The utilisation of bacterial species diversity as a bioindicator for biocide efficacy in water systems.

by

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DECLARATION

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ABSTRACT

The poor quality of cooling water, utilised by South African power plants, necessitates the use of biocides and bio dispersants to manage microbiological fouling. The two most common biocides used are isothiazolone and dibromonitrilopropionamide (DBNPA). Management of the dosing of these chemicals is historically not controlled or the efficacy thereof monitored. Cooling water microbiology is mostly performed on the planktonic (free-living) bacterial count in the water, which requires complex analyses in a microbiological laboratory. In order to manage the efficacy of biocide dosing, a simple, effective test is required, which does not need complex technical analysis.

The Darwinian concept of survival of the fittest can be applied to bacterial communities too. This implies that when a biocide is dosed into a cooling water system, there should be a change in the bacterial species diversity of the community (Koonin and Wolf, 2012, Spencer, 2018).

The monitoring of metabolic changes in the water offers insight to the bacterial species diversity present. In order to evaluate this Biolog Ecoplates ® were used to compare the carbon substrate utilisation (bacterial species diversity) of samples pre and post biocide dosing. In order to verify these results total bacterial plate counts were conducted as were denaturing gradient gel electrophoresis (DGGE) finger prints. The culturable analyses (total bacteria and Biolog Ecoplates®) showed good correlation when an appropriate concentration was dosed but there was little correlation when biocides were under dosed.

The effect of biocidal resistance was also evaluated to determine whether the metabolism of the resident bacterial community changes when impacted by sequentially increasing, sub lethal concentrations of biocide. These results, for both carbon substrate utilisation and DGGE, indicate a small change in bacterial species diversity. However the addition of a secondary biocide (DBNPA) post resistance development caused a decrease in the size of the bacterial community, to levels below detection limits for both the Biolog Ecoplate and the total plate count.

In an attempt to evaluate the concomitant development of biocidal and antibiotic resistance, Biolog Phenotypic Microarrays (PM) ® 1 to 20 were used. These plates offer an indication of variations in the metabolisms of bacterial communities in different samples. Biocidal resistance was again induced by sequential dosages of sub lethal concentrations of isothiazolone. Samples were collected at three specific times: 1) pre dosing, day 1, time 0 hrs (1-0), 2) when the because the non-resilient bacteria were all killed off and only the resilient bacteria survived, (lowest bacterial plate count) (day 9, 24 h

post dosing, 9 -24) and after the biocide residual was eliminated from the system (day 9, 48 h post dosing, 9-48). Each of the three samples was loaded onto a set of the Biolog PM® plates. The plates were incubated in an Omnilog® plate reader and the Omnilog® measurement units compared for the three samples. This technique, based on a colour change, indicated that microorganisms in the community were resistant to the antibiotics Rifamycin, Aztreonam and Ethionamide.

The study indicates that Biolog Ecoplates can be utilised to evaluate the efficacy of biocide dosing by monitoring changes in carbon substrate utilisation. The study highlights the need for optimal dosing of the various biocides. The possibility of antibiotic resistance development with biocidal resistance should be considered with the optimisation of dosing. However, the use of biocides with differing modes of action does minimise this risk.

Dedication

I dedicate this work to my father, who taught me I can do anything I my mind to and to my husband and mother for continually reminding me and believing in me all the time.

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Table of Content

List of abbreviations

ATP	Adenosine triphosphate
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming units
CW	Cooling water
DBNPA	Dibromonitrilopropionamide
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP's	Nucleotides for addition to PCR amplification
dsDNA	double stranded deoxyribose nucleic acid
Gel2K	DGGE electrophoresis gel analysis statistical comparison software
ITS	Internal transcribed spacer
MIC	Microbiologically influenced/induced corrosion
OTU	Operational taxonomic unit
PAH	Polyaromatic hydrocarbon
PCR	Polymerase chain reaction
PM	Phenotypic Microarray
rDNA	ribosomal deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
UV	Ultra violet
W/mK	Thermal conductivity, Watts per meter Kelvin
ZLED	Zero liquid effluent discharge

List of Figures

Page number

List of Tables

CHAPTER 1:
INTRODUCTION
Bacterial species diversity as a bio - indicator for biocide efficacy in water systems

South African power plants utilise surface water, from dams or rivers, for cooling (Kotze, 2015). Although the volume of the water supplied is guaranteed by government, the quality thereof is not. Increased industrialisation and urbanisation, around the water sources, led to a rapid deterioration of the quality of the water. This, poor quality, water requires treatment and management to limit problems on the power plant. This treatment includes the dosing of biocides and bio dispersants to minimise fouling in the system (van der Merwe-Botha, 2009) in addition to pH and scale control and management.

Historically, microbiological growth in the cooling water systems is managed by continuously dosing a bio dispersant and slug dosing a biocide on a two weekly basis. Although several biocides are used by the South African power generation industry, isothiazolone (a non-oxidising biocide) and dibromonitrilopropionamide (DBNPA) are the most common. Bacteria, however, may become resistant to isothiazolone (Zhou *et al.*, 2016). Hence, these biocides are usually applied in an alternating pattern to avoid possible development of bacterial resistance (Eskom *Legionella* Standard, 2017).

Bacterial numbers in the cooling water systems are primarily managed on the planktonic bacterial count which is analysed by culturing the total bacteria within the cooling water system. This analysis requires a basically equipped microbiological laboratory and specialised staff.

The efficacy of the biocide treatment is, however, normally not evaluated, beyond the initial 24 h post dosage sampling, and dosing volumes are often below effective concentrations. Furthermore, new legislation, governing *Legionella* and legionellosis, requires a formalised microbiological treatment regime and proof of efficacy. There is, however, no simple means of determining the efficacy of these biofouling control mechanisms. Thus a simple analysis is required to better understand the bacterial community and species composition within the

cooling water system and determine the efficacy of biocide treatments. Ideally, the analysis should not require a fully equipped microbiology laboratory.

In this study, two experimental methods were employed which do not rely on artificial (in a petri dish on a solid medium) culturing techniques and instead focus on species/community diversity through genetic and metabolic analysis.

It must be remembered that species diversity is not necessarily linked to total bacterial numbers (abundance) in an environment. For example, high numbers of bacteria may occur in extreme environments, although the species diversity is normally low (Sheng-Jin *et al.*, 2014). Therefore, it is proposed that bacterial species diversity is a better indicator of stress in an environment than the total count of bacteria in the environment (Reese *et al.* 2016).

Denaturing gradient gel electrophoresis (DGGE) is one of several molecular based techniques which may be used to determine bacterial species diversity. It uses the genetic sequences of bacteria to separate different species from one another (Mayrhofer *et al.*, 2014), allowing the various species within an environmental sample to be separated (Shah, 2015; Muyzer *et al.*, 1993). The sensitivity of the double stranded DNA to chemicals and heat cause them to denature and separate into a visual genetic fingerprint on an electrophoresis gel. This is thus a sensitive technique for the determination of bacterial species and community diversity.

For the metabolic approach Biolog Ecoplates® have been used to monitor bacterial communities in several environments including soil (Xu *et al.*, 2015), water (Lekhanya, 2010) and sewage (Gryta *et al.*, 2014). Utilisation of the Biolog Ecoplate® system allows for the evaluation of bacterial communities in terms of their carbon substrate utilisation i.e. metabolic fingerprint.

The concept of survival of the fittest can be applied to bacteria in cooling water systems (Martinez, 2013). If a stress (e.g. antimicrobial compound, high temperature) is applied to an environment, it is likely that the bacterial numbers and bacterial species diversity will change, selecting for the fittest. Tracking these changes could provide insight into the microbial ecology dynamics in a system. The type of biocide, dosage concentration and contact time will all affect this variation (Satpathy *et al.*, 2016).

For this study, carbon substrate utilisation will be considered indicative of bacterial species present. The understanding is that, for these trials, bacterial species diversity should decrease or change when subjected to a stressed environment. Therefore, the higher the bacterial diversity, the more Biolog Ecoplate® carbon substrates will be utilised while a lower diversity will lead to fewer substrates being respired. Based on the hypotheses, an effective biocide should lead to a reduction of both the bacterial count and the bacterial species diversity and hence fewer carbon substrates utilised.

To this end, the initial trials of this study aimed to determine whether Biolog Ecoplates® could be utilised as a potential simple indicator, to determine the efficiency of isothiazolone or DBNPA dosing regimens for the control of bacteria, in a cooling water system, by monitoring the changes in the microbial diversity.

Trials continued to determine whether the Biolog Ecoplates® could be used to detect bacterial species diversity changes due to development of isothiazolone susceptibility and to determine the effect of dosing a secondary biocide.

The development of biocidal resistance to non-oxidising biocides is well documented, as are the mechanisms by which this resistance is developed (Wales and Davies, 2015; Guest, 2016). As part of this study, changes in carbon substrate utilisation were evaluated in a bacterial community where biocidal resistance/susceptibility to isothiazolone had been developed. In addition, the effectiveness of an alternate, subsequently dosed DBNPA biocide on bacterial species diversity was investigated.

In both cases the effect determined on Biolog Ecoplates® was supported by conventional bacterial plate counts and molecular analysis on DGGE gels.

Antimicrobial products used in industries, as disinfectants, are called biocides while those used in for the treatment of man and livestock health are termed antibiotics (SNENIHR, 2009). In general, biocides have a broader range of activity as they target several sites in the bacterial cell while antibiotics have more specific targets (Blair *et al.*, 2015).

There are several studies indicating the concurrent development of antibiotic resistance with biocidal resistance. In an industrial cooling water environment, the possibility for concurrent

development of biocidal and antibiotic resistance could raise concerns about drug resistant bacteria being dispersed through cooling water aerosols.

Høiby *et al.* (2010) reported the development of antibiotic resistance in biofilms and there are several laboratory scale studies on the possible link between biocide use or exposure and antibiotic resistance (Kümmerer, 2009; Martinez, 2009; Allen *et al.* 2010, WHO 2014, Berendonk *et al.*, 2015).

Further investigation attempted to determine whether antibiotic resistance is developed in isothiazolone resistant cooling water systems. Biolog® Phenotype Microarray (PM) Plates (Biolog Ltd, Hayward, CA, USA) offered a simple technology for measuring bacterial cell respiration in specific substrates, either in pure culture or in a community.

This project aimed to determine whether there was any link between biocide and antibiotic resistance by utilising Biolog® PM Plates to compare untreated, resistant and recovering samples.

In an attempt to further understand the effect of sublethal biocide dosing; it is theorised that biocides may be bacteriostatic or bactericidal, depending on the concentration and regularity of the biocide dosed. This becomes important when the cooling water system has a possible safety impact such as in the case with *Legionella* bacteria in the planktonic phase of the system.

HYPOTHESIS

- The addition of a biocide to a cooling water system should change the microbial species within the microbial community and hence the metabolic profile of the microbial community. This change in metabolic profile could be used to act as a proxy for indicating changes in the species diversity of the microbial community and used to monitor biocide efficacy.

AIMS

1. To determine how bacterial counts and carbon substrate utilisation compare in environments treated with different concentrations of biocide.

2. To determine how bacterial counts and carbon substrate utilisation compare in an environment containing bacterial communities showing biocidal resistance to isothiazolone.
3. Determine whether a bacterial community, which is biocidally resistant, can develop antibiotic resistance.

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CHAPTER 2:
LITERATURE REVIEW
Bacterial species diversity as a bio - indicator for biocide efficacy in water systems

2.1 INTRODUCTION

The generation of electricity in South Africa is dominated by the use of wet cooled, coal fired power stations (Kotze, 2015). The water used for cooling is extracted from surface water sources (rivers or dams) in the areas surrounding the power stations (van der Merwe-Botha, 2009). Eskom utilises three main raw water sources, the Vaal, Usutu and Komati water systems. As surface water is a scarce commodity in South Africa, Eskom does not release water back to the environment and reutilises water in a system of cascading qualities until the water is allowed to evaporate, either from the ash dams or the parabolic cooling towers (Kotze, 2015). This process is known as zero liquid effluent discharge (ZLED) (Eskom Integrated Report, 2015). Due to ZLED, the water in the recirculating cooling water systems is cycled up in terms of elemental concentration within the water, often more than 10-15 times. This creates a saline environment in the cooling water system.

The cooling water systems have large capacities, approximately 148 Ml, and thus require continuous treatment and management (Kotze, 2015). The cooling water systems are routinely dosed with pH control chemicals in order to manage scaling (the formation of a hard, permanent coating of impermeable salts on the surfaces of the system). A 10% side stream from the system is subjected to lime clarification to precipitate suspended particles and dissolved elements (Kotze, 2015). However, the water still has a high turbidity, conductivity (1000 – 3600µS/cm) and suspended solids, making the system susceptible to biological build up known as biofouling (van der Merwe-Botha, 2009).

The Eskom power plants operate the cooling water systems at between 8 – 30 cycles of concentration, depending on the raw water quality. The main parameters analysed routinely in the system include pH, electrical conductivity, alkalinity, turbidity, total dissolved solids and sulphates (van der Merwe-Botha, 2009).

Biofouling is not as strictly managed as the physico-chemical parameters. Total aerobic bacteria counts are supposed to be analysed monthly on both sessile and planktonic samples as an indication of potential biofouling. However, this way of monitoring has proven to be far from ideal (Satpathy *et al.*, 2016). This chapter will discuss the basics of biofouling and biofilms and discuss how monitoring changes in diversity may be used as an indicator of environmental change – stress, pollution etc.

2.2 BIOFOULING AND BIOFILMS

Biofouling is the accumulation of suspended solids on the surfaces of a natural or industrial water system (Lear and Lewis, 2012), being trapped in a slimy secretion (biofilm) produced by the bacteria present. A biofilm is defined as "an assemblage of microbial cells that is irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material" and are complex, dynamic systems (Kostakioti *et al.,* 2013). Biofouling is known to decrease heat transfer i.e. 385 or 120 – 0.6 W/mK when compared with copper or brass, respectively (Al-Janabi, 2011). Biofouling blocks filters, strainers and narrow gauge piping in the cooling water system. However if the system flow rate is maintained above 1m/s biofouling will be less than in low flow systems (Goode *et al.,* 2013).

Problems associated with biofilm formation include:

- Decreased Heat transfer - Biofilm has a thermal conductivity of approximately 1.4 W/mK (depending on the formation of the biofilm) compared to 385 W/mK of the brass in the condenser tubes (Warsinger, 2015).
- Increased fluid frictional resistance and clogging - Due to thick biofilm development, decreases the flow rate, increasing energy consumption for pumping, decreases cooling and possibly deforms the metal surfaces due to increased pressure and possible biofilm related chemical effects (Goode *et al.,* 2013).
- Increased fouling and corrosion rates - The development of biofilm is known to increase other forms of fouling (scaling, corrosion) (Flemming and Wingender, 2010) and cause microbiologically influenced/induced corrosion (MIC) (Li *et al.,* 2016).

- Protection of pathogens - Biofilms are able to harbour potential pathogens (Pandin *et al.*, 2017).
- Increased resistance to antimicrobials - Biofilms offer increased resistance to antimicrobial compounds, because treatment chemicals cannot penetrate the biofilm quickly enough to be effective (Stewart, 2015) and internal EPS particulates may limit penetration into the biofilm (Pandin *et al.*, 2017).

2.2.1 Monitoring of Biofilms and Biofouling

Planktonic bacteria, in the cooling water system, are commonly enumerated by a grab sample (single point immediate sample) from the cooling water system and subjected to a classical total bacterial plate count. However, sessile bacteria, within the system, should also be monitored on an on-going basis, samples may be collected from in-situ or on-line biofouling monitors. There are several technologies available for both methodologies a comparison of which is given in Table 2.1.

Although there are a large number of monitors available they are scarcely used mainly due to a lack of consensus on biofilm monitoring techniques and a lack of information on concentrations of biocides required in industrial systems (Catto, 2014). On power plants the change in pressure (Vrouwenvelder *et al.*, 2011) and temperature (Ruiz *et al.*, 2011) across the heat exchanger are used to determine thermal resistance of fouling deposits. This offers an effective and accurate means of determining treatment efficacy but cannot differentiate between biotic and abiotic fouling. These techniques, however, all indicate the system cleanliness in regards to current dosing regimens but cannot mimic systems where there is accumulated biofouling present i.e. they indicate the surface condition not the nature of the fouling (Catto, 2014).

Table 2.1: Biofouling monitoring techniques.

Monitor	**Technique**	**Advantages**	**Disadvantages**	**Examples**
In-Situ Monitoring	Collection and analysis of sample from a known area.	Simple, No electricity required.	Labour intensive and require qualified laboratory personnel	Robbins devices Pedersen devices Murthy and Venkateson (2009)
On-line monitoring	Real-time Non-destructive Physical technique	Can be remotely accessed, Real time	Cannot determine whether a deposit is biotic or abiotic, Complex technology, Requires electricity.	Several different technologies
Differential turbidity	Two turbidity meters, one on clean stream one allowed to foul. Difference in turbidity correlates to growth.	Simple, inexpensive.	Cannot detect biofilms less than 0.1mm thick.	Bajpai (2015)
Optical fouling monitors	Applies light from below biofilm to head of fibre to determine light scattering.	Simple, widely used.	Not for thick biofilms.	Flemming and Ridgeway (2008) Rotoscope, Cloete and Maluke (2005)
Heat transfer	Build-up of biofilm increases resistance decreasing heat transfer.	Well suited to monitoring of heat exchangers, widely used.	Affected by hydrodynamics of system, wall temperature measurement uncertainties, cannot detect initial biofilm.	Yamashita (2017)

Pressure drop	Biofilm attachment increases surface roughness and decreases pipe width, increasing pressure in the system.	Simple, low cost method, widely used with heat exchangers.	Limited to a biofilm thickness of 30-35µm in a small bore pipe.	Yamashita (2017)
Metabolic by-products or test substrate measuring	Detecting and characterising metabolic by-products.	Wide range of possible by-products to analyse.	Does not separate between biofilm and planktonic communities.	Yamashita (2017)
Image analysis	Use of images to determine percentage fouling on a surface.	Very accurate.	Only proven in laboratory situations, complex and requires expensive software.	Yamashita (2017)
Electromagnetic radiation signals	Detection of visible, infrared or ultraviolet light with radio frequencies. Bioluminescence, Fluorometry, Spectroscopy	Widely used technology.	Environmental factors may influence light reactions.	Yamashita (2017)
Electrochemical monitors Resistance development	Measures changes in electrochemical reactions produced in biofilms on stainless steel electrodes	Used to monitor microbiologically influenced corrosion.	Used for monitoring corrosion rather than biofilms.	BioGeorge – Venhuis (2007)
Coaxial stub resonance (vibration)	Use of short resonators with high resonance frequencies. The development of biofilm decreases the vibration.	Can differentiate between old and new fouling. Can detect early biofilm formation.	Complex very new technology.	Hoog *et al*. (2015) Pereira *et al*. (2007)

2.2.2 Treatment of Biofilms and Biofouling

Biofouling in cooling water systems is managed by dosing bio dispersants and biocides. Bio dispersants are used to dislodge biofilms and bacteria from the surfaces of the system and biocides to kill the bacteria, both sessile (partly) and planktonic, in the system. However the current treatment regimens have not delivered the desired results. Dosing frequency and concentration are insufficient. Additionally, sites are often on the same chemical regime for extended time periods. This may cause the local bacterial community to become resistant to the chemicals.

Two non-oxidising biocides are commonly utilised for cooling water treatment on the power plants, isothiazolone and DBNPA. These chemicals will be considered in this study.

Isothiazolone biocides are heterocyclic, non-ionic, non-surface active (limiting interactions with other chemicals), ketones which utilise a two-step action mechanism: firstly rapid inhibition of growth and metabolism (respiration and energy generation), followed by irreversible cell damage (slow, several hours) (Williams, 2007). Inhibition is caused by disruption of metabolic pathways involving dehydrogenase enzymes, active in both aerobic and anaerobic bacteria. The cells die due to the destruction of protein thiols of the isothiazolone N-S bonds and the subsequent production of free radicals (Williams, 2007).

Isothiazolones are commonly used to treat cooling water systems as they combat a wide range of microorganisms. Additionally, due to their unique destruction mechanism microbes battle to develop biocidal resistance.

DBNPA inactivates enzymes thus disrupting metabolic pathways responsible for producing energy and transferring nutrients and waste (Huber *et al.,* 2010). It is an effective and fast acting bactericide against aerobic bacteria, especially slime formers, although less effective against anaerobes, fungi and algae, which can also act as slime formers (Huber *et al.*, 2010).

DBNPA is known to break down rapidly to form non-harmful by-products; it is not persistent and needs to be used in conjunction with a more persistent biocide. It is sensitive to UV light, alkaline conditions and nucleophilic substances (Huber *et al.,* 2010). It has an unpleasant odour and will be deactivated above pH 8.5 and in the presence of sulphide or

sulphite contamination (Huber *et al.*, 2010). It is also uncharged and non-surface active and thus unlikely to interact with other chemicals (Huber *et al.*, 2010).

2.3. MICROBIAL DIVERSITY CHANGES UNDER STRESS

Species diversity can be expressed in two or more ways, species richness, or the number of species in an environment, and species evenness, the distribution of the individual organisms between the relevant species (Tuomisto, 2010). Both must be considered when researching stressed environments, the species diversity may not change but the evenness of distribution may change when more resistant microorganisms are enhanced (Figure 2.1). Resistance in this environment is explained as the degree of change after a disturbance event (Ponge, 2013) and resilience as the ability of a system to return to its initial stage after the said disturbance (Thorén, 2014).

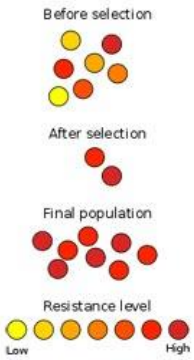

Figure 2.1: Diagram explaining the development of resistance in a bacterial community (Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), 2009).

2.3.1 Stresses on Environment

Microbial biodiversity can be defined as the set of microbial species detected within an ecosystem (Zeglin, 2015), the number of individual taxa and their distribution within the microbial community (Li *et al.*, 2012). Chase, 2009, detailed how environmental stresses and other factors affect ecosystem species diversity and functioning. The basis assumption is that if competition is allowed to continue, most species will be eliminated leaving only the resistant species resulting in a decrease in diversity (Shcherbakov, 2010). Environmental stress is considered an important factor for determining whether diversity change will alter the function and stability of an ecological community (Herren *et al.*, 2016). The effect of

stress will differ based on the type of pressure, whether it is positive or negative (Schmitz-Felten, 2017), the resident community and the degree of adaption of the community to this stress (Munita and Arias, 2016). When a community is continually placed under pressure, natural selection allows for changes in species diversity within a system, however, if stress is mild and intermittent the species diversity will rapidly return to normal once the stress is removed (Wiser and Lenski, 2017). The larger and more regular the stress, the more it negatively changes species diversity (Miller *et al.*, 2010).

When there are few stresses on an environment, stable biological communities develop. These environments have high species diversity while stress impacted sites have a lower species diversity (Miller *et al.*, 2011). As the stress gradient increases, the community changes to a point where the stress becomes greater than the adaptive abilities of the organism. This implies that the number of species decreases with increasing stress (Valladares *et al.*, 2015). Declines in species diversity have been shown to impact continued functioning and stability of ecosystems (Lopez *et al.*, 2013*)*. However species detected in stressed unstable environments have adapted to these conditions by maintaining a high level of genetic variability. These flexible species are opportunistic and do not speciate easily (Grant *et al.*, 2010). Studies conducted on extreme environments indicate low levels of bacterial species diversity (Tuomisto, 2010).

Industrialisation and urbanisation are leading to increased pollution in rivers, dams and underground water sources. This pollution or contamination, including heavy metals, sewage, toxic chemicals and genetically modified organisms, can be viewed as a stress on the system and will cause a change in the natural organisms inhabiting the specific environment. However, pollution is not the only form of stress which may impact the environment; there are several natural environmental stresses which will yield a similar effect. These include extreme temperatures, drought, increased salinity and sewage or heavy metal contamination.

2.3.1.1 Temperature

Valladares *et al.* (2015) indicated that tropical areas of high stress (increased temperatures in shallow waters) had very low levels of species diversity. While Sati *et al.* (2013) showed that species diversity is minimised in Indian soils covered in snow for 6 months but that those species show a community of moderate numbers (10^7 CFU/g).

In algal communities, Gruner *et al.* (2016) showed that stress due to cold temperatures limits community functionality, as species diversity is decreased.

A 16S ribosomal clone library developed from studies conducted on extremely cold, below 3°C, marine sediments indicates a limited number of main groups in the 353 clones tested. This indicates that there is minimal diversity in these samples (Tuomisto, 2010). Similarly, samples of the microbial mat from a deep-sea, hydrothermal vent were analysed by PCR amplification of conserved regions of 16S rDNA. RFLP analysis offered 12 different groups with different operational taxonomic units (OTU's). However, 72.9% of the 16S rDNA was contained in two OTU's. Of the remaining 21.1%, no OTU contained more than 3 clones, indicating low species diversity with several individuals in these main groups (Moyer *et al*., 1994).

This research indicates that the warmer the water the greater the bacterial species diversity, in the absence of other stresses. This is directly opposite to the finding by Valladares *et al.* (2015) in studies on tropical waters. This is believed to be due to the large variation in the water temperature. In tropical waters the temperatures will be relative to the environmental temperatures and be affected by day and night. In the power plant the temperature variation is less, power plant cooling water experiences temperatures between 18 and 45°C, which incorporate the optimal growth range of many bacteria. This leads to the expectation that the broad species diversity in the cooling water system will show definite species diversity changes in the presence of any stress.

2.3.1.2 Drought Conditions

If a slight or moderate stress (water limitation) is applied to the system the organisms often exhibit an improved facilitation between the species than when compared with a stable low stress environment. (Romanuk *et al.*, 2010). However, under high stress levels the organisms are mostly killed, only those with strong adaption ability are expected to survive.

Altermatt *et al.* (2009) showed that, although adapted to withstand desiccation, there was a substantial decrease in species composition of organisms in freshwater rock pools, during such a desiccation event, often killing the organisms off totally. While Romanuk *et al.*

(2010) showed that although species diversity, in rock pools, decreased with increasing stress, the functionality of the community was enhanced.

The effect of drying and rewetting stress on soil microbial communities was investigated by Schimel *et al.* (2007). They determined that soil drying and rewetting caused a decrease of 51% and 24% respectively on the microbial biomass. However, work conducted by Kumar *et al.* (2013) on the freeze thaw effects on Artic soils reported that a single freeze thaw event can cause the death of 50% of the microbiological biomass, with extreme events killing up to 60%. This indicates that the effect of freeze/thaw may be a greater stress to environmental bacteria than a wet/dry event.

2.3.1.3 Increased Salinity

Restriction digestion of samples taken from a multi-pond saltern (sodium chloride) system, covering a sodium chlorine concentration of 6.4 – 30.8%, show that the bacterial diversity decreases with increasing salt concentration but that the archaea diversity increased concomitantly (Fernández *et al.*, 2014).

2.3.1.4 Sewage and Toxic Chemical Contamination

When sewage sludge with low or high levels of heavy metal contamination was added to soil, the high heavy metal sludge caused a larger decrease in the microbial biomass than the low level contamination sludge (Singh *et al.*, 2011). This indicates that the practice of recovering treated sewage effluent should not have a negative effect of the bacterial species diversity.

Studies conducted by Yao *et al.* (2017) shows that heavy metal contamination of soil caused a decrease in bacterial diversity and caused many of these communities to lose their degradative abilities. Singer *et al.* (2016) report that waters exposed to toxic wastes have a higher degree of antibiotic resistance and contain more plasmids than clean or slightly contaminated waters. This concurs with Chen *et al.* (2015) who state that heavy metal resistance is linked to drug resistance. The presence of plasmids in these bacteria is indicative of increased transfer of genetic material which may improve the cells ability to survive in stressed environments (Kempf *et al.*, 2016).

2.3.2 Biocidal Stress

Bacterial resistance to non-oxidising biocidal action has been widely studied (Wales and Davies, 2015) and indicates that biocides are not equally active against all bacteria (Bridier *et al.*, 2011). There are several proposed resistance mechanisms to biocide action (Gnanadhas *et al.*, 2012). These included reduced uptake of the biocide by cellular impermeability (Barah, 2013) or efflux (Gnanadhas *et al.*, 2012). Since biocides have multiple bacteria targets, it follows that resistance results from cellular changes that limit or stop biocide accumulation.

Mutations, in the form of acquisition of resistance determinants on new genetic material e.g. plasmids (transference) are well documented (Bennett, 2008, Al-Marzooq *et al.*, 2015). The modification of the target site or enzyme has also been proposed as a mechanism of resistance (Garneau-Tsodikova and Labby, 2016) and the development of resistance mechanisms in biofilm bacteria has been widely studied (Singh *et al.*, 2017).

Resistance by biocide inactivation is known but rare, and specific to a few classes of biocides (Garneau-Tsodikova and Labby, 2016). Studies have shown that certain biocides may induce cross resistance to other biocides, indicating that a system may become more resistant to a given biocide after treatment with another (Bridier *et al.*, 2011). It is thus essential that any biocide treatment on the cooling water system include biocides with oxidising capabilities, to ensure the destruction of the bacterial cell.

