteria and are indicated as Emun3A3 (Quadrant 2). The concentration gradient of triclosan increases to the top right hand side of Figure 4.7 (Quadrant 1). The correlation between the triclosan concentration, vancomycin and erythromycin was greater than the correlation between triclosan concentration and penicillin G. To obtain a better understanding of these results the following isolate antibiotic diameters were plotted onto a graph (Appendix E). The results were noted in table 4.7.

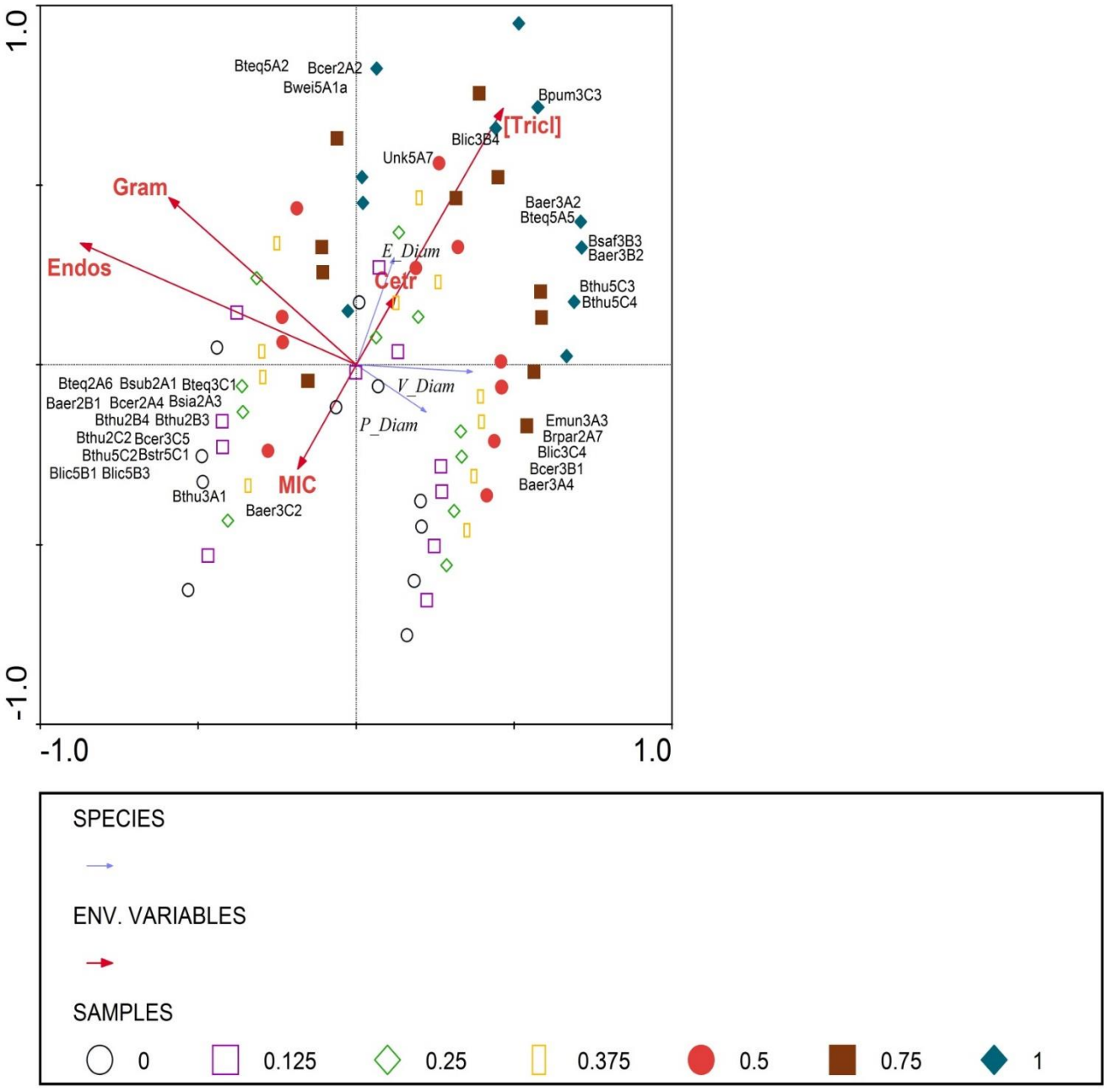

Figure 4.7: Redundancy analysis triplot (RDA) of *Bacillus* spp. highlighting Vancomycin, Penicillin G and Erythromycin. (Purple arrows indicate antibiotics used, e.g. P-_Diam – Penicillin G inhibition zone diameter, V_Diam – Vancomycin inhibition zone diameter, E_Diam – Erythromycin. Red arrows indicate other variables, e.g. Gram – Gram staining, MIC – Minimum inhibitory concentration, Cetr – Cetrimide agar, HPC bacteria are indicated by isolate name and number from original plate, e.g. Baer3B2 – *Bacillus aerius* 3B2)

Table 4.5 is an interpretation of the results presented in Figure 4.7. The table represents the data of the *Bacillus* spp and indicates synergistic or antagonistic

effect between the antibiotic and antimicrobial substance. Positive synergy as indicated in Table 4.5 was noted if the inhibition zone diameter enhances along with an increase in triclosan concentration. If the inhibition zone diameter decreases and the triclosan concentration increases the results were noted as antagonism. Results were noted as "no effect" if no pronounced effect could be observed between the antibiotic inhibition zone diameter and triclosan concentrations.

Table 4.5: Antibiotic resistant profiles in the presence of varying concentrations of triclosan indicating synergy/antagonism for the *Bacillus* spp group

S+ - positive synergism, A+ - positive antagonism, - - no effect

Isolate	**NR**	**[Low]**			**[High]**		
***Bacillus* spp**		**E**	**VA**	**PG**	**E**	**VA**	**PG**
Bacillus aerius	2B1	S+	S+	S+	S+	S+	-
Bacillus aerius	3A2	-	-	-	A+	A+	-
Bacillus aerius	3A4	S+	S+	S+	A+	A+	S+
Bacillus aerius	3B2	S+	S+	S+	S+	A+	A+
Bacillus aerius	3C2	-	-	-	-	-	-
Bacillus cereus	2A2	S+	S+	-	-	A+	-
Bacillus cereus	2A4	S+	-	-	S+	-	-
Bacillus cereus	3B1	S+	S+	-	A+	A+	-
Bacillus cereus	3C5	S+	S+	-	A+	A+	-
Bacillus licheniformis	3B4	S+	S+	S+	S+	A+	A+
Bacillus licheniformis	3C4	S+	A+	S+	A+	A+	-
Bacillus licheniformis	5B1	S+	-	-	S+	A+	-
Bacillus licheniformis	5B3	S+	S+	S+	S+	A+	-
Bacillus safensis	3B3	S+	S+	-	S+	S+	-
Bacillus siamensis	2A3	S+	S+	A+	A+	A+	-
Bacillus stratosphericus	5C1	-	S+	-	A+	A+	-
Bacillus subtilis	2A1	S+	S+	S+	S+	S+	A+
Bacillus tequilensis	2A6	S+	S+	S+	A+	A+	A+
Bacillus tequilensis	3C1	S+	S+	S+	S+	S+	A+
Bacillus tequilensis	5A2	S+	S+	S+	S+	A+	S+
Bacillus tequilensis	5A5	S+	S+	S+	A+	-	-
Bacillus thuringiensis	2B3	S+	S+	-	-	S+	-
Bacillus thuringiensis	2B4	S+	S+	-	S+	S+	-
Bacillus thuringiensis	2C2	S+	A+	-	A+	A+	-
Bacillus thuringiensis	3A1	S+	S+	-	A+	A+	-
Bacillus thuringiensis	5C2	S+	S+	-	A+	-	-
Bacillus thuringiensis	5C3	A+	A+	A+	S+	A+	-
Bacillus thuringiensis	5C4	S+	-	-	A+	-	-
Bacillus weihenstephanensis	5A1a	S+	S+	-	A+	S+	-
Brevibacillus parabrevis	2A7	S+	S+	-	S+	S+	S+
Enterococcus mundtii	3A3	S+	S+	S+	S+	S+	S+
Unknown	5A7	S+	S+	S+	A+	S+	A+

The above table contain the antibiotic resistant profiles in the presences of a combination of antibiotics of the *Bacillus* spp identified. Bar plots of the antibiotic inhibition zone diameters were constructed and are presented in Appendix (E). In the above table the range of concentrations was divided into lower (0 mg/l – 0.375 mg/l) and higher 0.375 mg/l – 1 mg/l) triclosan concentrations.

4.5.1.1 Triclosan and Erythromycin

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of erythromycin a synergistic effect can be observed within the *Bacillus* spp group (inclucing *Brevibacillus parabrevis, Enterococcus mundtii*, Unknown), with the exemption of *Bacillus aerius* (3A2 and 3C2) and *Bacillus stratophericus* which indicated no synergy or antagonism. *Bacillus thuringiensis* indicated an antagonistic effect in the presence of triclosan/erythromycin combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of erythromycin the *Bacillus* spp group (inclucing *Brevibacillus parabrevis, Enterococcus mundtii*, Unknown) indicated different profile patterns. *Bacillus aerius* (2B1, 3B2), *Bacillus cereus* (2A4), *Bacillus licheniformis* (3B4, 5B1 and 5B3), *Bacillus safensis* (3B3) *Bacillus subtilis* (2A1), *Bacillus tequilensis* (3C1, 5A2), *Bacillus thuringiensis* (2B4, 5C3), *Brevibacillus parabrevis* (2A7), *Enterococcus mundtii* (3A3) indicated a synergistic effect. *Bacillus aerius* (3A2, 3A4), *Bacillus cereus* (3B1, 3C5), *Bacillus licheniformis* (3C4), *Bacillus siamensis* (2A3), *Bacillus strato*sphericus (5C1), *Bacillus tequilensis* (2A6, 5A2, and 5A5), *Bacillus thuringiensis* (2C2, 3A1, 5C2, and 5C4), *Bacillus weihenstephanensis* (5A1b) and Unknown (5A7) indicated antagonism at higher concentrations of triclosan. No synergistic of antagonistic effect could be observed at *Bacillus aerius* (3C2), *Bacillus cereus* (2A2) and *Bacillus thuringiensis* (2B3) at higher triclosan concentrations.

4.5.1.2 Triclosan and Vancomycin

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of vancomycin a synergistic effect can be observed within the *Bacillus* spp group (inclucing *Brevibacillus parabrevis, Enterococcus mundtii*, Unknown). With the exemption of *Bacillus licheniformis* (3C4) and *Bacillus thuringiensis* (2C2 and 5C3) which indicated an antagonistic effect. *Bacillus aerius* (3A2 and 3C2), *Bacillus*

cereus (3B1) and *Bacillus thuringiensis* indicated no effect in the presence of triclosan/vancomycin combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of vancomycin the *Bacillus* spp group (inclucing *Brevibacillus parabrevis, Enterococcus mundtii*, Unknown) indicated different profile patterns. *Bacillus aerius* (2B1), *Bacillus safensis* (3B3) *Bacillus subtilis* (2A1), *Bacillus tequilensis* (3C1), *Bacillus thuringiensis* (2B4, 2B3), *Bacillus weihenstephanensis* (5A1b), *Brevibacillus parabrevis* (2A7), *Enterococcus mundtii* (3A3) and Unknown (5A7) indicated a synergistic effect. *Bacillus aerius* (3A2, 3A4 and 3B2), *Bacillus cereus* (2A2, 3B1 and 3C5), *Bacillus licheniformis* (3B4, 3C4, 5B1 and 5B3), *Bacillus siamensis* (2A3), *Bacillus stratosphericus* (5C1), *Bacillus tequilensis* (2A6, 5A2) and *Bacillus thuringiensis* (2C2, 3A1, and 5C3), indicated antagonism at higher concentrations of triclosan. No synergistic or antagonistic effect could be observed at *Bacillus aerius* (3C2), *Bacillus cereus* (2A4), *Bacillus tequilensis* (5A5) and *Bacillus thuringiensis* (5C2 and 5C4) at higher triclosan concentrations.

4.5.1.3 Triclosan and Penicillin G

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of penicillin G a synergistic effect can be observed within the *Bacillus* spp group (inclucing *Brevibacillus parabrevis, Enterococcus mundtii*, Unknown), with the exemption of *Bacillus siamensis* (2A3) and *Bacillus thuringiensis* (5C3) which indicated an antagonistic effect. *Bacillus aerius* (3A2 and 3C2), *Bacillus cereus* (2A2, 2A4, 3B1 and 3C5), *Bacillus licheniformis* (5B1), *Bacillus safensis* (3B3), *Bacillus stratosphericus* (5C1), *Bacillus thuringiensis (*2B3, 2B4, 2C2, 3A1, 5C2 and 5C4), *Bacillus weihenstephanensis* (5A1b) and *Brevibacillus parabrevis* (2A7) indicated no effect in the presence of triclosan/penicillin G combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of penicillin G the *Bacillus* spp group (inclucing *Brevibacillus parabrevis, Enterococcus mundtii*, Unknown) indicated different profile patterns. *Bacillus aerius* (3A4), *Bacillus tequilensis* (5A2), *Brevibacillus parabrevis* (2A7) and *Enterococcus mundtii* (3A3) indicated a synergistic effect. *Bacillus aerius* (3B2), *Bacillus licheniformis* (3B4), *Bacillus subtilis* (2A1), *Bacillus tequilensis* (2A6, 3C1) and Unknown (5A7) indicated

antagonism at higher concentrations of triclosan. No synergistic or antagonistic effect could be observed throughout the rest of the *Bacillus* spp group at higher triclosan concentrations.

4.5.2 *Paenibacillus* species

Figure 4.8 illustrates the redundancy analysis triplot highlighting *Paenibacillus* spp, triclosan concentration and antibiotics resistance patterns. Antibiotics are indicated by E_Diam (erythromycin), V_Diam (vancomycin) and P_Diam (penicillin G). Triclosan are indicated by [Tricl] and the concentrations thereof are indicated in the figure legend under samples. Furthermore the following variables are also present, minimum inhibitory concentration (MIC), Gram staining (Gram) and Endospore presence (Endos). Concentration of triclosan enhances from the centre of this figure towards the lower part of figure 4.8, and the different *Paenibacillus* spp with triclosan concentration enhances from the top of the figure towards the lower part of the figure along the triclosan concentration gradient.

Triclosan concentration and vancomycin showed a greater correlation than erythromycin and penicillin G in Figure 4.8. The correlation between the triclosan concentration Penicillin G and erythromycin is not that pronounced. To obtain a better understanding of these results the isolate antibiotic diameters were plotted onto a graph (Appendix E). The results were noted in Table 4.6.

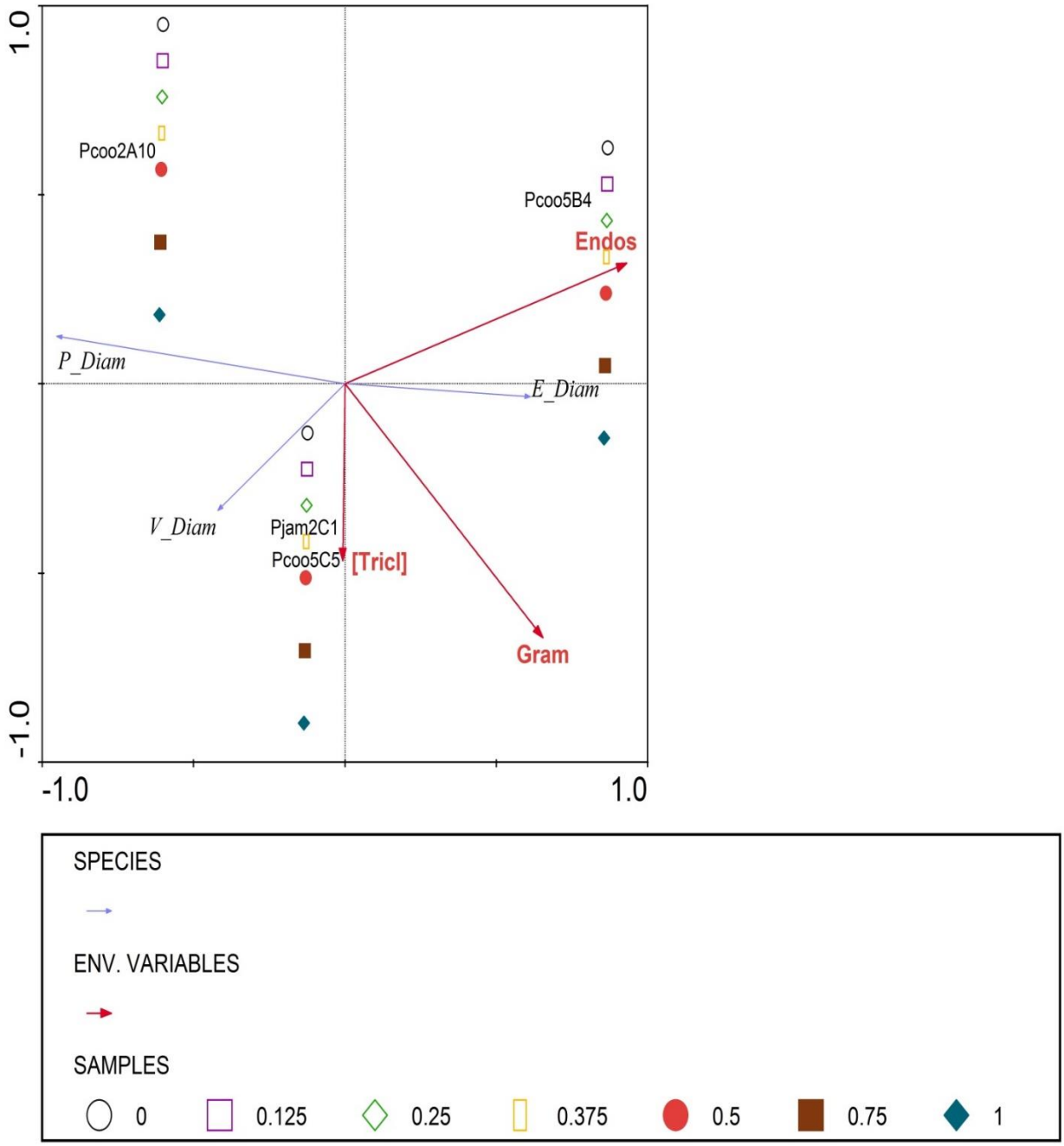

Figure 4.8: Redundancy analysis triplot (RDA) of *Paenibacillus* spp. highlighting Vancomycin, Penicillin G and Erythromycin. (Purple arrows indicate antibiotics used, e.g. P-_Diam – Penicillin G inhibition zone diameter, V_Diam – Vancomycin inhibition zone diameter, E_Diam – Erythromycin. Red arrows indicate other variables, e.g. Gram – Gram staining, MIC – Minimum inhibitory concentration, Cetr – Cetrimide agar, HPC bacteria are indicated by isolate name and number from original plate, e.g. Baer3B2 – *Bacillus aerius* 3B2)

Table 4.6 is an interpretation of the results presented in Figure 4.8. The table represents the data of the *Paenibacillus* spp and indicates synergistic or antagonistic effect between the antibiotic and antimicrobial substance. Positive synergy as

indicated in Table 4.6 was noted if the inhibition zone diameter enhances along with an increase in triclosan concentration. If the inhibition zone diameter decreases and the triclosan concentration increases the results were noted as antagonism. Results were noted as "no effect" if no pronounced effect could be observed between the antibiotic inhibition zone diameter and triclosan concentrations.