2.3.2.1 Cellular Impermeability

Many non-oxidising biocides attack cell wall structure forming a stressed environment for the bacteria (Wales and Davies, 2015). The outer membrane of Gram-negative bacteria acts as a permeability barrier to the ingress of biocides, limiting uptake (Gnanadhas *et al.*, 2012). There are several papers supporting that changing permeability is responsible for acquired resistance in Gram-negative bacteria, offering them a higher resistance to biocides than Gram-positive bacteria (Garneau-Tsodikova and Labby, 2016). These proposed changes include changes in surface hydrophobicity (Exner *et al.*, 2017), outer membrane ultrastructure (Zgurskaya *et al.*, 2015), outer membrane protein composition (Exner *et al.*, 2017), and

changes in outer membrane fatty acid composition (Zgurskaya *et al.*, 2015). Bacterial spore cells have double coats which limit permeability (Gnanadhas *et al.*, 2012). Some commercial biocides actively attack the cell wall structure, which enhances biocidal action (Wales and Davies, 2015).

2.3.2.2 Efflux

Efflux is the mechanism of moving compounds out of the cell. The efflux systems require an energy source (e.g. ATP) to transport unwanted substances through efflux pumps (Wilkens, 2015). Efflux systems recognise compounds based on physiochemical properties. As antimicrobials are normally amphiphilic, they are easily recognised by efflux pumps. This has a significant effect on biocide resistance, attributed to the following:

- Genes coding for the efflux pumps are encoded on chromosomes or plasmids giving natural or acquired resistance, respectively.
- Antimicrobials act as inducers or regulators for the expression of efflux pumps.
- The presence of several efflux pumps in a bacterial species lead to a broad spectrum of resistance (Munita and Arias, 2016).

2.3.2.3 Target Site Mutation

Biocidal resistance may be developed by mutation or amplification of chromosomal genes or by acquiring plasmids. Biocides have several target areas (cellular components) within bacteria cells and site mutations are rare in biocide resistant organisms (Garneau-Tsodikova and Labby, 2016).

2.3.2.4 Biofilms

It is hypothesised that biofilms may concentrate biocides within their structure, as they would accumulate nutrients, this would make the bacterial cells within a biofilm more susceptible to biocidal action (Guest, 2016). However, these effects are not well comprehended or routinely observed in practice. It is more likely that biocide resistance of bacterial cells, in biofilms, is due to the decreased permeability of extracellular polymeric substances (EPS) that form the biofilm matrix (Liu *et al.*, 2011; Flemming, 2016). Beyond chemical interactions with EPS,

slow diffusion through the biofilm matrix also reduces delivery of disinfectants to bacteria deep in the biofilm (Liu *et al.*, 2011). Biofilm bacterial cells have been shown to be 10 – 1000 times more resistant to biocidal action than planktonic cells of the same species (Flemming, 2016).

Biocide treatment is based on understanding the bacteria present, the allowable period of contact, required concentration, temperature, pH and available nutrients (Gnanadhas *et al.*, 2012). Each non-oxidising biocide has its own mechanism of action and the use thereof therefore selects for a different set of resistant bacteria (Wales and Davies, 2015). It is a known fact that systems dosed with a single non-oxidising biocide will develop into a system with low bacterial species diversity, populated with species that can tolerate the biocide (Wales and Davies, 2015). Studies conducted by Guest (2016) showed that bacteria cannot become resistant to oxidising biocides. Thus, an oxidising biocide should be used as part of a treatment regime to prevent the development of microbial resistance.

Although (Bridier *et al.*, 2011) published information on disinfectant and biocide selectivity in different environments and for different applications, there are few studies detailing how bacterial communities react to the dosing of various biocides in cooling water systems and even less that identify the changes in species diversity in such systems (Yurudu, 2013).

2.3.2.5 Stress vs Species Diversity Hypothesis

For the purpose of this study, the bacterial count – species diversity relationship is explained in Hudson's model shown graphically in Figure 2.2 (Huston, 2014). It is predicted that the addition of a biocide to the cooling water system will cause a change from a high species diversity - high numbers environment to a low species diversity- high numbers environment. Category A is indicative of an environment with a high level of species diversity but with few individuals of each species as seen in an environment with limited nutrient availability. A category B habitat includes a high level of species diversity with a high number of individuals per species. This is found in a stable community with sufficient nutrients and limited or mild external stresses. An environment in category C has low species diversity and low numbers of individuals per species. This is indicative of a pioneer community or a heavily disinfected environment. Category D indicates an extreme environment, where an

external stress (pressure, temperature, salinity, toxin concentration) limits the bacterial species capable of surviving in the conditions.

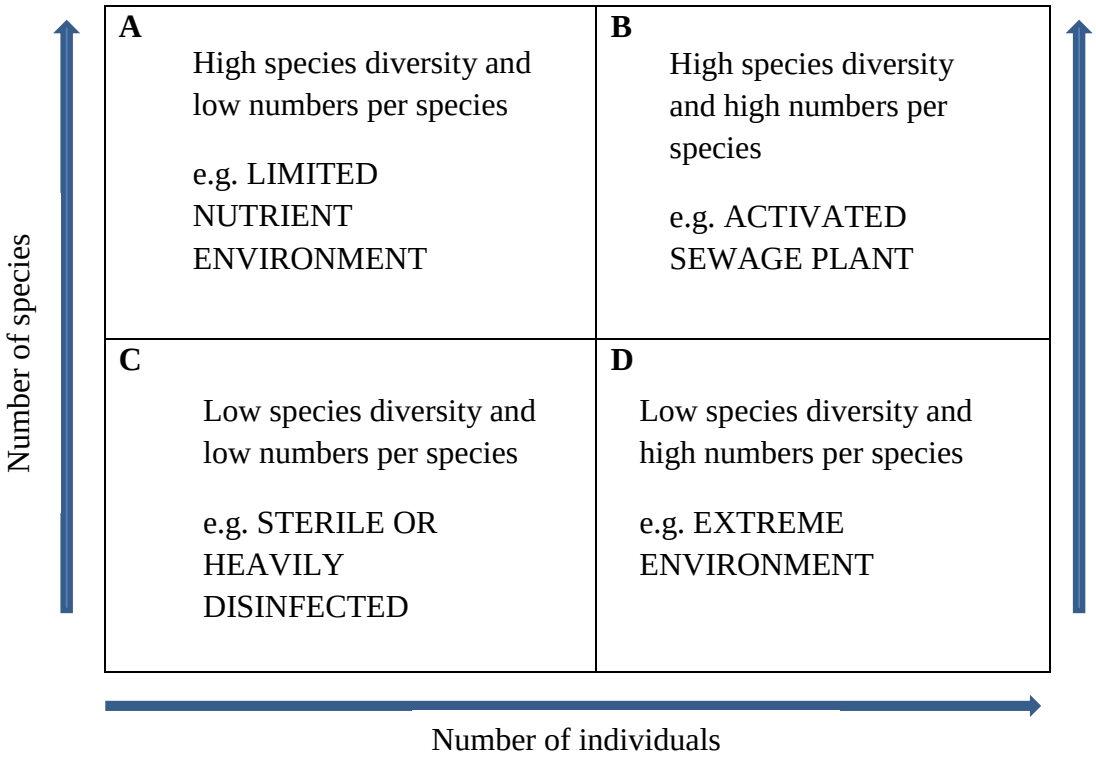

Figure 2.2: Theoretical indication of the relationship between different microbial environments based on the species diversity (derived from Hudson, 2014).

The conditions within various environments are able to change and this will cause a corresponding change in the species diversity. As biocide concentration within a water system decreases the environment should change from a C to B, as the bacteria re-establish. If a D environment experiences a decrease in the relevant extreme parameter the environment will tend towards category B, allowing the suppressed or unadaptable bacteria to grow.

2.3.3 Viable but non-culturable state (VBNC) and Persistor cells

The concept of dormancy plays an important role in the metabolism of bacteria. Some bacteria are able to become dormant during times of stress and Li *et al.* (2014) suggested that at least 85 bacterial species have been found in a viable but not culturable (VBNC) state in order to survive times of stress (Li *et al.*, 2014), while allowing for a "resurrection" once the conditions become more favourable (Ayrapetyan *et al.*, 2014). Bacterial cells are capable of

entering a novel, physiological, viable but non-culturable (VBNC) state, in response to stressful conditions (Ramamurthy *et al.*, 2014). Although the exact role of the VBNC state in bacteria is not fully understood, it is believed to be a survival technique against these stressed conditions (Olivier, 2010). These stresses include *inter* alia, factors such as antibiotic/biocidal pressure, high/low temperature, starvation, chlorination, change in the pH, and oxygen stress (Patrone *et al.*, 2013; Pasquaroli *et al*, 2013; Pawlowski *et al.*, 2015). Although this strategy is believed to be a survival strategy, research has shown that bacteria in the VBNC state act as a reservoir for bacteria in the environment (del Mar Lleo' *et al.*, 2007), which has important implications in several fields including environmental monitoring (Ramamurthy *et al.*, 2014).

These VBNC cells differ from dormant cells in that they remain viable; maintaining a low, measurable, level of metabolic activity (Ramamurthy *et al.*, 2014), but they can no longer be cultured on standard laboratory media (Oliver, 2010). Bacterial cells in the dormant state, however, will display metabolic activity which is below detection levels (Olivier, 2010). Due to the inability to culture VBNC bacteria with conventional methods, community densities are often underestimated (Ramamurthy *et al.*, 2014). This leads to the requirement for alternative non-culturing techniques, which may require direct examination for the detection of an intact cell membrane (Olivier, 2010) or the use of reverse transcriptase (RT)-PCR, which detects gene expression (Conway & Schoolnik, 2003)

In the VBNC state bacterial cells are often resistant to biocides and antibiotics, which often cause treatment failure in human health (Hu and Coates, 2012). This is likely because the VBNC cells have such low metabolic activity which limits the antimicrobial's activity (del Mar Lleo *et al.*, 2007). VBNC cells can be resuscitated once the stressor is removed and will reacquire their infective abilities.

Persister cells are similar to VBNC cells but are separated based on their ability to be cultured as soon as the stress is removed (Ayrapetyan *et al.*, 2015a), while VBNC cells require time and optimum conditions to resuscitate (Li *et al.*, 2014). VBNC cells have been detected during persister experiments indicating that the cells co-exist and are formed under the same conditions (Ayrapetyan *et al.*, 2015a). In addition, Ayrapetyan *et al.* (2015b) proposed that persister cells may be the transitory cells leading to the formation of VBNC cells, that they

are closely related and have shared mechanisms which control their existence. The relationship between persister and VBNC cells is expanded in Figure 2.3.

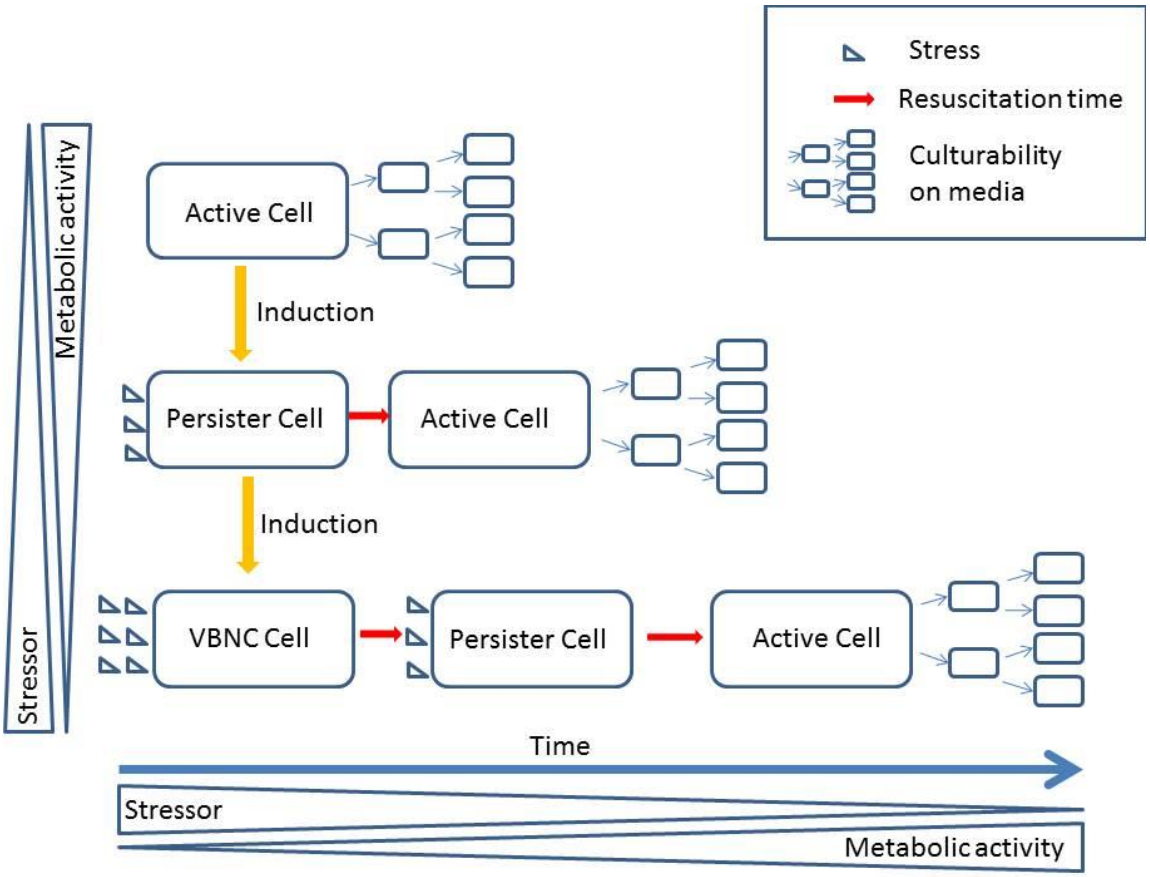

Figure 2.3: The relationship between Active, Persister and VBNC cells. Adapted from Ayrapetyan *et al.* (2015a).

2.4. INDICATORS OF CHANGE

2.4.1 Species Diversity as Environmental Indicators

The use of aquatic diversity changes in community structure has been used as a tool to detect pollution in and health of various environments (Martinez, 2013). There are several macroscopic biological indicators (fish, frogs and daphnia) that are utilised to determine the toxicity of an aquatic environment, but microorganisms are not widely used. Although macroscopic organisms may miss small changes in the environment due to their resilience at low concentrations, bacteria, however, respond to slight changes in environment, with large phenotypic variations in enzymatic activity, cell wall composition and cell surface structure

(Zrelli *et al.*, 2013), often involving molecules that control transfer over these barriers (Dang and Lovell, 2016). This makes bacteria a more sensitive indicator of environmental changes.

The concept of survival of the fittest is not new (Spencer, 2018); this theory is used to understand changes in various environments including water, soil, forests, coral reefs and ground water. This idea proposes that only those organisms able to adapt to the new environment will survive, although the environmental changes may be either positive (increased nutrients) or negative (drought, toxins). Changes in ecosystem diversity have been used to indicate the presence of atypical environmental conditions acting on the environment (e.g. pollution). These changes will kill off non-adaptable species and allow the species that adapted to survive.

A rapid decrease in aquatic diversity may indicate *inter alia* ineffective water protection (Solomon, 2008) and maintaining this biodiversity is essential to ensure correct system function (Oliver *et al.*, 2015). Exploitation of water sources has led to a shift in the ecological balance (Oliver *et al.*, 2015). These shifts result in species diversity changes as more tolerant species survive (Gruner *et al.*, 2016).

Species diversity changes within aquatic ecosystems can be brought about by chemical changes (Gruner *et al.*, 2016), however, turbidity, total suspended solids, temperature, pH, salinity, inorganic salts, decreased dissolved oxygen and increased nutrient concentration have all been shown to cause diversity changes in ecosystems (Oliver *et al.*, 2015).

Increasing the available nutrient concentration allows uncontrolled growth of algae and water plants which decrease the dissolved oxygen, leading to eutrophication (Abdel-Raouf *et al.*, 2012). It also allows non-native and tolerant organisms to thrive under the new conditions (Logue and Lindstom, 2008).

Microbes found in extreme environments have adapted in order to live and often require these conditions to be maintained to ensure continued survival (Rampelotto, 2010). However, they do not necessarily adapt well to more classically favourable environments (Pikuta and Hoover, 2007). Generally, the more extreme the environment, the lower the microbial species diversity within the community (Sati *et al.*, 2013). Historically bacterial communities in extreme environments were believed to have limited species diversity, with a higher

number of individuals, but recent research has indicated that extremophile species diversity, in extreme environments may be as diverse as the diversity at more "conventional" conditions (Rasuk *et al.*, 2016).

2.4.2 Diversity Indices

The indices used to determine microbial diversity were derived from models developed for plant or animal studies. The clear species definition and unambiguous individual identification, required by the Shannon or Simpson indices, are difficult to apply when studying bacteria (Atlas and Dartha, 1993), although they are utilised as standard practice.

Ideally a bacterial diversity index should: (Atlas and Bartha, 1993)

- Include three diversity dimensions (species diversity, species abundance and taxonomic distance between individuals).
- Be based on a statistically justified parameter.
- Be insensitive to possible errors or varying analytical results.
- Not be overly sensitive in relation to sample size. (Atlas and Bartha, 1993)

However, bacterial species diversity is difficult to define because it incorporates species richness (number of species) and species abundance (number of individuals in each species) (Locey and White, 2013).

2.4.2.1 Shannon Diversity Index (Shannon-Weaver, Shannon- Weiner)

The Shannon diversity index was developed by Shannon and Weaver (1948) and is commonly used for environmental studies combining richness and abundance (Morris *et al.*, 2014; Rocchini *et al.*, 2016) as it is easy to use and is sensitive to slight variations. It quantifies the uncertainty in the species identification of an individual randomly taken from the community, has a range of 0 to 5 and is calculated by:

$$H' = -\sum p_i(\log p_i) = -\sum (n_i/N)\log(n_i/N) \qquad \text{(Odum, 1969)}$$

Where n_i is the number of individuals of each species, N is the total number of individuals for the site and p_i is the importance probability of each species (Odum, 1969).

This formula has been used subsequently by White and Pickett (2012) and Pianka (2011).

2.4.2.2 Simpson Index

The Simpson index measures dominance (λ). It quantifies the probability that two individuals drawn at random from an infinite community belong to the same species (Simpson, 1949). This index was used by Doan-Nu *et al.* (2016) for the evaluation of anthropogenic impact on phytoplankton in coastal waters, while Dörsch *et al.* (2012), used the Simpson index to evaluate pH responses of cells extracted from denitrified organic soils.

$$\lambda = \sum \frac{n_i(n_i-1)}{N(N-1)}$$

Where n_i is the number of individuals of each species and N is the total number of individuals for the site (Simpson, 1949).

This index is also known as the Hunter-Gaston index in microbiology. It is less sensitive than the Shannon index and is less sensitive to species richness and tends to most abundant species (Hunter and Gaston, 1988; Morris *et al.*, 2014).

In order to calculate true species diversity (1-λ), the Gini-Simpson index must be calculated.

$$1 - \lambda = 1 - \sum p_i^2$$

Where p_i is the the importance probability of each species (Jost, 2006). This technique was revitalised by Guiasu and Guiasu (2012).

2.4.2.3 Renkonen Percentage Similarity Indices

The Renkonen percentage similarity index is used to measure the similarity between communities based on species composition (Powney *et al.*, 2014)

$$P = \sum \text{minimum}(p1_i, p2_i)$$

where: P = Percentage similarity between sample 1 and 2, $p1_i$ = Percentage of species *i* in community sample 1, $p2_i$ = Percentage of species *i* in community sample 2 (Renkonen, 1938).

It can be used to compare communities over long time periods and is particularly useful when communities are very different and other diversity indices would be irrelevant (Internet, 2015a). Although it is a simple calculation, it is a good quantitative technique as it is not affected by potential differences in abundance but is sensitive to additive changes (Giere, 2008).

2.4.3 Methods for analysing changes in species diversity

2.4.3.1 Classical Plate Count Culturing

Conventional classical plate count methodologies utilise a suite of different nutrient growth media to enumerate viable microorganisms (Parshionikar *et al.*, 2009). These tests are often combined with subsequent physiological and/or biochemical analyses for further identification of the bacteria (Sohier *et al.*, 2014). Plate count enumeration has been commonly utilised for the study of bacterial communities in various aquatic environments. (Gensberger *et al.*, 2015) and is the basic technique for many international water standards (Allen *et al.*, 2004).

Culturing offers fast, easy and relatively inexpensive results of the viable heterotrophic bacteria in the study sample (Lagier *et al.*, 2015). However the majority (90 – 99.9%) of microbes, in their natural environments, are unculturable and thus undetected with this technology (Parshionikar *et al.*, 2009). For this reason culturing is seen as inaccurate and only used as a supporting analysis.

2.4.3.2 Molecular Fingerprints.

Molecular (nucleic acid) techniques have been developed to overcome the problems with classical culture techniques (Kuchta *et al.*, 2014). These methods are generally qualitative not quantitative, time consuming, complex and require a trained analyst (Ali *et al.*, 2017). Metagenomics focuses on the analysis of genetic material (DNA or RNA) recovered directly

from environmental samples (Thomas *et al.*, 2012). While classical culture microbiology techniques and microbial genome sequencing/genomics rely heavily on culturing cultures, environmental DNA sequencing often relied on either directly extracted DNA or cloned specific genes (often the 16S rRNA gene) to produce a profile of community diversity from an environmental sample. This work revealed that the vast majority of microbial biodiversity is not accounted for in culture-based methods (Thomsen and Willerslev, 2015). Recovery of DNA sequences from environmental samples, which are longer than a few thousand base pairs, was very difficult in the past. However, metagenomics allows for the construction of genetic libraries in bacterial artificial chromosomes (BACs) that provide better vectors for use in molecular cloning. However, shotgun sequencing strives to ensure that many organisms that would be missed through classical culturing techniques are represented in sequence segments (Eisen, 2007).

2.4.3.2.1 16S Ribosomal (r)RNA

Certain regions of rRNA gene sequences are conserved across all organisms while other regions vary (Srinivasan *et al.*, 2015). The 16S gene of the bacterial genome contains the rDNA code for the 16S subunit of the ribosome (Srinivasan *et al.*, 2015) and is most often used as the target site for bacterial, molecular studies due to its usefulness in phylogenetic/species studies (Land *et al.*, 2015) allowing for phylogenetic comparisons between different microorganisms (Srinivasan *et al.*, 2015). In addition, the sequencing of this gene facilitates the identification of both cultured and as yet uncultured bacteria (Kuchta. 2014).

2.4.3.2.2 Polymerase chain reaction (PCR)

Thermocycle polymerase chain reaction (PCR) was developed in 1983 by Dr Kary Mullis, (Garibyan and Avashia, 2013). This process enabled the amplification of micro- or nano-gram amounts of short, well-defined DNA sequences by many orders of magnitude (Ohad *et al.*, 2016).

PCR requires several basic components:

- DNA template – This can range from total genomic DNA or DNA fragments containing the target region of DNA to be amplified.

- Two primers – Primers are short, artificial DNA strands 18-25bp long that are complementary to the beginning and end of the target region of DNA to be amplified, where they anneal to the template and allow *Taq* polymerase to bind and begin synthesis of the new strand. Degenerate primers may also be used - these are mixtures of similar, non-identical, primers. As several codons may code for the same amino acid, it may be necessary to use a primer that has a varying base composition. For example, the tri-base primer sequence for a certain amino acid may be ATH. This means that an adenine and a thymine are required, followed by an adenine, thymine or cytosine, designated by the letter H. By varying this base, the primers allow for amplification of the same gene from different organisms with differing codon usages (Garibyan and Avashia, 2013).
- dNTPs – nucleotides, the building blocks of DNA, which are incorporated into the reaction for utilisation during synthesis of a complementary DNA strand.
- A thermostable DNA polymerase – which catalyses the extension of a complimentary DNA strand. DNA polymerases may cause errors when copying DNA by mismatching nucleotide bases, leading to mutations in the sequence. *Taq* polymerase lacks 3'-5' proof-reading exonuclease activity, and therefore has a fairly high error rate (McInerney *et al.*, 2014). A commercial combination of the two enzymes is available for PCR (Fermentas, Life Sciences).
- Buffer – this maintains a stable chemical environment for the DNA polymerase and the DNA template when it is being duplicated.
- $MgCl_2$ – The addition of this salt affects the specificity of primer binding. Increased concentrations lead to lower specificity. Degenerate primers have a low specificity; therefore a moderate to high concentration of $MgCl_2$ is used.

There are three basic steps in PCR (Wang, 2014):

i. The target DNA is denatured at a temperature of 90-96°C. The hydrogen bonds between the two DNA strands are broken and the double-stranded helix unwinds to form single stranded DNA. This initial step lasts for 1-2 minutes to ensure all DNA is in single stranded form.

ii. The temperature is reduced to between 45 and 55°C where hybridisation or annealing takes place. The temperature at this stage is usually 5°C below the melting

temperature of the primers. The primers hybridise with the single strand DNA at a region of high homology.

iii. The temperature is raised to 72°C where the *Taq* polymerase binds to the 3' end of the double stranded DNA region and adds dNTPs (deoxyribonucleic triphosphates) to the 5` end of the primer sequence, forming an exact and complimentary copy of the template. This step is known as extension.

This cycle is repeated 30-40 times, which allows for an exponential increase in the template concentration. A final extension at 72°C allows for final and complete extension. The reaction is then cooled rapidly to 4°C to preserve the amplified DNA (Ohad *et al.*, 2016; Ali *et al.*, 2017).

2.4.3.2.3Molecular identification techniques

These techniques normally involve the comparison of DNA nucleotide sequences that have been amplified by PCR. There are several techniques that utilise this technology but DGGE was used in this study.

DGGE involves the separation of DNA fragments of the same length but differing sequences (Carmona *et al.*, 2012). DGGE technology was first introduced by Muyzer *et al.* (1993) as a molecular fingerprinting technique. It is an ideal method for the separation of DNA fragments of the same length but different sequences. 16S ribosomal DNA is suitable for DGGE as it can be specifically amplified from target organisms. It is the most widely studied bacterial gene and has sufficient heterogeneity for good phylogenetic resolution. Additionally, a large database of 16S rRNA genes sequences is available (over 30 000 variations stored in Genbank) (Land *et al.*, 2015). In the process of DGGE, double-stranded (ds) DNA PCR products (200-1500bp) undergo electrophoresis through a polyacrylamide gel that contains an increasing denaturant gradient (Mohammadi *et al.*, 2015). The most common denaturants are heat (constant 60°C), formamide (0-40%) and urea (0-7 M) (Muyzer *et al.*, 1993)

Double stranded DNA fragments initially move according to their molecular mass; i.e., the larger the molecule the slower it will move. As the gradient increases the dsDNA fragments begin to denature (separate into two strands suddenly within a narrow concentration range), slowing their progress through the gel (Surridge, 2007). The strands do not separate in a

zipper-like or bubble fashion (Surridge, 2007), but are halted by the scissor-like action of the strands separating; the whole strand is stabilized in the gel by a GC clamp. The GC clamp is a GC-rich sequence incorporated into one of the primers, during PCR, to modify the melting behaviour of the fragment and allow for the majority of sequence variation to be detected in the denaturing gel (Rettedal *et al.*, 2010; Lourenz, 2012). The denaturing range of the strand is determined by the G-C bonding within the strand; the higher the GC value the more stable the strand. A GC-clamp attached to the 5' end of a PCR product prevents complete melting during fragment separation in a denaturing gradient, and sensitizes DGGE enough to detect single base changes in PCR fragments of 500 bp (Lourenz, 2012). Rettedal *et al.* (2010) found that attaching a GC-clamp of 40-45 bp to primers allowed for the determination of single-base-mutations, previously only 40% distinguishable in DGGE analysis, to increase to 100%. Genetic variations of microorganism's genes and possible mobility shifts cause differentiation of genetic sequences, allowing species in environmental samples to be separated (Surridge, 2007; Mohammadi *et al.*, 2015). The sensitivity of DGGE can be increased by narrowing the concentration gradient. This allows strands of similar structure to be denature over a wider range of denaturant conditions, making the process more sensitive. Under optimal conditions, DGGE can separate oligonucleotides differing by as little as one base pair (Old *et al.*, 2012).

Once the sample has been allowed to pass through the gel, the gel is stained with a fluorescent DNA-binding agent such as Cyber Gold or Ethidium bromide and viewed under UV-light. When DNA preparations from environmental samples, amplified by PCR using primers specific for any marker gene, are analysed on DGGE, a ladder-like set of bands is formed. Individual bands on a gel indicate DNA fragments of different sequence. When phylogenetic markers such as the 16S rRNA gene are used, each band is generally thought to reflect the presence of an individual microbial species (Srinivasan *et al.*, 2015). These bands can be excised and after a further PCR can be sequenced for tentative species identification (Rettedal *et al.*, 2010). This methodology allows for a rapid, high resolution, visual indication of changes in communities (Everman and Wang, 2017). One of the problems associated with DGGE is that of co-migration. Although the technique is sensitive enough to detect single base pair differences, multiple variations are more difficult to detect. This is due to similar electrophoretic mobilities in phylogenetically related sequences and similar melting temperatures in separate sequences (Stewart, 2012). As a result, an amplified product from more than one species may be represented in a single band (Surridge, 2007).