Table 4.6: Antibiotic resistance profiles in the presence of varying concentrations of triclosan indicating synergy/antagonism for the *Paenibacillus* spp group

S+ - positive synergism, A+ - positive antagonism, - - no effect

Isolate	**NR**	**[Low]**			**[High]**		
***Paenibacillus* spp**		**E**	**VA**	**PG**	**E**	**VA**	**PG**
Paenibacillus cookii	2A10	-	-	-	-	-	A+
Paenibacillus cookii	5B4	-	S+	-	S+	A+	-
Paenibacillus cookii	5C5	S+	-	-	-	-	-
Paenibacillus jamilae	2C1	A+	S+	A+	-	A+	S+

The above table contains the antibiotic resistance profiles in the presence of a combination of antibiotics of the *Paenibacillus* spp identified. Bar plots of the antibiotic inhibition zone diameters were constructed and is presented in Appendix (E). In the above table the range of concentrations were divided into lower (0 mg/l – 0.375 mg/l) and higher 0.375 mg/l – 1 mg/l) triclosan concentrations. Antibiotics are indicated by the following abbreviations: Erythromycin – E; Vancomycin – VA; Penicillin G – PG.

4.5.2.1 Triclosan and Erythromycin

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of erythromycin only one species indicated a synergistic effect (*Paenibacillus cookii*, 5C5). *Paenibacillus jamilae* (2C1) indicated antagonism at lower triclosan concentrations in the presence of erythromycin. *Paenibacillus cookii* (2A10 and 5B4) indicated no antagonism or synergy effect in the presence of triclosan/erythromycin combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of erythromycin, with the *Paenibacillus* spp group there was no synergism or

antagonism in the presence of triclosan/erythromycin combination with the exemption of *Paenibacillus cookii* (5B4) which indicated a synergistic effect.

4.5.2.2 Triclosan and Vancomycin

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of vancomycin no antagonistic effect could be observed within the *Paenibacillus* spp group. *Paenibacillus cookii* (5B4) and *Paenibacillus jamilae* (2C1) indicated a synergistic effect. *Paenibacillus cookii* (5B4 and 5C5) indicated no effect in the presence of triclosan/vancomycin combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of vancomycin no synergistic effect could be observed within the *Paenibacillus* spp group. *Paenibacillus cookii* (5B4) and *Paenibacillus jamilae* (2C1) indicated an antagonistic effect. *Paenibacillus cookii* (5B4 and 5C5) indicated no effect in the presence of triclosan/vancomycin combination.

4.5.2.3 Triclosan and Penicillin G

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of penicillin G an antagonistic effect can be observed within *Paenibacillus jamilae* (2C1). The remaining *Paenibacillus* spp group (*Paenibacillus cookii*) indicated no effect in the presence of triclosan/penicillin G combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of penicillin G the *Paenibacillus* spp group indicated different profile patterns. *Paenibacillus cookii* (2A10) indicated an antagonistic effect at higher triclosan concentrations. *Paenibacillus jamilae* indicated synergy at higher triclosan concentrations. The rest of the *Paenibacillus* spp group indicated no effect in the presence of triclosan/penicillin G combination.

4.5.3 *Pseudomonas* species

Figure 4.9 illustrates the redundancy analysis triplot highlighting *Pseudomonas spp*, triclosan concentration and antibiotics resistance patterns. Concentration of triclosan enhances from the centre of this figure towards the lower part of figure 4.9 (third quadrant) It can be observed in the RDA plot that the Triclosan concentration,

Vancomycin and Erythromycin showed correlation between each other. Penicillin G and triclosan concentration showed no correlation between each other. To obtain a better understanding of these results the following isolate antibiotic diameters were plotted onto a graph (Appendix E). These results were noted in Table 4.7.

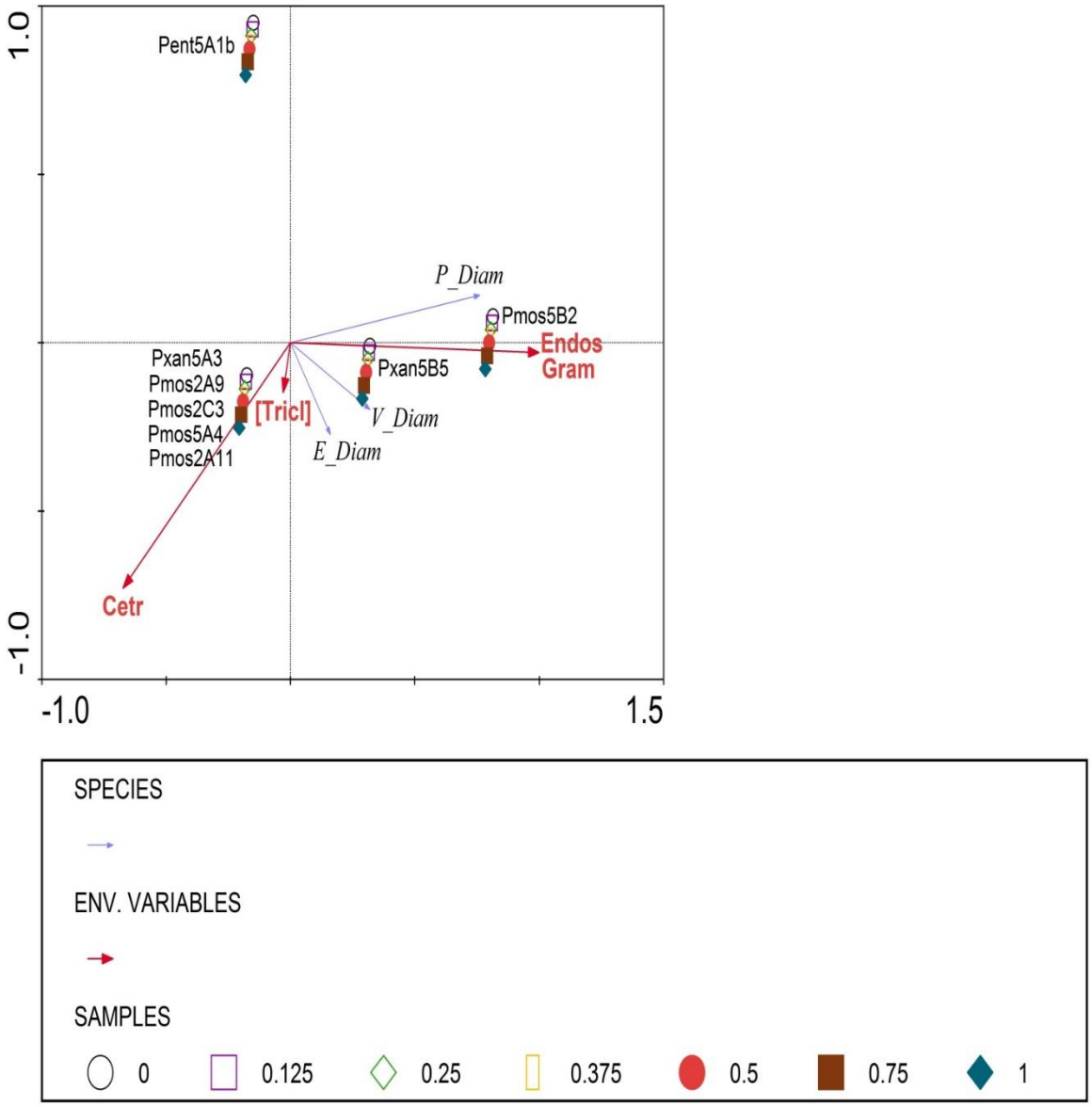

Figure 4.9: Redundancy analysis triplot (RDA) of *Pseudomonas* spp. highlighting Vancomycin, Penicillin G and Erythromycin. (Purple arrows indicate antibiotics used, e.g. P-_Diam – Penicillin G inhibition zone diameter, V_Diam – Vancomycin inhibition zone diameter, E_Diam – Erythromycin. Red arrows indicate other variables, e.g. Gram – Gram staining, MIC – Minimum inhibitory concentration, Cetr – Cetrimide agar, HPC bacteria are indicated by isolate name and number from original plate, e.g. Baer3B2 – *Bacillus aerius* 3B2)

The following table (Table 4.7) is an interpretation of the results presented in Figure 4.9. The table represents the data of the *Pseudomonas* spp and indicates synergistic or antagonistic effect between the antibiotic and antimicrobial substance. Positive synergy as indicated in Table 4.7 was noted if the inhibition zone diameter enhances along with an increase in triclosan concentration. If the inhibition zone diameter decreases and the triclosan concentration increases the results were noted as antagonism. Results were noted as "no effect" if no pronounced effect could be observed between the antibiotic inhibition zone diameter and triclosan concentrations.

Table 4.7: Antibiotic resistance profiles in the presence of varying concentrations of triclosan indicating synergy/antagonism for the *Pseudomonas* spp group

S+ - positive synergism, A+ - positive antagonism, - - no effect

Isolate	**NR**	**[Low]**			**[High]**		
***Pseudomonas* spp**		**E**	**VA**	**PG**	**E**	**VA**	**PG**
Pseudomonas entomophila	5A1b	S+	S+	A+	-	-	-
Pseudomonas mosselii	2A9	S+	S+	-	A+	-	-
Pseudomonas mosselii	2A11	S+	S+	S+	-	-	-
Pseudomonas mosselii	2C3	-	-	-	S+	A+	-
Pseudomonas mosselii	5A4	S+	S+	-	A+	A+	-
Pseudomonas mosselii	5B2	S+	S+	S+	A+	-	A+
Pseudomonas xanthomarina	5A3	S+	S+	S+	A+	A+	-
Pseudomonas xanthomarina	5B5	S+	S+	-	A+	A+	-

Table 4.7 contains the antibiotic resistance profiles in the presence of a combination of antibiotics of the *Pseudomonas* spp identified. Bar plots of the antibiotic inhibition zone diameters were constructed and are presented in Appendix (E). In the above table the range of concentrations were divided into lower (0 mg/l – 0.375 mg/l) and higher 0.375 mg/l – 1 mg/l) triclosan concentrations. Antibiotics are indicated by the following abbreviations: Erythromycin – E; Vancomycin – VA; Penicillin G – PG.

4.5.3.1 Triclosan and Erythromycin

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of erythromycin only one species indicated no effect (*Pseudomonas mosselii*, 2C3). The remaining isolate in the *Pseudomonas* spp group indicated a synergistic effect at lower triclosan concentrations in the presence of erythromycin.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of erythromycin within the *Pseudomonas* spp group most of the isolates indicated an antagonistic effect in the presence of triclosan/erythromycin combination, with the exemption of *Pseudomonas mosseliii* (2C3) which indicated a synergistic effect. *Pseudomonas entomophila* (5A1b) and *Pseudomonas mosselii* (2A11) indicated no effect at higher concentrations of triclosan in the presence of erythromycin.

4.5.3.2 Triclosan and Vancomycin

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of vancomycin no antagonistic effect could be observed within the *Pseudomonas* spp group. All of the isolates indicated synergy at lower triclosan concentration with the exemption of Pseudomonas mosselii (2C3). No antagonistic effect could be observed.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of vancomycin no synergistic effect could be observed within the *Pseudomonas* spp group. *Pseudomonas mosselii* (2C3, 5A4, 5A3 and 5B5) indicated an antagonistic effect. *Pseudomonas entomophila (5A1b), Pseudomonas mosselii (*2A9, 2A11 and 2A9 and 5B2*)* indicated no effect in the presence of triclosan/vancomycin combination.

4.5.3.3 Triclosan and Penicillin G

At lower triclosan concentrations (0 mg/l – 0.375 mg/l) and in the presence of penicillin G an antagonistic effect can be observed within *Pseudomonas entomophila* (5A1b). *Pseudomonas mosselii* (2A11 and 5B2) and *Pseudomonas xanthomarina* (5A3) indicated a synergistic effect at lower triclosan concentrations. The remaining *Paenibacillus* spp group (*Pseudomonas mosselii and Pseudomonas xanthomarina*) indicated no effect in the presence of triclosan/penicillin G combination.

At higher triclosan concentrations (0.375 mg/l – 1 mg/l) and in the presence of penicillin G the *Pseudomonas* spp group indicated different profile patterns. *Pseudomonas mosselii* (5B2) indicated an antagonistic effect at higher triclosan

concentrations. The remaining *Pseudomonas* spp indicated no effect in the presence of triclosan/penicillin G combination.

4.5.3 Summary of antibiotic resistant profiles in the presence of varying concentrations of triclosan

The *Bacillus* spp group indicated more synergy at lower triclosan concentrations and in the presence of erythromycin. At higher triclosan concentrations synergy and antagonism was observed. A more synergistic effect was observed at lower triclosan concentrations and in the presence of vancomycin. Antagonism was more present in the higher triclosan concentrations and in the presence of vancomycin. The *Bacillus* spp group indicated more synergy at lower triclosan concentrations and in the presence of penicillin G and more antagonism at higher triclosan concentrations. The penicillin G/triclosan combination indicated a more "no effect" throughout the triclosan concentration range than the other antibiotics.

The *Paenibacillus* spp group indicated that there was a more "no effect" than a synergistic or antagonistic effect. But more synergy could be observed at lower triclosan concentrations and antagonism at higher triclosan concentrations and in the presence of vancomycin. The *Pseudomonas* spp group indicated synergy at lower triclosan concentrations in the presence of erythromycin and antagonism at higher triclosan concentrations. Vancomycin indicated the same patterns as erythromycin for the *Pseudomans* spp group. The *Pseudomonas* spp group had a greater "no effect" at higher and lower triclosan in the presence of penicillin G but some synergy was observed at lower triclosan concentration.

4.6 High performance liquid chromatography

Water samples were taken from the wastewater treatment plant and treated according to the methods mentioned in chapter 3.7. Four water samples were taken from each treatment site to perform the method in triplicate and one water sample was also spiked with 500ppm triclosan. This was done to determine triclosan efficiency.

The method was optimised to ensure specific retention time (according to literature) for triclosan by injecting different concentrations of triclosan in the HPLC. Time was observed as 10.196 min (Figure 4.10).

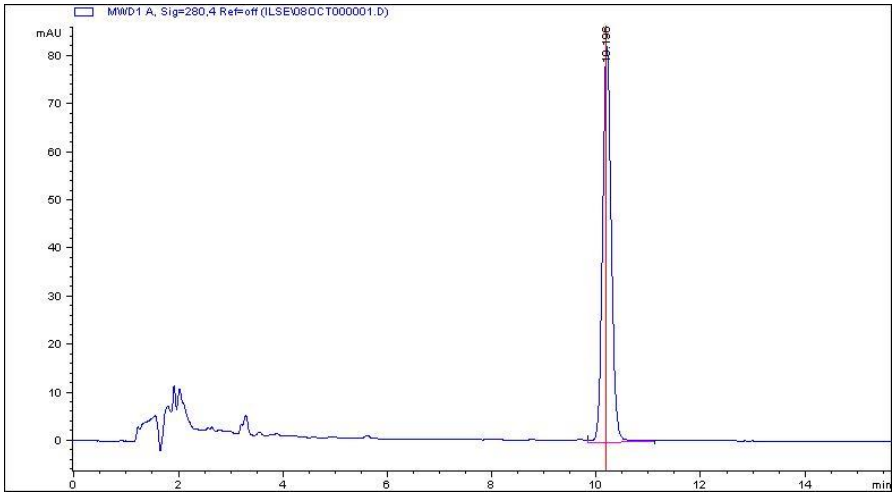

Figure 4.10: Chromatogram showing the retention time peak at 10.196 min

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

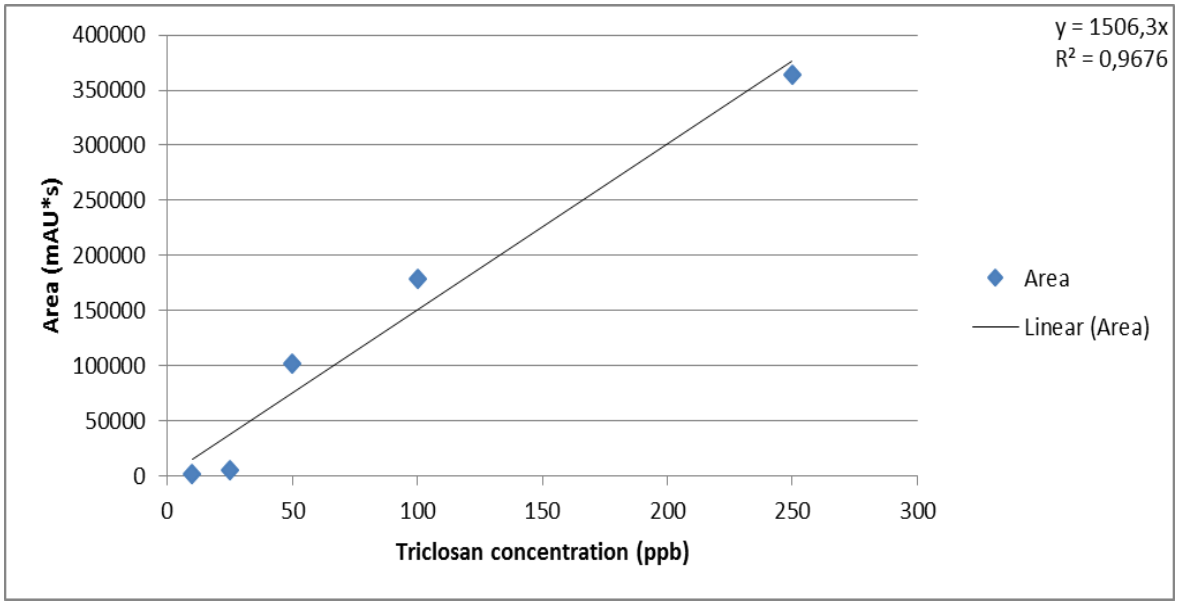

Figure 4.11: Triclosan concentration gradient (0, 25, 50, 100, 250 ppm)

Water Samples were taken from the wastewater treatment plant in Potchefstroom, North-West province, South Africa. The different sites were as follows:

1. Inflow – This site is situated after filtration of the wastewater. This specific site was chosen to ensure that only water is sampled and to measure the triclosan concentration before treatment of the wastewater.
2. Before chlorination – Water samples were taken after sedimentation before any chlorination to determine if there is a reduction in triclosan after flocculation.
3. Effluent – This site was sampled twice so as to determine what the levels of triclosan are during a rainy and dry season. It is also essential to know what the levels of triclosan are before the treated wastewater leaves the wastewater treatment plant as it enters our water system.