DGGE has been used for the determination of genetic diversity and predominant communities in environmental samples, without further analysis (Shah, 2015). DGGE has been used to compare soil populations in different agro-ecosystems and under different afro-economic treatments, with one soil being contaminated with polycyclic aromatic hydrocarbons (PAH) (Srinivasan *et al.*, 2015). This research indicated large species diversity in all the soils treated but lower diversity in the PAH contaminated soils. Similar research conducted by Wemheuer *et al.* (2016) studied bacterial community diversity in fertilised and unfertilised grasslands. This research showed that more highly fertilised grasslands had a lower bacterial diversity. This suggests that bacterial communities have evolved and stabilised to this nutrient level.

Individual bands in DGGE gels may be sequenced to extract more detailed phylogenetic information. The resulting sequences can then be used for comparative phylogenetic analysis to determine the evolutionary relationships between organisms in the community being analysed. Everman and Wang (2017) investigated a soil fungal community by DGGE of the ITS region (ITS1-F with a GC-clamp and ITS2 yielding a 300 bp fragment), sequencing of bands, and BLAST analysis of the resulting sequences. Phylogeny gives an indication of species diversity and not richness, since each band indicated the presence of a single species and does not indicate the richness of the community (Villalobos and Vamosi, 2016). By determining the closest relatives of unknown organisms, characteristics of the relatives may be inferred upon the unknown organisms (Chen, 2012). The sequence data can also be used in the design of primers and probes for *in situ* identification of selected organisms.

Software such as Gel2K has been designed, by Norland (2004), to facilitate the analysis of gel images by estimating the relative position of peaks/bands in a lane (Surridge, 2007). Subsequent optimization of the image will allow the formation of dendrograms (indicating the relatedness of the species) and the comparison of band intensity, which is roughly proportional to the species richness (Shah, 2015).

2.4.3.3 Biolog Ecoplates®

Biolog Ecoplates® are 96 well, plastic, microtiter plates with 3 x 31 carbon substrates in the wells (Table 2.2). One well in each set is utilised as a control blank, with no carbon substrate

(Mahrous, 2012). Tetrazolium dye, included in each well, is reduced by the respiration of the bacteria, and turns to violet formazan.

The substrates utilised form a pattern (metabolic fingerprint) that, when measured spectrophotometrically, can be used to assess or characterise bacterial presence (Gryta *et al.*, 2014) or determine the functionality of the environment (Zhang *et al.*, 2013), the bacterial species diversity (H') (Yao *et al.*, 2017), the presence, absence or rate of utilisation of substrates (Gryta *et al.*, 2014) or to compare the impact of changes in communities (Zhang *et al.*, 2013). The technique can also be used to conduct studies of community level physiological profiles (CLPP) (Gryta *et al.*, 2014).

Although the Biolog Ecoplate technique offers a fast and simple means to study community diversity and metabolic changes, however it has some limitations.

- The community function is implied as actual catabolic activity and the Biolog offers only the potential activity.
- It is considered a culture dependant technique (Concha-Guerrero *et al.*, 2014) as there are some bacteria which will not be able to grow on the substrates available on the plates (Zhu *et al.*, 2016). However there is an argument that the Biolog cannot be considered culture dependant, but rather a set of metabolic tests (Locey and White, 2013).
- Several bacteria do not reduce tetrazolium dye (Concha-Guerrero *et al.*, 2014) and results obtained may be indicative of only part of the community (Kteily, 2014).

Biolog plates have been used to evaluate, amongst others:

- The effect of tilling on microbial community composition (Chaer *et al.*, 2008).
- Bacterial diversity in soils irrigated with mine water (Lekhanya, 2010).
- The effect of plant species diversity on soil bacterial communities (Muñiz *et al.*, 2014).
- Microbiological communities on stone ballast at oil depots (Surridge, 2007).
- The differing microbial activity in various zones of sewage plants (Gryta *et al.*, 2014).
- The effect of temperature on sewer sediment (Biggs *et al.*, 2011)

For this study the purpose is not to detect each reaction of the bacterial community but rather to give a pattern of a community. It is believed that this pattern will change when the system is placed under stress (Gryta *et al.*, 2014; Huston, 2014). The pattern may show:

- A high number of substrates being utilised indicating a high species diversity.
- Evenness of species counts which would be shown by the same substrates being utilised even when diluted.
- When diluted some bacteria exit the community the species diversity will decrease. The extend reduction on dilution should be indicative of the community structure (Gryta *et al.*, 2014).

Table 2.2: Carbon substrate configuration and groups on Biolog Eco-plates® (configuration repeated 3 times on each plate) (Chazarenc *et al.*, 2010).

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

NOTE:

Carboxylic acids
Phosphate carbon
Amino acids
Carbohydrates
Amines
Complex carbon sources

Jousset *et al.* (2017) developed a model for the interpretation of the microbial community patterns of a diluted sample (Figure 2.4). Line A theoretically shows homogeneity as the bacteria ratio remains unchanged on dilution. However, as homogeneity is unlikely it is accepted that line A indicates the presence of a predominant bacterium in the environment. Line B shows a heterogeneous species distribution within the environment. As the sample is diluted the species with lower numbers are diluted out and do not consume the relevant substrates. Line C is indicative of an environment where the bacteria are suppressed by non-fermentative bacteria in the community. Once these are diluted out, the unsuppressed species utilise the different substrates.

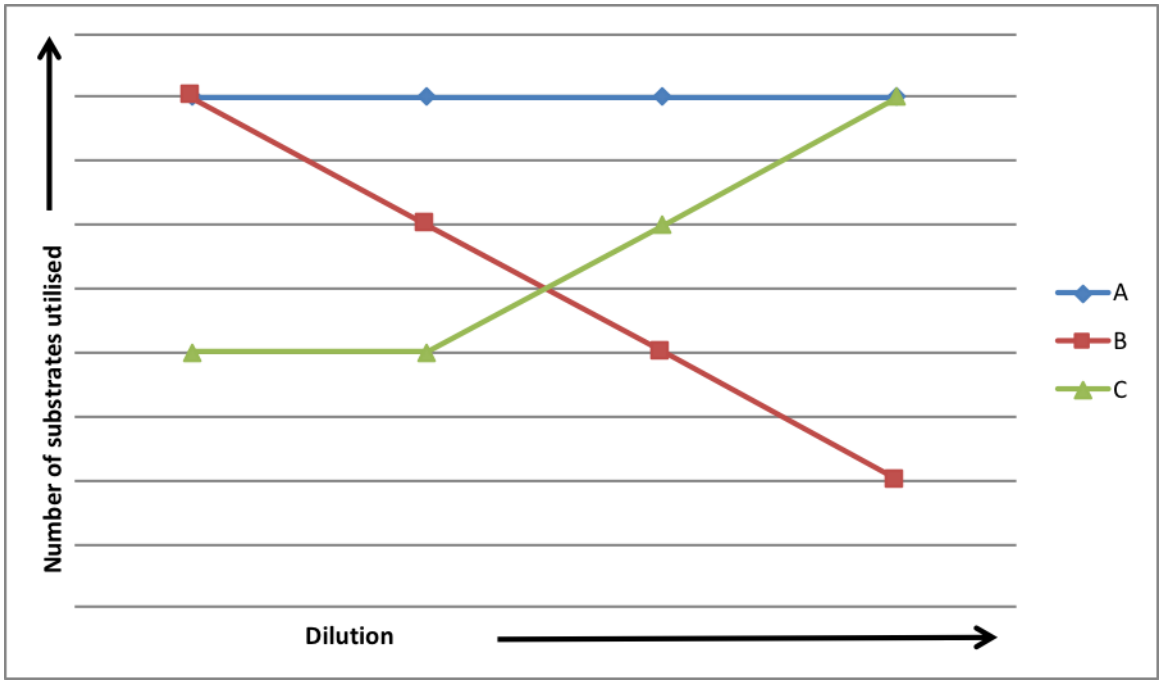

Figure 2.4: Model for bacterial community interpretation by Jousset *et al.* (2017).

2.5. BACTERIAL AND ANTIBIOTIC RESISTANCE RELATIONSHIPS

Antimicrobial products used in industries, as disinfectants, are called biocides while those used in for the treatment of man and livestock health are termed antibiotics (SNENIHR, 2009). In general biocides have a broader range of activity as they target several sites in the cell while antibiotics have more specific targets (Garneau-Tsodikovo and Labby, 2016). Although both products kill bacteria, the use of antibiotics is strictly controlled and biocides can be used without any form of monitoring (SNENIHR, 2009). However, the abuse and

over-prescription of biocides and antibiotics led to an increase of antimicrobials resistance in bacteria, especially in aquatic environments (Choudury *et al.*, 2012), necessitating treatment with other more expensive chemicals. Bacteria are considered resistant to antibiotics and/or biocides when the following situations occur (Bridier *et al.*, 2011).

- When the bacterial species is not killed or inhibited at antimicrobial concentrations commonly used in practice.
- When the bacterial species is not killed or inhibited by a concentration where most species are affected.
- When the bacteria are not affected by a concentration which acts on the other cells of the culture.

Although antibiotics have been proven to be effective against pathogens, there are several studies that indicate they severely damage the host microbial community forming an environment inductive of the growth of opportunistic pathogens, increasing the development of antibiotic resistance (Ubeda *et al.*, 2010).

Høiby *et al.* (2010) reported the development of antibiotic resistance in biofilms and there are several laboratory scale studies on the possible link between biocide use and antibiotic resistance (Martinez, 2009; Allen *et al.*, 2010; WHO, 2014). While others, have shown a link between biocide exposure and antibiotic resistance (Kummerer, 2009; Berendonk *et al.*, 2015). Biocide-antibiotic cross-resistance is mainly due to efflux pumps which work for both agents (Berendonk *et al.*, 2015) and numerous studies indicate that Gram-negative bacteria are more likely to develop this cross resistance (Berendonk *et al.*, 2015; Wales and Davies, 2015).

Bacteria become increasing resistant as antimicrobial concentrations increase and, in some cases, biocidal resistance mechanisms can contribute to antibiotic resistance as the mechanisms for development are similar (Bridier *et al.*, 2011). These include impermeability (Exner *et al.*, 2017), multi-drug efflux pumps (Randall *et al.*, 2007), over expression of operons (Berendonk *et al.*, 2015), plasmid transfer (Choudhurg *et al.*, 2012) quorum sensing (Fernandes and Hancock, 2013) and the alteration of a target site (Blaire *et al.*, 2015).

Although there have been several studies which determine an association between plasmids and antibiotic resistance (Dantas *et al.*, 2008), D'Costa *et al.* (2011) did not find any correlation when studying agricultural soils.

The, above-mentioned, common mechanisms are all utilised in the development of antibiotic resistance in biofilm bacterial cells (Fernandes and Hancock, 2013) Studies show that biofilm bacteria are 100 – 1000 times more resistant to antimicrobials than their planktonic counterparts (Høiby *et al.*, 2010; Flemming, 2016). Berhe *et al.*, (2017) proposed 3 possible mechanisms explaining antibiotic resistance in biofilms.

These hypothesised mechanisms are:

1. Possible slow or incomplete penetration into the biofilm
    - Studies on *in* vitro biofilm penetration indicate that some antibiotics can penetrate bacterial biofilms (Otto, 2008).
    - If the antibiotic is inactivated in the biofilm, penetration will be severely limited. The antibiotic may be inactivated in the surface layers before it is able to penetrate.
    - Antibiotics which adsorb into the biofilm matrix may have limited penetration.
2. Altered chemical environment in the biofilm
    - Nutrient gradients in the biofilm may limit growth.
    - Anaerobic regions in thick biofilms limit oxygen availability.
    - The accumulation of waste products may alter the pH (Stewart and Franklin, 2008).
    - These changes may cause the bacteria to enter a non-growing state.
    - Osmotic changes may lead to an osmotic stress response (Stewart and Franklin, 2008).
3. Speculated mechanism that only subpopulations of bacteria in the biofilm form a highly protected, spore-like state.
    - Supported by findings that indicate that newly formed biofilms show resistance, although they are too thin to act as a barrier to penetration (Ragon *et al.*, 2011).
    - Most bacteria in a biofilm are killed by antibiotics but approximately 1% on the initial community continues even if continually exposed to the antibiotic (Wood *et al.*, 2013).

The development of biofilms in cooling water systems is well documented (Flemming and Ridgeway, 2008; Lear and Lewis, 2012) as is the use of biocides in their treatment and management (Satpathy *et al.*, 2016; Liu *et al.*, 2011). It is thus pertinent that biocide resistant bacteria in cooling water biofilms may resist antibiotics. This is of concern since bacteria which naturally reside within biofilms e.g. *Legionella* and *Pseudomonas*, may have developed antibiotic resistance while within the biofilm. When the biofilms slough off the surfaces these bacteria are released into the bulk water which may come into contact with personnel, raising a significant health risk.

Biolog® Phenotype Microarray (PM) Plates (Hayward, CA) offer a simple technology for measuring bacterial cell respiration in specific substrates, either in pure culture or in a community. PM's are available in a commercially available set of 20, 96 well microtiter plates (Borglin *et al*, 2012). The set consists of around 200 assays of C-source, 400 assays of N-source, 100 assays of P-source and S-source metabolism, 100 assays of biosynthetic pathways, 100 assays of ion effects and osmolarity, 100 assays of pH effects and pH control with deaminases and decarboxylases, and 1000 assays of chemical sensitivity. In the chemical sensitivity assays, there are 240 diverse chemicals, each at four concentrations. The chemicals selected are toxic to most microorganisms and are toxic by interfering with diverse cellular pathways. A range of common antibiotics is included. For purposes of including chemicals found in natural environments, Biolog have also incorporated into this set, tests to measure the sensitivity of bacteria to numerous inorganic chemicals, such as cations (Na, K, Fe, Cu, Co, Zn, Mn and anions (chloride, sulphate, chromate, phosphate, vanadate, nitrate, nitrite, selenite and tellurite) (Zingue *et al.*, 2017). The sample is loaded into the wells with a tetrazolium dye and cultured either aerobically or anaerobically (Borglin *et al.*, 2009) Reduction of the tetrazolium dye due to increased cell respiration results in formation of a purple colour in the well. The colour change is measured colorometrically on the OmniLog® reader (Biolog, Hayward, CA) (Borglin *et al.*, 2012). The PM plates have been used to assess the bacterial communities in stress response trials (Decorosi *et al.*, 2011), to compare the growth and metabolism of bacteria exposed to metal stressors (Tremaroli *et al.*, 2009) and to compare the growth of *Campylobacter jejuni* 1168 at different temperatures for food safety (Line *et al.*, 2010). Decorosi *et al.* (2011) used the PM plates to assay antibiotic resistance changes in the bacteria present in yoghurt and pasteurised milk.

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Chapter 3

Bacterial Species Diversity as an Indicator of Dibromonitrilopropionamide (DBNPA) Biocide Efficacy

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Abstract

Microbial growth in industrial systems is controlled through the use of biocides and bio dispersants. There is, however, no simple means of determining the efficacy of these control mechanisms which are currently conducted through complex bacterial culturing techniques.

Biolog Ecoplates® have been used to detect bacterial community changes in various communities. These microtiter plates comprise of 31 different carbon substrates (in triplicate) in wells. When a sample is added to the wells, bacteria capable of metabolising the

relevant carbon sources, respire the substrates causing the tetrazolium dye in the well to turn purple, indicating a positive result.

Hypothetically, the higher the microbial diversity, the more substrates will be utilised and *vica versa*.

The objective of this study was to test this hypothesis, using Biolog Ecoplates® as a potential simple indicator to determine the efficiency of a biocide to control microbial growth in cooling water systems by monitoring the changes in the microbial metabolic pattern.

This study proved the hypothesis using Biolog Ecoplates® indicating that the addition of biocides at various concentrations resulted in fewer substrates being utilised, indicative of a decrease in microbial species diversity.

3.1 INTRODUCTION

South African power plants are mostly wet cooled, obtaining their water from various surface water sources including dams and rivers (Kotzer, 2015). Due to the poor water quality of these surface waters, chemical treatment of the cooling water is required to prevent biofouling and corrosion. Treatment includes the dosing of bio dispersants and biocides to minimise and control the microbial growth and biofouling within the cooling water system (Satpathy *et al.*, 2016).

Bacteria, like all organisms, are affected by stress on their environment (Martinez, 2013). It is theorised that the addition of a biocide (stress) to the environment should decrease the number of species present (the species diversity) and numbers of bacteria. Dosage concentration, the type of chemical dosed and contact time will have an effect on this decrease (Satpathy *et al.*, 2016).

Although several biocides are used by the South African power generation industry, isothiazalone and dibromonitrilopropionamide (DBNPA) are the most common. Bacteria however may become resistant to isothiazolone (Zhou *et al.*, 2016). Hence, these biocides are usually applied in an alternating pattern to avoid possible development of bacterial resistance (Eskom Legionella Standard, 2017). DBNPA is a fast acting, non-oxidising

(despite the bromine) biocide that inactivates enzymes, destroying metabolic pathways responsible for energy production and transfer of wastes and nutrients. It is neutral and degrades to relatively innocuous by-products (Williams and McGinley, 2010). Due to its lack of persistence it is most often used as an alternate biocide. It becomes deactivated above pH 8.5 or in the presence of sulphide or sulphite contamination (Huber *et al*., 2010).

Historically at ESKOM, biocide dosing was conducted on a two weekly cycle with a continuous bio dispersant dosage. Biocide efficacy testing was done by conducting classical plate counts on the planktonic bacterial community in cooling water (Eskom Standard). However, many bacterial species are non-culturable through classical microbiology methods, thus an inaccurate indication of the bacterial community in the treated water is likely (Parshionikar *et al.*, 2009). In this study, two experimental methods have been employed that do not rely on artificial (in a petri dish on a solid medium) culturing techniques and instead focus on implying species/community diversity through metabolic and genetic means.

By way of a conventional metabolic based microbiology approach, Biolog Ecoplates® have been successfully used to monitor changing communities in water (Lekhanya, 2010), sewage (Gryta *et al.*, 2014) and soil (Xu *et al.*, 2015). Biolog Ecoplates® contain a triplicate set of 31 carbon substrates, with a blank well in each set. When the bacteria utilise the substrate in the well they respire and cause a colour change in the dye present in the well.

The premise of this, as a method to determine microbial species diversity within different samples, relies on the organism's ability to utilise various carbon substrates/sources for nutrition. If bacterial species, in a certain environment, are killed or altered through the addition of a stress to the environment (biocide), the number of substrates utilised should decrease (Zhou *et al.*, 2016).

Cellular genomic based methods may be employed in the detection of bacterial diversity and potentially the full description of complex microbial communities. Among these techniques is denaturing gradient gel electrophoresis (DGGE), which uses the genetic sequences of organisms in a target environment in order to detect microbial community diversity (Mayrhofer *et al.*, 2014). Genetic variations within microorganism's genes and possible mobility shifts cause differentiation of genetic sequences, allowing species in environmental samples to be separated (Carmona *et al.*, 2012; Shah, 2015). Based on the sensitivity of

double stranded DNA (dsDNA) to heat and chemicals, target populations within a sample can be separated into a visual genetic fingerprint. This method thus allows for a highly sensitive means of determining community diversity.

Species diversity is not necessarily linked to the total bacterial numbers (abundance) in an environment. It is therefore obvious that bacterial species diversity is a better indicator of stress in an environment than the total count of bacteria in the environment.

Hypothetically, the higher the bacterial diversity, the more substrates will be utilised and a lower diversity will lead to fewer substrates being utilised. An effective biocide should, when applied at an appropriate concentration lead to the reduction of the microbial species diversity and abundance and hence fewer substrates utilised. The objective of this study was to test this hypothesis, using Biolog Ecoplates® as a potential simple indicator to determine the efficiency of a biocide to control microbial growth in cooling water systems by monitoring the changes in the microbial diversity.

3.2 MATERIALS AND METHODS

A 500 L cooling water system simulator (CWSS) (Figure 3.1) was used to evaluate changes in bacterial species diversity after the addition of DBNPA biocide at concentrations of 8 mg/L and 20 mg/L, representing a sub lethal and lethal dose according to Buckman Laboratories. The CWSS included a storage drum (1), a circulating pump (4), a heating vessel (3), which heats the water to 42 °C; an ICT 850 cooling tower supplied by Industrial Cooling Towers, Alrode, Johannesburg (5) and a pressure stabilisation drum (3), for water level maintenance. A water level stopcock (2), was used to ensure that any water losses incurred, either by evaporation or windage, were corrected. The CWSS could be drained through a drain valve to a collection drum (6) if the water in the system needed to be replaced.

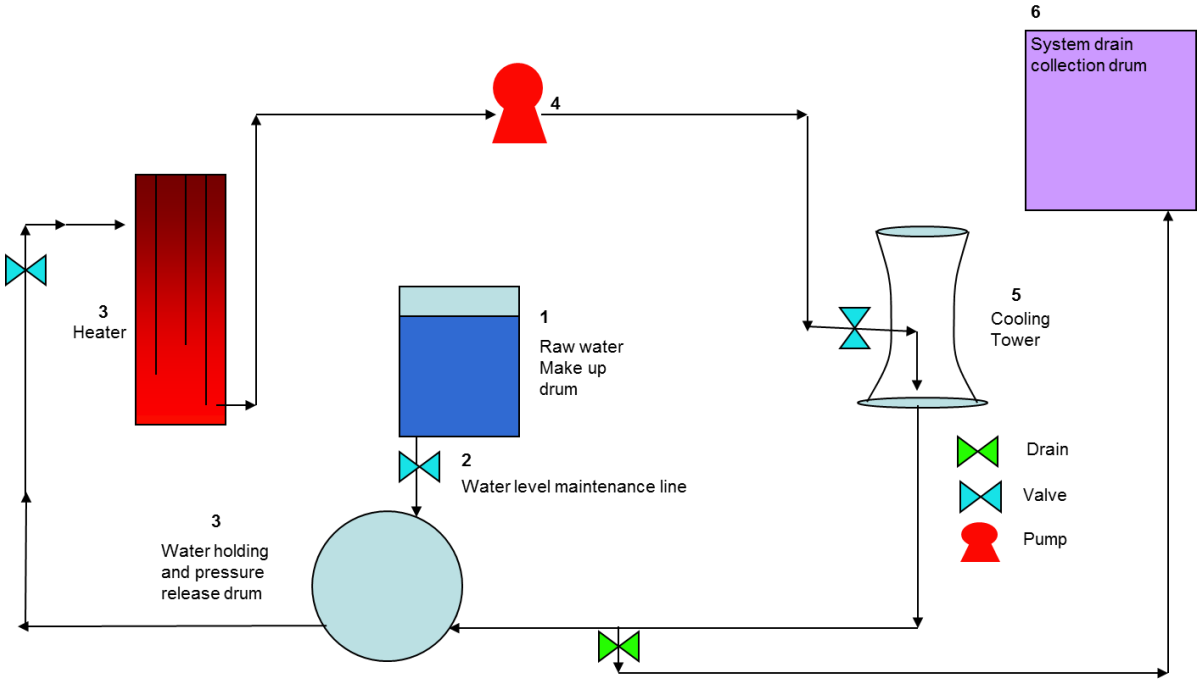

Figure 3.1: Flow diagram of the Cooling water simulator system (CWSS).

The CWSS was filled with cooling water, from an operational power plant, and the water circulated, without treatment, for 3 days prior to any biocide dosage. DBNPA biocide was added after taking an initial pre dose sample (time 0). Samples were then taken after 15 and 30 minutes, and 1, 2, 3, 4, 6, 24 and 48 h post dosing. The classical microbiology and Biolog Ecoplate® analyses were conducted at Eskom Research, Testing and Demonstration, Microbiology laboratory and the molecular work at the MicroSci Consulting laboratories.

3.2.1 Classical Microbiology

Total aerobic bacteria plate counts were conducted, in duplicate, by completing ten fold serial dilutions in sterile Ringers solution; 1 mL of each dilution was aseptically transferred into a sterile 90 mm petri-dish. Approximately 15 mL of cooled, molten Plate Count Agar (Oxoid Ltd, Basingstoke, Hampshire, England) was added to the petri-dish, after which the petri-dish was swirled gently to mix the sample into the agar and allowed to set. The petri-dishes were incubated, inverted, at 37°C for 48 h. This method was utilised because it is the methodology most commonly employed on the power plants to determine total bacterial counts. All colonies that formed on the agar were counted, and this count was multiplied by the dilution factor to determine the final count.

3.2.2 Substrate Utilisation – Biolog Ecoplates®

Each of the 96 wells (triplicate test) on the Biolog Ecoplate® (Biolog Inc, Hayward, CA, USA) was filled with 150 µL of sample. The Biolog Ecoplates® were then incubated at 35°C and examined after 24 and 48 h respectively. Any purple colouration, regardless of intensity, was marked and counted as a positive reaction. These reactions were logged (binary) and used to construct a digital graphic image of the plates for statistical analysis through Gel2K.

3.2.3 Molecular Analysis

For molecular analysis, 300 mL of sample was filtered through a 0.45 µm sterile filter and then aseptically transferred into 20 mL of sterile saline solution.

Total DNA was extracted using the BIO101 Fast DNA Spin kit (Soil)® (Qbiogene Molecular Biology products, Pretoria, South Africa).

One g sample was added to Lysing Matrix E tubes thereafter, sodium phosphate buffer (978 µl) and MT buffer (122 µl) were added, the tube was vortexed for 30s and centrifuged at 20 817 g (10 000 rpm) for 10 min.

The supernatant was transferred to a clean tube, 250 µl PPS reagent was added and mixed by inversion. The suspension was then centrifuged for 5 min at 20 817 g (10 000 rpm) to pellet the precipitate. The supernatant was transferred to a clean tube and 1 mL of Binding Matrix suspension added.

After settling, approximately 500 µL of supernatant was removed and discarded. The Binding Matrix was then resuspended in the remaining supernatant and 600 µL of the suspension was added to a SPIN™ Filter and centrifuged at 20 817 g (10 000 rpm) for 1 min. The catch tube was emptied and the remaining supernatant added to the SPIN™ Filter and centrifuged.

Subsequently 5 µL of SEWS-M was added to the SPIN™ Filter and centrifuged 20 817 g (10 000 rpm) for 1 min. The flow-through was decanted and the SPIN™ Filter replaced in the catch tube. The pellet was centrifuged at 20 817 g (10 000 rpm) for 2 min to dry the matrix.

The SPIN™ Filter was placed in a fresh Catch tube and air dried for 5 min at room temperature. A total of 50 µL DES (DNase/Pyrogen Free water) was added to the matrix and gently stirrred with a pipette tip. The resuspension was centrifuged at 20 817 g (10 000 rpm) for 1 min to transfer the eluted DNA to the catch tube.

3.2.4 Polymerase chain reaction (PCR) amplification

A portion of the bacterial 16S rRNA gene was amplified by PCR using K and M primers.

K (PRUN518R) : 5'ATT-ACC-GCG-GCT-GCT-GG3' (Surridge, 2007; Siciliano *et al.* 2003)
M (pA8f-GC) : 5'CGC-CCG-CCG-CGC-GCG-GCG-GGC-GGG-GCG-GGG-GCA-CGG-GGG-GAG-AGT-TTG-ATC-CTG-GCT-CAG3' (Surridge, 2007; Fjellbirkeland *et al.* 2001)

A reaction with no template DNA was included as a negative control. Each PCR tube contained a total volume of 20 µL: 10.8 µL sterile SABAX water, 2.5 µL PCR buffer (10x), 2 µL $MgCl_2$ (25mM), 2 µL dNTPs (2.5 µM), 1 µL primer K (50 µM), 1 µL primer M (50 µM), 1 µL template DNA (27 ng/µL), 0.2 µl *Taq* polymerase (5 U/µL). Prokaryotic DNA amplification was performed in a PCR thermal cycler using the following programme: 10 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 58°C and 1 min at 72°C, followed by 10 min at 72°C, and then held at 4°C. PCR product was analysed on a 1.5 % TAE agarose gel.

3.2.5 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were subjected to DGGE according to the method described by Carmona *et al.* (2012). In short, 10 µl (ca. 250 ng) of each PCR product was loaded per lane onto a 25-55% urea/formamide denaturing gradient gel. Gels were run at 70 V for 17 h at a constant temperature of 60°C. Image analysis was performed using the Gel2K programme (Norland 2004; Surridge, 2007). The fingerprint comparisons were analysed using CLUST (Norland 2004; Surridge, 2007). Dominant bands were compared and analysed to determine

community diversity. Several comparison options are available but Jaccard and Simple indices (matchings) were used in this study.

These comparisons differ in how they compare the results obtained. Simple matching involves the comparison of the number (diversity) of species within a sample (DGGE lane) in comparison to other samples (lanes). Jaccard matching (Jaccard index/similarity coefficient) is a statistical comparison of similarity and diversity of sample sets. It is defined as the size of the intersection divided by the size of the union of the sample sets (Jaccard, 1901; Palmer *et al.*, 2015).

3.3 RESULTS AND DISCUSSION

3.3.1 Substrate Utilisation and Classical Microbiology

After dosing 20 mg/L of DBNPA the total planktonic bacterial numbers decreased from 5.6 x 10^5 CFU/mL to 1,06 x 10^2 CFU/mL after 15 min (Figure 3.2). After dosing 8 mg/L of DBNPA the total planktonic bacterial numbers decreased from 1.74 x 10^5 CFU/mL to 2.3 x 10^2 CFU/mL after 1 h (Figure 3.3). This indicated that the higher biocide concentration (20 mg/L) resulted in a shorter time period (15 min) to reduce the bacterial numbers than at the lower concentration (8 mg/L) where the same reduction was achieved only after 1 h (Figure 3.3). The higher dosage also maintained the low counts for a longer period and slowed the relative regrowth over the 48 h trial (Figure 3.2). These results confirm previous studies indicating the relationship between biocide concentration, contact time and efficiency (Netshidaulu, 2015). An increase in biocide concentration normally results in a more rapid killing of microorganisms in a system (Williams, 2007). Nevertheless, there is not necessarily a linear relationship between biocide concentration and kill rate (Wales and Davies, 2015). The fact that the higher biocide concentration had a longer lasting effect than the lower concentration was due to the larger concentration of active ingredient of the biocide at the higher concentration (Taylor Industrial, 2016). These results indicate the importance of dosing the correct concentration, of a given biocide, to control microorganisms in water systems and especially cooling water systems that are by design dynamic systems. Calculating the theoretical dosage level of a given biocide, taking into consideration the minimum inhibitory concentration (MIC) of the biocide is easy when based on the hydrodynamics of a system (i.e. system volume, dilution rate due to system losses, make-up

water added etc.) (Kanga, 2010). However the efficiency of the biocide is also affected by non-hydraulic factors like pH, interaction with organic matter in the system and overall system chemistry (Gnanadhas *et al.*, 2012). Measuring the bioactivity of a biocide (the ability of the biocide to kill bacteria in a particular system) is therefore more useful than doing only a theoretical hydrodynamic calculation, since it takes all system parameters into account (Tidwell and Broussard, 2014). In order to measure how bacterial communities varied, it was necessary to determine the holistic variation of the bacterial community. This was the reason why the carbon substrate utilisation approach was followed as part of this study.

In terms of the Huston (2014) species diversity model, these results indicate that the bacterial community, of the 8 ppm DBNPA dosage, moves from a Category B environment (high bacterial numbers and high species diversity) to a Category A environment (low bacterial numbers and high species diversity). Once again, indicating an inadequate dosage concentration while the bacterial community from the 20 ppm trial showed a bacterial community shift from Category B to Category C (low bacterial numbers and low species diversity), showing an effective biocide dose.

The reduction in carbon substrate utilisation at 15 min, 30 min and 1 h after the 20 mg/L dose (Figure 3.2), is indicative that the bacterial species, capable of utilising the substrates were metabolically inhibited. There were however, still a small number of surviving bacteria present in the system at these times not reflecting as metabolically active based on the metabolic analysis (Figure 3.2). The reason for this is, speculated to be, due to the reduced bacterial numbers being below the detection level (sensitivity) of the Biolog Ecoplate® system. To date, the exact detection level of the Biolog Ecoplate® has not been established, although this should form part of the proposed future work. This may be due to bacteria remaining in the treated water not being able to utilise/metabolise the carbon substrates available.

After 2 h the surviving bacteria again began to multiply, showing an increase in both the total aerobe count and increased substrate utilisation to the original levels (Figure 3.2). These results are in agreement with previous studies in cooling towers (Liu, 2011; Brözel and Cloete, 1992). Brözel and Cloete (1992) indicated that the bacterial numbers in cooling towers after regrowth, as in this study, could exceed the bacteria number before biocide

dosage. The reason for this was given as selecting for a more resistant species of bacteria that was selected for by the particular biocide used (Chien *et al.*, 2013, Brözel and Cloete, 1992).

Carbon substrate utilisation results for the 8 mg/L trial indicated the presence of metabolic diversity throughout the trial period after biocide dosage (Figure 3.3). This suggests that, although the DBNPA was effective at reducing the planktonic counts, there were still a variety of bacterial species that survived capable of utilising a variety of the various carbon sources. Similarly results were observed by Du Toit (2007) in paper effluent treated by DBNPA. This indicated that the biocide was ineffective at 8 mg/L in reducing the species diversity in the system. This was supported by the more rapid recovery of aerobic planktonic bacterial counts in the system compared to the higher dosage (Figure 3.2).

In order to generate a statistical comparison amongst the substrates utilized over the experimental period, simulated gel diagrams were developed for analysis with Gel2K.

Substrate utilisation patterns, for the 20 mg/L trial, indicate a rapid decline in substrate utilisation between time 0 and 15 min after biocide addition, indicating the efficiency of the biocide (Figure 3.4). However, after 2 h some substrate utilisation is evident, increasing through 4 h, 6 h, 24 h and eventually 48 h (Figure 3.4). This substantiates the recovery of microbial communities in the community. Substrate utilisation patterns for time 0 and 48 h showed only a 30% difference and clustered together distinctly different from the other time periods during the 48 h trial (Figure 3.4).

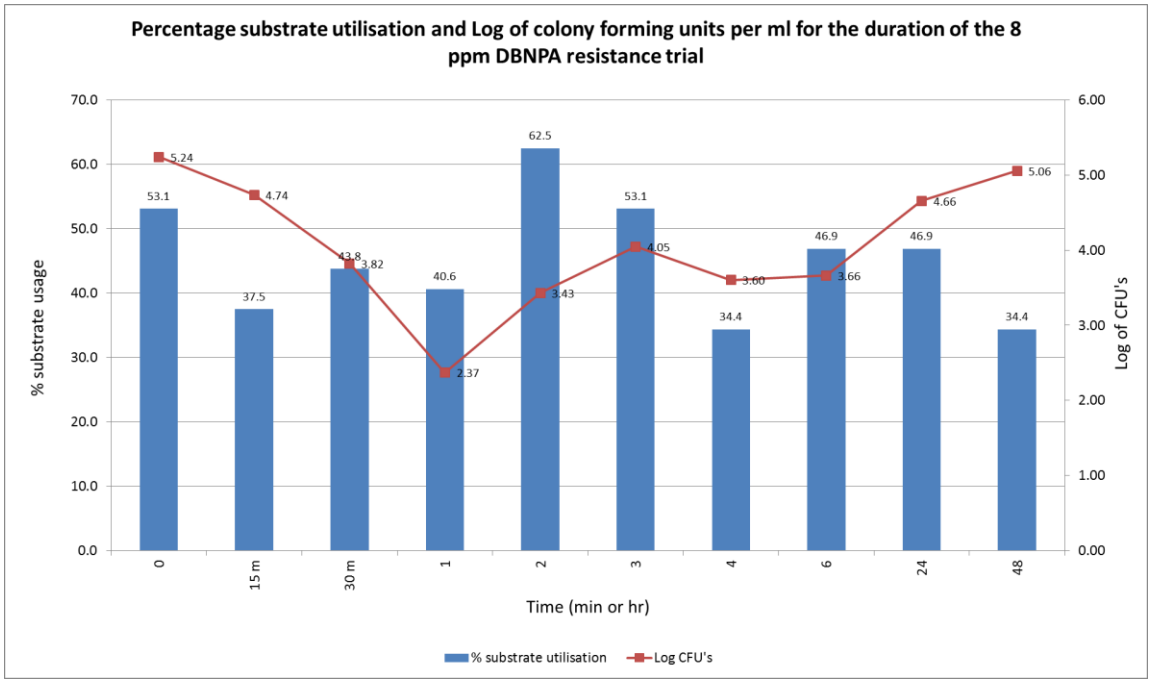

Figure 3.2: Biolog Ecoplate® substrate utilisation versus log of aerobic bacterial counts per ml for the duration of the 20 mg/L DBNPA trial.

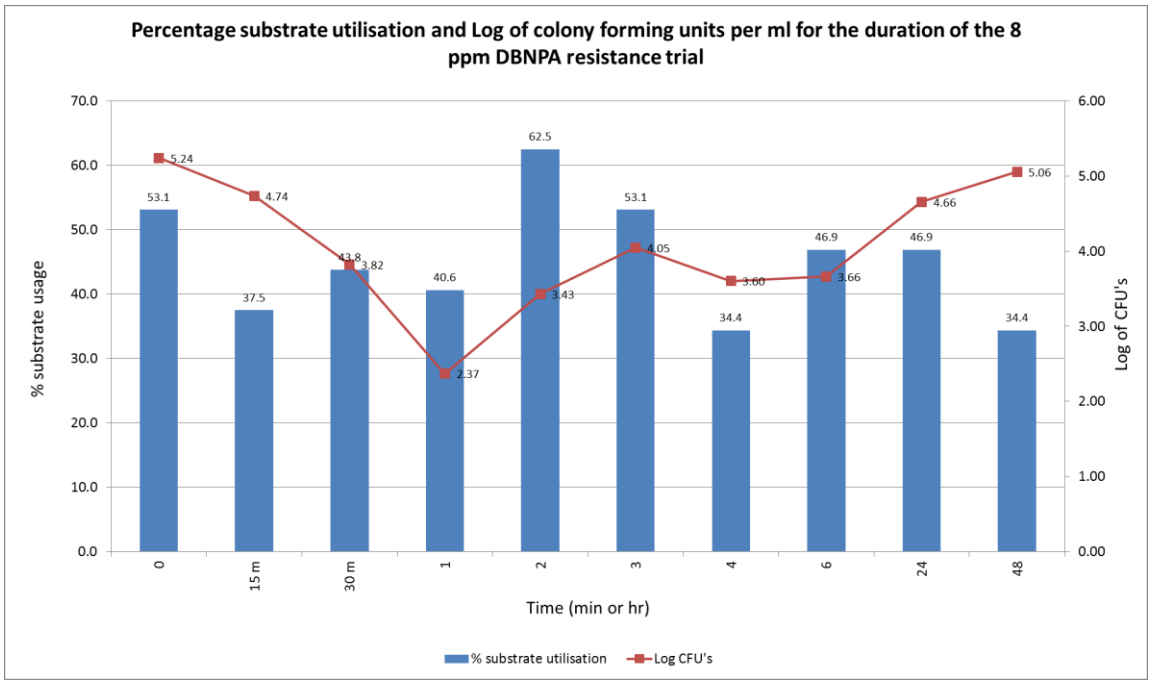

Figure 3.3: Biolog Ecoplate® substrate utilisation versus log of aerobic bacterial counts per ml for the duration of the 8 mg/L DBNPA.

This might account for the 30% difference that still existed in terms of substrate utilisation between time 0 and 48 h. The results nevertheless indicate that the bacterial community was busy recovering to the original microbial diversity and community composition within the community at the start of the trial. It was concluded that the biocide did not eliminate the bacteria species present, but merely reduced the numbers to below the detection threshold of the Biolog Ecoplate® system. This result was also reported by Gryta *et al.* (2014), in an evaluation of the treatment of dairy sewage sludge.

In the 8 mg/L trial, substrate utilisation never differed more than 30 % during the different sampling times over the trial period, with the exception of the sample taken after 48 h (Figure 3.5). The sample taken after 48 h displays the lowest substrate utilisation and a 35% variation from the other samples, implying a species diversity change and decrease, plausibly either as a result of biocide efficacy or a stabilisation of the bacterial population once the dominant bacteria re-establish in the system, as proposed by Forbes *et al.* (2017). This indicated that the biocide at a concentration of 8 mg/L had a limited effect on the metabolic diversity and hence the species diversity in the system and that bacterial community changes in the community were marginal (Figure 3.5).

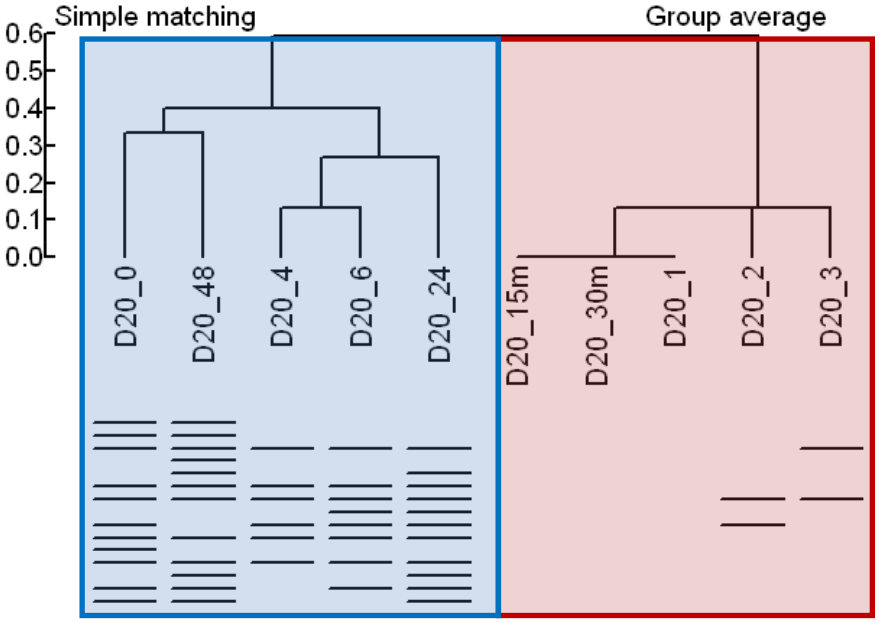

Figure 3.4: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands, at various times throughout the 20 mg/L DBNPA trial period. Refer to paragraph 3.2.2

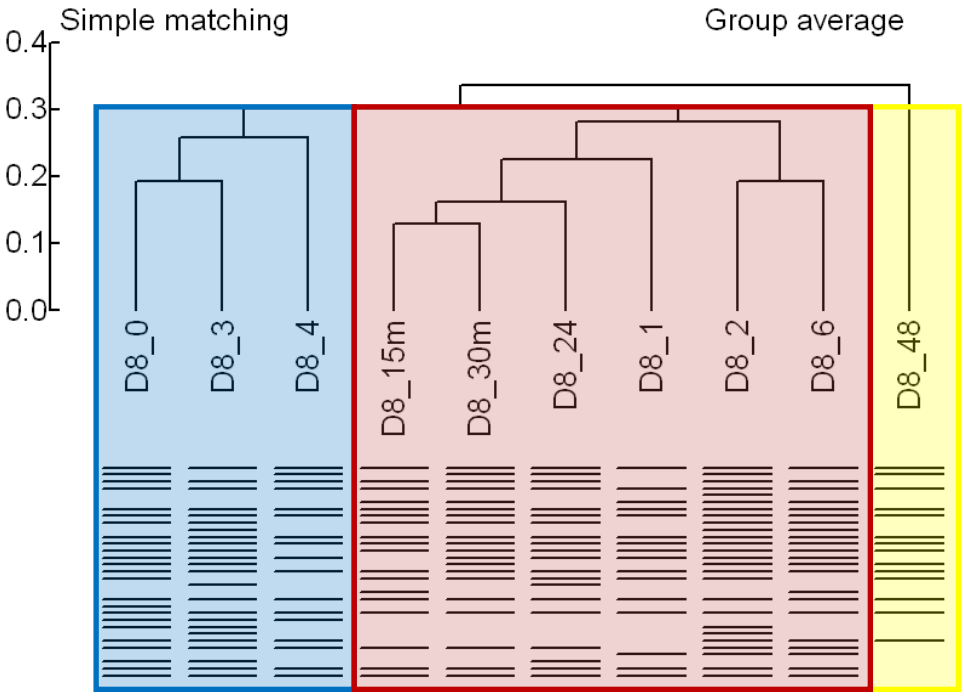

Figure 3.5: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands, at various times throughout the 8 mg/L DBNPA trial.

3.3.2 Molecular analyses results

Gel2K was used to generate a DGGE gel pattern for statistical analysis of the 20 mg/L trial. The DGGE results indicate that there was no complete elimination of all of the bacterial species after treatment at 20 mg/L of DBNPA over the trial period (Figure 3.6). The DGGE results indicate that the bacteria diversity in the system was retained throughout the trial after biocide addition. This is in contrast with the Biolog Ecoplate® results that showed a complete inhibition of metabolic activity within 15 min of biocide dosage (Figure 3.2). However, it must be remembered that the PCR amplification of the samples for DGGE will affect all DNA in the sample, whether it is contained within a viable bacterial cell or floating loose in the water, as a result of bacterial cell lysis. The DGGE analysis supports the previous conclusion, that the biocide reduced bacterial numbers to below the detectable threshold required for substrate utilisation of the Biolog Ecoplate® system, but not the species diversity as indicated by the genetic diversity remaining in the system. This result is supported by, previously mentioned, work completed by Gryta *et al.* (2014). This explains the recovery of bacterial communities in the system and increased metabolic activity during

the recovery stage within the 48 h trial period (Figure 3.2). These results suggest that a viable but non-culturable state was induced at the lower biocide concentration. DGGE analysis hence may be a useful tool to indicate a VBNC state in bacteria, but will require further investigation.

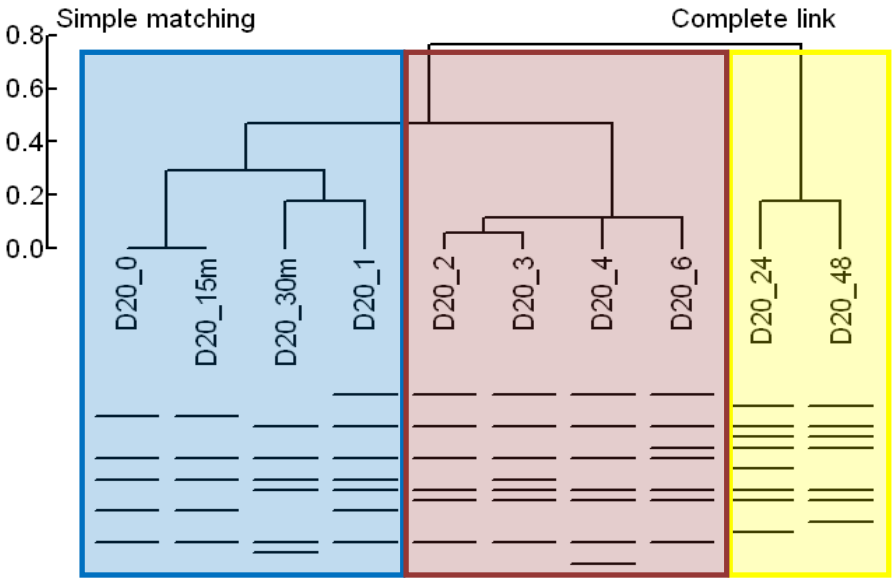

Figure 3.6: The Gel2K statistical relationship between the DGGE banding patterns at the various times throughout the 20 mg/L DBNPA trial

As with the previous analyses, Gel2K was used to generate a DGGE gel fingerprint pattern, for statistical analysis, of the 8 mg/L trial. These DGGE results indicate the presence of a diverse group of bacteria throughout the trial period after treatment with 8 mg/L of DBNPA (Figure 3.7). This is in agreement with the Biolog Ecoplate® results that showed continued metabolic activity after biocide treatment (Figure 3.3). This explains the recovery of bacterial communities in the system and increased metabolic activity during the recovery stage within the 48 h trial period (Figure 3.3). These results also imply that the 8 mg/L biocide concentration was sub-optimal to use as a cooling water treatment regime and therefore ineffective against the resident bacterial community. A higher dosage concentration of DBNPA is thus required to maintain the cooling water system.

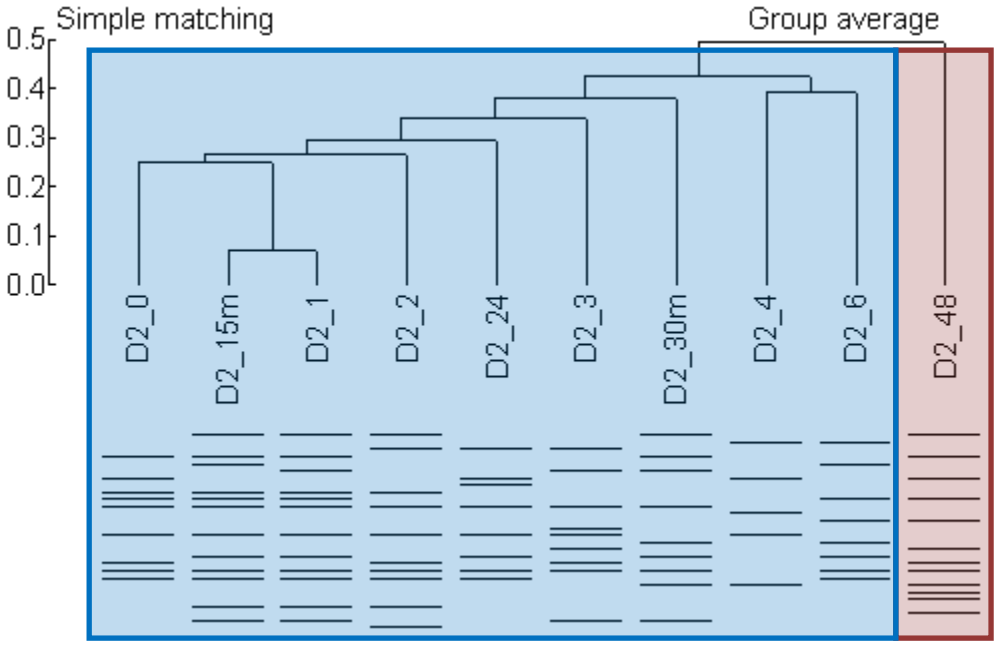

Figure 3.7: The Gel2K statistical relationship between the DGGE banding patterns at the various times throughout the 8 mg/L DBNPA trial

3.4 CONCLUSIONS

The addition of 20 mg/L of DBNPA resulted in a decrease in the number of bacteria within 15 min and reduced the metabolic activity of the bacteria to below the threshold of the Biolog Ecoplate®. DGGE analysis indicated that the species diversity was not reduced to the same extent as the substrate utilisation, resulting in a recovery of the metabolic diversity with the increase in bacterial numbers. However, it must be considered that the DGGE species diversity may be skewed due to the uncontrolled amplification of possible free DNA in the PCR amplification. The microbial ecosystem dynamics therefore shifted from a scenario of high species diversity and a high number of individuals within each species to a scenario of high species diversity and low numbers of individuals within each species. This does not reflect an ideal outcome for an effective biocide program (in this case DBNPA used at 20 mg/L). An ideal biocide, used at the optimal concentration and contact time, should have resulted in a reduction of both the number of species and the number of individuals within a species, within the planktonic phase. This result was exacerbated when a lower concentration of the biocide (8 mg/L) was used, where only the number of individuals within each species was reduced but not the diversity of species as indicated by both the Biolog Ecoplate® system and DGGE results.

In terms of the hypothesis that the Biolog Ecoplate® system could potentially be used as a simple method for determining the efficiency of a biocide, the results indicated that it could indeed be used with some caution when interpreting the results. The significant decrease in metabolic activity using the Biolog Ecoplate® system did not reveal the fact that many of the species were still present in the system after biocide addition, but at levels below the sensitivity of the system as supported by the DGGE results. However, the recovery of the bacterial numbers and metabolic diversity within 48 h did confirm the latter. In addition, the DGGE results may indicate that the addition of a biocide induces a VBNC state within the bacteria and suggests that DGGE may be a useful tool to detect and monitor bacteria in a VBNC state.

This study indicated that monitoring of the metabolic activity (as an indirect indicator of species diversity), using a system such as the Biolog Ecoplate® in a water cooling tower ecosystem, is a more useful technique to monitor the efficacy of a biocide, than determining the total number of bacteria in a system, since the recovery of the metabolic diversity can be directly linked to the resilience of a system against a natural (extreme environment) and/or manmade induced stress such as adding a biocide.

This means of determining biocide efficacy can therefore only be utilised when the biocide is being dosed at optimal levels for the system being tested. It is therefore essential that the supplier's recommendations be correctly followed both in terms of concentration and regularity of product dosing. Additionally, it must be noted that the various cooling water systems will have different dosage requirements and the recommended 20 mg/L dose is effective but for a limited period. This emphasised the need for more regular dosing.

The effect of other biocides used in cooling water treatment plants must be evaluated, and the accuracy tested on an operational plant. Testing for bacterial count and Biolog Ecoplate substrate utilisation should be consistent, however, the variability on the cooling water system, in terms of water chemical quality, volumes of make-up water recovered, flow rates, ambient temperature and cooling water system design may affect the biocidal efficacy.

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Chapter 4:
Bacterial Species Diversity as an Indicator of Isothiazolone Biocide Efficacy

Abstract

The use of natural surface water as a source for cooling water raises the need for microbiological control in cooling water systems. This is normally controlled by the use of biocides and bio dispersants. Presently, however, there is no simple means of determining the efficacy of these dosing regimens. Currently this is managed through bacterial culturing techniques.

Bacterial populations, in all systems, are affected by the concept of survival of the fittest. When an environment is subjected to stress, be it temperature, pressure or biocide dosage, it is theorised that the bacterial species diversity will change in response. The bacterial species that remain will be resistant to the stress. Monitoring these variations in bacterial populations could offer a means of understanding the bacterial dynamics in the environment.

Bacterial population variations have been studied, in several environments, utilising Biolog Ecoplates®, these include water, sewage and soils. These microtitre plates contain wells with 31 different carbon substrates, repeated in triplicate (Preston-Mofham, 2002; Lekhanya, 2010). Once a sample is added to the wells, bacteria capable of metabolising the carbon substrate begin to grow; they respire the substrates and cause the tetrazolium dye within the wells to turn purple. For the purpose of this study this indicates a positive result.

Theoretically a greater number of substrates will be utilised if there is high bacterial species diversity while fewer substrates will be utilised if the bacterial species diversity is low. Effective dosing of a biocide should lead to a decrease in bacterial species diversity and fewer substrates being used over time.

This study indicated that Biolog Ecoplates® could be used as an analytical tool to predict the efficacy of biocide dosing to manage bacterial growth in an active cooling water system.

4.1. INTRODUCTION

Power plants often use surface natural water (rivers and dams) as a cooling medium in the power production process (Kotze, 2015). Due to increased industrialisation and urbanisation around the water sources, the quality of the water is deteriorating rapidly. This poor quality water requires treatment and management to limit problems on the plant. Treatment includes the dosing of biocides and bio dispersants to minimise fouling in the system (van der Merwe-Botha, 2009).

The concept of survival of the fittest can be applied to bacteria too (Martinez, 2013). When subjected to a stress (biocide) the natural bacterial species diversity and numbers should decrease. The type of biocide, dosage concentration and contact time will all affect this variation (Satpathy *et al.*, 2016).

The South African power generation industry primarily utilises Isothiazolone (a non-oxidising biocide) and dibromonitrilopropionamide (DBNPA) for the treatment of cooling water. Due to the risk of possible biocidal resistance development to isothiazolone (Zhou *et al.*, 2016), biocides are dosed in a varying pattern to minimise this resistance (Eskom Cooling Water Standard).

Isothiazolone based biocides are heterocyclic, non-ionic, non-surface active (limiting interactions with other chemicals), ketones which utilise a two-step action mechanism: firstly rapid inhibition of growth and metabolism (respiration and energy generation), followed by irreversible cell damage (slow, several hours) (Williams, 2007). Inhibition is caused by disruption of metabolic pathways involving dehydrogenase enzymes, active in both aerobic and anaerobic bacteria. The cells die due to the destruction of protein thiols by the isothiazolone N-S bonds and the subsequent production of free radicals (Williams, 2007).

Isothiazolones are commonly used to treat Eskom cooling water systems as they combat a wide range of microorganisms.

Historically the power plants dose biocide on a two weekly cycle with a continuous addition of bio dispersant. Biocide efficacy testing was conducted by classical bacterial pour plate counts on the planktonic community of the cooling water (Eskom Standard). This technique

has been proven ineffective because a large number of the bacterial species in the cooling water are non-culturable with classical methodology (Parshionikar *et al.*, 2009).

Although, there is an argument that the planktonic bacterial community is a reflection of the sessile community (Cloete *et al.*, 1989), due to the continuous exchange between them (Costerton *et al*., 1986) thus offering a simpler means of trending the sessile community without *in-situ* testing. In addition, the efficacy testing is conducted 1 or 24 h post dosing, which is not indicative of the effective time range of the biocides and does not evaluate the period between dosages.

This study focused on two non-classical techniques utilising species/community diversity through metabolic and genetic means. The metabolic approach included the utilisation of Biolog Ecoplates®, which is a convenient method to study variations in bacteria communities in soil (Xu *et al.*, 2015), water (Lekhanya, 2010) and sewage (Gryta *et al.*, 2014).

The carbon substrates act as indicators for bacterial metabolism as the respiration of substrates lead to a change in colour of the tetrazolium dye within the wells. The theory of this technique is to determine bacterial species diversity in samples depends upon the bacteria's ability to utilise various carbon substrates for nutrition. Figure 2.2 explains the theory; that if bacterial species within an environment are changed by the addition of a stress (biocide), the number of substrates used should decrease (Zhou *et al.*, 2016).

Several molecular based methodologies may be used to determine bacterial species diversity. Denaturing gradient gel electrophoresis (DGGE) is one such technique. It uses the genetic sequences of bacteria to separate different species from one another (Mayfhofer *et al.*, 2014), allowing the various species within an environmental sample to be separated (Carmona *et al.*, 2012; Shah, 2015). Separation on the DGGE gel is based on the concentration of G-C pairs in the DNA code; the more G-C pairs present the more stable the DNA strand. The sensitivity of the double strand DNA strands to chemicals and heat cause them to denature and separate further down the electrophoresis gel. This is thus a sensitive technique for the determination of bacterial species diversity.

Bacterial species diversity does not correlate to the total bacteria count in a given environment. (Stanish *et al.*, 2016). In extreme environments there may be many individuals

of a small number of species (low diversity) or a large number of individuals of a large group of species in a nutrient rich environment. It is thus proposed that changes in bacterial species diversity may be a better indicator of environmental stress that the total bacteria count (Stanish *et al.*, 2016).