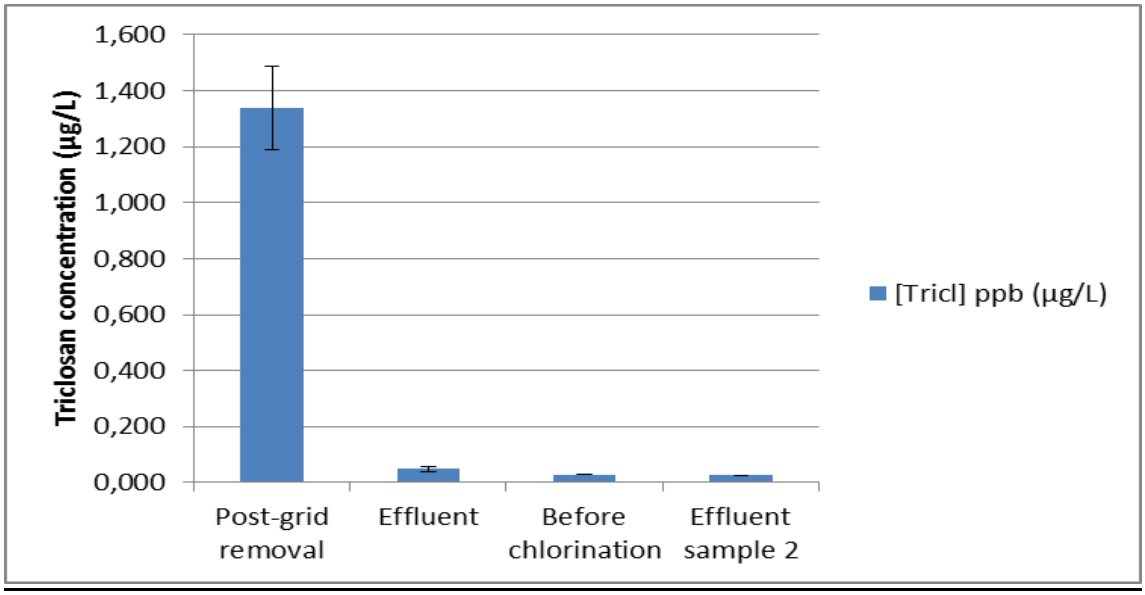

Figure 4.12: Concentration of triclosan measured in the wastewater treatment plant

The levels of triclosan were determined by plotting the area obtained after HPLC onto the triclosan standard curve (Figure 4.11). Water sampled after post-grid removal had high concentrations of triclosan whereas post-grid removal 1 had the highest concentration of 1.488 ppb of triclosan (Figure 4.12). Effluent water samples taken during the rainy season had a much lower concentration of triclosan whereas Effluent 1 was measured at 0.058 ppm and the lowest was Effluent 2 of 0.026 ppb (Figure 4.12).

Water samples were taken after sedimentation and before chlorination to determine if the triclosan settles in the sludge and or reacts with chlorine to produce other products. The concentration of triclosan ranged between 0.027 ppb and 0.029 ppb. Samples were collected from the wastewater treatment plant effluent as a replicate sample. The triclosan concentrations ranged between 0.026 ppb and 0.027 ppb.

The triclosan concentration of the inflow was higher than the rest of the sampling areas and varied between 1.488 ppb (as the highest concentration). During the rainy season the effluent values (Effluent 1 and 2) were slightly higher than the second sampling of the effluent (2Effluent 1 – 2Effluent 3). The highest concentration of triclosan was in Effluent 1 (0.058 ppb). Triclosan concentration of the samples taken before chlorination was less than the Effluent 1-2 samples but more or less the same concentration of the second effluent samples (2Effluent 1-3).

4.6 Summary of results

Effluent samples were taken from the WWTP in Potchefstroom. Heterotrophic plate count bacteria were grown in the presence of varying concentrations of triclosan. Out of the forty four isolates obtained twenty seven tested Gram positive and seventeen Gram negative. Morphology of the isolates indicated that most of the heterotrophic plate count bacteria were bacillus shaped. Twelve isolates tested positive on cetrimide agar for possible *Pseudomonas* spp. Identification of the heterotrophic plate count bacteria isolated indicated that twenty nine of the isolates were *Bacillus* spp. Four isolates were identified as *Paenibacillus* spp, eight *Pseudomonas* spp, one *Brevibacillus* spp, one *Enterococcus* spp and one isolate could not be identified.

The minimum inhibitory concentration of most of the isolates indicated greater than 1 mg/l triclosan. *Bacillus aerius* (3A2), *Bacillus licheniformis* (5B1) and *Bacillus tequilensis* (5A5) had a MIC value of 1 mg/l triclosan. *Bacillus aerius* (3B2), *Bacillus thuringiensis* (5C3) and *Pseudomonas xanthomarina* (5A3) had a MIC value of 0.375 mg/l triclosan. *Paenibacillus cookii* (5B4) had the lowest MIC value of 0.125 mg/l triclosan.

Out of the seven antibiotics tested, erythromycin, vancomycin and penicillin G indicated greater antibiotic resistance profiles. The resistance profiles of these antibiotics tests correlate to the triclosan concentrations. Antibiotic resistance profiles in the presence of varying concentrations of triclosan indicated that the *Bacillus* spp group indicated more synergy at lower triclosan concentrations and in the presence of erythromycin. At higher triclosan concentrations synergy and antagonism were observed. A greater synergistic effect was observed at lower triclosan concentrations and in the presence of vancomycin. Antagonism was more present in the higher triclosan concentrations and in the presence of vancomycin. The *Bacillus* spp group indicated more synergy at lower triclosan concentrations and in the presence of penicillin G and more antagonism at higher triclosan concentrations. The penicillin G/triclosan combination indicated a more "no effect" throughout the triclosan concentration range than the other antibiotics.

The *Paenibacillus* spp group indicated that there was more "no effect" than a synergistic or antagonistic effect. But more synergy could be observed at lower triclosan concentrations and antagonism at higher triclosan concentrations and in the presence of vancomycin. The *Pseudomonas* spp group indicated synergy at lower triclosan concentrations in the presence of erythromycin and antagonism at higher triclosan concentrations. Vancomycin indicated the same patterns as erythromycin for the *Pseudomonas* spp group. The *Pseudomonas* spp group had a greater "no effect" at higher and lower triclosan in the presence of penicillin G but some synergy was observed at lower triclosan concentration. HPLC values indicated the presence of triclosan in the wastewater. The triclosan concentration decreases through the WWTP but small concentrations enter our water bodies.

CHAPTER 5 – DISCUSSION

5.1 Screening and isolation of heterotrophic plate count bacteria

Triclosan resistant heterotrophic plate count bacteria was found in the effluent stream of the wastewater treatment plant. These isolates was able to grow in the presence of triclosan up to a concentration of 0.5 mg/l and no growth was observed at 1 mg/l triclosan concentration. A total of forty-four (44) isolates were obtained and identified. Gram staining indicated that there were a wide spread of Gram positive (27 isolates) and Gram negative isolates (17 isolates). Colony morphology varied from small white granular colonies, round ruffled edge clear colonies, small white round colonies, white round flat colonies to big white concave colonies.

Twelve possible *Pseudomonas* candidates were found when a cetrimide screening test was done. However, identification of the isolates indicated that only eight of the isolates were positive *Pseudomonas* spp. The remaining isolates were identified as *Bacillus* spp. Studies by Tkachenko *et al.,* (2007) indicated that *Pseudomonas* spp and *E.coli* showed correlation between triclosan and quinolone resistance and this resistance encodes for the multi-efflux pump in these bacteria. *Pseudomonas* spp isolated from sewage water only grew on triclosan concentration of 0.25 mg/l and 0.5 mg/l.

5.2 Identification of isolates obtained

Phenotypical and molecular methods were used to determine the identification of the isolates. Phenotypical methods had inconclusive results thus molecular methods were used to determine the identifications of the isolates. Table 4.2 indicates the identification of these isolates and five different genus groups could be identified as *Bacillus* spp*, Paenibacillus* spp*, Pseudomonas* spp*, Brevibacillus* spp and *Enterococcus* spp*.*

Pseudomonas spp were identified as the following species, *Pseudomonas entomophila*, *P. mosselii*, *and P. xanthomarina*. Chains of carbohydrates on the

outside of the region give specific antigen identity to the whole cell (Brenner *et al*., 2005). This creates an effective barrier against the passage of molecules over the membrane, such as antibiotics, although it is permeable to nutrients affected by the presence of porins (Brenner *et al*., 2005). *Pseudomonas* plasmids can either act as fertility factors or may impart resistance to various agents. Resistance to antibiotics and other antibacterial compounds are some of the properties which these plasmids encode for. Compounds include: carbenicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, tetracycline, tobramycin and sulfanomides. Plasmids also encode for resistance to chemical and physical agents, bateriophage propagation and bateriocins (Brenner *et al*., 2005). This resistance to antibiotics is of importance in human health because of the fact that *Pseudomonas* spp are serious opportunistic pathogens.

Bacillus are rod-shaped cells which can be straight or curved. They can survive in a variety of conditions which include aerobic, anaerobic and facultative anaerobic conditions and are also psychrophilic, thermophilic, acidophilic, alkaliphilic and some are halophilic. Most of this genus are isolated from soil, but can occur in water, food and clinical specimens. *Bacillus* endospores are resistant to heat, radiation, disinfectants and are therefore problematic in hospitals, pharmaceutical products and food. These bacteria are in most cases not pathogenic except for *Bacillus anthracis* and *Bacillus thurgiensis*. *Bacillus* spp isolated in this study were identified as *Bacillus subtilis, Bacillus cereus, Bacillus siamensis, Bacillus tequilensis, Bacillus aerius, Bacillus thuringiensis, Bacillus safensis, Bacillus licheniformis, Bacillus weihenstephanens* and *Bacillus stratophericus*.

Only one isolate obtained indicated faecal origin and was identified as *Enterococcus mundtii*. This species was isolated from the effluent water at the wastewater treatment plant. *Enterococcus* spp are commonly found in the human intestinal flora, and are also important pathogens causing serious infections. They are facultative anaerobes and can function in a wide variety of environments. They contain intrinsic and acquired resistance to antibiotics because they contain penicillin-binding proteins and therefore are able to synthesize cell components in the presence of vancomycin (Deshpande *et al*., 2007).

5.3 Minimum inhibitory concentration (MIC)

Overall the minimum inhibitory concentrations of the organisms indicate the ability to survive in the presence of varying concentrations of triclosan. The minimum inhibitory concentration of most of the isolates indicated greater than 1 mg/l triclosan. Further studies need to be done in order to determine the precise MIC of the isolates. Only three isolates had a minimum inhibitory concentration of 1 mg/l, three isolates had a MIC of 0.375 mg/l and the lowest MIC was 0.125 mg/l (one isolate). This indicates that the correct use of triclosan concentration is of importance.

Different organisms react differently to a specific concentration of triclosan and therefore the precise concentration needs to be determined to ensure correct application of antibiotics. Previous studies done by Brenwald & Fraise (2003), indicated that at lower concentration, triclosan appears bacteriostatic and that isolates of MRSA with a low triclosan resistance had a MIC value between 2 mg/l to 4 mg/l. But in an article published by Tuffnell *et al*., (1987) it was stated that MRSA was sensitive to a concentration of triclosan between the values of 0.1 – 2 mg/l. In 2007 an article published by Rodriquez *et al.,* (2007) stated the importance of the correct use of antimicrobials and that the applied concentration of these products is of great importance and that the incorrect concentration use will result in antimicrobial resistance (Rodriquez, 2007).

5.4 Triclosan and antibiotic resistance patterns.

5.4.1 Antibiotic resistance

Out of the seven antibiotics tested, erythromycin, vancomycin and penicillin G indicated greater antibiotic resistance profiles. The resistance profiles of these antibiotics tested correlate to the triclosan concentrations. The vancomycin and penicillin G are cell synthesis inhibitors and erythromycin is a protein synthesis inhibitor. Literature indicates that triclosan acts as a biocide with multiple cytoplasmic and membrane targets (Brenwald & Fraise, 2003). Redundancy analysis triplot highlighting Penicillin G and triclosan indicates that triclosan concentrations of the isolates obtained from the original plates increase from the left

to the right hand side of the triplot. Penicillin G is a cell wall synthesis inhibitor and has a cidal effect on bacteria. Penicillin G only targets Gram positive organisms and was thus only applied on these organisms. Resistant bacteria were scattered over the figure but most of the resistance patterns lie to the right hand side of the figure indicating that there is a relation between the increasing triclosan concentrations and penicillin G.

Resistance profiles of the isolates in the presence of vancomycin are indicated in the Redundancy analysis triplot highlighting vancomycin and triclosan. Triclosan concentrations of the isolates obtained from the original plates enhance from the left to the right hand side of the triplot. Vancomycin is a cell wall synthesis inhibitor and has a cidal effect on bacteria. Vancomycin only targets Gram positive organisms and was thus only applied on these organisms. The increase in triclosan concentration enhances from the left towards the right hand side of the figure. Most of the resistance patterns lie to the right hand side of the figure indicating that there is a relation between the increasing triclosan concentrations and vancomycin.

Redundancy analysis triplot highlighting erythromycin and triclosan indicates that triclosan concentrations of the isolates obtained from the original plates enhance from the left to the right hand side of the triplot. Erythromycin is a protein synthesis inhibitor and has a static effect on bacteria. Erythromycin targets Gram positive and Gram negative organisms and was thus applied on all the isolates. Most of the resistance patterns lie to the right hand side of the figure indicating that there is a relation between the increasing triclosan concentrations and vancomycin.

As stated by Rodriquez *et al*., (2007) the importance of the correct use of antimicrobials and applied concentration of these products is of great importance and incorrect concentration used results in antimicrobial resistance. Brenwald & Fraise (2003) indicated isolates become bacteriostatic at lower triclosan concentrations. The antibiotic resistance observed indicates a relation between triclosan concentration and antibiotic use. Cell wall targeting antibiotics (penicillin G and vancomycin) show more pronounced relation with the triclosan concentration. Relation to triclosan concentration is not as apparent with the antibiotic (erythromycin), targeting protein synthesis.

5.4.2 Antibiotic resistance profiles with varying concentrations of triclosan

5.4.2.1 *Bacillus* species

Antibiotic resistance profiles in the presence of varying concentrations of triclosan indicated that the *Bacillus* spp group exhibited more synergy at lower triclosan concentrations and in the presence of erythromycin. This indicates that applying the antibiotic and antimicrobial simultaneously enhances the cidal effect. In a study by Tabak *et al*. (2009), it was observed that exposure of *Salmonella typhimurium* to triclosan before exposure to antibiotics resulted in a reduction of viable cells and that this reduction was dependent on the concentrations of triclosan and the antibiotic. It was also noted that applying the drugs simultaneously an additive effect was observed at lower concentrations and synergy at higher concentrations of triclosan and the applied antibiotic (Tabak *et al*., 2009). Synergy between antibiotics and antimicrobials were also studied by Tong *et al*., (2011). These authors observed that the combination of these drug and the concentrations thereof will determine the efficacy of the treatment. It was also observed that different antimicrobial combinations will deliver different synergy results because of the antimicrobial target mechanisms (Tong *et al*., 2009). In the 1 mg/l triclosan the susceptibility towards erythromycin was the highest. This demonstrates that these antimicrobial combinations were most effective at inhibiting these microorganisms. At higher triclosan concentrations synergy and antagonism was observed.

A synergistic effect was observed at lower triclosan concentrations and in the presence of vancomycin. Antagonism was more pronounced at higher triclosan concentrations and in the presence of vancomycin. Thus the effect of antimicrobials and antibiotics lowered the inhibition zone diameter. It could be speculated that the bacteria were able to remove these substances from the cell. This might be due to the efflux pump mechanism that enabled the bacteria to pump these substances from the cell. According to a review by Schweizer (2001) triclosan and antibiotics share multidrug efflux systems and the combination of these two drugs causes multiple efflux systems to be switched on. In contrast to multidrug efflux pump mechanisms, antagonism could also be due to combining antimicrobials that have different antimicrobial mechanisms (Tong *et al*., 2009). It was observed by Tong *et al*., (2009) that the use of different antimicrobials with different target mechanisms

enhances the cidal effect on the bacteria. The authors observed that vancomycin alters the permeability of the cell wall and according to Brenwald & Fraise (2003) triclosan targets multiple cytoplasmic and membrane targets. In the present study it could thus be speculated that an additive effect is present between the antibiotic and antimicrobial. At 0.75 mg/l triclosan the susceptibility towards vancomycin was the highest. This demonstrates these antimicrobial combinations were most effective at inhibiting these microorganisms (BIRG1).

The *Bacillus* spp group indicated more synergy at lower triclosan concentrations and in the presence of penicillin G and more antagonism at higher triclosan concentrations. The penicillin G/triclosan combination indicated that a more "no effect" existed throughout the triclosan concentration range than the other antibiotics.

5.4.2.2 *Paenibacillus* species

The *Paenibacillus* spp group indicated that there was more "no effect" than a synergistic or antagonistic effect. But more synergy could be observed at lower triclosan concentrations and antagonism at higher triclosan concentrations and in the presence of vancomycin. This indicates that applying the antibiotic and antimicrobial simultaneously enhances the cidal effect. In a study done by Tabak *et al*., (2009) it was stated that exposure of the bacteria to triclosan before exposure to antibiotic resulted in a reduction of viable cells and that this reduction was dependent on the concentrations of triclosan and the antibiotic. It was also noted that applying the drugs simultaneously an additive effect was observed at lower concentrations and synergy at higher concentrations of triclosan and the applied antibiotic (Tabak *et al.,* 2009). Synergy between antibiotics and antimicrobials was also studied by Tong *et al*. (2011). These authors observed that the combination of these drugs and the concentrations thereof will depend the efficacy of the drug. It was also observed that different antimicrobial combinations will deliver different synergy results because of the antimicrobial target mechanisms (Tong *et al*., 2009). In the 1 mg/l triclosan region, the susceptibility towards erythromycin was the highest. This demonstrates that these antimicrobial combinations were most effective at inhibiting these microorganisms.

An antagonistic effect between vancomycin (30 µg) and triclosan was observed at higher concentrations. Thus the effect of antimicrobials and antibiotics lowered the inhibition zone diameter. It could be speculated that the bacteria were able to remove these substances from the cell. This might be due to the efflux pump mechanism that enabled the bacteria to pump these substances from the cell. According to a review by Schweizer (2001) triclosan and antibiotics share multidrug efflux systems and the combination of these two drugs causes multiple efflux systems to be switched on. In contrast to multidrug efflux pump mechanisms, antagonism could also be due to combining antimicrobials that have different antimicrobial mechanisms (Tong *et al*., 2009).

The *Paenibacillus* spp group exhibited resistance towards penicillin G (10µg) throughout the triclosan concentration range and no additive effect between the antibiotic and antimicrobial could be observed. It could be speculated that the bacteria were able to remove these substances from the cell. This might be due to the efflux pump mechanism that enabled the bacteria to pump these substances from the cell. According to a review by Schweizer (2001) triclosan and antibiotics share multidrug efflux systems and the combination of these two drugs causes multiple efflux systems to be switched on. In contrast to multidrug efflux pump mechanisms, antagonism could also be due to combining antimicrobials that have different antimicrobial mechanisms (Tong *et al*., 2009).