Theoretically, more substrates will be used in a highly diverse bacterial community and less in a lower diversity environment. The effective dosing of a biocide should lead to a reduction in the bacterial species diversity and therefore a reduced number of substrates would be used. In order to test this hypothesis, Biolog Ecoplates® were used as a potential indicator to evaluate the efficiency of isothiazolone biocide in the control of bacterial growth in a cooling water system but monitoring changes in bacterial species diversity.

4.2. MATERIALS AND METHODS

A 500 L cooling water system simulator (CW Rig) (Figure 3.1) was used to evaluate changes in bacterial species diversity after the addition of isothiazolone biocide at concentrations of 8 mg/L, 20 mg/L and 50 mg/L. The CW rig was filled with cooling water, from an operational power plant, and the water level maintained by stopcock to ensure that any water losses incurred, either by evaporation or windage, were corrected.

The water in the CW rig was circulated, without treatment, for 3 days prior to any biocide dosage. Isothiazolone biocide was added after taking an initial pre dose sample (time 0). Samples were then taken after 1, 2, 4, 6, 24, 48 and 72 h post dosing. The classical microbiology and Biolog Ecoplate® analyses were completed at the Eskom RT&D Microbiology laboratory and the molecular work at the MicroSci Consulting laboratories. Classical microbiology, substrate utilisation – Ecoplates and molecular analyses were conducted as mentioned in Section 3.2.

4.3. RESULTS AND DISCUSSION

4.3.1 Substrate Utilisation and Classical Microbiology

The results for the three concentration trials are shown in Table 4.1.

After dosing 8 mg/L of isothiazolone, the total planktonic bacteria count decreased from a log count of 4.48 to 2.97 after 6 h. After dosing 20 mg/L and 50 mg/L isothiazolone the planktonic counts decreased from a log of 4.88 to 3.38 after 24 h and 4.17 to 1.93 after 4 h respectively. The higher dosage (20 and 50 mg/L) also maintained the low counts for a longer period and slowed the relative regrowth over the 48 h trial.

Table 4.1: Results of average Biolog Ecoplate® substrate utilisation versus average aerobic bacterial counts for the duration of the 8, 20 and 50 mg/L Isothiazolone trials.

Biocide concentration	8 mg/L		20 mg/mL		50 mg/mL	
Time (hr)	Substrates used	Log_{10} CFU/mL	Substrates used	Log_{10} CFU/mL	Substrates used	Log_{10} CFU/mL
0	19.0	4.48	13.0	4.88	6.0	4.18
1	16.3	3.07	16.0	4.18	1.0	2.27
2	13.7	3.18	20.0	4.03	1.0	2.43
4	14.7	3.41	20.0	3.80	0.0	1.93
6	16.0	2.97	15.0	3.53	2.0	2.45
24	17.7	4.43	2.0	3.38	3.0	2.48
48	15.0	4.11	9.0	5.00	11.0	4.52
72	16.0	4.16	8.0	5.73	9.0	4.91

Results obtained in this study concur with other studies that indicated a correlation between biocidal concentration and efficacy (Netshidaulu, 2016; Brözel and Cloete, 1992). The fundamental work conducted by Brözel and Cloete (1992) evaluated the effect of various biocides on the planktonic community in an open recirculating cooling water system by monitoring the planktonic bacterial count and determining the Shannon-Weaver Index at set times. Although each biocide had a different effect on the planktonic bacterial community all indicated that the bacterial counts decreased significantly after the biocide dose. This was attributed to the mechanisms of cell damage relative to the biocide action. However, at the time these mechanisms were not fully understood.

Cloete *et al.* (1989) hypothesised that biocide inactivation (loss of biocidal effect) was a function of the holding time index (HTI) of the system being treated; the longer the HTI the quicker the biocide inactivation. This will be relevant on the operational full recirculating cooling water systems, where water is lost only through evaporation, thus all salts and nutrients concentrate. However, although the biocide concentration should, theoretically,

also increase the bacterial counts do not decrease. This suggests that the biocides become inactivated although how this occurred was not known.

Netshidaulu (2016) considered the effect of biocide, contact time, biocide residual and efficiency of mixing on biocide efficacy. His work indicated that chlorine efficiency was related to concentration of chlorine dosed, however due to the two phase effect of chlorine, attributed to the development of a residual chlorine concentration, the first initial kill effect was greater than the delayed action. Although isothiazolone has a similar two-phase effect, the mechanism is reversed as bacteria are initially inactivated (biostatic), before the action of the biocide can be effectively biocidal after 6 hr. This emphasises that it is essential to understand the mechanism of action of the biocide being used. In many cases post dosing efficacy testing is conducted before the biocide is optimally functional. In addition, a single test post dosing may not detect the re-growth of bacteria post inactivation of the biocide.

Increasing biocidal concentrations in the system results in a more rapid kill rate of the bacteria which is in line with a review conducted by Williams (2007) on the mechanism of action of isothiazolone biocides. However, this relationship is not linear as explained by Johnson *et al.* (2003) who determined whether the damage caused to *Staphylococcus aureus* using phenolic biocides, either singly or in combination, gave a linear or non-linear reaction. Cell damage was only noted after long contact times or with high dosage concentrations. This is due to the extended contact time of the biocide and the bacterial cell, as well as the increased concentration of active ingredients able to react with the bacterial cell. The extended duration of the biocidal effect with higher biocide concentration is indicative of the effective time of the biocide (Dagher *et al.*, 2017). This was reported by Dagher *et al.* (2017) relative to their work on the evaluation of peracetic acid, which indicated that the dosage of higher concentrations of biocides will extend the time of the biocide efficacy. Although this is a valuable result, the cost effectiveness of dosing high concentrations of biocides must be evaluated against the biocidal benefits. Biocides are costly and as the cooling water systems are not required to operate under sterile conditions, the lower the bacterial count the less the risk of biofouling. The results obtained, in these trials, emphasise the need for dosing at optimal concentrations of biocides, especially when designed dynamic systems are involved. Calculation of the optimal concentration for biocide dosing is relatively simple and can be based on the hydrodynamics of the system (Kanga, 2010). However, non-hydraulic parameters such as pH, organic concentration and system chemistry also affect biocide

efficacy (Gnanadhas *et al.*, 2012). Thus, determining efficacy of the biocide to kill bacteria in a water system is a more accurate than a purely hydrodynamic calculation (Tidwell and Broussard, 2014). For this reason, a carbon substrate utilisation approach, signifying the species diversity, was followed in this study.

When evaluating these results against the Huston (2014) model (Figure 2.2), it can be deduced that the bacterial community, in the 8 ppm trial, moves from a Category B (high bacterial numbers and high species diversity) to a Category A (low bacterial counts and high species diversity), indicating an inefficient biocide dose. Similar results were obtained with the 20 ppm trial which maintained high species diversity (Appendix B). However, in the 50 ppm trial, the bacterial community moved from a Category B to a Category C (low bacterial numbers and low species diversity). This implies that the 50 ppm dosage was more effective as a biocide in the cooling water system, as the biocide dosage decreased the carbon substrate utilisation and the bacterial count.

Carbon substrate utilisation results for the 8 mg/L trial indicated the presence of metabolic diversity throughout the trial period after biocide dosage (Table 4.1) (Appendix B). The reduction in carbon substrate utilisation after 1 and 2 h, post the 8 mg/L dosage, (Table 4.1) indicated the effect of the biocidal action. However the rapid recovery after 4, 6 and 24 h showed that the concentration of the biocide dose was insufficient to maintain the reduced community and consequently the surviving bacteria recovered rapidly (Table 4.1).

The above results suggested that, although the isothiazolone was effective at reducing planktonic counts, there were still a variety of bacterial species that survived, capable of utilising an assortment of carbon sources. Results indicated that the biocide was ineffective at 8 mg/L in reducing the species diversity in the system. This was supported by the recovery of aerobic planktonic bacterial counts in the system (Table 4.1). Cloete *et al.* (1989) proposed bacterial succession in the dosed system. It was theorised that an initial bacterial community may be killed by the addition of a biocide but the bacterial community which succeeds this community will be unaffected by the biocide. This hypothesis may be used to explain the results reported by Muñiz *et al.* (2014), where a rapid recovery of bacterial community was noted when a cadmium stress was no longer utilised to irrigate soil, post 32 days of contaminant irrigation. This implies that a stress must be maintained in order for bacterial counts and species diversity to be maintained at a low level. In the power plant cooling water

system this means that once off bulk dosing of biocides is insufficient to maintain a low bacterial community and a regular dosing regime is required.

The Biolog Ecoplate® results after the 20 mg/L dose indicated a slight increase in the first 6 h followed by a substantial decrease after 24 h (Table 4.1). This may be due to the suppressed bacteria becoming viable when primary competitors are eliminated. However, the biocidal action of isothiazolone is shown after 24 h when bacterial counts and carbon substrate utilisation is at its lowest (Table 4.1).

Table 4.1 shows the carbon substrate utilisation during a 50 mg/L dose trial. The carbon substrate utilisation decreases immediately and becomes undetectable after 4 h. This may be due to a lack of sensitivity of the Biolog Ecoplates® to detect the species at the lower counts.

Another possibility is the development of viable but non-culturable cells (VBNC) by the application of the biocide stress. These cells lose their ability to be cultured on standard media when subjected to stress (Ramamurthy *et al.*, 2014), they are however still viable, maintaining a low metabolic activity which can be measured by other non-culturing techniques (Oliver, 2010). This concept however, cannot be proven by changes in the bacterial count and Biolog Ecoplate® (culturing techniques) relative to the DGGE analysis (molecular technique). The reason is that DGGE analysis evaluates all DNA present in the sample and cannot differentiate between viable and non-viable.

However, in all cases the bacterial counts recovered within 48 h (Table 4.1). It is indicative of the need for more regular dosing of the biocide to maintain the lower counts required in the cooling water system. Liu *et al*, (2011) offered similar conclusions in his study on the treatment of cooling tower biofilms. In this work, a consortium of *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Flavobacterium* sp. in both sessile and planktonic phases was subjected to continuous and delayed slug dosing of chlorine. The bacterial counts in both environments were lower when a regular continuous dosage was used. Increasing bacterial counts post biocide inactivation was also reported by Cloete *et al.* (1989) when researching the use of planktonic bacterial communities to evaluate biocide efficacy. Doubling rates of between 6.6 and 2 h was reported, which are dependent on the make-up volume and the retention time in the system. This implies that a single slug dose on a weekly or two weekly

basis will actually allow a period of higher bacterial counts in the system before the next dose.

In order to generate a statistical comparison amongst the substrates utilised over the experimental period, simulated gel diagrams were developed for analysis with Gel2K (Appendix B).

Carbon substrate utilisation pattern comparison dendrograms are shown in Figures 4.1, 4.2 and 4.3 for the 8, 20 and 50 mg/L trials respectively. For the 8 mg/L trial (Figure 4.1) the substrates utilised at time 0, 24 and 72 h cluster together (blue clade), although the 24 and 72 h samples cluster independently of the time 0 sample. All the other samples cluster separately (red clade), although the difference is only 30%. An interesting observation is that the 48 h sample clades with the second cluster. This indicates that the carbon substrates utilised at 48 h are more similar to those used after 1, 2, 4 and 6 h than the 24 and 72 h samples. This correlates to the total bacterial plate count determined in the 8 mg/L trial (Table 4.1).

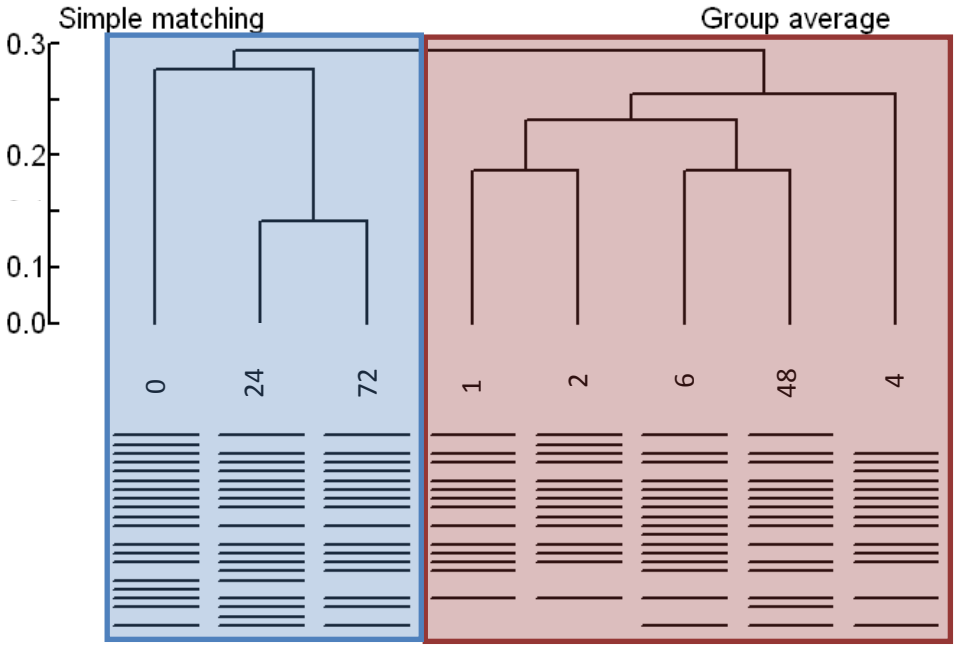

Figure 4.1: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands at various times (hr) throughout the 8 mg/L isothiazolone trial period.

The carbon substrate utilisation dendrogram (Figure 4.2), for the 20 mg/L trial shows two independent clades which are 50% different. The carbon substrate utilisation for 24, 48 and 72 h (red clade) samples clade independently from those in the earlier part of the trial (blue clade). This indicates that the biocide dose did alter species diversity within the cooling water system. It is also noted that fewer carbon substrates were utilised in the red clade. This may indicate a re-developing community once the biocide stress was removed. This result was also reported by Muñiz *et al.* (2014) who conducted research using Biolog Ecoplates® to determine the effect of cadmium on soil bacterial communities. They showed that the bacterial count and carbon substrate utilisation in the cadmium irrigated soils were significantly lower than the control soils. However, the bacterial count and carbon substrate utilisation increased once irrigation with cadmium was stopped. This shows that the bacterial community can recover from a stressed environment. This will cause the bacterial counts in the cooling water to increase once the biocidal stress has been exhausted.

During the 50 mg/L isothiazolone trial, the carbon substrate utilisation split into two initial clades, one of which further separated into two sub-clades (Figure 4.3). In this trial, the carbon substrate utilisation decreased immediately after dosing so the sample at T=0 is in a clade alone (blue). However, the carbon substrate utilisation in this sample appeared low in comparison to the previous tests. The reason for this is unclear as the substrate utilisation on the recovering samples (yellow) was higher. The next clade comprises of samples from 1, 2, 4, 6 and 24 h (red), which all had substantially decreased carbon substrate utilisation but were a maximum of 20% different; a maximum of 4 carbon substrates were utilised. The sample at 4 h showed no carbon substrate utilisation at all, this might be indicative that the Biolog Ecoplates® are not sensitive enough to detect the low bacterial counts (Table 4.3). The samples in the yellow clade (Figure 4.3) show the increasing carbon substrate utilisation as the community re-establishes in the cooling water system. The differing bands in these samples lead to the idea that the species diversity is continually changing and re-adapting to the environment. This impact was also identified in research conducted on rhizosphere soils from Mafikeng, South African (Masenya, 2013). The soils showed varying bacterial species communities from the different sites impacted by different fertilisers. In addition, Sati *et al.* (2013) showed different species diversity in Himalayan soils from environments that experience differing temperatures. This implies that the environment will affect the bacterial community present. This may be of concern when make-up water sources change or are contaminated. If the water in the catchment is impacted by agricultural run off or mining the

bacterial community in the water will differ and may require increased treatment. This effect is more noticeable with seasonal temperature variations. In the warmer months, more biocide dosing is required than in the winter.

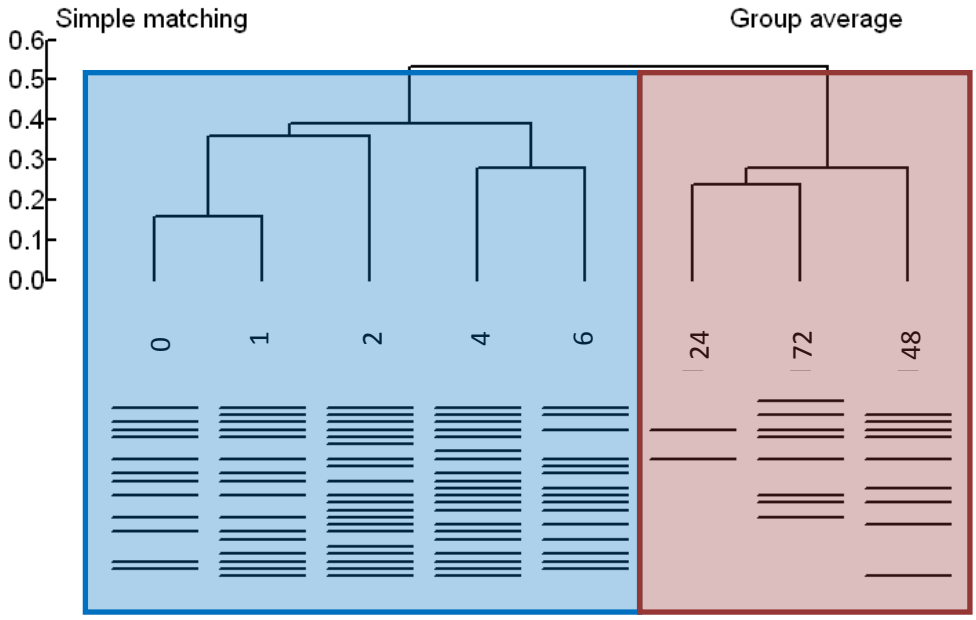

Figure 4.2: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands at various times (hr) throughout the 20 mg/L isothiazolone trial.

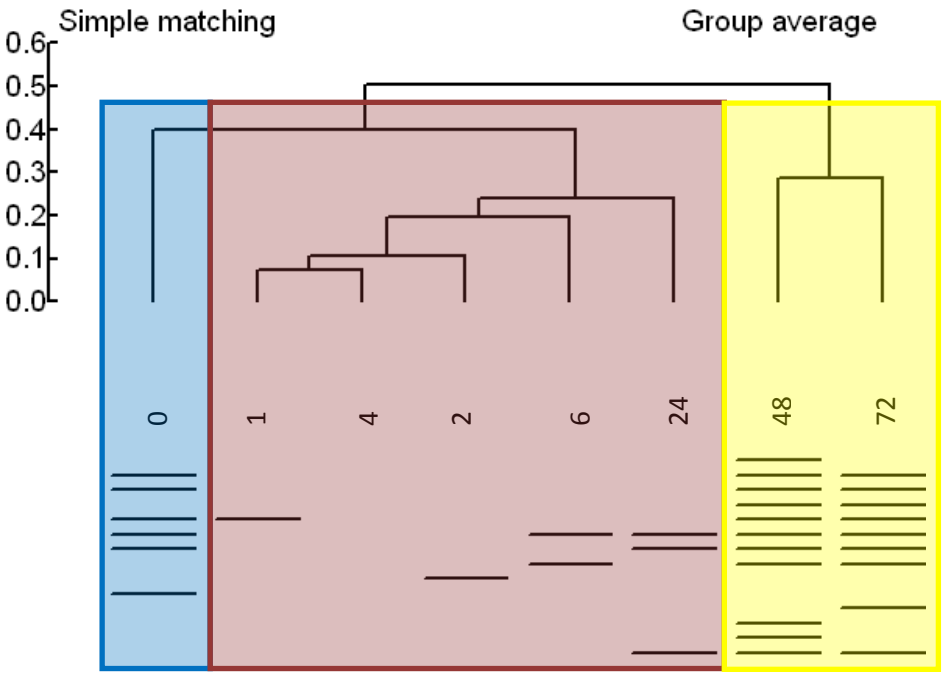

Figure 4.3: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands at various times (hr) throughout the 50 mg/L isothiazolone trial.

4.3.2 Molecular analyses results

Gel2K was used to generate a DGGE gel pattern for statistical analyses of the 8 mg/L trial samples and a subsequent dendrogram comparing the band patterns was generated (Figure 4.4). The DGGE results show that there was little change in the bacterial species diversity after the 8 mg/L dosage. This is in agreement with the Biolog Ecoplate® results (Table 4.1) which also showed minimal changes to the carbon substrate utilisation over the 8 mg/L trial. In contrast, the bacterial counts do decrease, meaning that although the bacterial species diversity has not varied greatly the number of individual cells of these species has been decreased. Franklin *et al.* (2001) research disagreed with this finding in their research on the effect of dilution on community structure and functional ability. They hypothesised and proved that dilution of the sample would cause a shift in the bacterial species diversity. However, the results continued that community composition and number of individual cells relative to the concentration also decreased. This may be concerning when samples are diluted to conduct plate counts. It is thus essential that alternative simple means of biocide efficacy testing be developed.

The DGGE dendrogram (Figure 4.4) splits into two clades that are approximately 50% different with only the 6 h post dosage sample separating from the other samples. This is in line with the mechanism for isothiazolone which is biostatic for the first 6 h and then biocidal. The analysis of the DGGE supports the conclusion that although the biocide dosage reduces the bacterial numbers a significant amount of the bacterial diversity remains in the system. Samples from 24, 48 and 72 h post dosing compare most closely with the pre-dosing sample (T=0), indicating that the bacteria that remain post dosage re-populate the system to initial concentrations. This is corroborated by Liu *et al.* (2011) who showed that the remaining bacteria in a cooling tower recolonise the system once the biocide residual is complete. This was evident by the initial decrease in bacterial count immediately following glutaraldehyde dosage and the subsequent bacteria regrowth over 4 to 5 days, once the biocidal effect was exhausted. This reaction was also noted by Cloete *et al.* (1989) in work on biocidal effect on planktonic communities. They suggested that bacterial success may allow for increased growth post biocide dosage, as previously mentioned. This again emphasised the need for regular optimised dosing of the cooling water system.

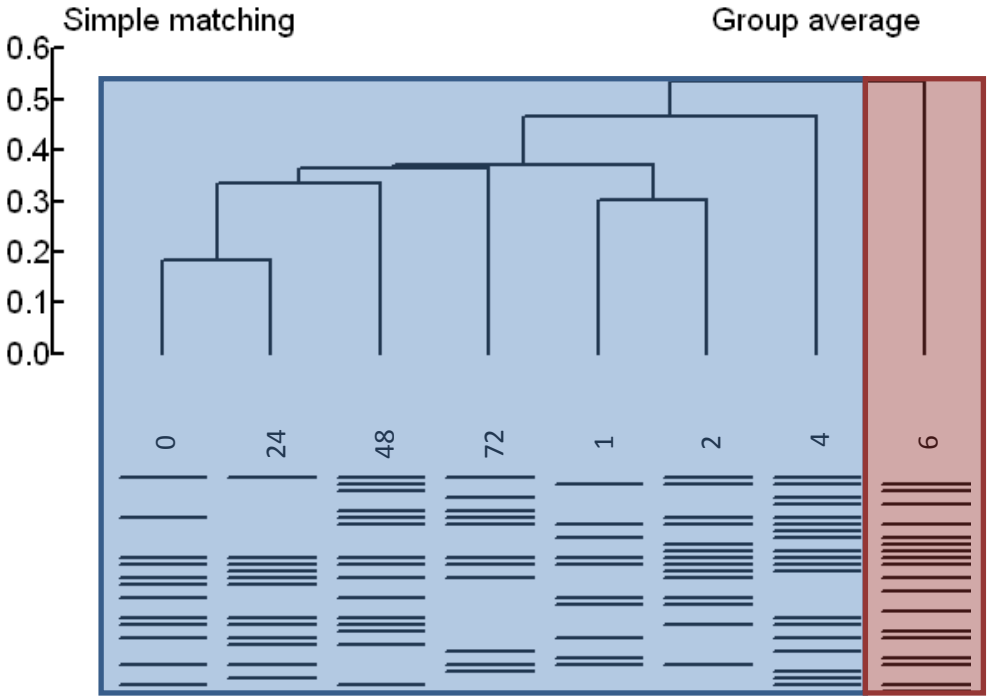

Figure 4.4: The Gel2K statistical relationship between the DGGE banding patterns at the various times (hr) throughout the 8 mg/L isothiazolone trial

The Gel2K dendrogram of the DGGE results of the 20 mg/L trial is shown in Figure 4.5. Once again there is little variation in the bacterial species diversity, which is opposite to the bacterial counts obtained for the samples (Table 4.2), which decreased over the trial. This again highlights that although the bacterial counts were reduced, the species diversity remained relatively consistent as was also mentioned by Liu *et al.* (2011), in his studies on the use of chlorine and glutaraldehyde on a standard consortium. The research indicates that the community species diversity remains consistent to the initial consortium but the counts show a log 3 decrease in counts. In addition, this result indicates less than 30% difference for all samples with the exception of the 48 h sample. This sample falls basal to the blue clade and is approximately 50% different. It is believed that this may be due to previously suppressed bacterial species becoming viable during a period with higher available nutrients post-dosing. This cannot be attributed to genetic changes due to the short time interval between the dosing and the sample time. In addition, the bacteria community readily returns to a state similar to the pre-dosing after 72 hr. Gilbert and McBain (2003) discussed the issue of the possible growth of less susceptible bacterial post biocide dosage in their review of the potential impacts of increased use of biocides on consumer products. This implies that the dosage concentration was insufficient to affect the entire community and just eliminated the large number of individuals from each species allowing other species to grow briefly before the dominant species reasserted themselves.

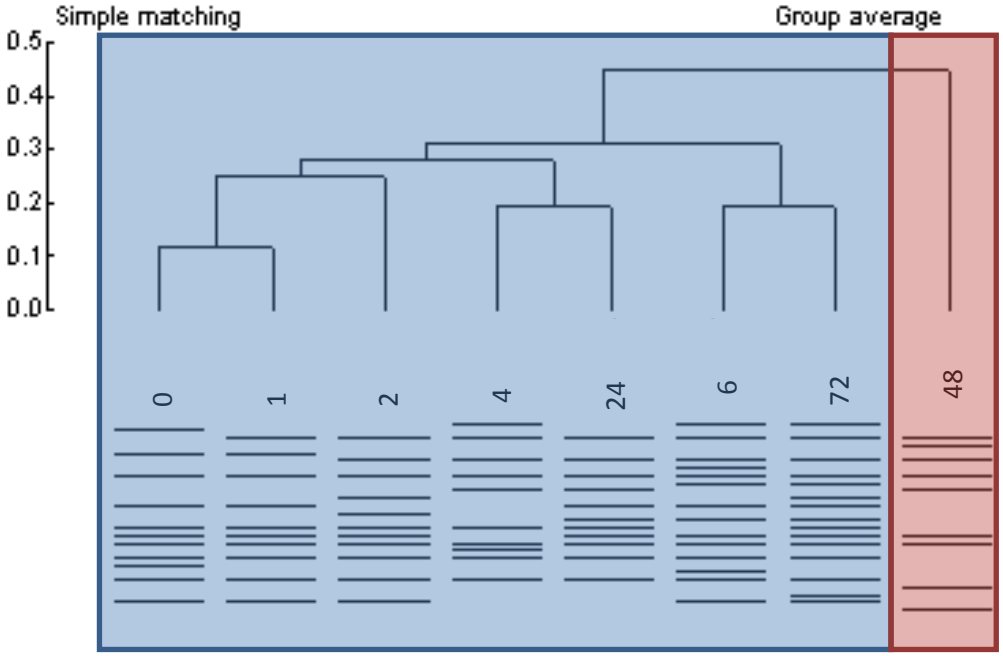

Figure 4.5: The Gel2K statistical relationship between the DGGE banding patterns at the various times (hr) throughout the 20 mg/L isothiazolone trial

In contrast to the 50 mg/L Biolog Ecoplate® results, which show no carbon substrate utilisation after 4 h, the DGGE gel for the trial indicated that the bacterial species diversity is maintained throughout the trial. This may be an indication of the PCR bias, which was reviewed by Congelosi and Meschke, (2014), where it was noted that PCR could not identify between a viable bacterial cell and an inactive one or merely a free DNA fragment. DGGE separates DNA amplified by PCR, which makes it therefore, also bias towards a viable or non-viable DNA identification. This concern impacts all molecular amplification and care must thus be taken when attempting to derive quantitative results from this technique.

Figure 4.6 gives the DGGE dendrogram of the 50 mg/L trial. The samples separate into two clades, which are 45% different from each other. Samples from time 0, 1, 2 and 4 h group in the blue clade and 6, 24, 48 and 72 h group together in the red clade.

The analyses from the DGGE supports the conclusion that although the 50 mg/L biocide dosage reduces the bacterial count and carbon substrate utilisation below the detection limit for the Biolog Ecoplate®, in sample 4 h, a significant amount of the bacterial diversity remains in the system. These bacteria recolonise in times 48 and 72 h.

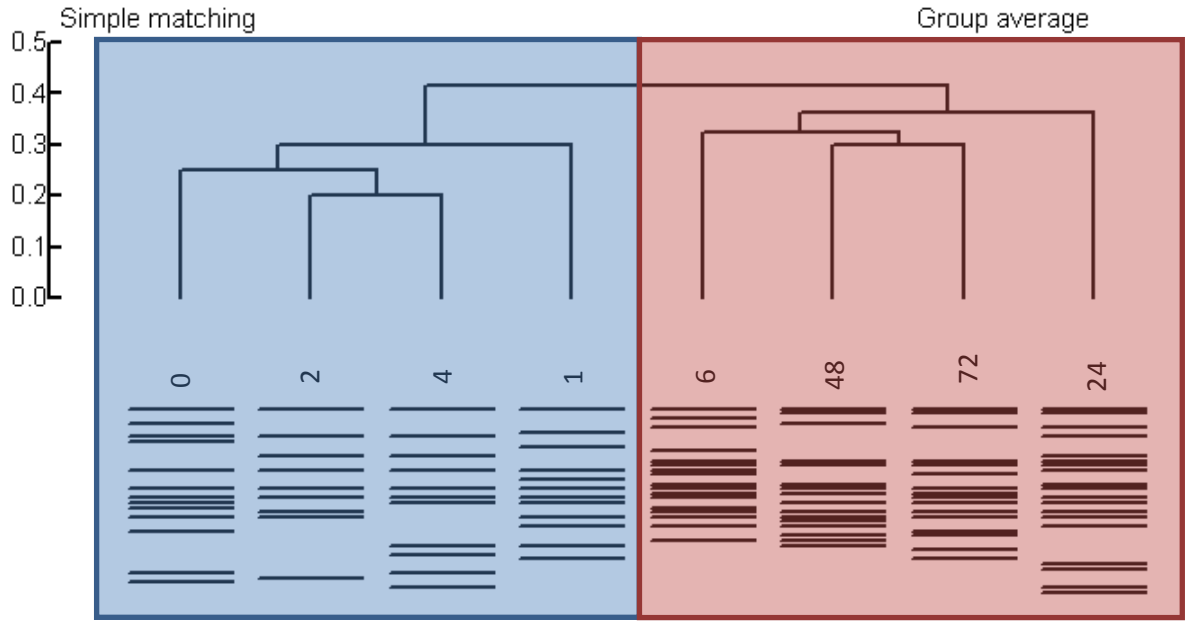

Figure 4.6: The Gel2K statistical relationship between the DGGE banding patterns at the various times (hr) throughout the 50 mg/L isothiazolone trial

4.4 CONCLUSION

All concentrations of isothiazolone dosed decreased the bacterial plate counts. However the extent of this decrease and the duration of the reduction depend on the concentration of biocide dosed, the higher the concentration of biocide the longer the kill duration. Although the bacterial counts decreased, the carbon substrate utilisation remained relatively constant in the 8 mg/L trial, indicating that the biocide concentration was insufficient to alter the carbon substrate utilisation (less than 30% change) of the bacterial species community in the cooling water. When a 20 mg/L dose was added, the carbon substrate utilisation varied more widely (50% variation) in the samples 24, 48 and 72 h post dosage, while the 50 mg/L dose caused a similar variation in the carbon substrate usage but the changes were detected sooner and lasted for a longer time.

In the 50 mg/L trial the bacterial count was decreased more rapidly than the other trials. In addition the metabolic activity of the bacteria reduced to below the threshold of the Biolog Ecoplate® after 4 h. The DGGE analysis did not show the same extent of reduction but rather a change in the bacterial community. It has been hypothesised that viable but non-culturable cells may be formed when exposed to the biocidal stress. These cells become culturable

again once the biocide stress is depleted and there is thus a recovery of metabolic diversity with the increase in bacterial numbers over the trial duration. Although the change in DGGE bands indicated that there was also a change in the species diversity.

The bacterial ecosystem dynamics shift from an environment with high species diversity and a high number of individuals within each species to an environment with high species diversity and a low number of individuals per species.

These trials did not show an ideal outcome for an effective biocide dosing regimen. An ideal biocide should be used at the optimal concentration and should result in a reduction of both the bacterial plate count and the bacterial species diversity resulting in a near sterile environment (Figure 2.2).

This study showed that monitoring metabolic activity (as an indirect indicator of species diversity) with the Biolog Ecoplate® system, is a more effective means of monitoring the efficacy of a biocide than by conducting bacterial plate counts. This is partially due to the fact that metabolic diversity can also be related to bacterial persistence in a system under stress either as persister cells or by forming VBNC cells. The shortcoming of using the Biolog Ecoplate® system is the lack in sensitivity to indicate the presence of bacterial numbers less than 2 logs. Effective biocide dosage regime is also site specific and therefore the Biolog Ecoplate® system for each site need to be validated as a tool to measure efficient biocide dosage.

However this Biolog Ecoplate® technique can only be used when biocide dosage is optimal for the system being treated. It is thus essential that the biocide supplier's recommendations are followed.

The effect of the biocide dosage will be site and biocide specific and will need to be tested for accuracy on an operational plant. This will also determine which plant specific factors may affect biocidal efficacy.

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Chapter 5:
The development of biocidal resistance to Isothiazolone and the effect on subsequent treatment and bacterial species diversity

Abstract

The development of biocidal resistance to non-oxidising biocides is well documented, as are the mechanisms by which this resistance is developed. These mechanisms may be effective at either a molecular level (DNA mutations) or at a structural level (cell wall structure changes).

This study attempts to determine the effect of biocidal stress on bacterial species diversity. It is envisaged that the bacterial population should vary as the biocidal resistance of the population increases. Theoretically, the Darwinian concept of survival of the fittest will allow only bacterial species that have developed biocidal resistance to survive. Monitoring these populations and understanding the fluctuations in bacterial species present in resistant populations is expected to offer a means of understanding effective biocide dosing.

In the past, Biolog Ecoplates® have been utilised to monitor changing bacterial populations in several environments, including water and soil. These microtitre plates have a triplicate set of 31 wells, each containing a different carbon substrate. When the bacteria population is added to the wells, the bacteria begin to grow utilising the substrate and respire the Tetrazolium dye, included in the plates, causing a purple colour change. For the purpose of this study this change indicates a positive result.

For this study, the number of substrates utilised should decrease as the population diminishes in size. In addition, the effectiveness of an alternate, subsequently dosed dibromonitrilopropionamide (DBNPA) biocide on bacterial species diversity was investigated. The effect determined on Biolog Ecoplates® was supported by conventional classical plate counts and DGGE analysis.

5.1. INTRODUCTION

Bacterial resistance to non-oxidising biocidal action has been widely studied (Barah, 2013) and indicates that biocides are not equally active against all bacteria (Bridier *et al.*, 2011). There are several proposed resistance mechanisms to biocide action (Gnanadhas *et al.*, 2012). These included reduced uptake of the biocide by cellular impermeability (Barah, 2013) or efflux pumps (Gnanadhas *et al.*, 2012). Since biocides have multiple bacterial targets, it follows that resistance results from cellular changes that limit or stop biocide action.

Mutations, in the form of acquisition of resistance determinants on new genetic material e.g. plasmids (transferance) are well documented (Bennett, 2008; Al-Marzooq *et al.*, 2015). The modification of a target site or enzyme has also been proposed as a mechanism of resistance (Garneau-Tsodikova and Labby, 2016) and the development of resistance mechanisms in biofilm bacteria has been widely studied (Singh *et al.*, 2017).

Resistance by biocide inactivation is known but rare and specific to a few classes of biocides (Garneau-Tsodikova and Labby, 2016). Studies have shown that certain biocides may induce cross resistance to other biocides, indicating that a system may become more resistant to a given biocide after treatment with another; this is normally related to the biocide mechanism of action (Bridier *et al.*, 2011). It is thus essential that any biocide treatment on the cooling water system include biocides with oxidising capabilities, to ensure the disruption of the bacterial cell membrane and wall and the subsequent death of the cell (Finnegan *et al*., 2010; Guest, 2016).

For the purpose of this study, the bacterial count – species diversity relationship is explained in Figure 2.2 and is explained in the same section. It is predicted that the addition of a biocide to the cooling water system will cause a change from a high species diversity- high numbers to a low species diversity - low numbers environment.

Theoretically, as biocidal resistance develops, microbial species diversity and abundance should reduce and hence fewer substrates will be utilised. However, total bacterial count is not synonymous with species diversity within an environment; there may be many

individuals of a specific species or several species with a small number of individuals. It is therefore proposed that bacterial species diversity is a better indicator of stress in an environment than the total count of bacteria in the environment.

The use of techniques such as denaturing gradient gel electrophoresis (DGGE) allows for the determination of bacterial species diversity within target bacterial communities. DGGE uses the DNA sequence of these bacteria to separate various species in a community (Mayrhofer *et al.*, 2014). Based on the sensitivity of DNA to chemicals used in the concentration gradient gel, various species are separated to give a fingerprint of the specific community. This is a very sensitive way of determining bacterial species diversity in a community.

The objective of this study was to test whether Biolog Ecoplates® could be used as a simple indicator to detect species diversity changes in an environment with developing biocidal resistance, by monitoring the changes in the bacterial ecology. These studies were supported through classical plate counts and molecular techniques.

5.2. MATERIALS AND METHODS

A 500 L cooling water system simulator (CW Rig) (Figure 3.1) was used to evaluate changes in carbon substrate utilisation and therefore possible bacterial species diversity during the development of isothiazolone biocidal resistance, by subsequent increasing, sub-lethal dosages of isothiazolone and after normal treatment with 20 mg/L isothiazolone biocide. The CW Rig was managed as described in previous chapters.

A selected bacterial community with implied isothiazolone resistance was developed by the sequential addition of increasing concentrations of sub-effective levels of isothiazolone. On day 1 of the trial, 4 mg/L of isothiazolone was dosed into the Rig. A sample was taken pre-dosing (day 1, time 0 hr) and after 6 h (day 1, 6 h post dosing). This was repeated with 6 mg/L on day 3, 10 mg/L on day 5 and 16 mg/L on day 8. On day 9, 20 mg/L isothiazolone was dosed, representing a normal concentration for treatment in the cooling water system. The system was then sampled at time 0, 1, 2, 4, 6, 24, 48 and 72 h after dosing. On the figures, the day and time of sampling are indicated as (Day_Time).

In the alternating biocide trial, the same protocol was followed but on day 12, a 20 mg/L dose of an alternate biocide (DBNPA) was dosed. Samples were taken at time 0, 15 min, 30 min, 1, 2, 3, 4, 6 and 24 h.

Classical microbiology, substrate utilisation – Biolog Ecoplates® and molecular analyses were conducted as described in section 3.2.

5.3 RESULTS AND DISCUSSION

5.3.1 Isothiazolone resistance trial

The results of the isothiazolone resistance trial are summarised in Table 5.1. In this trial, the dosing of 4 mg/L isothiazolone decreased the total planktonic bacterial log counts from 5.08 to 4.60 after 48 h and then further to 4.01 6 h after the 6 mg/L dose. This was followed by a slight increase in log of total bacterial numbers after a further 48 hrs to 4.31. The 10 mg/L dose decreased the log count to 3.80 after 6 h, although the 16 and 20 mg/L doses did not show a marked change in the log bacterial counts that reached a minimum of 3.70, 48 h after the 20 mg/L dose. The bacterial counts began to increase marginally 72 h after the dose to a log count of 3.87.

Huston (2014) model shows that the bacterial community in the initial resistance trial moved from Category B (high bacterial numbers and high species diversity) to Category A (Low bacterial numbers and high species diversity). It was maintained in Category A for as long at isothiazolone residual was present. In the alternate biocide trial the same change from Category B to Category A for the duration of the isothiazolone trial was noted. However, once DBNPA was dosed, the bacterial population moved into Category C (low bacterial count and low species diversity), again indicating the efficacy of the alternating biocide regime.

This is in line with research findings from studies conducted on sea water biocidal treatment (Binmahfouz, 2011) and cooling water biocides by Cloete *et al.* (1998). Results obtained in these studies all show an initial decrease as biocidal action takes effect, when continued sub-lethal dosages are regularly repeated at short time intervals, bacterial counts remain low. However, once the biocide dosing ceases and the residual concentration is exhausted the

bacterial count then begins to increase. All of the studies recommended dosing a second alternative biocide for management of bacterial counts and to minimise development of biocidal resistance. When a secondary biocide is dosed, bacteria that have become resistant to the initial biocide dosages are impacted and the counts decrease further. If this alternating pattern can be maintained at optimal time intervals, bacterial counts will be maintained at a low level. Although these results are expected, the regularity of alternative dosing must be investigated on the power plant. At present, the two weekly dosing will not result in the required reduction and maintenance of bacterial counts as the time interval is longer than the biocide reactivity time. This is exacerbated by the continual addition of make-up water to the system, which continuously replenishes the bacterial cells and nutrients within the system.

Carbon substrate utilisation results for the initial resistance trial indicated the presence of metabolic diversity throughout the trial period after all biocide dosages (Table 5.1). This suggests that, although isothiazolone was effective at reducing the planktonic counts, there were still a variety of bacterial species that survived, capable of utilising an assortment of carbon sources. These results follow the work conducted by Muñiz *et al.* (2014) on the effect of stressors on soil bacterial communities, as mentioned in Section 4.3.1 of Chapter 4. This research showed an increase in bacterial count once the stressor was removed from the environment, indicating the present of bacterial species that remained in the treated system which were capable of proliferating when favourable conditions returned. This implies that once the biocide is inactivated a successor community of less sensitive bacteria will thrive. These bacteria may use as similar set of carbon substrates to the initial community. This means that any result obtained on the Biolog Ecoplate® is indicative of variations in the community not specifically a single species.

Table 5.1: Biolog Ecoplate® average substrate utilisation versus average aerobic bacterial counts for the duration of the isothiazolone resistance trial.

Time (hr)	Code for Figures	Biocide concentration dosed (mg/L) isothiazolone	Substrates used	Log_{10} CFU/mL
Day 1 Time 0 hr	1_0	4	9.0	5.08
Day 1 Time 6 hr	1_6		10.0	4.80
Day 3 Time 0 hr	3_1	6	12.0	4.60
Day 3 Time 6 hr	3_6		10.0	4.01
Day 5 Time 0 hr	5_0	10	10.0	4.31
Day 5 Time 6 hr	5_6		12.0	4.00
Day 8 Time 0 hr	8_0	16	8.0	4.00
Day 8 Time 6 hr	8_6		10.0	3.80
Day 9 Time 0 hr	9_0	20	12.0	3.81
Day 9 Time 1 hr	9_1		13.0	3.83
Day 9 Time 2 hr	9_2		11.0	3.84
Day 9 Time 4 hr	9_4		19.0	3.71
Day 9 Time 6 hr	9_6		14.0	3.77
Day 9 Time 24 hr	9_24		12.0	3.84
Day 9 Time 48 hr	9_48		10.0	3.70
Day 9 Time 72 hr	1_0		10.0	3.87

In order to generate a statistical comparison amongst the substrates utilised over the experimental period, simulated gel diagrams were developed for analysis with Gel2K (Appendix C).

A dendrogram detailing the statistical relationship of substrate utilisation during the isothiazolone resistance trial is shown in Figure 5.1. The results separate into two distinct clades, which are more than 50% different. The first clade further separates into two sub-clades, which are 30% different. The samples are differentiated into the changes linked to biocide dosage concentration of the treatment. The second clade (yellow) contains all the samples post the 20 mg/L isothiazolone dose. Although there are a similar number of substrates utilised, the combination thereof is significantly different. Within the yellow clade samples 9-4 and 9-6 are marginally (30%) separating out. This distinct separation indicates that once a lethal concentration of isothiazolone is dosed into the system, the carbon utilisation of the bacterial community that remains is significantly different from those used by the bacterial communities in the pre-dosing and sub-lethal concentration dosages. This may be due to the initial biostatic and secondary biocidal action of the isothiazolone which

initially cause rapid (minutes) disruption of metabolic pathways involving dehydrogenase. This causes rapid inhibition of growth, respiration and energy generation. The second, slower (6 – 24 hr) action involves the destruction of protein thiols in the cell wall and the production of free radicals as explained by Williams (2007) in a review on the mechanism of isothiazolone biocide. The grouping of the 9-0, 9-1, 9-2 and those of the later samples (9-24, 9-48 and 9-72) indicates that a 20 mg/L dose is not sufficient to alter the community on a permanent basis indicating biocidal inefficacy.

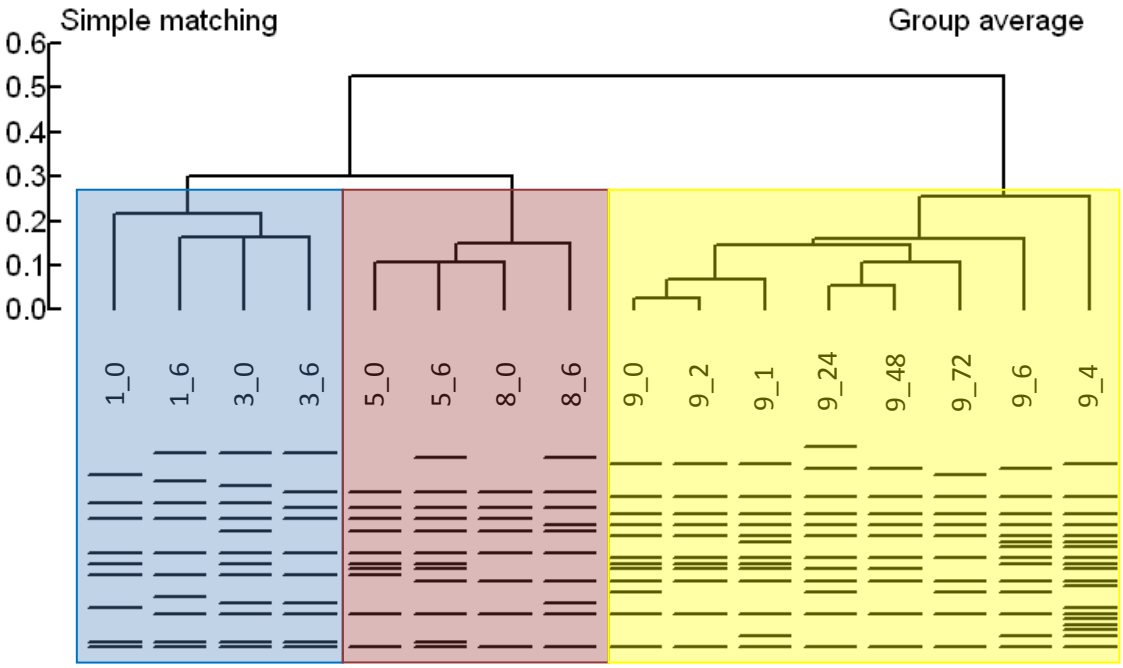

Figure 5.1: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands at various times (day_time, as explained in Table 5.1) throughout the isothiazolone resistance trial period.

Gel2K was used to develop a DGGE gel pattern of the isothiazolone resistance trial period (Figure 5.2). These results indicate a slow change in bacterial species composition over the resistance development trial. The samples separate into two main clades. The first contains the blue and burgundy subclades, while the yellow green and pink subclades form clade 2. There is a maximum of 40% difference between the clades, indicating a slight variation in species diversity. This is in conflict with the Biolog Ecoplate® dendrograms that indicated

large variations throughout the trial. This may be due to DGGE analysis detecting DNA from dead or lysed cells, post biocide dosage as previously mentioned.

There is very little change in bacterial species diversity in samples 9-24 and 9-72 post dosage (Figure 5.2). Sample 9-2 separates alone in the pink subclade, indicating an approximately 40% variation in species diversity; however the substrate utilisation (Figure 5.1) shows a close correlation between the 9-0, 9-1 and 9-2 samples. This implies that the culturable bacterial community did not utilise different substrates but the PCR amplification of the 9-2 sample may have contained other free stranded or non-viable DNA, which was separated on the DGGE gel as mentioned previously by Cangelosi and Meschke, 2014). The substrate and DGGE dendrograms do show some correlation as the initial samples, days 1 to 8 tend to cluster together and the day 9 and later samples fall into the second clade. The exception to this is sample 9-0, which falls into the first clade (burgundy sub-clade). This position is theoretically more accurate as sample 9-0 is the water pre the 20 mg/L isothiazolone dosing.

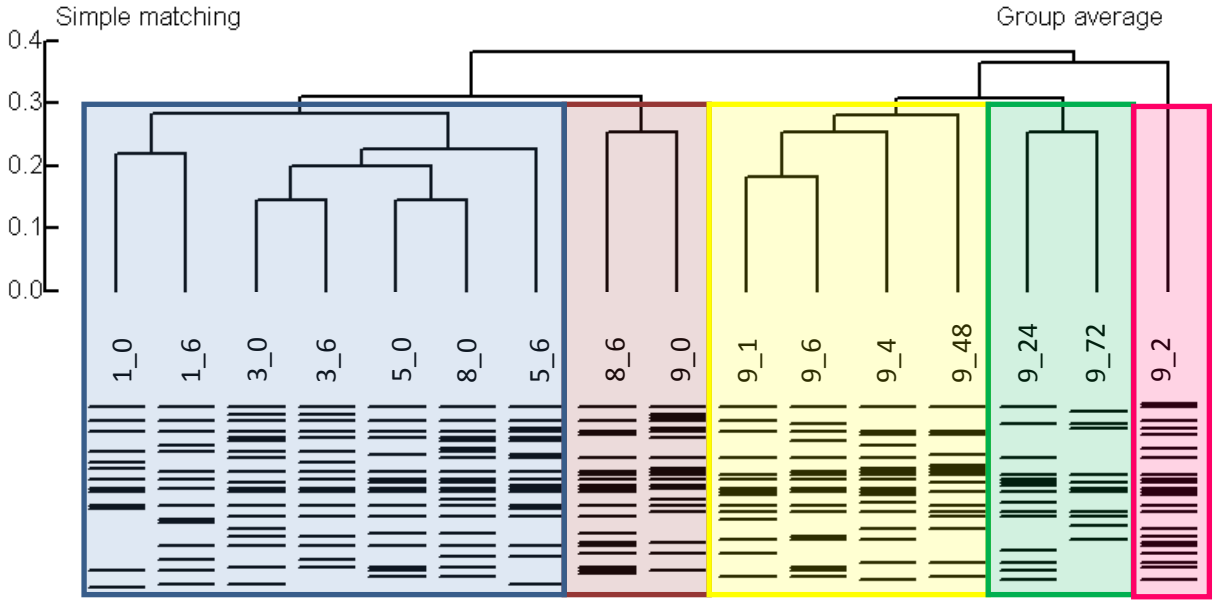

Figure 5.2: The Gel2K statistical relationship between the DGGE banding patterns at various times (daytime, as explained in Table 5.1) throughout the isothiazolone resistance trial period

5.3.2 DBNPA alternative biocide trial

The alternating DBNPA / isothiazolone biocide resistance trial (Table 5.2) indicated that the initial dosing of 4 mg/L isothiazolone decreases the total log of planktonic bacterial counts 4.48 to 4.43 after 48 h. After the 6 mg/L isothiazolone dose the log counts decreased to 3.97 after 6 h and then increased to 4.31 after 48 h. This is due to the presence of bacteria in the water as a result of inefficient biocide dosing. The log of the bacterial count decreased to 3.75 6 h after the 10 mg/L isothiazolone dose and the counts remained low after 48 h. The subsequent 16 and 20 mg/L isothiazolone doses decreased the total log of bacteria count to a minimum of 2.11, 4 h after the 20 mg/L dose. However 48 h post dosing the log of the bacterial count increased to 4.13. This is possibly due to a decreased competition for nutrients due to the eradication of more sensitive bacterial species. This result was corroborated by Forbes *et al.* (2017) as a conclusion from their research on the effect of biocide formulations on bacterial communities. They noted that bacterial which were less susceptible to certain biocides proliferated when other more sensitive species were killed. This indicates that a generic isothiazolone biocide may not be the most effective means of bacterial control in the power plant cooling water system. It suggests that, although the systems are site specific, the bacterial communities should be evaluated and a more "selective" biocide developed. Once these available nutrients are utilised, log of bacterial counts returned to a level similar to that of the initial pre-dosing sample (4.55). This reaction was reported by Kiuru *et al.* (2010), in research evaluating the effect of different biocides in paper manufacture. They evaluated batch versus continuous dosing of biocide and concluded that batch dosing has a risk of unacceptable increased bacterial counts between doses. This is of concern because increased times between dosing, allow the proliferation of bacteria in a system. This is of concern because increased times between dosing, allow the proliferation of bacteria in a system. This leads to an increased risk of biofouling and subsequent loss of heat transfer efficiency and subsequently to power generation ability.

The alternate biocide dosing of 20 mg/L DBNPA further decreased the total bacterial counts to a minimum of log 1.2, 30 min post dosing. This shows that the bacterial community with decreased sensitivity to isothiazolone was further reduced through dosing DBNPA biocide. For cooling water maintenance, the planktonic bacterial counts should be as low as possible; so dosing of the DBNPA, as an alternating biocide, reduced the bacterial count because the bacteria that were less sensitive to isothiazolone did not appear to adapt to the different stress

mechanism of DBNPA. These bacterial counts however then increased considerably after 48 h to a level that was similar to the pre-dosing log count of 4.55. This is once again due to the inactivation of the biocide over time and the subsequent re-establishment of the bacterial community from dormant or VBNC cells which may remain.

Carbon substrate utilisation results, for the alternating biocide section of the trial, indicated the presence of metabolic diversity throughout the isothiazolone trial period, after all sub-optimal biocide dosages (Table 5.2) (Appendix C). This leads to the understanding that the low concentrations simply reduced the individual numbers of the bacterial species but that the species mostly remained viable through this period.

Once again, this indicated that isothiazolone, although effective at decreasing bacterial counts, does not sustainably impact the carbon substrate utilisation and implied species diversity of a system. However, the dosing of 20 mg/L DBNPA biocide decreases substrate utilisation to less than 1 in all samples between day 12, 15 min post dosing to 6 h after dosing. After 24 h there is a significant increase in carbon substrate utilisation that further increases after 48 h, exceeding utilisation of the pre-dosing community. This means that bacterial communities in the post treatment sample display changed community diversity based on carbon utilisation on Biolog Ecoplate® plates and that there are different bacterial species in the final sample. These findings were also reported by Martínez (2017), who obtained similar results on the effect of antibiotics on bacterial communities. He noted that the release of antibiotics into the environment has an immediate effect on the microbiome, which is gradually recovered over a long time. However, the bacterial species present in the new community were different. He also stated that once an organism has been displaced, the habitat will rarely be re-colonised by the same species. The biocidal susceptibility of this new, redeveloped community may be different to the initial community. Leading to the need for an alternative biocide to be dosed which was able to manage the new community. This variability will require a more in-depth understanding of the variability of the cooling water which is to be optimally dosed. It may be necessary to use a variety of biocides, with different mechanisms of action to ensure that no single community develops in the system.

The dendogram detailing substrate utilisation in the alternative secondary biocide trial, derived from Appendix C, is depicted in Figure 5.3. The samples again separate into several clades, however a single sample, sampled 48 h after the DBNPA dosing on day 12 (12-48)

falls basal to the remainder of the dendogram. The location of this sample indicates that the bacterial species diversity in it utilises a completely different set of carbon substrates to that of the sample pre-dosing. This implies that the bacterial community has completely changed.

Table 5.2: Biolog Ecoplate® substrate utilisation versus aerobic bacterial counts for the duration of the extended isothiazolone resistance and alternate DBNPA biocide trial.

Time (hr)	Code for Figures	Biocide concentration dosed (mg/L) isothiazolone	Substrates used	Log_{10} CFU's/mL
Day 1 Time 0 hr	1_0	4	10.0	4.48
Day 1 Time 6 hr	1_6		10.0	4.48
Day 3 Time 0 hr	3_1	6	11.0	4.43
Day 3 Time 6 hr	3_6		7.0	3.97
Day 5 Time 0 hr	5_0	10	7.0	4.31
Day 5 Time 6 hr	5_6		8.0	3.75
Day 8 Time 0 hr	8_0	16	3.0	3.66
Day 8 Time 6 hr	8_6		2.0	3.23
Day 9 Time 0 hr	9_0	20	5.0	3.13
Day 9 Time 1 hr	9_1		3.0	2.80
Day 9 Time 2 hr	9_2		8.0	2.91
Day 9 Time 4 hr	9_4		6.0	2.11
Day 9 Time 6 hr	9_6		7.0	2.90
Day 9 Time 24 hr	9_24		5.0	3.24
Day 9 Time 48 hr	9_48		11.0	4.13
Day 12 Time 0 hr	12_0	20 (DBNPA)	5.0	3.48
Day 12 Time 15 min	12_15m		0.0	1.23
Day 12 Time 30 min	12_30m		0.0	1.20
Day 12 Time 1 hr	12_1		0.0	1.28
Day 12 Time 2 hr	12_2		0.0	1.38
Day 12 Time 3 hr	12_3		0.0	1.28
Day 12 Time 4 hr	12_4		0.0	1.90
Day 12 Time 5 hr	12_6		0.0	2.06
Day 12 Time 6 hr	12_24		8.0	4.20
Day 12 Time 7 hr	12_48		20.0	4.55

All the other samples from the trial fall into six subclades (blue, burgundy, yellow, green, pink and orange). Within these clades the green, pink and orange clades each house a single sample which is marginally different from the bulk of the samples in the blue, burgundy and yellow subclades. Sample 3_0 (orange) is from the third day of isothiazolone resistance

development. The reason for its independent location cannot be explained, as it appears to utilise an entirely different set of substrates to the sample both before (1_6) and after (5_0) which are in the same, separate subclade (blue). Sample 9_4 is similarly misplaced, theoretically it should fall between 9_2 and 9_6 which fall next to each other in the yellow clade, however, the difference between these samples is a maximum of 30%. The sample in the pink clade was taken 48 h after the dosing of 20 mg/L isothiazolone and differs by approximately 30%. This sample indicates that the bacterial community has altered the carbon substrate utilisation at this time. This implies that bacterial species diversity has shifted, possibly due to excess nutrients available from bacteria lysed by the isothiazolone dose. In addition, it correlates to a large increase in total bacterial count (Figure 5.3). Guest (2016) noted similar shifts in bacterial communities when dosing, planktonic *Salmonella*, laboratory scale water treatment trials, with oxidising biocides. The majority of the samples group in the blue, burgundy and yellow subclades. Each sub-clade houses samples from each stage of the trial, although the total variation between the sub-clades is less than 25%.