5.4.2.3 *Pseudomonas* species

The *Pseudomonas* spp group indicated synergy at lower triclosan concentrations in the presence of erythromycin and antagonism at higher triclosan concentrations. This indicates that applying the antibiotic and antimicrobial simultaneously enhances the cidal effect. In a study done by Tabak *et al*., (2009) it was observed that exposure of *Salmonella typhimurium* to triclosan before exposure to antibiotic resulted in a reduction of viable cells and that this reduction was dependent on the concentrations of triclosan and the antibiotic. It was also noted that applying the drugs simultaneously an additive effect was observed at lower concentrations and synergy at higher concentrations of triclosan and the applied antibiotic (Tabak *et al*., 2009). Synergy between antibiotics and antimicrobials was also studied by Tong *et al.* (2011). These authors observed that the combination of these drugs and the

concentrations thereof will determine the efficacy of the drug. It was also observed that different antimicrobial combinations will deliver different synergy results because of the antimicrobial target mechanisms (Tong *et al*., 2009). In the present study at 0.75 mg/l triclosan the susceptibility towards erythromycin was the highest. This demonstrates that these antimicrobial combinations were most effective at inhibiting these microorganisms.

At higher triclosan concentrations an antagonistic effect is observed. It could be speculated that the bacteria were able to remove these substances from the cell. This might be due to the efflux pump mechanism that enabled the bacteria to pump these substances from the cell. According to a review by Schweizer (2001) triclosan and antibiotics share multidrug efflux systems and the combination of these two drugs causes multiple efflux systems to be switched on. In contrast to multidrug efflux pumps mechanisms, antagonism could also be due to combining antimicrobials that have different antimicrobial mechanisms (Tong *et al*., 2009).

Vancomycin indicated the same patterns as erythromycin for the *Pseudomonas* spp group. The *Pseudomonas* spp group had a greater “no effect” at higher and lower triclosan in the presence of penicillin G but some synergy was observed at lower triclosan concentration. This might be due to the efflux pump mechanism that enabled the bacteria to pump these substances from the cell. According to a review by Schweizer (2001), *Pseudomonas aeruginosa* is resistant to triclosan but resistance could further be enhanced when adding other antimicrobials. It is speculated that this might be due to the efflux pump mechanism that enabled the bacteria to pump these substances from the cell. According to a review by Schweizer (2001) triclosan and antibiotics share multidrug efflux systems and the combination of these two drugs causes multiple efflux systems to be switched on. In contrast to multidrug efflux pumps mechanisms, antagonism could also be due to combining antimicrobials that have different antimicrobial mechanisms (Tong *et al.,* 2009).

To summarize the antibiotic resistance profiles in the presence of varying concentrations of triclosan, it was observed that the susceptibility of the bacteria towards the antimicrobial/antibiotics enhances. This is due to the fact that bacteria

might develop resistance mechanisms in the presence of more than one antimicrobial. Literature states (McBain & Gilbert, 2011) that the main resistance mechanism which bacteria develop is the *fabI* gene that encodes the enol reductase for fatty acid synthesis (McBain & Gilbert, 2011). Bacteria are also able to use efflux pumps and in the presence of unwanted substances more efflux pumps can be activated (McBain & Gilbert, 2011). The more the bacteria are exposed to the antimicrobials the more likely they become to acquire resistance mechanisms (Rodriquez, 2007) and it was stated by Aiello (2003) that triclosan acts as a substrate for multidrug efflux pumps and selection of pump mutations.

5.5 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography was used not only to determine if triclosan is present in our wastewater, but also to quantify the levels present. The levels of triclosan at the inflow of the Tlokwe WWTP were determined to quantify the concentrations of triclosan entering the plant. According to Glaser (2004) 95 % of uses for triclosan are in consumer products and personal care products and ends up in wastewater systems. Thus triclosan in the WWTP of Tlokwe could be from domestic wastewater. Levels measured indicate a triclosan concentration in the influent at the wastewater treatment plant up to 1.488 ppb. It was reported by Adolfson-Erici *et al*., (2002); Lindström *et al.,* (2002); Singer *et al*., (2002); Bester, (2005) and cited in Chen *et al.,* 2011 that triclosan concentrations between 1 – 10 bbp enters wastewater treatment plants.

Effluent samples taken during the rainy season were slightly higher than the samples taken in the dry season. Rain could have caused this increase in triclosan concentration but the highest effluent triclosan concentration was 0.04 ppb. Although a reduction of triclosan is visible there is still levels of triclosan that enter the river after treatment of the wastewater. The removal of triclosan was reported up to 90% in WWTP's by using conventional activated sludge processes (Adolfson-Erici *et al*., 2002; Lindström *et al.,* 2002; Singer *et al*., 2002; Bester, 2005 and cited in Chen *et al.,* 2010). Inefficient removal of triclosan could result in accumulation of these antimicrobials in fresh water bodies.

A reduction in triclosan concentrations is observed through the WWTP. It could be speculated that the triclosan is partly removed by the WWTP processes. Alternatively triclosan remains in the sludge of the WWTP. According to Tsai (2008) triclosan is soluble in ethanol, methanol, diethyl ether and strong basic solutions and is only slightly soluble in water (Tsai, 2008). Therefore triclosan could remain in the sludge after the sedimentation process. But literature also stipulates that triclosan can react with chlorine through photodegradation to form end products such as dioxins and dibenzofurans (Aranami & Readman, 2007). From the data obtained it could be speculated that certain levels of triclosan remain in the sludge and that certain levels react with chlorine, but further investigation is required to clarify the fate of triclosan in the water at the WWTP.

As previously stated, five main genera were isolated from the Tlokwe WWTP in Potchefstroom. They were *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Enterococcus* and *Brevibacterium*. Within the genus *Bacillus*, some of the species may be regarded as opportunistic pathogens and have been associated with bacteremia, meningitis, wound infections and eye and ear infections. These species include *Bacillus subtilis* and *Bacillus licheniformis* whereas *Bacillus cereus* causes food poisoning (Brener *et al*., 2005). *Paenibacillus* spp are not regarded as pathogenic organisms but research by Roux *et al*. (2008) observed that this genus was isolated from human urine and cerebrospinal fluid. *Paenibacillus*, though isolated from these medical samples, was not clearly associated with an infection or disease condition. Species from this genus included *Paenibacillus massiliensis*, *P. illinoisensis*, *P. xylanilyticus*, *P. barcinonensis*, *P. pabuli* and *P. amylolyticus (*Roux *et al.,* 2008*).* Further study regarding the potential pathogenicity of *Paenibacillus* may be warranted.

Pseudomonas spp are opportunistic pathogens and cause severe infections in immunocompromised hosts, pulmonary disease, bronchiectasis and fibrosis (Feldman *et al*., 1998). Whereas the other isolates can be considered as opportunistic pathogens it is worthwhile to note that *Enterococcus mundtii* could be used as a probiotic control agent in the prevention of mastitis in cows Repizo *et al*., 2014). *Brevibacillus parabrevis* is not considered pathogenic and several studies

indicate application of this species in biocontrol (Che *et al.,* 2015; Zimmer *et al.,* 2013) and bioremediation (Bao *et al*., 2012; Reddy *et al.,* 2010).

These bacteria are exposed to varying levels of triclosan at wastewater treatment works. Literature states that bacteria develop antibiotic resistance mechanisms in the presence of triclosan or other antimicrobials. This is cause for concern as these HPC bacteria as well as levels of triclosan enter our natural water bodies.

CHAPTER 6 –CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion:

In the present study the aim of the study was to identify and determine antibiotic resistance profiles of triclosan tolerant heterotrophic plate count bacterial isolates from sewage influent and effluent. Five objectives were made to reach the aim of this study and are discussed according to the objective.

6.1.1 Isolation and identification of triclosan tolerant bacteria by using phenotypical and molecular methods

Triclosan tolerant heterotrophic plate count bacteria were isolated at the effluent of the Tlokwe WWTP in Potchefstroom, South Africa. These bacterial species are resistant to high concentrations of triclosan and can grow in the presence of triclosan up to a concentration of 0.5 mg/l. A total of forty four isolates that were resistant to triclosan levels were obtained. Isolates were identified to Species level by means of 16S rDNA amplification (molecular methods). Isolates could not be identified by phenotypical methods as the results obtained were inconclusive. Five main genera were identified namely, *Bacillus, Paenibacillus, Pseudomonas, Brevibacillus* and *Enterococcus*. It is concluded that triclosan tolerant heterotrophic plate count bacteria are able to grow in the presence of varying concentrations of triclosan and that they are resistant to these levels of triclosan. The main cause of concern is thus that they enter our natural water bodies together with levels of triclosan.

6.1.2 Determination of minimum inhibitory concentrations (MIC)

A great deal of the heterotrophic plate count bacteria isolated from the WWTP had MICs greater than 1 mg/l triclosan. MICs were observed for seven isolates out of the forty four bacteria isolated. Three had a MIC of 1 mg/l, three had a MIC of 0.375 mg/l and one isolate indicated a MIC of 0.125 mg/l triclosan. The remaining bacteria had MICs >1 mg/l. Conflicting results were observed for isolates that showed no growth at certain triclosan concentrations and growth at latter concentrations. Previous

research stated that applying the incorrect concentration might result in bacteria altering resistance mechanisms. It is therefore essential to determine the MIC of specific bacteria to apply the correct antimicrobial concentrations.

6.1.3 Assay for cross-resistance to selected antibiotics

Antibiotic resistance patterns observed indicated that resistance towards selected antibiotics enhances in the presence of triclosan. It was determined that cell wall synthesis inhibitor antibiotics and protein synthesis inhibitor antibiotics indicated enhanced resistance in the presence of varying triclosan concentrations. The main antibiotics used were penicillin G, vancomycin and erythromycin.

6.1.4 To determine if antibiotic resistance patterns enhance in the presence of varying concentrations of triclosan

The combination of antimicrobials indicated that at certain triclosan concentrations synergism or antagonism is observed. Synergism and antagonism is observed within the bacteria investigated at higher and lower triclosan concentrations. The importance of applying the correct concentration and combination of antimicrobials is observed. Antibiotic resistance profiles in the presence of varying concentrations of triclosan indicated that the susceptibility of the bacteria towards the antimicrobial/antibiotics enhances. This might be due to the fact that bacteria develop resistance mechanisms in the presence of more than one antimicrobial. It is speculated that the presence of antimicrobials in wastewater contributes to bacteria altering resistance mechanism.

6.1.5 Measurement of triclosan concentrations and reduction in sewage water by using HPLC methods

Levels of triclosan were found at the WWTP and are cause for concern. Levels at the influent were higher than the effluent which is an indication that a certain level of triclosan was removed. A decrease in triclosan concentrations was observed through the WWTP. Levels of triclosan decreased at sedimentation. Due to the fact that triclosan is insoluble in water, it is speculated that triclosan could be present in

the sludge. Furthermore triclosan might react with chlorine as stated previously in the literature study. This might lead to decreased levels of triclosan at the effluent. Thus we conclude that triclosan is present in our WWTP and that a certain level of triclosan leaves the WWTP. Triclosan concentrations end up in our natural water bodies and are cause for concern for the environment and human health. This presence of triclosan might cause antibiotic resistance in bacteria.

6.1.6 Conclusion

In conclusion triclosan tolerant heterotrophic plate count bacteria were isolated from the water at the WWTP and are resistant to antibiotics at certain levels of triclosan. Triclosan was also found in the WWTP and is released into our natural water bodies. These levels are causing bacteria to become resistant to antibiotics and are a risk to the environment. Further studies need to be conducted to find possible methods to remove triclosan out of the WWTP before the water is released into the environment.

6.2 Recommendations

6.2.1 Isolation and identification of triclosan tolerant bacteria by using phenotypical and molecular methods

In the present study heterotrophic plate count bacteria were sampled from WWTP effluent water. These HPC bacteria were isolated in the presence of varying concentrations of triclosan. It is recommended that further investigation is necessary to observe tolerance towards other antimicrobials e.g. chlorhexidine, chloroxylenol, ethyl alcohol and triclocarban. It is recommended that the other antimicrobials need to be investigated to determine the antimicrobial resistance patterns.

6.2.2 Determination of minimum inhibitory concentrations (MIC)

It was observed in the present study that minimum inhibitory concentrations of HPC bacteria isolated had MICs >1 mg/l triclosan. Growth curves of bacteria were obtained after 24 hours. It is recommended that the triclosan concentrations applied in the present study should be enhanced to obtain the exact MIC. Furthermore it is

recommended that the optical density is measured over longer periods of time in order to obtain a better growth curve.

6.2.3 Assay for cross-resistance to selected antibiotics and to determine if antibiotic resistance patterns enhance in the presence of varying concentrations of triclosan

Antimicrobial resistance mechanisms are present in bacteria. It is recommended that further investigation should be done to determine the precise resistance mechanisms the bacteria use to alter their resistance towards antimicrobials. These resistance mechanisms could contribute to determine the correct antimicrobial combinations for treatment of patients. Better understanding of these mechanisms will be useful to determine correct concentrations and combinations of antimicrobials.

6.2.4 Measurement of triclosan concentrations and reduction in sewage water by using HPLC methods

Tlokwe WWTP in the present study obtained a high Green drop rating in the previous audit done by Department Water and Sanitation. The present study might assist WWTPs nationally, to determine triclosan levels in the wastewater. Furthermore the role of chlorine and organic matter needs to be investigated as to determine the fate of triclosan within a WWTP. Better understanding of the fate of triclosan could assist in altering treatment processes accordingly. It is also recommended that more sample points should be investigated at various steps in the WWTP to determine the triclosan levels. These sampling points should include the water bodies as triclosan is released into the environment. It is recommended that further investigation should be done so as to determine the effect of triclosan on environmental water bodies and the aquatic ecosystem.

Previous literature states that sludge from a WWTP contains triclosan and is used as agricultural fertilizer. Therefor it is recommended that the fate of triclosan is determined in agricultural practices as well as the effect of triclosan on the environment.

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APPENDIX A

Table A: Isolates obtained from R2A supplemented with 0.25mg/l ticlosan plated onto Cetrimide agar to test for *Pseudomonas* species.

Sample nr	Cetrimide
2A1	-
2A2	+
2A3	-
2A4	-
2A5	-
2A6	-
2A7	-
2A9	+
2A10	-
2A11	+
2B1	-
2B2	-
2B3	-
2B4	-
2C1	-
2C2	-
2C3	+

APPENDIX B:

Table B: Isolates obtained from R2A supplemented with 0.375mg/l ticlosan plated onto Cetrimide agar to test for *Pseudomonas* species.

Sample nr	Cetrimide
3A1	+
3A2	-
3A3	-
3A4	-
3A5	-
3B1	-
3B2	+
3B3	+
3B4	-
3C1	-
3C2	-
3C3	+
3C4	-
3C5	-

APPENDIX C:

Table C: Isolates obtained from R2A supplemented with 0.5mg/l ticlosan plated onto Cetrimide agar to test for *Pseudomonas* species.

Sample nr	Cetrimide
5A1a	+
5A1b	-
5A2	+
5A3	+
5A4	+
5A5	-
5A7	-
5B1	-
5B2	-
5B3	-
5B4	-
5B5	-
5C1	-
5C2	-
5C3	+
5C4	-
5C5	-

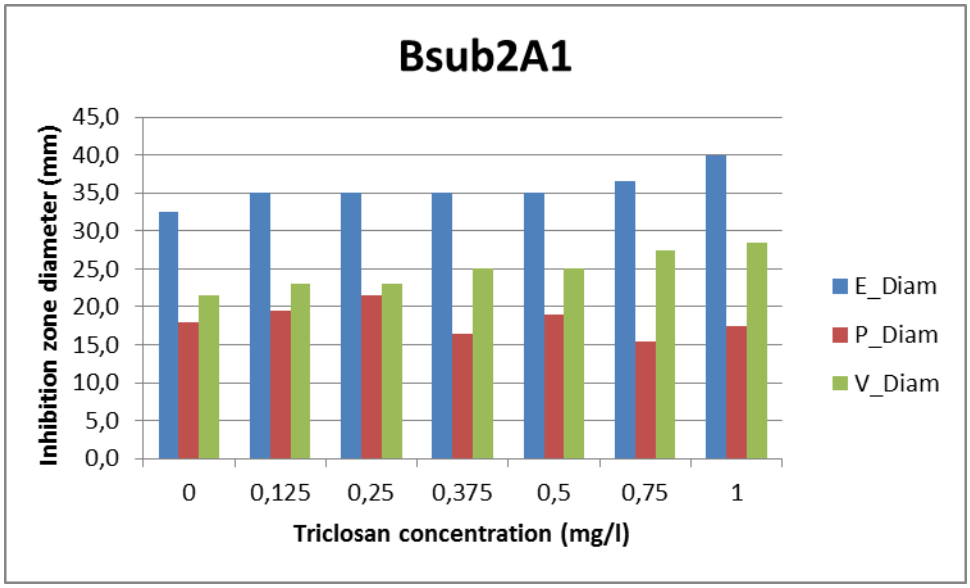
APPENDIX D:

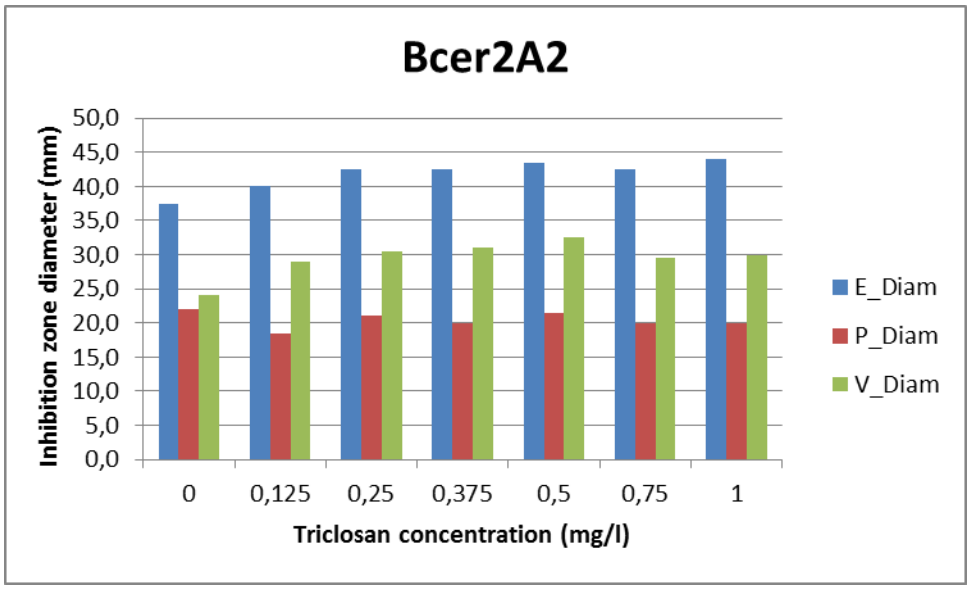
Table D: Concentration of triclosan measured in the wastewater treatment plant