The blue subclade contains mostly the isothiazolone resistance development samples, while the samples in the burgundy sub-clade are associated with the DBNPA dosage. All samples for the DBNPA trial group together from the 15 min to the 6 h post dosing sample, when no substrate utilisation was detected. This correlates to the active reaction time for DBNPA. Subsequent changes in substrate to the 12 - 24 sample (yellow clade) and the 12 - 48 sample indicates that substrate utilisation was significantly changed, inferring that bacterial species diversity was completely changed post-DBNPA dosing.

Gel2K was used to generate a DGGE dendrogram for the statistical comparison of the alternating DBNPA biocide trial (Figure 5.4). The samples again separate into two main clades approximately 35% different. The main clades again separate into several subclades, the blue, burgundy and yellow in the initial clade and the green and pink subclades in the second clade.

The notable similarity between the DGGE (Figure 5.4) and the substrate (Figure 5.2) dendrograms is that sample 12-48 separates from the other samples; however in the DGGE dendrogram (Figure 5.4), sample 12-2 is in the same subclade. It is believed that this due to residual DNA detection from the post dosage kill off (Table 5.1). The remainder of the samples separate randomly into the various subclades, this is indicative of non-viable DNA

remnants, extracted in the PCR process, being detected in the DGGE analysis. This phenomenon was expanded by Congelosi and Meschke (2014), in a mini review on molecular assessment of microbial viability. They stated that PCR amplification is unable to differentiate between viable and non-viable DNA in sample. Therefore the PCR product which is subjected to DGGE has both sets of DNA present and both are separated, leading to a false species count.

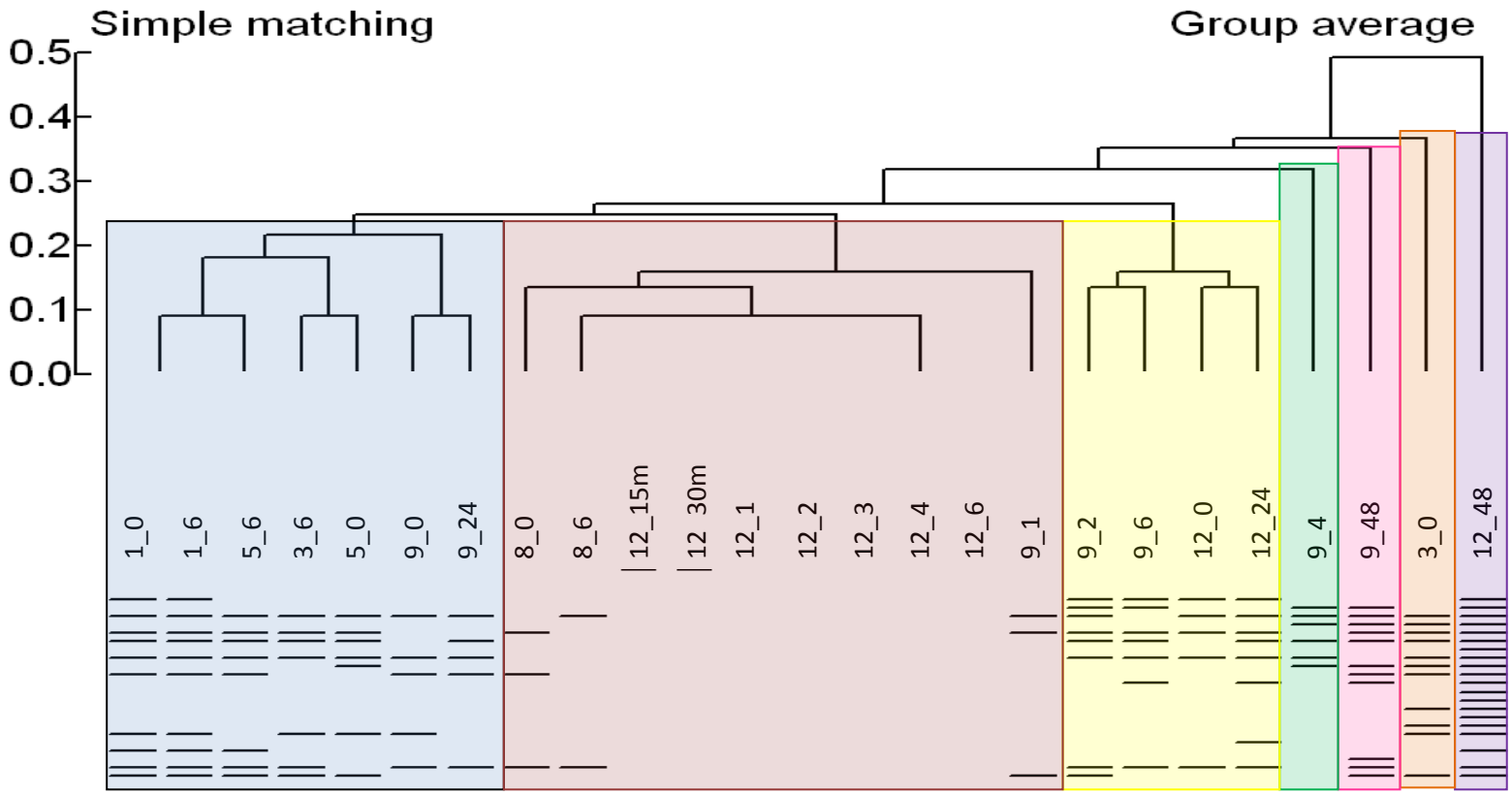

Figure 5.3: Gel2K statistical relationship of an average of three replicate results of each positive substrate reaction on Biolog Ecoplates® represented by bands at various times (Day_hour / min, as explained in Table 5.2) throughout the extended isothiazolone with DBNPA alternate biocide resistance trial.

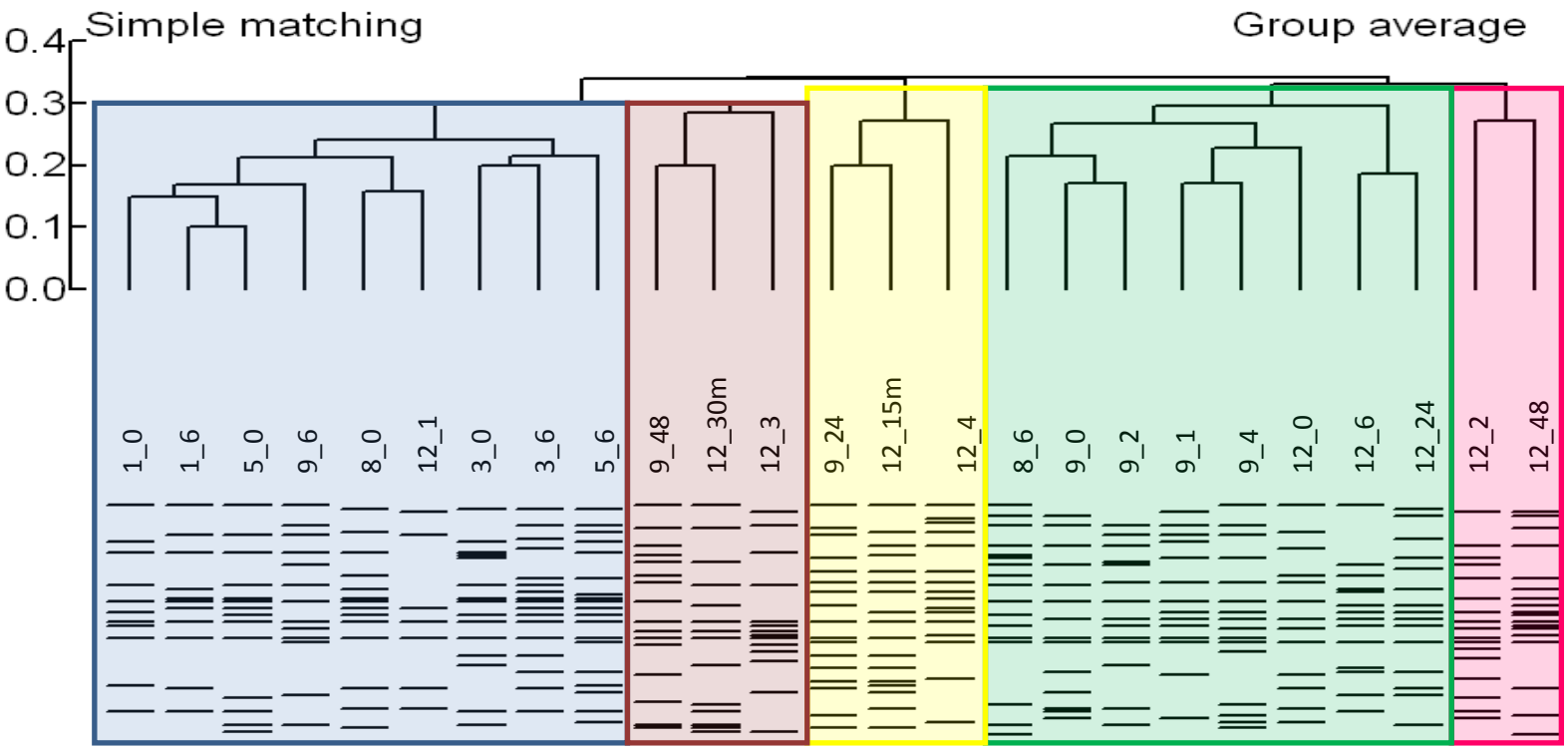

Figure 5.4: The Gel2K statistical relationship between the DGGE banding patterns at various times (Day_hour /min, as explained in Table 5.2) throughout the extended isothiazolone with DBNPA alternate biocide resistance trial.

5.4 CONCLUSIONS

As biocidal resistance was evolved by the regular dosing of sub-lethal concentrations of isothiazolone, carbon substrate utilisation on Biolog Ecoplates® showed an increase. This is indicative of an increase in bacterial species diversity, which implies that there are more species in the resistant sample. This may be attributed to the elimination of previously dominant species which allow suppressed species to grow.

However when DBNPA is dosed as a secondary alternate biocide, both bacterial count and carbon substrate utilisation decreased. It is thus deduced that the DBNPA kills the new bacterial community which had developed resistance to isothiazolone. The results also indicated the short active life of the DBNPA as both bacterial counts and carbon substrate utilisation increased 24 h after dosing.

Substrate utilisation in the resistance trial varies significantly between resistance development (pre dosing Day 9_0) and after the 20 mg/L isothiazolone dose (after 9_1), however this variability is substantially less when compared to the carbon substrate utilisation with secondary biocide dosing.

DBNPA dosage decreased the log of bacterial counts to 1.2 and no carbon substrate utilisation was detected, this indicates that the Biolog Ecoplate® system is not sensitive enough to detect the low bacterial counts. Both bacterial count and carbon substrate utilisation increase 24 h after DBNPA dosing however, using a significantly different set of carbon substrates.

There is also a possibility that the dosing of a biocide may cause the development of viable but non-culturable cells (VBNC) in the cooling water. This would cause the results on the Biolog Ecoplates® to be undetectable as the cells would not be culturable in the carbon substrate wells. In addition, this would allow for the subsequent redevelopment of the bacterial community once the stress had been removed from the system. This requires further research to determine whether VBNC bacterial cells are maintained in the cooling water system.

Molecular dendrograms correlate to the carbon substrate dendrograms for the resistance trial, indicating that the Biolog Ecoplate® may offer a means of tracking biocide efficacy. The molecular DGGE results are suspected to indicate a higher level of banding than the carbon substrate comparison because the Biolog Ecoplate® system relies on the culturability of the species present in the system.

In terms of the hypothesis that the Biolog Ecoplate® system could potentially be used as a simple method for determining the efficiency of a biocide, the results indicated that it could indeed be used with some caution when interpreting the results. The significant decrease in metabolic activity using the Biolog Ecoplate® system did not reveal the fact that many of the species were still present in the system after biocide addition, but at levels below the sensitivity of the system as supported by the DGGE results.

This study indicated that the use of an indirect indicator of bacterial species diversity, such as the Biolog Ecoplate®, could be an effective technique to evaluate the efficacy of a biocide dosed into a cooling water system. In the Eskom environment, it may be preferable to conduct a total bacterial count as the carbon utilisation can be correlated to the bacterial species community in the induced stress environment. This can lead to a more indicative biocide efficacy because monitoring bacterial plate counts will show an increase with biocide dosing but if the implied species diversity does not change we now understand that the biocide is not optimally dosed. This study confirms that more work needs to be conducted on each power plant to develop a base line of their substrate utilisation with and without dosing. In addition monitoring will need to be conducted more regularly than once 24 h post biocide dosing.

On-site evaluations will also have to include under and proposed optimal dosing. In this way each plant will know what degree of substrate utilisation change is necessary to indicate acceptable biocide efficacy.

The use of other biocides in cooling water treatment needs to be evaluated and the accuracy of the test evaluated on an operational plant, where other external factors may impact the biocide efficacy.

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Chapter 6:
The concomitant development of antibiotic resistance and biocidal resistance to Isothiazolone and the effect on bacterial species diversity.

Abstract

The development of biocidal resistance to non-oxidising biocides is well documented, as are the mechanisms by which this resistance is developed. These mechanisms may be effective at either a molecular level (DNA mutations) or at a structural level (cell wall structure changes).

This study attempts to determine the effect of biocidal stress on bacterial species diversity. It is envisaged that the bacterial population should vary as the biocidal resistance of the population increases. Theoretically, the Darwinian concept of survival of the fittest will allow only bacterial species that have developed biocidal resistance to survive. Monitoring these populations and understanding the fluctuations in bacterial species present in resistant populations is expected offer a means of understanding effective biocide dosing.

In the past, Biolog Ecoplates® have been utilised to monitor changing bacterial populations in several environments, including water and soil. These microtitre plates have a triplicate set of 31 wells, each containing a different carbon substrate. When the bacteria population is added to the wells, the bacteria begin to grow utilising the substrate and respire the Tetrazolium dye, included in the plates, causing a purple colour change. For the purpose of this study this change indicates a positive result.

It is hypothesised that the greater the diversity of bacterial species in the sample, the more carbon substrates will be utilised while fewer different bacterial species will use a smaller number of substrates.

For this study, the number of substrates utilised should decrease as the population is induced to become more resistant to the biocide isothiazalone. In addition, the effectiveness of an alternate, subsequently dosed biocide on bacterial species diversity is investigated. The effect

determined on Biolog Ecoplates was supported by conventional classical plate counts and molecular analysis with NextGen sequencing on certain samples.

6.1. INTRODUCTION

The use of antimicrobial products in the form of antibiotics and disinfectants is increasing internationally to minimise possible infections or contamination in human health and industry respectively (Gnanadhas *et al.*, 2012). Antimicrobial products used in industries, as disinfectants, are called biocides while those used in for the treatment of man and livestock health are termed antibiotics (Scientific Committee on Emerging and Newly Identified Health Risks SCENIHR, 2009). In general, biocides have a broader range of activity, compared to antibiotics, as they target several sites in the bacterial cell while antibiotics have more specific targets (Garneau-Tsodikova and Labby, 2016). Although both products kill bacteria, the use of antibiotics is strictly controlled while biocides can be used without any significant form of monitoring and/or control (Scientific Committee on Emerging and Newly Identified Health Risks, 2009). However, the abuse and over-prescription of biocides and antibiotics has led to an increase in antimicrobial resistance in bacteria, especially in aquatic environments (Singer *et al.*, 2016), necessitating alternative treatment regimes. Bacteria are considered resistant to antibiotics or biocides in the following situations (Bridier *et al.*, 2011):

- When the bacterial species is not killed or inhibited at antimicrobial concentrations commonly used in practice.
- When the bacterial species is not killed or inhibited by a concentration where most species are affected.
- When some bacteria are not affected by a concentration that acts on the other cells of the culture.

Although antibiotics have been proven to be effective against pathogens, there are several studies that indicate that they severely damage the host microbiome forming an environment inductive for the growth of opportunistic pathogens, increasing the development of antibiotic resistance (Ubeda *et al.*, 2010)

Høiby *et al.* (2010) reported the development of antibiotic resistance in biofilms and there are several laboratory scale studies on the possible link between biocide use and antibiotic

resistance (Martinez, 2009; Kummerer, 2009; Allen *et al.*, 2010; WHO, 2014; Berendon *et al.*, 2015). This phenomenon is generally referred to as cross – resistance. Studies indicate that Gram negative bacteria are more likely to develop this cross resistance (Slama, 2008; Exner *et al.*, 2017).

Biocide-antibiotic cross-resistance is mainly due to efflux pumps that work for both agents (Fernández and Hancock, 2013; Wales and Davies, 2015; Slipski *et al.*, 2018). Other mechanisms include impermeability of the cell wall/membrane (Exner *et al.*, 2017), multi-drug efflux pumps (Randall *et al.*, 2007), over expression of operons *inter alia ompB, IS 1, IS 10, mexR* and *nfxB* (Fernández and Hancock, 2013), plasmid transfer (Singer *et al.*, 2016), quorum sensing (Fernández and Hancock, 2013) and the alteration of a target site (Blair *et al.*, 2015). In addition, there have been several studies which determine an association between plasmids and antibiotic resistance (Johnson *et al.*, 2012; Ramirez *et al.*, 2014; Jaran, 2015).

Biofilm bacteria are 100 – 1000 times more resistant to antimicrobials than their planktonic counterparts (Høiby *et al.*, 2010; Flemming, 2016). Fernández and Hancock, (2013), developed a comprehensive review of common mechanisms of biocide and antibiotic resistance development in biofilms. These include that the antibiotic may penetrate into the biofilm slowly or incompletely. Studies on *in vitro* biofilm penetration indicate that few antibiotics can actually penetrate bacterial biofilms (Otto, 2008). In addition, antibiotics may be inactivated by surface layers of the biofilm, completely limiting biofilm penetration. Antibiotic adsorption into the biofilm matrix would also allow only limited penetration into the biofilm (Otto, 2008).

Chemical variability through the biofilm could cause bacteria to enter an inactive (spores) state, which would affect the efficiency of the antibiotics. These changes may include decreased nutrient gradients, decreased oxygen availability in anoxic or anaerobic regions, possible changes in pH and osmotic concentrations due to the accumulation of waste products, which become trapped within the biofilm (Stewart and Franklin, 2008).

There is speculation that sub communities of bacteria in the biofilm form highly protected, spore-like states. Research to date indicates that newly formed biofilms show resistance, although they are too thin to act as a barrier to penetration (Ragan *et al.*, 2011), indicating

that the bacterial cells must be resistant in their own right. Wood (2013) showed that most bacteria in a biofilm are killed by antibiotics but approximately 1% of the initial community continues even if continually exposed to the antibiotic. This 1% is believed to be comprised of persister cells, which are capable of living in the toxic environment caused by the antibiotics. These cells are capable of re-establishing active growth as soon as the stressor is removed (Ayrapetyan *et al.*, 2015)

The development of biofilms in cooling water systems is well documented (Flemming and Ridgeway, 2008; Lear and Lewis, 2012) as is the use of biocides in their treatment and management (Liu *et al.*, 2011; Satpathy *et al.*, 2016). It is thus pertinent that biocide resistant bacteria in cooling water biofilms may also resist antibiotics. This is of concern to bacteria that naturally reside within biofilms e.g. *Legionella* and *Pseudomonas*, which may have developed antibiotic resistance while within the biofilm. When the biofilms slough off the surface these bacteria are released into the bulk water which may come into contact with personnel, raising a significant health risk (Cloete *et al.*, 1998).

In this particular study, Biolog PM® plates were utilised to compare the metabolic variability (bacterial species variability) of a pre-dose, biocidally resistant and recovering cooling water sample. Particular emphasis was placed on the response of the sample microbiome to three antibiotics, namely rifamycin, azetreonum and ethionamide. The data was compared and validated to microbial plate counts, DGGE and metagenomic next generation sequencing.

6.2. MATERIALS AND METHODS

6.2.1 Pre-dosage of isothiazolone to cooling water system

A 500 l cooling water system simulator (CW Rig) was operated as described in 3.2. A bacterial community with implied isothiazolone resistance was developed by the sequential addition of increasing concentrations of sub-lethal levels of isothiazolone. On day 1 of the trial, 4 mg/L of isothiazolone was dosed into the Rig and a sample was taken pre-dosing (1_0). This dosing was repeated with 6 mg/L on day 3 (3_0 and 3_6), 10 mg/L on day 5 (5_0 and 5_6) and 16 mg/L on day 8 (8_0 and 8_6) but no samples were taken based on the isothiazolone resistance trials conducted in Chapter 5. On day 9, 20 mg/L isothiazolone was dosed, representing a normal concentration for treatment in the cooling water system. The

system was then sampled at time 0, 1, 2, 4, 6, 24 and 48 h (9_0; 9_1; 9_2; 9_4; 9_6; 9_24 and 9_48) after dosing.

Total bacterial plate counts were used to determine the pre-dosing bacterial count and sample times where isothiazolone dosing was most effective i.e. the sample with the lowest bacterial count and when the isothiazolone residual concentration was completed and the bacterial counts began to increase again.

Bacterial culturing analyses and Biolog EcoPlates® were completed as mentioned previously in Section 3.2. The metabolic variations were monitored on Biolog PM® Plates, using the Biolog OmniLog® plate reader at Onderstepoort Biological Products laboratories.

6.2.2 Metagenomic Next Generation Sequencing

Samples were sent to Inqaba Biotec for 16S bacterial DNA metagenomics analysis, where they were amplified through unbiased/shotgun PCR and subjected to high throughput next generation sequencing. Once the sequence data were acquired and assembled, every sample was subjected to BLAST (Basic Local Alignment Search Tool) analysis to attain a qualitative and quantitative result.

6.2.3 The use of Biolog PM® Plates to evaluate changes in bacterial species metabolism.

Each sample was distributed into a set of 20 Biolog PM Plates by adding 100 µl of sample, with added Biology Dye H, to each well. These were incubated at 35°C for 48 h. The dye in the sample causes the sample in the well to change colour when the substrate is utilised. This colour change was monitored every 15 min for 48 h. The reading in OmniLog® units is indicative of the rate of the substrate usage. The readings were plotted for each sample and compared. The first 10 Biolog PM® Plates (1-10) are known nutrients or substrates for bacteria while the second 10 (11 to 20) are toxins or inhibitory compounds, including antibiotics, each at four concentrations (Lei and Bochner, 2013).

6.3 RESULTS AND DISCUSSION

6.3.1 Bacterial plate counts

Three samples were used for this trial, pre-dosing; 24 h after dosing on day 9 (lowest bacterial count) and 48 h after dosing on day 9 (increased count post dosing). Bacterial count results, from the trial (Table 6.1), indicated resistance development after 20 mg/l of isothiazolone dosing, as the planktonic plate count decreased from a log count of 5.11 to 4.58 after day 9, pre-dosing (this correlates to the previous resistance results reported in Chapter 5) and, after dosing a further 20 mg/l isothiazolone, decreased to 3.53 24 h after dosing on day 9 (Table 6.1). Forty-eight hours after dosing on day 9, the total bacterial count increased again to log count 5.00, indicating that any biocide residual had been depleted. This reaction was highlighted by Cloete *et al.* (1998) in investigations on the use of biocides in cooling water treatment, as explained in Chapter 5, Section 5.3 and by Brözel and Cloete (1992) in their report on the effect of biocide treatment on planktonic communities. In both studies, once biocides had been dosed, the total bacterial count decreased. After between 36 and 48 h however, the counts had increased above the initial, pre-dosing count. This implies that a secondary community of more resilient bacterial species populates the system and grows at a rapid rate due to an excess of nutrients available from lysed and dead cells. Once this surplus is depleted the bacterial count returns to a stable, pre-dosing, count.

6.3.2 Determining metabolic activity using Biolog Ecoplate® substrate utilisation

The number of substrates (Table 6.1) utilised in the Biolog Ecoplate® inoculated with sample from day 9, 24 h post dosing, reduced from 25 to 5. The sample taken 24h later, showed an increase in utilisation of 10 substrates. A lowered substrate utilisation such as shown by the day 9, 24 h sample indicates a decrease in metabolic activity which could serve a proxy for a decrease in bacterial species diversity in the sample. This, substrate utilisation, is supported by Erbilgin *et al.* (2017) who investigated the use of substrate preferences to predict metabolic properties and abilities to carry out specific biochemical functions, in a bacterial consortium. Using *Bacillus* and *Pseudomonas* sp; they grew mixed cultures on specifically formulated media and generated different growth curves based on the order of substrate utilisation by the consortium. This was used to create an opportunity to integrate the results into a predictive model indicating resource utilisation within a bacterial community.

Table 6.1: Biolog Ecoplate® average substrate utilisation versus average aerobic bacterial counts of the biocide/ antibiotic cross resistance trial.

Time	Code for Figures	Biocide dosage (mg/L)	Substrates utilised	Log_{10} CFU/mL
Day 1, Time 0 hr	1_0	4	25.0	5.11
Day 3, Time 0 hr	3_0	6		
Day 5, Time 0 hr	5_0	10		
Day 8, Time 0 hr	8_0	16		
Day 9, Time 0 hr	9_0	20		4.58
Day 9, Time 1 hr	9_1			3.60
Day 9, Time 2 hr	9_2			3.59
Day 9, Time 4 hr	9_4			3.72
Day 9, Time 6 hr	9_6			3.97
Day 9, Time 24 hr	9_24		5.0	3.53
Day 9, Time 48 hr	9_48		10.0	5.00

Their results confirm that it is possible to use substrate utilisation as an indicator of bacterial metabolism and thus inferred diversity. However, it also raises the fact that bacteria may prefer certain substrates over others. This must be considered when evaluating the pattern of carbon substrates used on the Biolog Ecoplates®, and should only be used to trend responses of the bacterial community in the system rather than using the result as a definitive quantity. This research may also be considered limited in that the bacterial cells are still classified culturable, in that they have to grow in a manmade environment with set nutrient availability. Increasing isothiazolone concentrations eliminated many of the species present, which is in agreement with research conducted on biocidal treatment of cooling water (Brözel *et al.*, 1993) and paper making (Ullah, 2011). The research conducted by Brözel *et al.* (1993) expanded on the use of non-oxidising biocide for the treatment of water. The research focused on the development of decreased susceptibility in several bacterial cultures to biocides *inter alia* isothiazolone. It concluded that isothiazalone is more effective at higher concentrations. This is in agreement with Ullah (2011) who consolidated information relative to biocides utilised in the papermaking industry. The study gives a comprehensive overview of several biocides including isothiazolone and concurs that increased concentrations of isothiazolone does eliminate a wide range of species when optimally dosed.

The results of this study, theoretically, offer the possibility to deal with higher bacterial counts in the cooling water by dosing a higher concentration of the biocide. Although this may seem self-explanatory, it is essential that dosing of the biocides is conducted in a manner that will optimise the efficacy of the biocide. This means that the correct concentration of biocide must be dosed at the correct frequency to maintain the bacterial community. If this is not managed the bacterial count varies widely between dosages and will allow the system to rapidly foul in these periods of low biocidal activity. Therefore, the next biocide dose will have to deal with the new fouling and whatever bacterial communities were in the water initially. This is a financial waste as the biocide is costly to procure and will be continuously utilised to control an ever increasing community.

6.3.3 Molecular Analyses Results

Gel2K was used to generate a DGGE gel pattern for statistical analysis of the trial. The DGGE results indicate that the number of bacterial species (bands in the gel) decreased after development of resistance from 26 bands in day 1, predosing sample to 18 bands in day 9, 24 h post dosing sample. These bacterial species recovered, but not substantially 24 h later, when the bacterial species band count increased to 19 bands (Figure 6.1). This indicated that the stressed environment selected for specific bacterial communities.