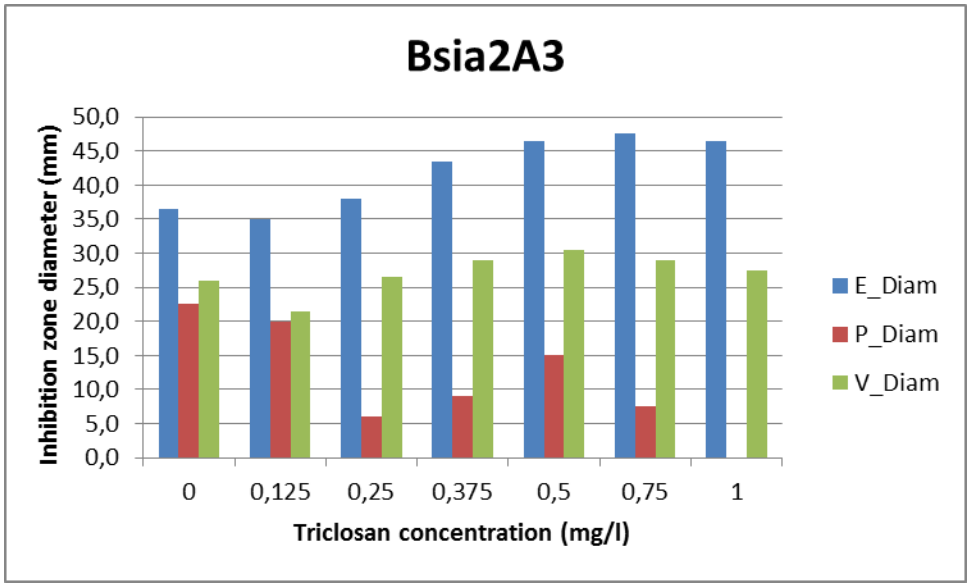
Sample	Area	[Tricl] ppb (µg/L)
Post-grid removal	2240,888	1,488
Post-grid removal	1788,875	1,188
Effluent	86,83	0,058
Effluent	60,814	0,040
Before chlorination	40,9019	0,027
Before chlorination	43,09042	0,029
Before chlorination	43,26363	0,029
Effluent sample 2	40,93499	0,027
Effluent sample 2	39,39392	0,026

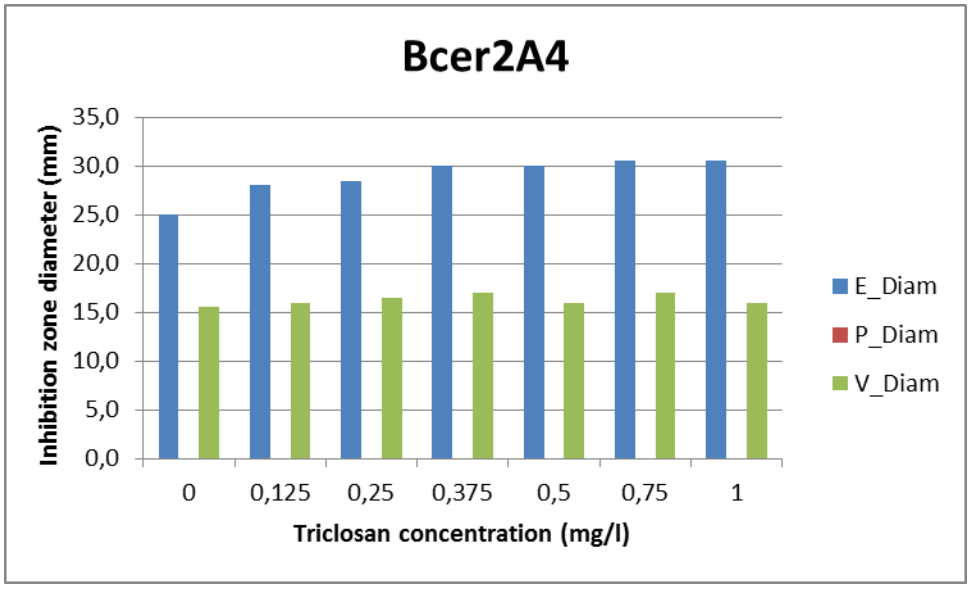
APPENDIX E:

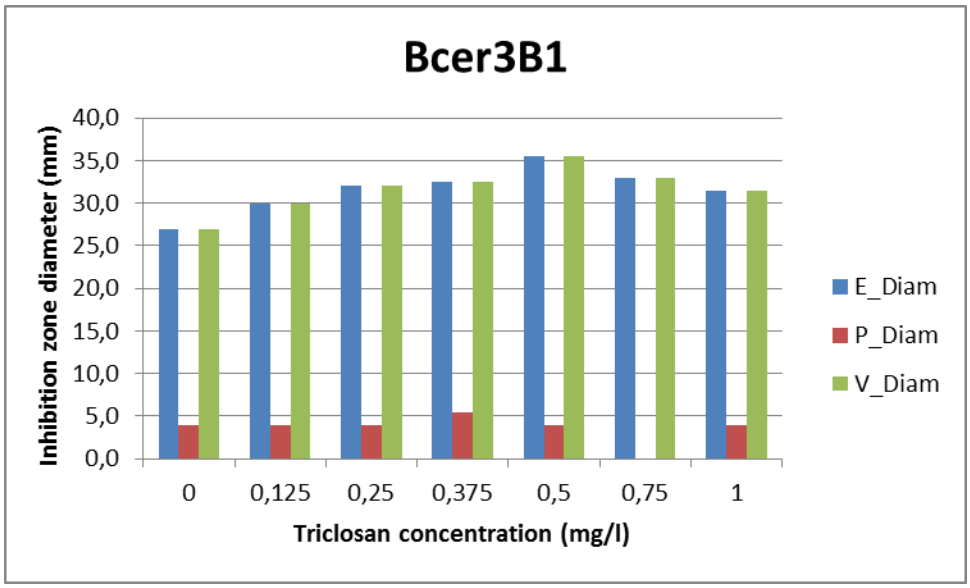
Table D: Bar plots of the inhibition zone diameters measured of the *Bacillus* spp group






Bsia2A3
Inhibition zone diameter (mm)
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bcer2A4
Inhibition zone diameter (mm)
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bcer3B1
Inhibition zone diameter (mm)
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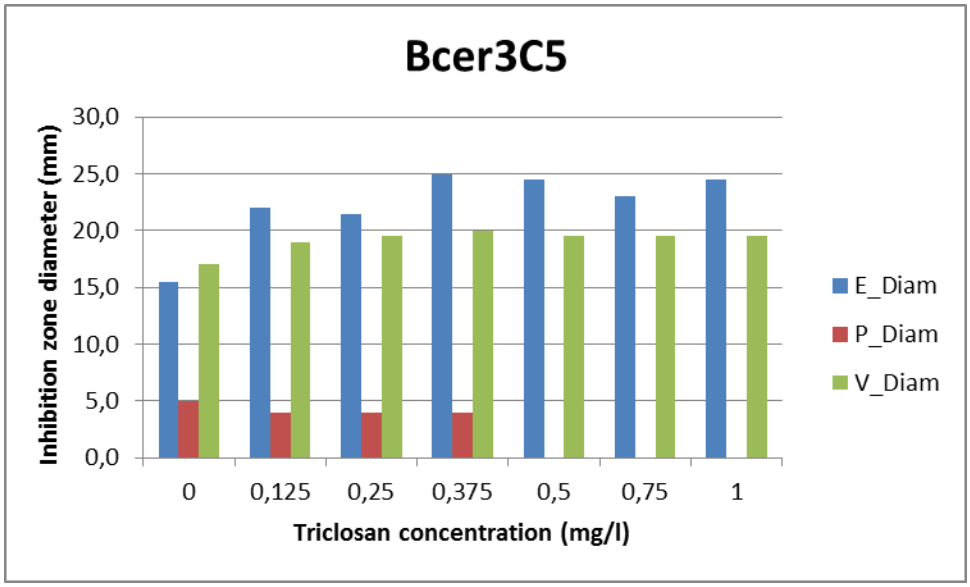
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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Inhibition zone diameter (mm)
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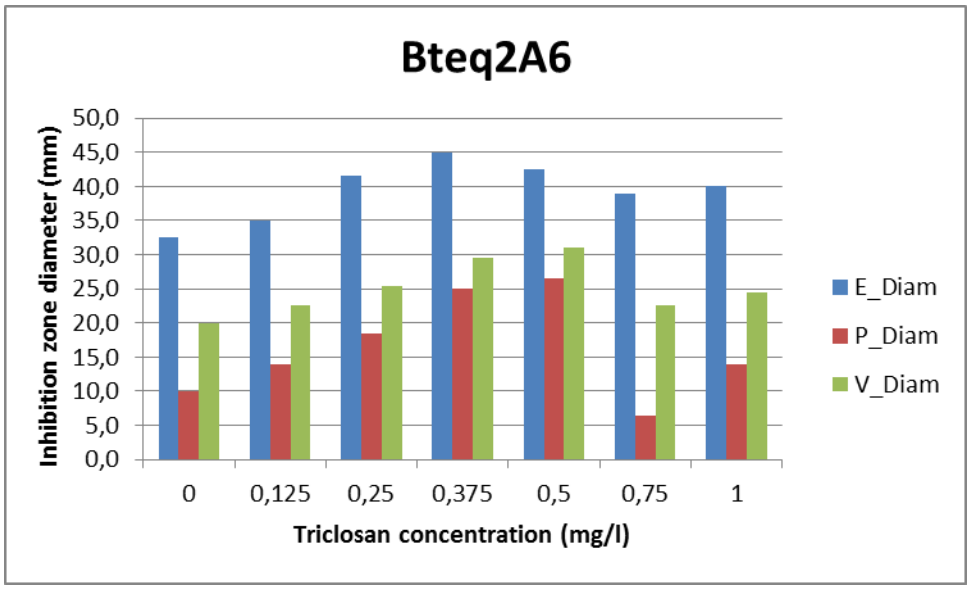
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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Inhibition zone diameter (mm)
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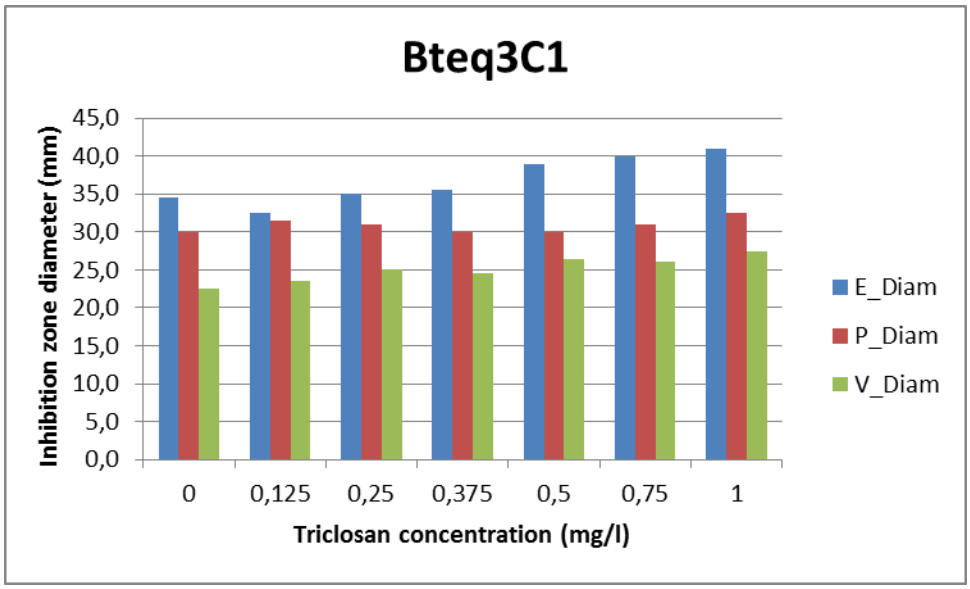
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bteq3C1
Inhibition zone diameter (mm)
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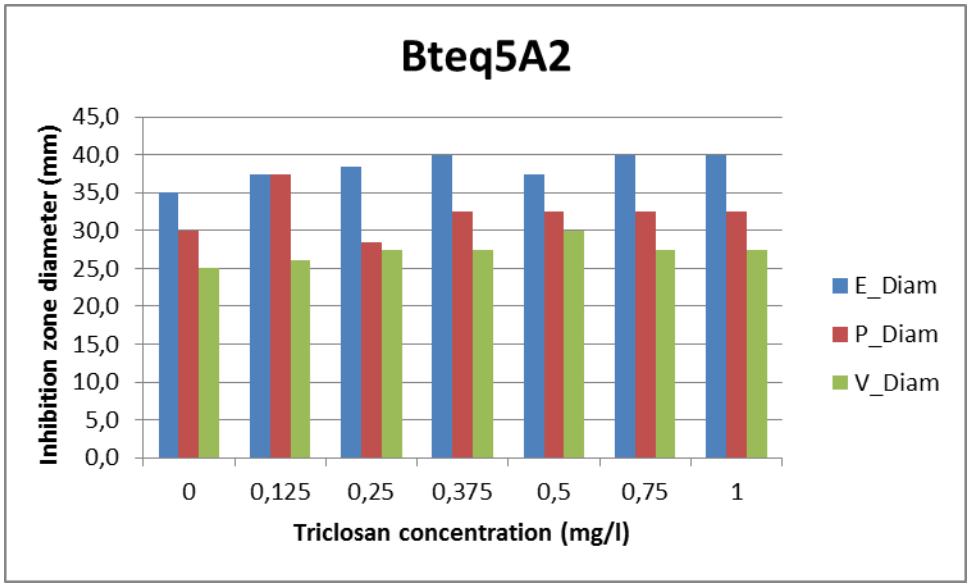
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bteq5A2
Inhibition zone diameter (mm)
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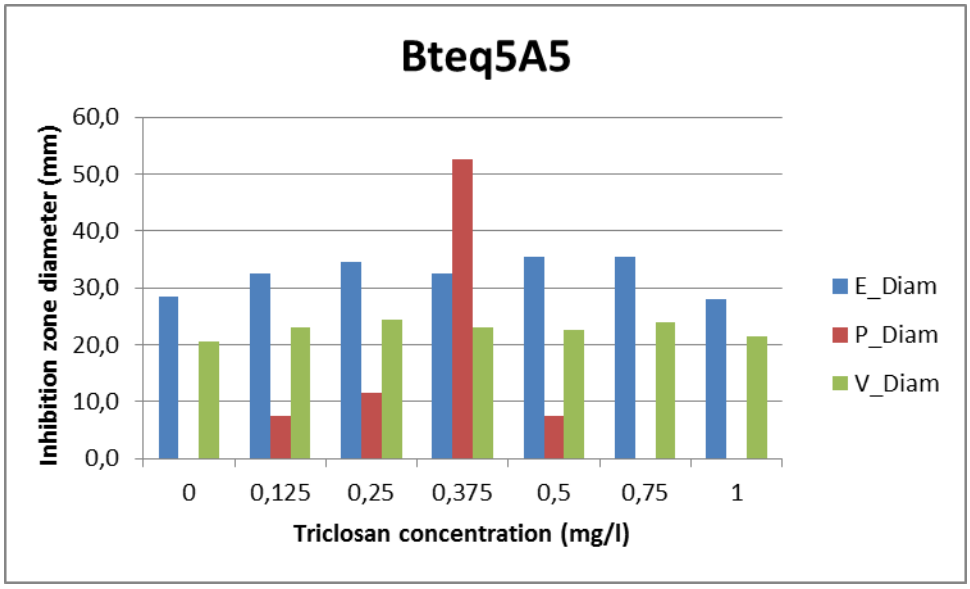
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bteq5A5
Inhibition zone diameter (mm)
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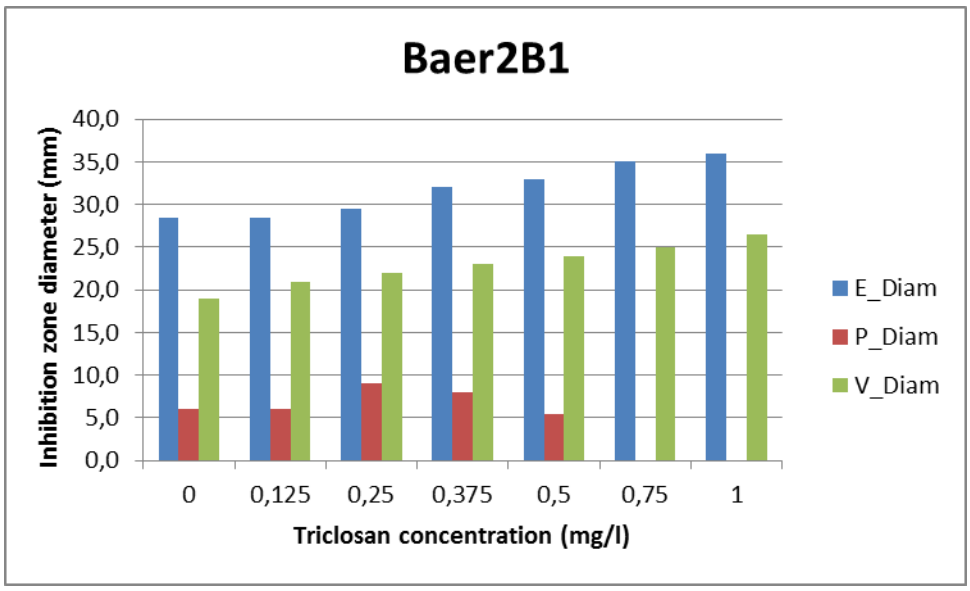
40,0
30,0
20,0
10,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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Inhibition zone diameter (mm)
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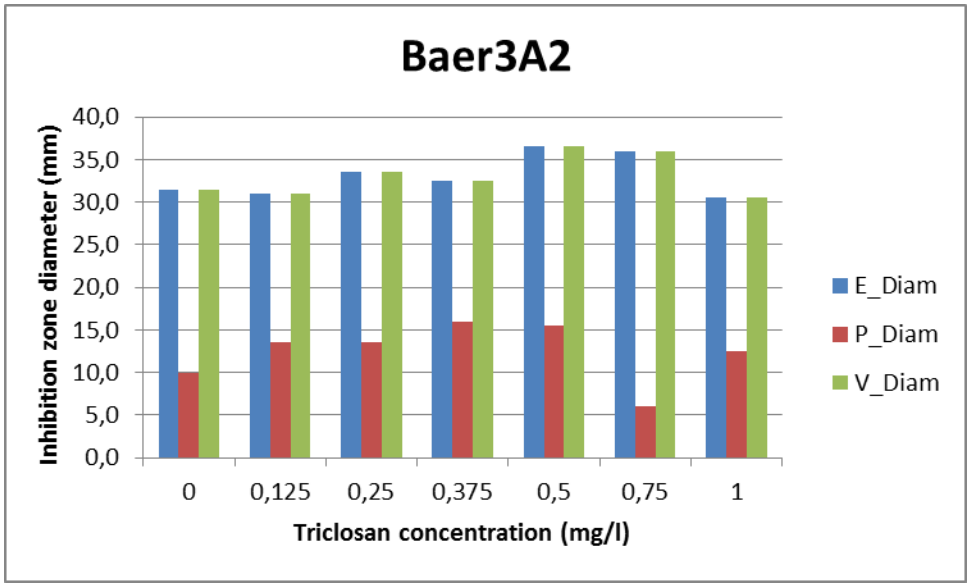
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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10,0
5,0
0,0
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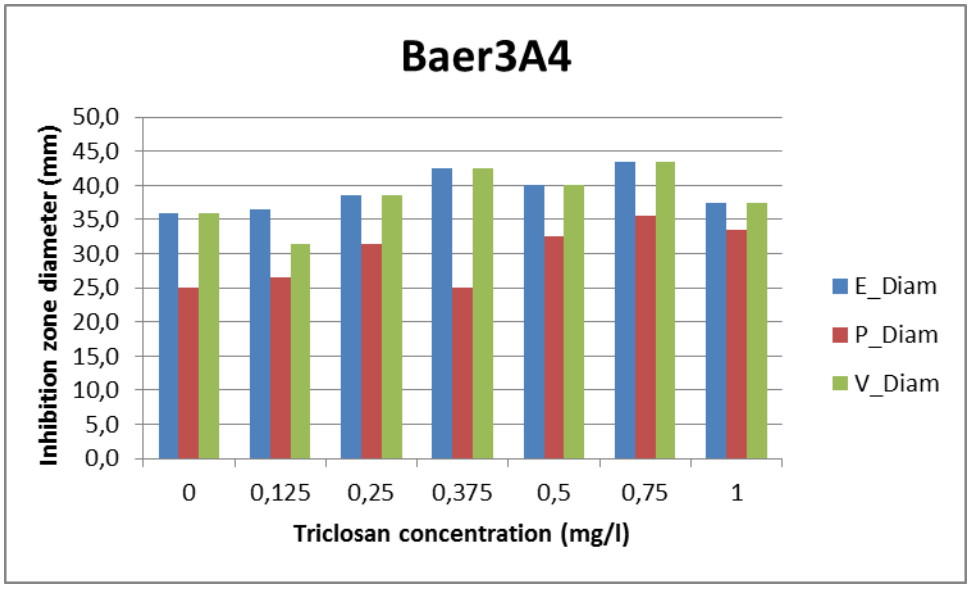
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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20,0
15,0
10,0
5,0
0,0
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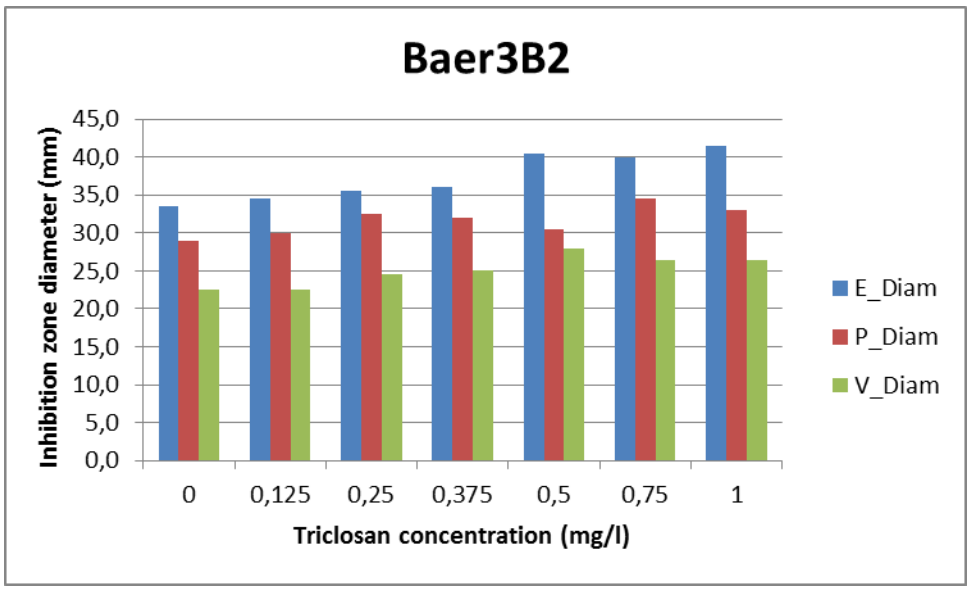
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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Inhibition zone diameter (mm)
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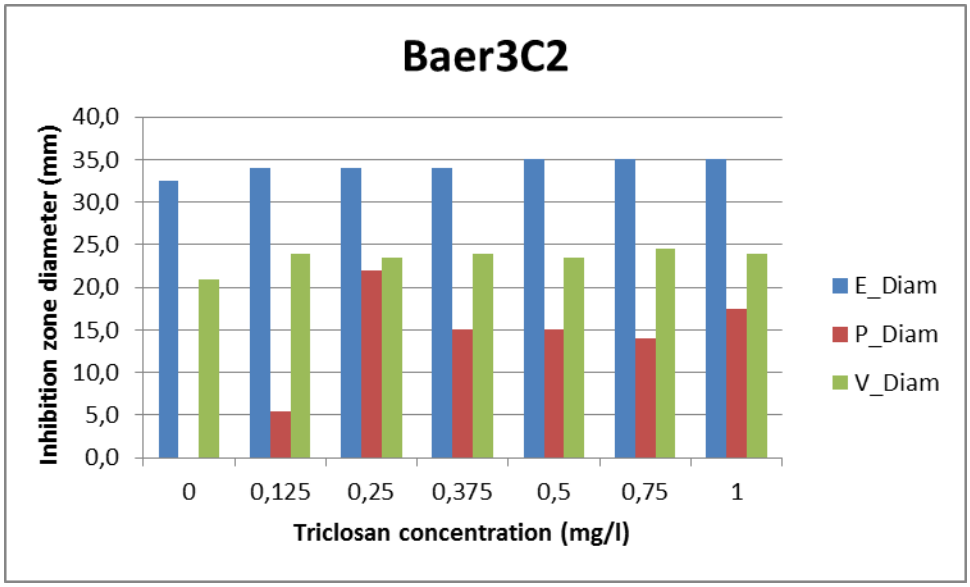
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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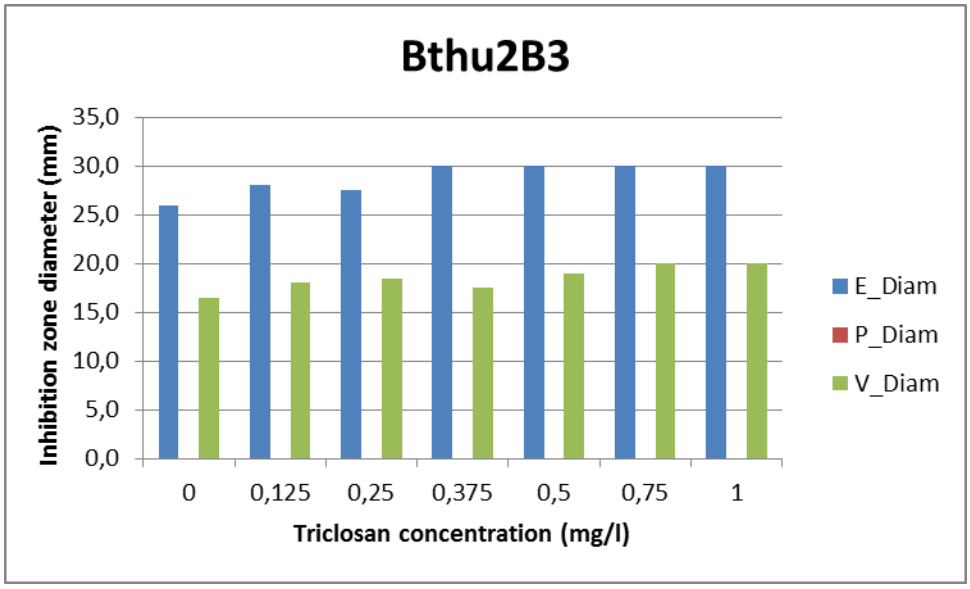
P_Diam
V_Diam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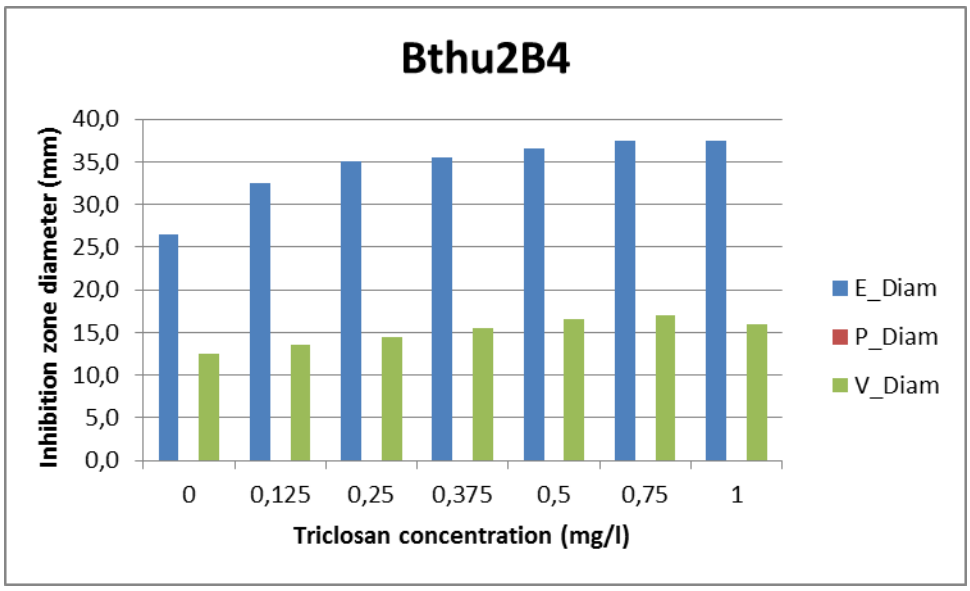
P_Diam
V_Diam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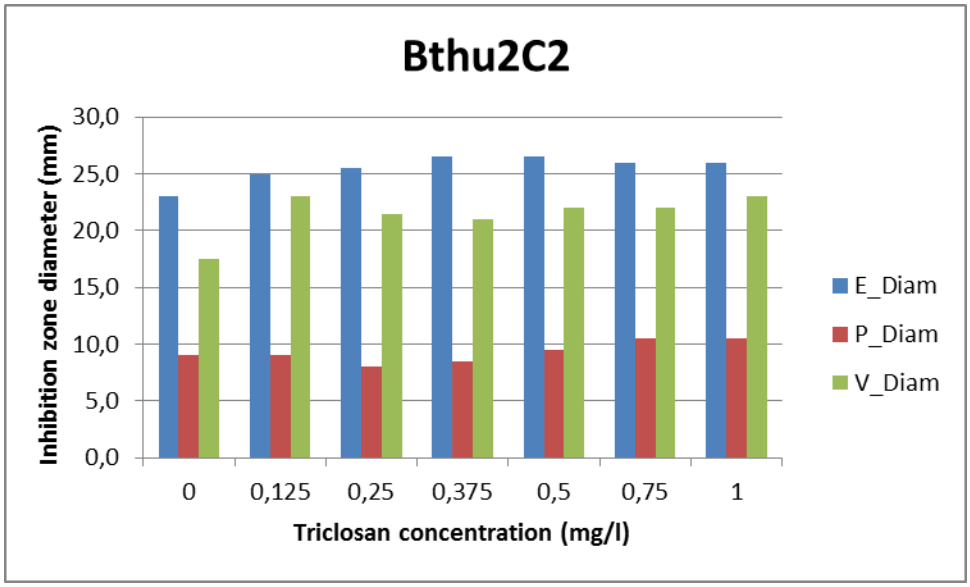
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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Inhibition zone diameter (mm)
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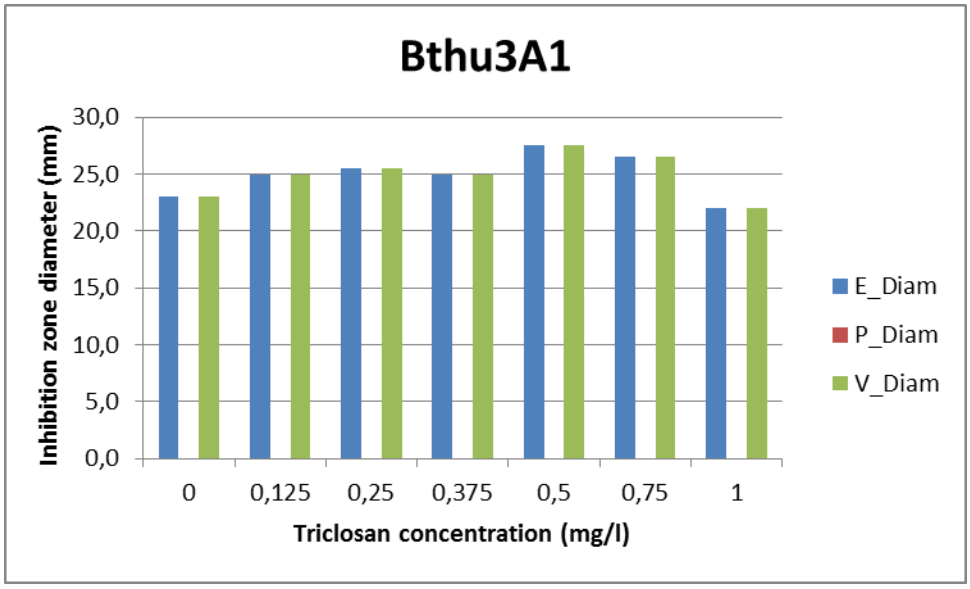
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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Inhibition zone diameter (mm)
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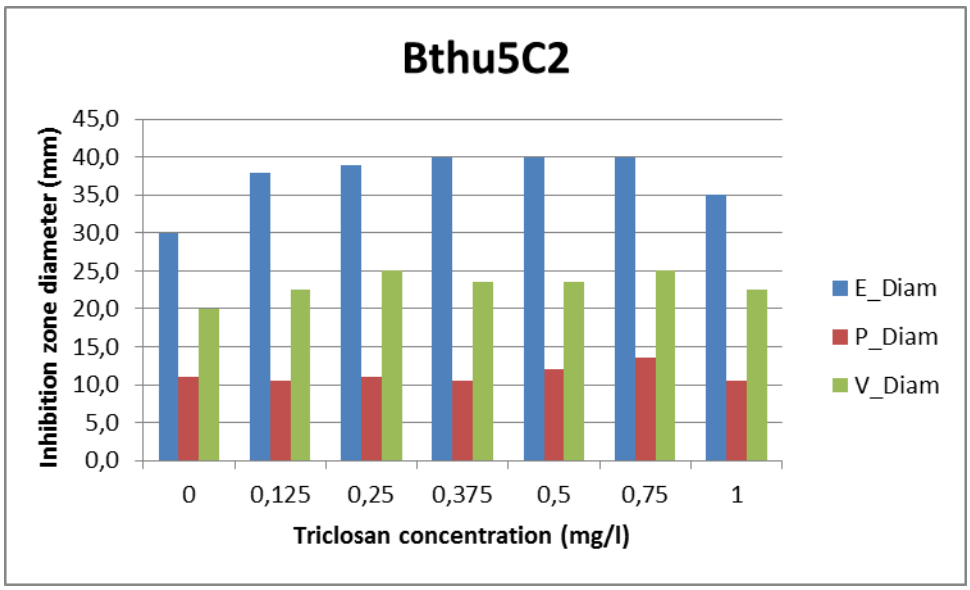
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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25,0
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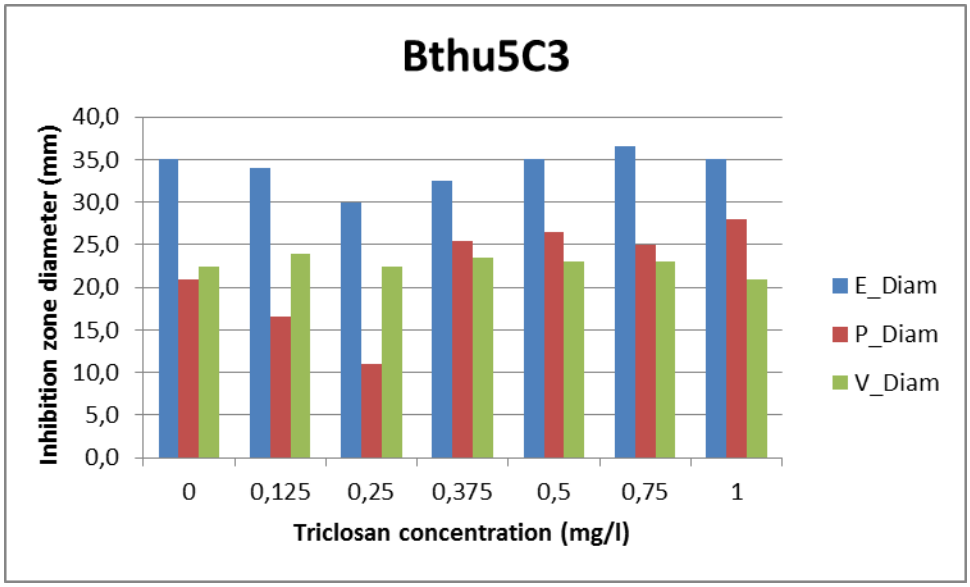
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam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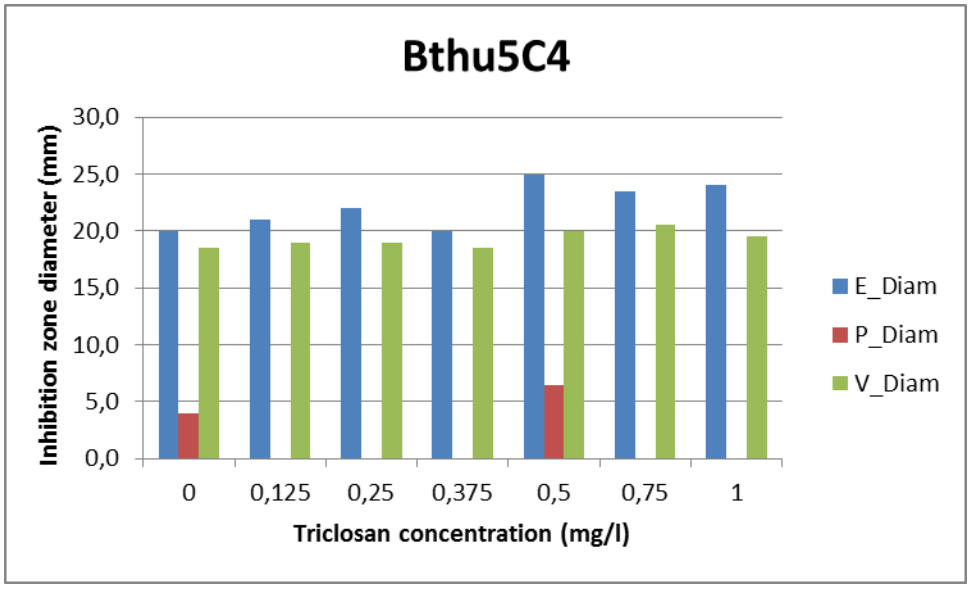
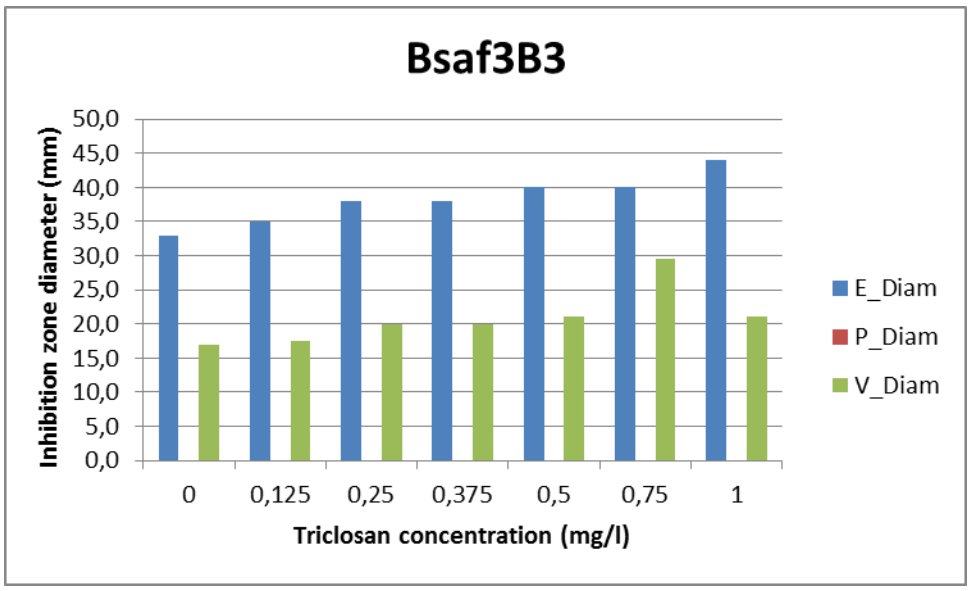
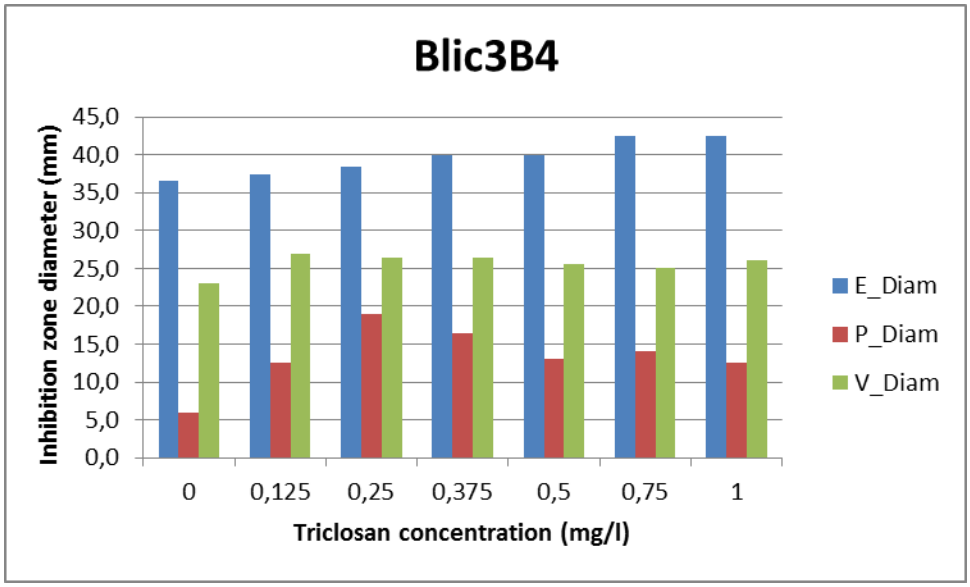
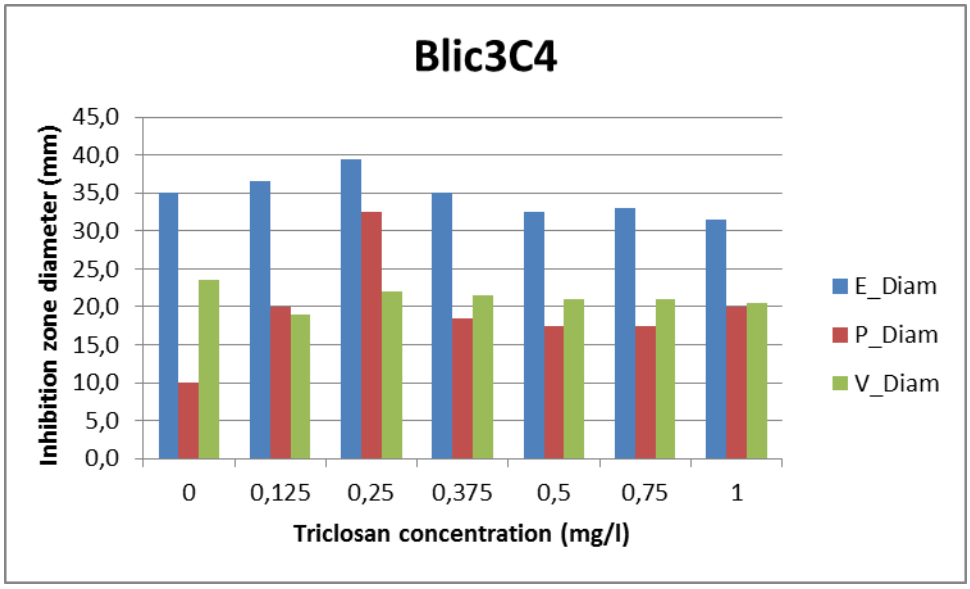
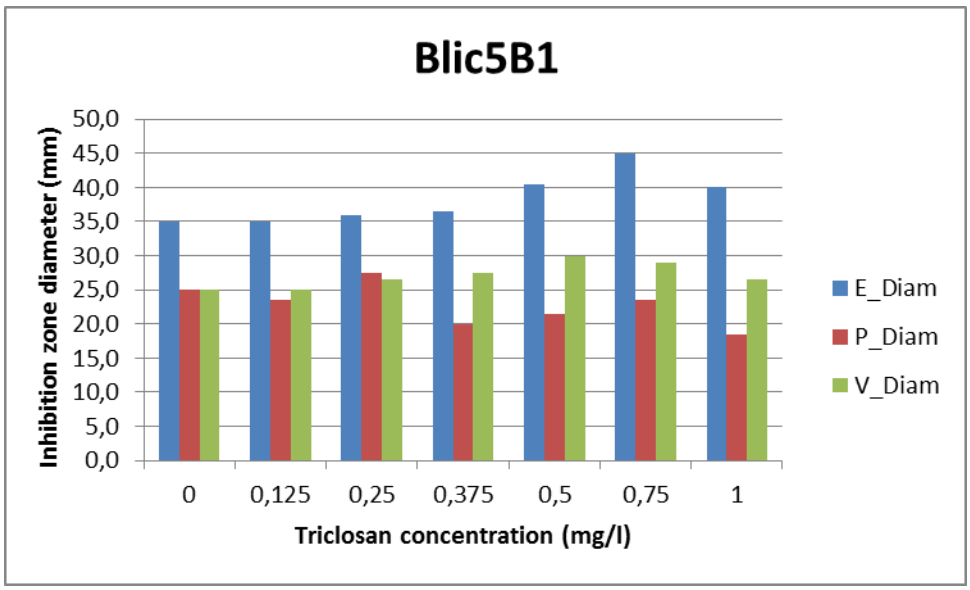
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

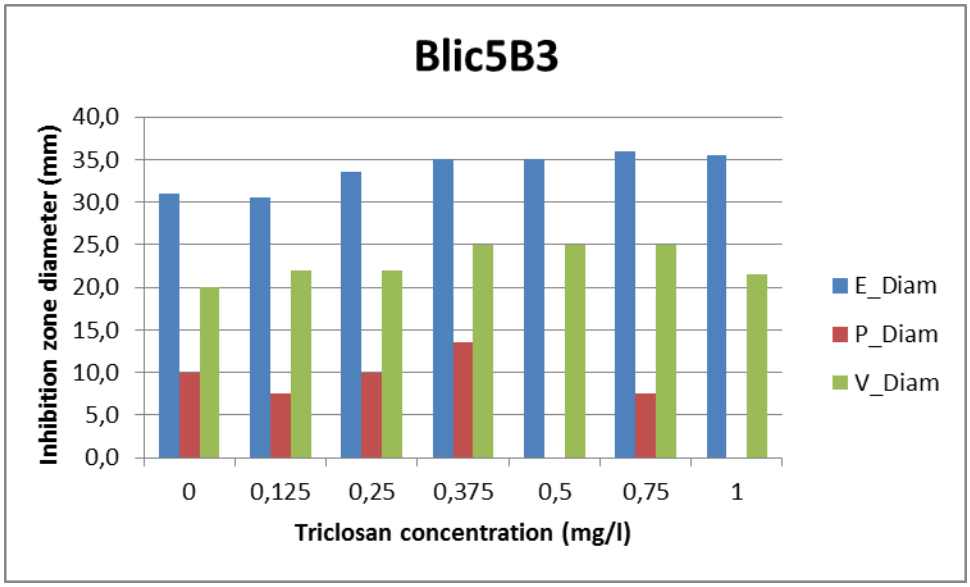

Bthu5C4
Inhibition zone diameter (mm)
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

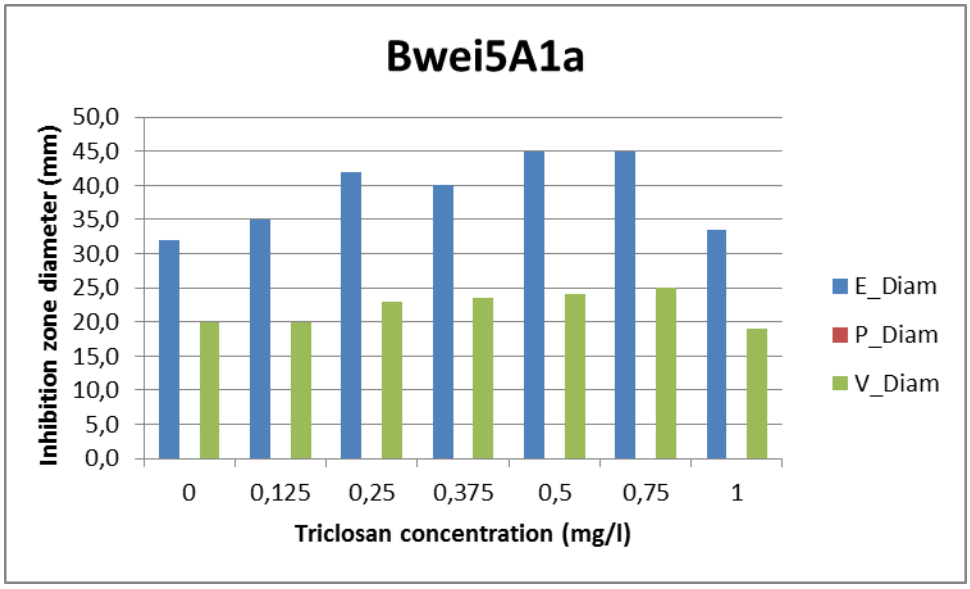

Bsaf3B3
Inhibition zone diameter (mm)
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

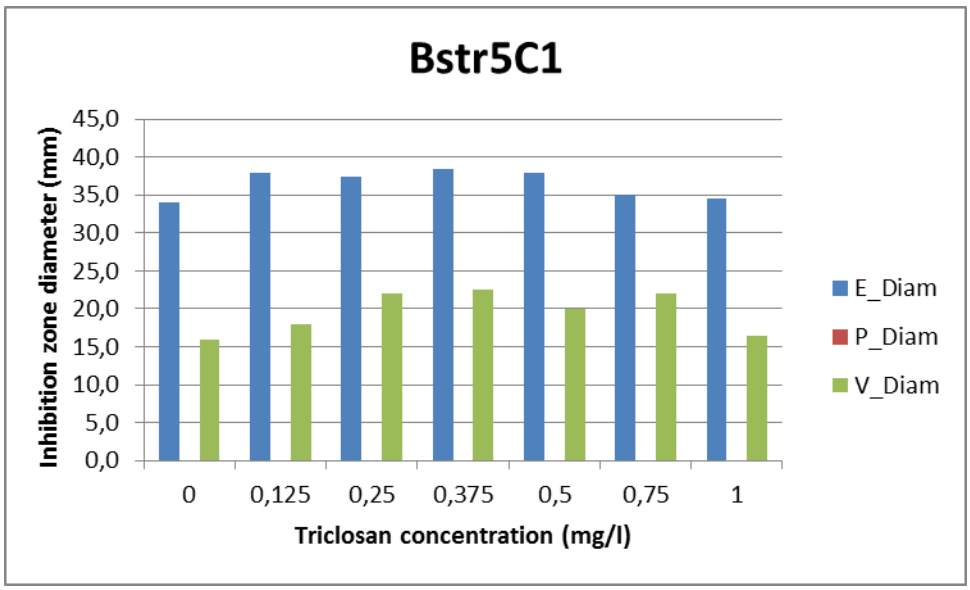

Blic3B4
Inhibition zone diameter (mm)
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

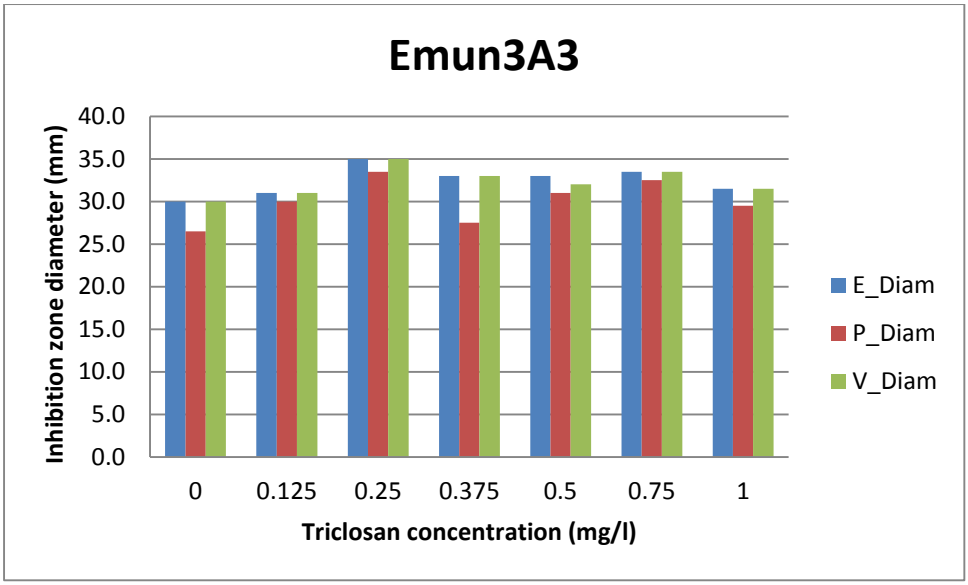

Blic3C4
Inhibition zone diameter (mm)
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

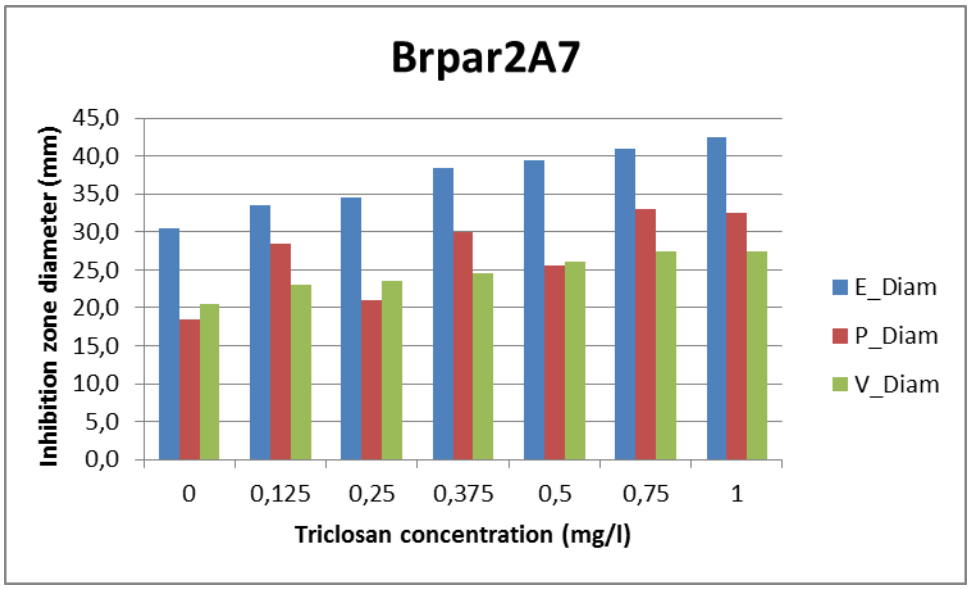

Blic5B1
Inhibition zone diameter (mm)
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Blic5B3
Inhibition zone diameter (mm)
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bwei5A1a
Inhibition zone diameter (mm)
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Bstr5C1
Inhibition zone diameter (mm)
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Emun3A3
Inhibition zone diameter (mm)
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
0.125
0.25
0.375
0.5
0.75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


Brpar2A7
Inhibition zone diameter (mm)
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

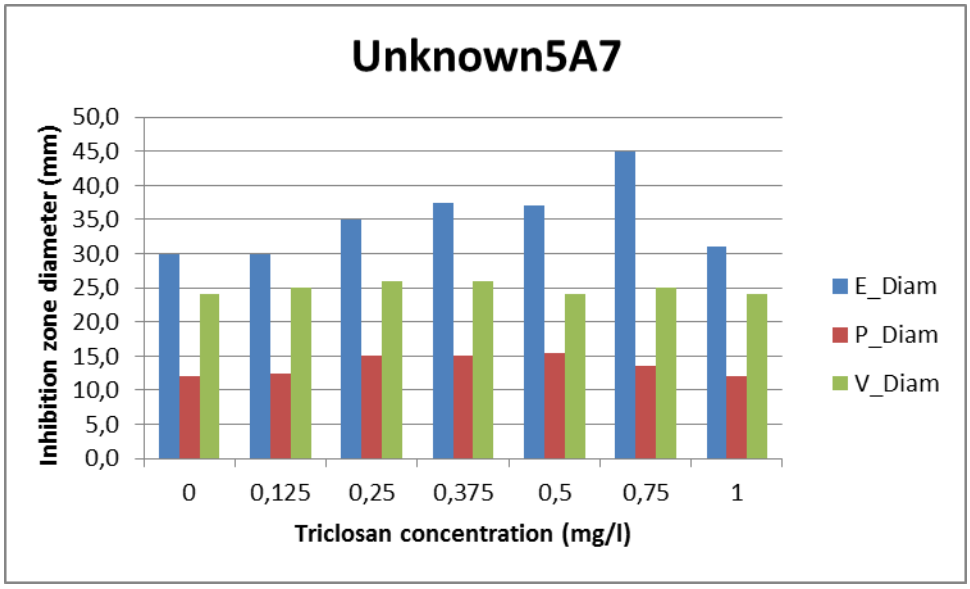
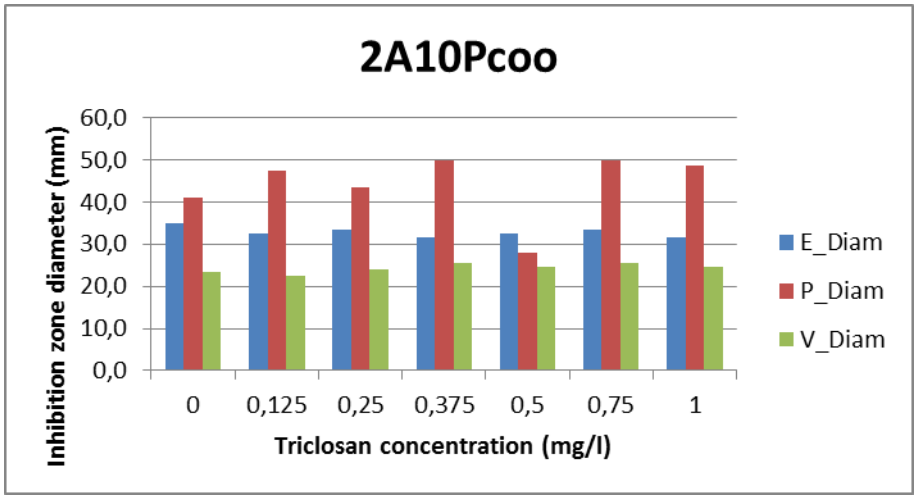
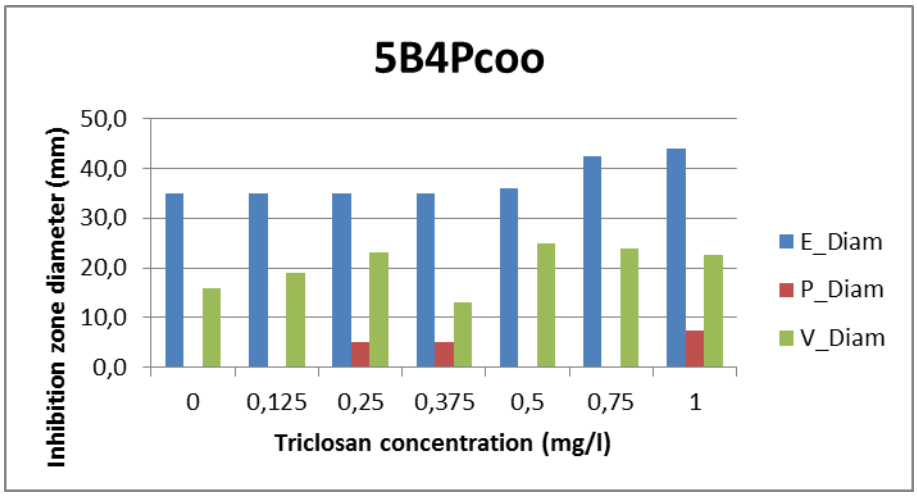
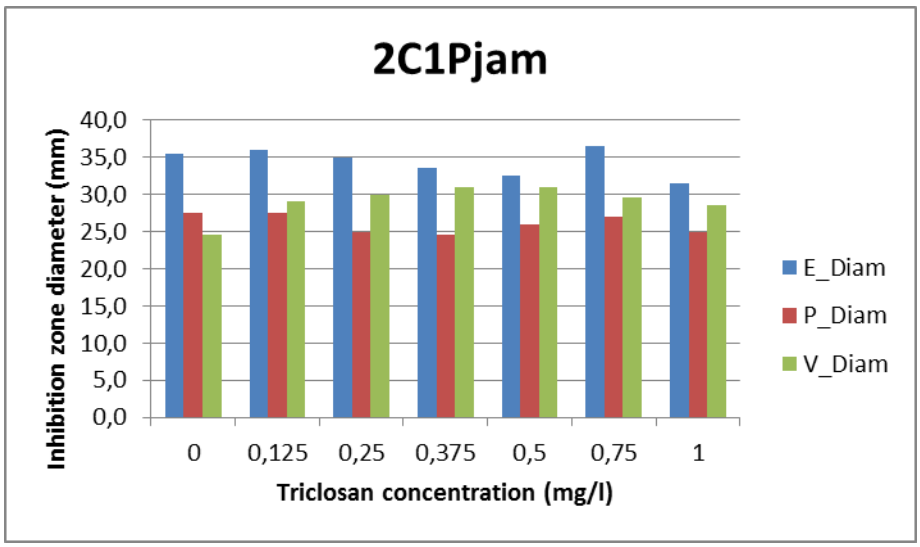

Unknown5A7
Inhibition zone diameter (mm)
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
0
0,125
0,25
0,375
0,5
0,75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

Table E: Bar plots of the inhibition zone diameters measured of the Paenibacillus spp group







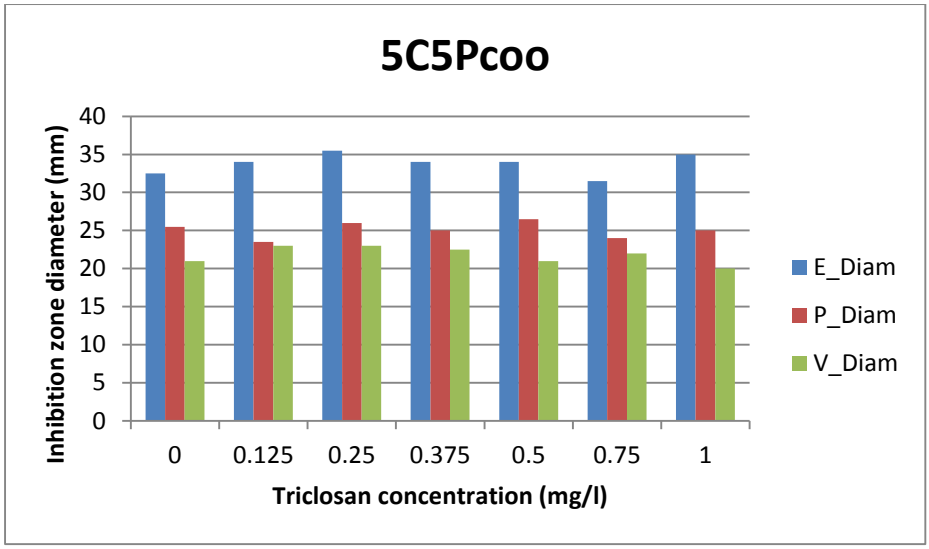
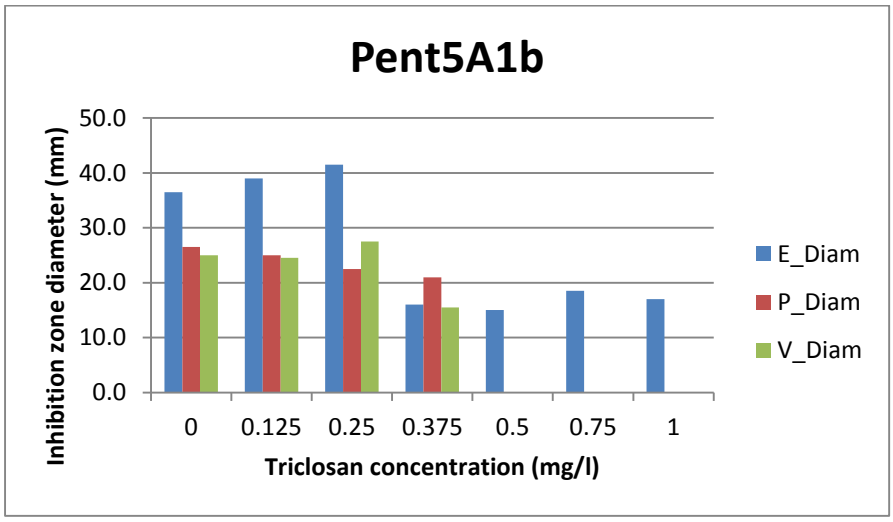
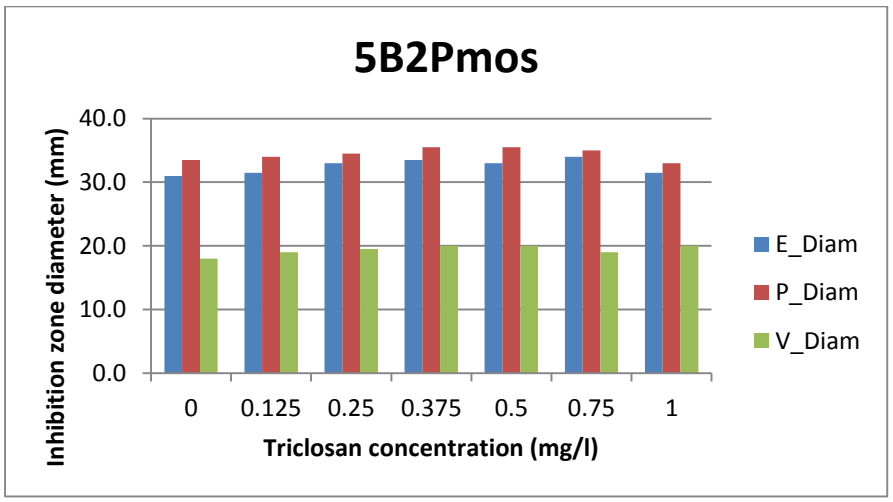
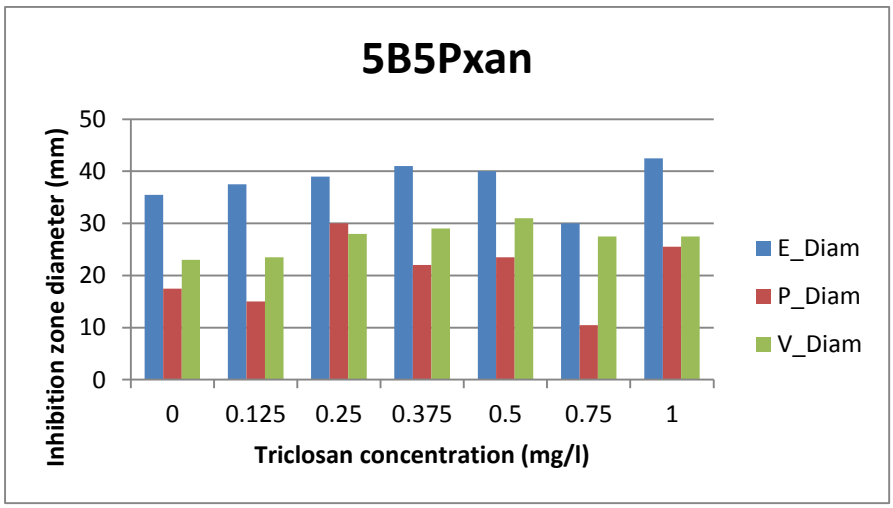
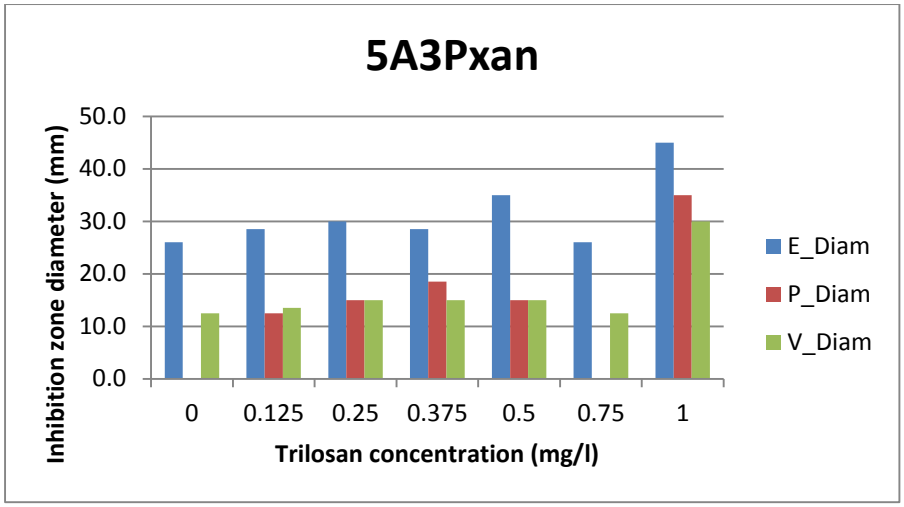

5C5Pcoo
Inhibition zone diameter (mm)
40
35
30
25
20
15
10
5
0
0
0.125
0.25
0.375
0.5
0.75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam

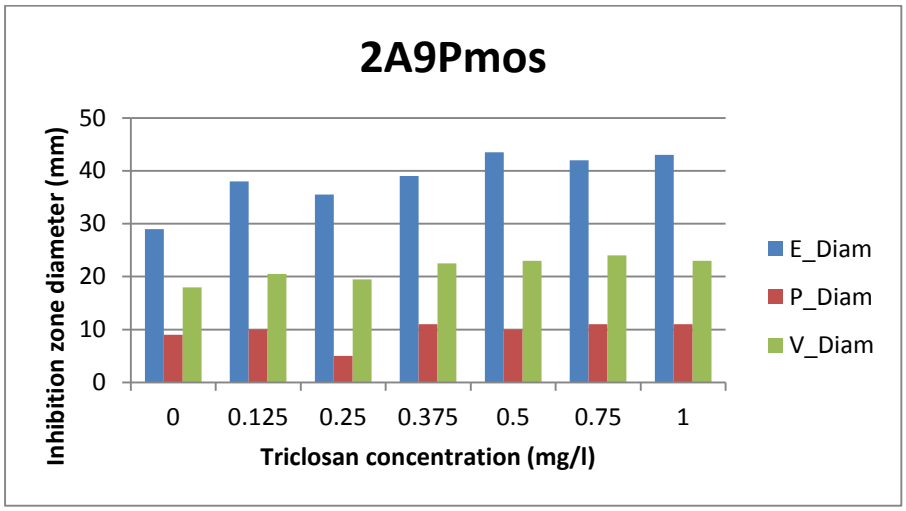
Table F: Bar plots of the inhibition zone diameters measured of the *Pseudomonas* spp group

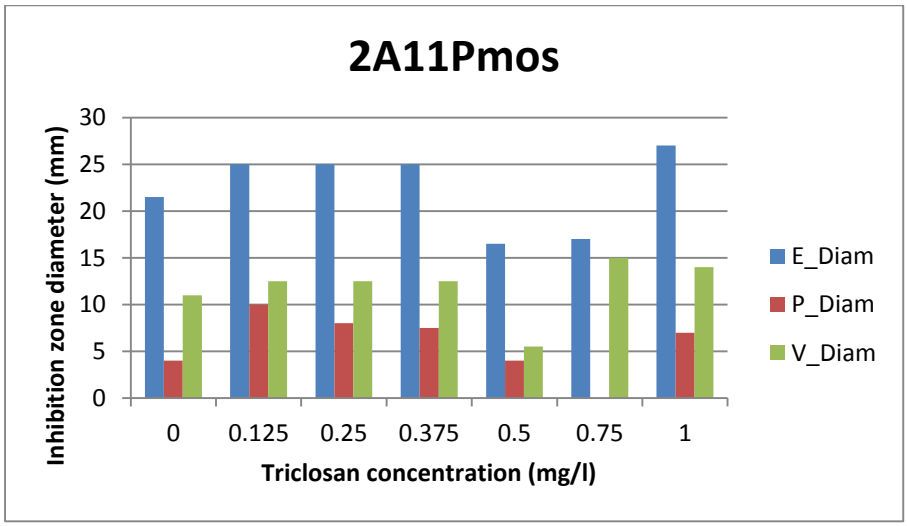


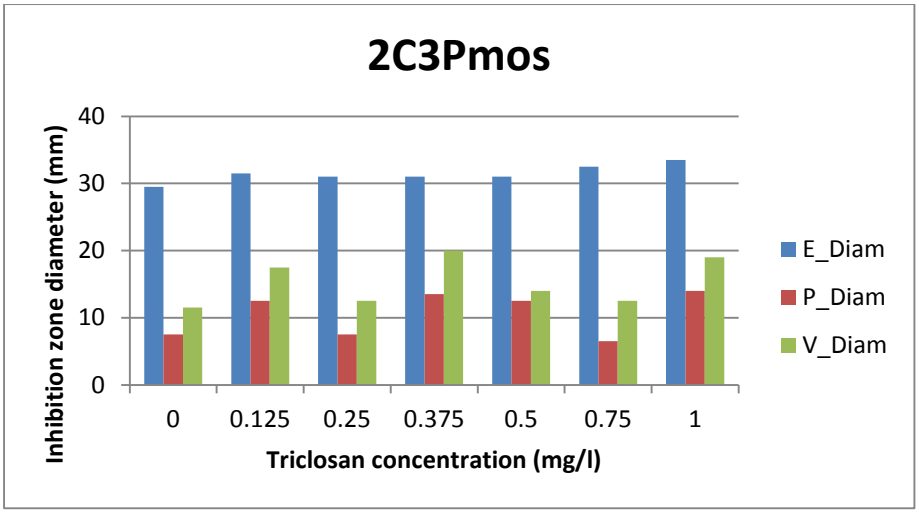


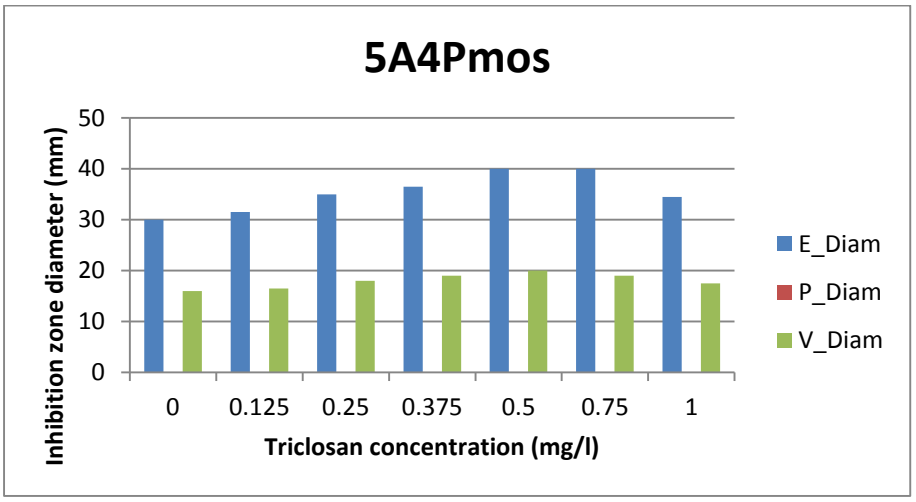



5A3Pxan
Inhibition zone diameter (mm)
50.0
40.0
30.0
20.0
10.0
0.0
0
0.125
0.25
0.375
0.5
0.75
1
Trilosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


2A9Pmos
Inhibition zone diameter (mm)
50
40
30
20
10
0
0
0.125
0.25
0.375
0.5
0.75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


2A11Pmos
Inhibition zone diameter (mm)
30
25
20
15
10
5
0
0
0.125
0.25
0.375
0.5
0.75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


2C3Pmos
Inhibition zone diameter (mm)
40
30
20
10
0
0
0.125
0.25
0.375
0.5
0.75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam


5A4Pmos
Inhibition zone diameter (mm)
50
40
30
20
10
0
0
0.125
0.25
0.375
0.5
0.75
1
Triclosan concentration (mg/l)
E_Diam
P_Diam
V_Diam