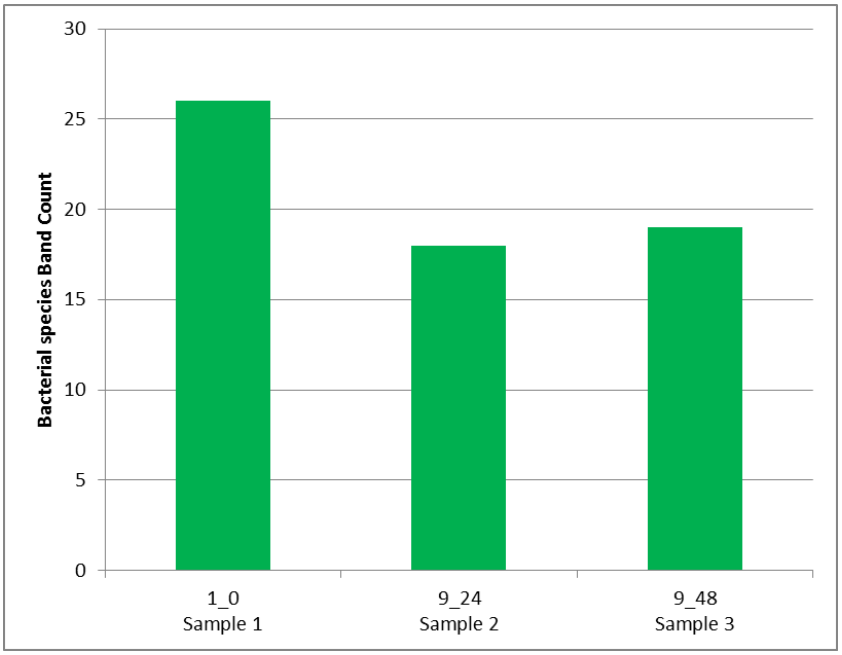

Figure 6.1: Bar graph indicating DGGE band count for each Sample. The bars represent the number of bands detected per sample on the DGGE gel.

However, once biocidal stress was removed, species able to tolerate this stress increased and conditions selected for new species present in day 9, 48 h post dosing, evident by unique new bands. DGGE analysis supports previous conclusions that the biocide reduced bacterial numbers, but the diversity only changed when the community was allowed to develop resistance to biocide concentrations. Wales and Davies (2015) made a similar conclusion on bacterial cross resistance to both biocides and antibiotics. They noted that cross resistance adaptations may continue to have an adverse effect on the bacterial community when the biocide/antibiotic has been removed. This may cause concern where a system is repeatedly treated with isothiazolone. As it implies that the bacterial community may change to a new community with an inherent cross resistance, even once the treatment is stopped. In addition, Singer *et al.* (2016) concluded similar findings on antimicrobial resistance in the environment. They attempted to explain the need for antimicrobial resistance plans emphasising that the treatment of a bacterial community and subsequent development of cross resistance allows for the persistence of common, small mutations, which will allow for a variation in community. They suggested that a risk based assessment of resistance development in a range of biocides be developed. This would offer guidance to the selection of biocides that offered the least risk of cross resistance development. The development of this risk assessment would offer, the power plants, guidance to the selection of biocides that offered the greatest treatment of the relative cooling water with the least risk of cross resistance development. The development of stable slimy biofilms can be noted on many of the power plants despite, admittedly infrequent, biocide dosing.

6.3.4 Metagenomics Next Gen sequencing results

The Next Gen sequencing results are depicted in Figure 6.2. The results are shown at family classification level and only results above 1% are shown on the graphs.. Additionally, the total number of BLAST results, decreased from 448 in the predosing sample to 350 in the day 9, 24 h post dosing sample and subsequently increased to 418 24 h later. The species diversity graph shows an increase in percentage of unknown DNA. The reason for this is unclear but may be inherent to the method of analysis. The bacterial family composition of the pre-dosing sample shows the presence of a 62.3% unknown, 20.8% *Comamonadaceae*, 11.1% *Sphingomonadaceae* and 2.0% *Microbacteriaceae*. However, in day 9, 24 h post dosing sample, the family groups present in the bacterial community contains a percentage of

Pseudomonadaceae (13.9%), a reduced percentage of *Comamonadaceae* (7%), unknown (75.8%) and an exclusion of *Sphingomonadaceae* and *Microbacteriaceae.* This is due to isothiazolone sensitivity of *Comamonadaceae*, *Sphingomonadaceae* and *Microbacteriaceae* that were all negatively affected by biocide dosage, indicating, that these bacterial families do not develop complete resistance to isothiazolone biocide. The sensitivity of *Microbacteriaceae* to isothiazolone was studied by Kapoor and Yadav (2010) for use in development of bioluminescence assays. They monitored ATP development of various *Microbactrium* cultures and determined that different species of *Microbactrium* sp. have different sensitivities to isothiazolone dosage concentrations. This implies that any biocide dosed has to cover the most resistant species of each bacteria, which is not the case as biocides are normally active against as broad, but limited spectrum as possible. A biocide efficacy evaluation of water treatment biocides conducted by Brözel and Cloete, (1991) showed that six different isothiazalone products dosed into the same cooling water at 50 mg/L are all less effective at treating *Pseudomonas* species. As isothiazalone is a primary biocide used for power generation water treatment, this implies that the bacterial community of the cooling water should have a high number of *Pseudomonas* species, which are known slime formers. As many sites do not alternate biocides, they are likely to have a large slime forming community in the cooling water systems. In addition, some *Pseudomonadaceae* are reported to be resistant to isothiazolone (Zhou *et al.*, 2015). This characteristic was utilised by Zhou *et al.* (2015) to determine what changes occurred in the protein composition of the bacterial cell wall which afford the cell improved resistance to isothiazolone. Ideally this form of study should be conducted on bacteria which are proven resistant to antimicrobials, this will allow for the development of treatments that work against these proteins. This may offer the development of a more effective biocide for the treatment of *Pseudomonas* species biofilms in particular.

In day 9, 48 h post dosing sample, the bacterial family community begins to revert to its initial composition. The *Sphingomonadaceae* population increased (11.0%) as did the unknowns (77.5%). This proves that *Sphingomonadaceae* are sensitive to isothiazolone but are not completely killed off by the biocide and are capable of recovery once the biocide residual concentration is sufficiently minimised. The *Pseudomonadaceae* population decreased significantly to 5.6%, indicating that other families are becoming more dominant, however, *Microbacteriaceae* did not re-establish. This implies that isothiazolone was more effective against the *Microbacteriaceae* than the *Sphingomonadaceae*, although the

susceptibility of *Microbacteriaceae* to isothiazolone was highlighted by Kapoor and Yadav (2010) as previously mentioned. *Comamonadaceae* percentages (3.2%) were marginally lower but this is offset by the increased unknown percentage (77.5%) in the sample.

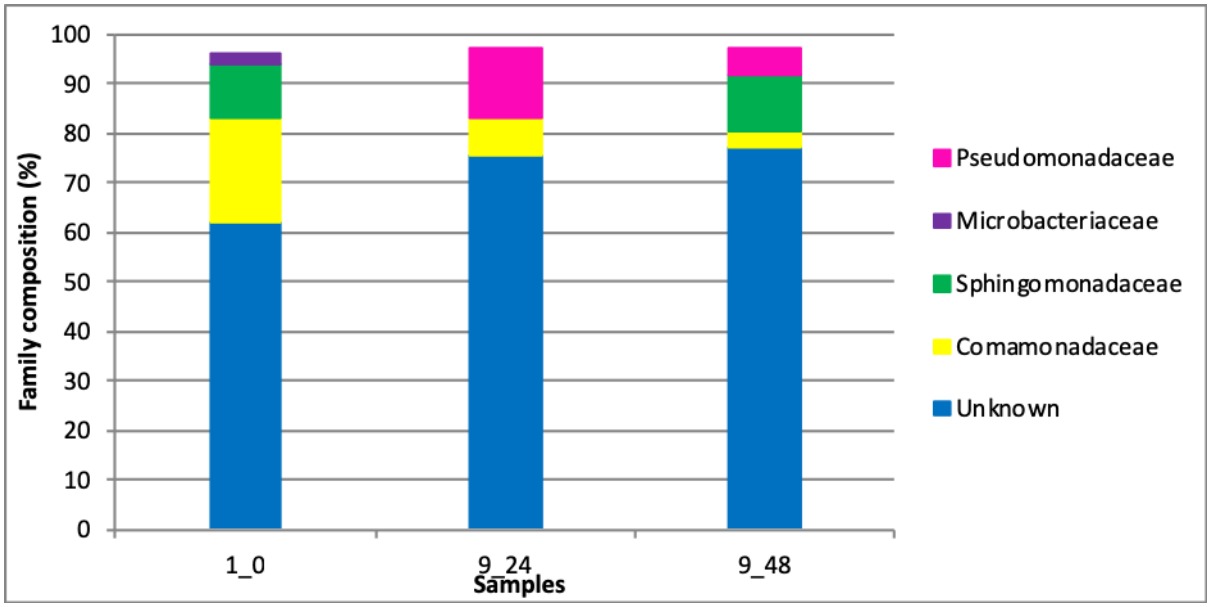

Figure 6.2: NextGen Sequencing percentage compositions at family level. This graph indicates the family groups which constitute a minimum of 1% of the total bacterial population.

6.3.5 Results obtained on Biolog PM Plates for the monitoring of antibiotic resistance

Kinetic data was compared across samples (1_0; 9_24 and 9_48), OmniLog® software, OmniLog®-OL_PM_FM/Kin 1.30-: File Management/Kinetic Plot Version, Jun 16, 2003, was utilised to compare kinetic outputs, indicating bacterial cell growth through increased respiration, from each set of plates. This software compares the results for each substrate (well) on each plate taken at the 3 different sample times. Kinetic plot data, producing respiration rate curves, was investigated and showed notable variations in the antibiotic usage of the 9_24 sample, when subjected to Rifamycin, Azetreonum and Ethionamine.

The above-mentioned technique was also used by Lei and Boucher (2013) to determine changes that improved or repressed *Clostridium difficile* toxin production, allowing for the comparison of the bacterial metabolisms within the samples. The team utilised Biolog PM

Plates to determine which substrates were utilised by each strain (toxin producers or not). They used the Omnilog reader to automatically collect kinetic data of colour development, due to tetrazolium dye respiration by the bacteria and compared each well's colour development for the various *Clostridium difficile* cultures. This led to the development of a new assay for the evaluation of *Clostridium difficile* toxicity. Although this research utilised the metabolic substrate pathway monitoring for a different outcome, the generation of the data will allow one to determine how bacterial communities with biocidal and/or antibiotic resistance differ from their wild type counterparts. Additionally, this may be used to see how the bacteria's metabolisms change when exposed to biocidal stresses.

6.3.5.1 Antibiotics

a) Rifamycin.

The Rifamycin group antibiotics can be produced by *Amycolatopsis rifamycinia* or artificially. They are particularly effective against mycobacteria and are used to treat HIV-related tuberculosis and leprosy (Forrest and Tamura, 2010). Their antibiotic action is based on the inhibition of bacterial DNA-dependent RNA synthesis (Artsimovitch *et al.*, 2012). Development of high level resistance in bacteria occurs due to a single amino acid change in bacterial DNA-dependent RNA synthesis (Munita and Arias, 2016).

Bacterial communities in all of the samples were capable of utilising rifamycin in their metabolism (Figure 6.3). This concurs with a review from Zhou *et al.* (2015), who stated that wild types of both *Pseudomonas* and *Burkholderia* have outer cell membranes which are susceptible to the development of isothiazolone resistance. The resistant cells lack proteins (42 and 35 kDa respectively), which transport antimicrobials across the cell membrane. They hypothesised that the action of the isothiazolone causes the protein to be damaged, which stops the rifamycin being transported across the membrane, allowing the development of cross resistance. From this conclusion it can be hypothesised that any antimicrobials which utilise the same transport mechanism across the cell wall will also have limited effect. It is therefore essential to understand the mechanisms of action of the biocides and be aware which antibiotics share the same mechanism for effective delivery.

The sample respiration rate (colour development), across all three samples was similar and as the concentration of rifamycin increases, the growth of the sample communities is enhanced (Figure 6.3). This means that whether biocidal resistance is absent, induced or declining, the bacterial communities in the isothiazolone resistant cooling water samples utilise rifamycin in its metabolism. It appeared therefore that rifamycin is not acting as a growth inhibitor but rather as a substrate for bacterial growth.

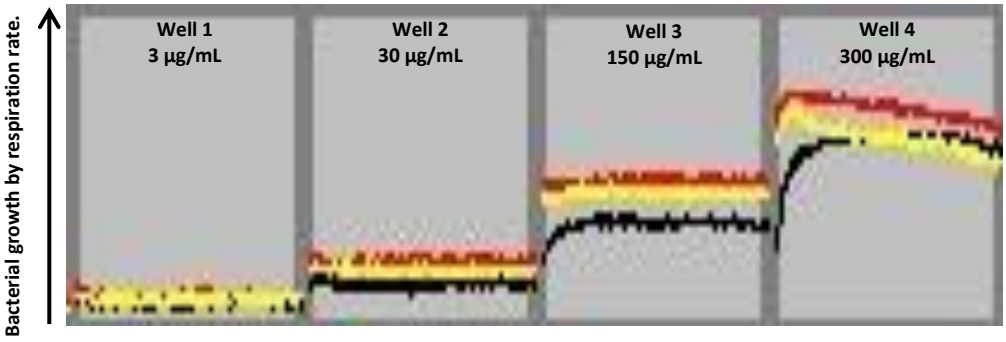

Figure 6.3: Each block represents a different concentration of rifamycin (µg/mL) over an incubation time of 48 h. OmniLog® kinetic data for rifamycin (scale at maximum of 250 OmniLog® units) at four increasing concentrations as indicated, black = taken at time 0 before inducing resistance; red = taken 24 h after inducing resistance over a 9 d period; yellow = taken 48 h after inducing resistance over a 9 d period.

b) Aztreonam

Aztreonam is a member of the beta-lactam class of antibiotics and was selectively effective against Gram-negative aerobic bacteria having no effect against Gram-positive or anaerobic bacteria. It is effective, at low concentrations, against many *Enterobacteriaceae* and against 90 % of *Pseudomonas*, while some staphylococcal species, *Aeromonas hydrophila*, *Citrobacter diversus*, *Enterobacter agglomerans*, *Haemophilus* spp. and *Streptococcus pyogenes* have developed various levels of resistance (Spellerberg and Brandt, 2016). It is interesting to note that aztreonam is reportedly effective against *Pseudomonas* species but that the bacterial community in the biocide resistant sample has a significant number of *Pseudomonas* species present as indicated in Figure 6.2. This implies that the antibiotic resistance is in some way induced by the development of biocidal resistance. Aztreonam is used to treat urinary tract infections, septicaemia, gonorrhoea, intra-abdominal and lower

respiratory tract infections especially pneumonia and bronchitis, by inhibiting cell wall biosynthesis (Quon *et al.*, 2014).

The bacterial community of the day 9, 24 h post dosing sample was able to utilise aztreonam better than those of the pre-dosing or day 9, 48 h post dosing communities (Figure 6.4). The day 9, 24 h post dosing sample showed an increase in growth compared, across each single-concentration-well, with the pre-dosing and day 9, 48 h post dosing samples, which remained sensitive to the antibiotic over the same time period. The development of cross-resistance between aztreonam and isothiazolone was documented by Charrier *et al.* (2016). By using a two dimensional chequerboard, minimum inhibitory concentration test, combinations of antibacterials were evaluated. The results indicate that *Enterococcus* and *Acinetobacter* sp developed cross resistance between isothiazolone and several antibiotics *inter alia* aztreonam. This form of testing may become necessary when developing a risk assessment of the health risks of the cooling water system, as required by the South African *Legionella* standard.

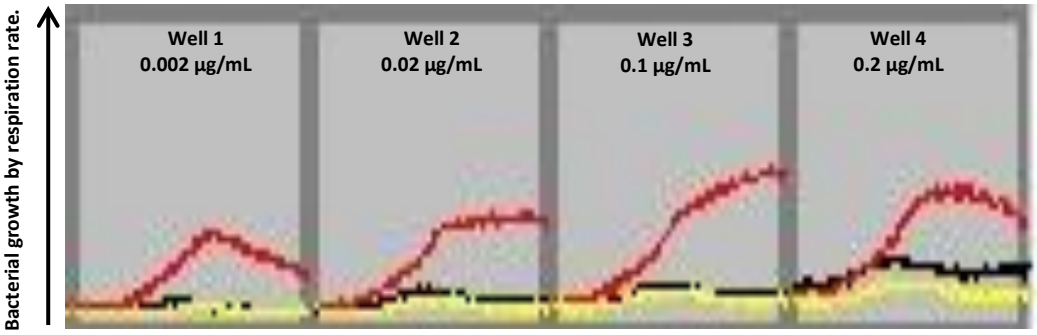

Figure 6.4: Each block represents a different concentration of aztreonam over an incubation time of 48 h. OmniLog® kinetic data for aztreonam (scale at maximum of 250 OmniLog® units) at four increasing concentrations as indicated, black = taken at time 0 before inducing resistance; red = taken 24 h after inducing resistance over a 9 d period; yellow = taken 48 h after inducing resistance over a 9 d period.

c) Ethionamide

Ethionamide is a pro-drug, this means it is administered into the patient in an inactive form and then metabolised to an active drug (Hacker *et al.*, 2009). Activation is by enzyme ethA, a mono-oxygenase in *Mycobacterium tuberculosis*, which then binds NAD+ to form an

adduct that inhibits InhA. The mechanism of action is thought to be through disruption of mycolic acid (Grzegorzewicz *et al.*, 2012). Ethionamide is traditionally used in conjunction with other anti-tuberculosis medications to treat active multidrug-resistant tuberculosis (ASHP, 2016; WHO, 2016*)*. Since increasing concentrations of ethionamide seem to enhance the growth of bacterial communities in cooling water, it is understood that an individual that comes into contact with a similar cooling water bacterial community could be unresponsive to treatment with this antibiotic.

The bacterial communities of all the isothiazolone resistant cooling water samples displayed an ability to metabolise ethionamide, this appeared to increase with an increase in concentration of the drug (Figure 6.5). This result can be supported by the biocide cross resistance review by Gnanadhas *et al.* (2013), which indicated that ethionamide is transported into the bacterial cell through water filled porins. However, isothiazolone operates on a similar pathway and thus the sample which is resistant to isothiazolone should be resistant to ethionamide too. Cross resistance development may occur due to many parameters, however in this study, it appears that transport mechanisms across the cell wall, are the common cause. A development of an understanding of these similarities could assist industry in managing this risk. In addition, several antimicrobials may have more than one mode of treatment which should be considered in the study. Samples 9_24 and 9_48 showed a similar increase in growth rate, across each single-concentration-well, and remained resistant to the antibiotic over the same time period.

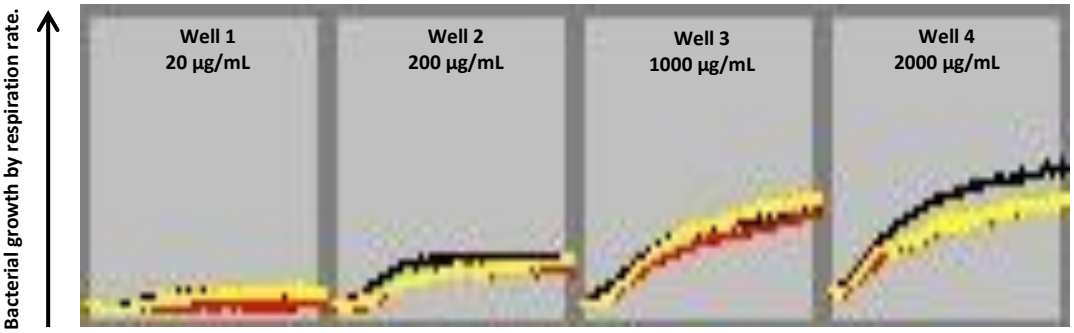

Figure 6.5: Each block represents a different concentration of ethionamide over an incubation time of 48 h. OmniLog® kinetic data for ethionamide (scale at maximum of 250 OmniLog® units) at four increasing concentrations as indicated, black = taken at time 0 before inducing resistance; red = taken 24 h after inducing resistance over a 9 d period; yellow = taken 48 h after inducing resistance over a 9 d period.

6.4. CONCLUSIONS

This study indicates that development of biocidal resistance to isothiazolone reduces the bacterial species diversity and the total bacterial count as seen in bacterial culturing and molecular species diversity analyses. The overall trend displayed shows that bacterial community diversity and counts begin high before biocide treatment begins, drop at 9 d to a minimum following the development of biocidal resistance through a dosing regime, and then recover from 24 h, post final biocide dosing, onwards showing a trend towards expected full bacterial community recovery after 48 h, post final biocide dosing.

A decrease in bacterial plate counts, initially taken before biocide dosing was observed during the isothiazolone treatment regime and remained at a minimum for 24 h post-dosing. These counts then increased between the samples on day 9, 24 and 48 h post dosing due to nutrient release from cells lysed during the biocide treatment. Similarly, Biolog Ecoplate® substrate utilisation decreased from a maximum of 25 substrates in the pre-dosing sample to a minimum of 5 substrates, 24 h post the day 9 dosing. It then marginally, albeit significantly, increased to 10 substrates utilised 24 h later. This indicates a decreased species diversity that persists post-biocide dosing and then recovers to some extent within 24 hr.

DGGE analyses mirror the substrate utilisation seen in Biolog EcoPlate® results. There is no significant difference between bacterial diversity observed in the post dosing samples, although this recovery seems imminent if extrapolated over time. Bacterial community dynamics thus changed from a scenario of high species diversity and a high number of individuals within each species, to a scenario of high species diversity and low numbers of individuals within each species. DGGE analysis indicated that species diversity was not significantly redeveloped after the biocide stress was removed although the total bacterial count did show some recovery.

Biolog PM® plates offered an insight into metabolic changes that occurred within biocidally resistant bacterial communities. The antibiotics considered in this study, all acted as nutrient substrates for bacterial growth, which increased with an increasing antibiotic concentration. Rifamycin was the most highly utilised antibiotic, displaying the maximum bacterial growth of the selected test antibiotics at highest concentration. Aztreonam was the antibiotic that displayed least bacterial growth but still acted as a suitable growth substrate for the bacterial communities over the trial. Ethionamide showed a growth and substrate utilisation level between the previous two antibiotics considered.

The Biolog PM Plates indicate that it is possible to develop cross resistance to isothiazolone and various antibiotics in bacterial communities of cooling water. In the case of rifamycin and ethionamide, the development of bacterial biocidal resistance in the cooling water did not change the antibiotic resistance of the bacterial community. However, in the aztreonum Biolog PM® test 24 h post dosing on day 9, showed increased bacterial resistance and growth, which was lost once the biocidal stress was removed. These results indicate that biocide and antibiotic resistance are linked in the tests performed in this study.

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Chapter 7:
CONCLUSIONS

Due to the strategic importance of electrical power supply, Eskom coal fired power stations are allocated a guaranteed water volume through the Department of Water and Sanitation (DWS), however the quality thereof is not specified. Unfortunately, rapid urbanisation and industrialisation has led to a swift decrease in the quality of this water. Due to Eskom's zero liquid effluent discharge (ZLED) policy, this water remains in the plant, often in the cooling water system. This, combined with the continuous evaporation through the cooling towers, causes the system to concentrate, in terms of nutrients, until it is blown down (or diluted) by releasing concentrated cooling water into the ash handling systems and replacing this volume loss with fresh make-up water. This excess of nutrients allows for microbiological growth in the system that may cause bio-corrosion or biofouling, leading to a decrease in heat transfer, implying a loss in power station efficiency.

Microbiological communities in the cooling water are managed through the addition of biocides and dispersants. There is, at present, no means of evaluating the efficacy of this treatment, without complex microbiological analyses, conducted in an analytical laboratory. As a result of increasing legislative human safety and environmental pressures, the efficacy of cooling water treatment must be monitored. It was therefore necessary to develop a simple test that will evaluate biocide treatment efficacy in cooling water treatment on power plants.

Several research studies have proven that, the concept of survival of the fittest can be applied to bacterial communities. This implies that when a stress (biocide) is applied to a community, species capable of adaption to the stress will survive while others will die off. This does not however necessarily mean that the bacterial species diversity will drop, it should however change. This change, in bacterial species, was evaluated by the monitoring of metabolic changes over the biocide trial period. Biolog Ecoplates® were used to monitor this change in metabolism by the variation in carbon substrate utilisation. Theoretically the addition of a biocide should alter the metabolism (carbon substrate utilisation) of the community, so the pattern of substrates utilised and changes thereof was used to evaluate biocidal efficacy.

Dibromonitrilopropionamide (DBNPA) and isothiazolone were selected for these evaluation trials due to their wide usage within power production cooling water systems. The cooling water system was dosed with various concentrations of the biocides signifying effective and ineffective doses. Sampling was conducted relative to the action of the biocide. These samples were analysed for bacterial counts and their ability to utilise various carbon substrates. In an attempt to compare the results obtained to the actual population, the samples were submitted for molecular analysis and phenotypic separation by DGGE.

Bacterial counts decreased in all trials, even with a low concentration of biocide, however the efficacy of the kill rate and the duration of the effect were directly affected by the concentration dosed. The higher the biocide dose concentration the higher the kill rate and the longer the duration of biocide efficacy. This may cause some concern as biocide efficacy is normally evaluated within a short time post dosing and will thus indicate that the biocide had decreased the bacterial counts. These bacteria however repopulated the system within 48 h for DBNPA and 72 h for isothiazolone. Indicating that the system is at risk and that biocide should be re-dosed.

The evaluation of the Biolog Ecoplates® did not however show the same decrease in carbon substrate utilisation for all trial dosages. Lower dosage concentrations appeared to have little effect on the substrate utilisation while the higher and over dosed concentrations caused the substrate utilisation to mirror the bacterial counts. In the overdosed trials (50 mg/L) no substrate utilisation was detected if the bacterial counts were below 10^4 CFU/mL (log 2.04), indicating that the bacterial community remaining, in the over dosed trials, was unable to respire the carbon substrate or that the Biolog Ecoplates were not be sensitive enough to detect low bacterial numbers. For the purpose of this trial, a decrease in Biolog Ecoplate® carbon substrate utilisation indicated a decrease in the bacterial species diversity in the cooling water. In addition, this result indicated the possible development of viable but not culturable (VBNC) or persister cells, although this aspect was not investigated thoroughly and is an area for continued research.

Although bacterial plate counts decreased, this did not always equate to a decrease in bacterial species community, as was seen in the lower biocide concentration dosages. It rather means that the number of individuals of each species decreased but the biocidal concentration was insufficient to eliminate all the cells. This explains why an increased in

carbon substrate utilisation was noted with a low bacterial count. It was this premise that was the basis for the use of Biolog Ecoplates® as a simple test to evaluate the efficacy of biocide treatment. If the biocide was dosed at optimal concentrations then both the bacterial count and the Biolog Ecoplate® carbon substrate utilisation decreased, but where sub-lethal concentrations of biocide were dosed, the carbon substrate utilisation did not necessarily decrease with the total bacterial counts. Results generated by this technique would therefore need to be interpreted with caution, however, the bacterial species community should move from an environment with high species diversity and a high number of individual cells to an environment with low species diversity and a low bacterial cell count, as was seen in the higher biocide dosages, from region B to region C on the Hudson (2014) species diversity relationship chart.

A decrease in metabolic activity on the Biolog Ecoplates® was not completely corroborated by DGGE results, which showed either a decrease but not a complete elimination in species numbers or a significant change in the bacterial community post. This implied that the DGGE technique may have been detecting free or inactive bacterial cell DNA, that VBNC cells were present in the biocide stressed environment or that the Biolog Ecoplate® was not sensitive enough to detect low bacterial counts and that the DGGE analysis was correct.

When isothiazolone resistance was selected, by regular dosing of sub-lethal concentrations, the bacterial counts reduced to a lower stable count but the carbon substrate utilisation showed an increase. This was considered to be indicative of increased bacterial species diversity, which is believed to be caused by previously suppressed species being able to grow due to available nutrients as a result of the bacterial count reduction. When DBNPA was dosed as a secondary, alternate biocide, carbon substrate utilisation dropped to zero and the bacterial plate count decreased to below 20 CFU/mL; indicating that the biocide was effective against the remaining bacterial community. This may have been indicative of the development of VBNC cells because the community re-developed once the biocide stress was removed.

The DGGE analysis correlated to the carbon substrate utilisation for the biocide resistance trials, it showed a significant decreased in bacterial species diversity post dosing of the secondary biocide.

The evaluation of biocidal – antibiotic cross resistance indicated that isothiazolone resistant bacterial communities developed antibiotic resistance to rifamycin, ethionamide and aztreonam. This was attributed to the similar methodologies of action, porin transport across the cell membrane, of the antimicrobials.

This study showed that the use of an indirect indicator of bacterial species diversity, such as the Biolog Ecoplates®, could offer an effective means of evaluating biocide efficacy in a cooling water system. In the Eskom environment, it may be preferable over total bacterial counts as carbon substrate utilisation can be correlated to the bacterial species community in the induced stress environment. This could lead to more indicative biocide efficacy monitoring because bacterial plate counts would have shown a decrease with increased biocide dosing but if the implied species diversity does not change we now understand that the biocide is not optimally dosed.

The effectiveness of monitoring metabolic activity rather than bacterial plate counts is enhanced by the fact that metabolic diversity can be related to bacterial persistence in a stressed system either as persister cells or by forming VBNC cells. The shortcoming of using the Biolog Ecoplate® system is the lack in sensitivity to indicate the present of bacterial numbers less than 2 logs. Effective biocide dosing regimens are site specific and therefore the Biology Ecoplate® technique will need to be validated for each site.

7.1 IMPACT ON ESKOM COOLING WATER TREATMENT

The results of this research indicated that the cooling water treatment regimens, currently conducted on the power stations, are not effective or efficient and do not protect staff health in the long term. This is emphasised by the rapid increase in total aerobic bacterial counts 24 – 48 h post biocidal treatment. As the bacterial counts increase to above initial counts in this time, this could lead to a period of fouling risk, in the heat exchangers.

The current, two weekly dosing was proven insufficient and ineffective as the biocides active range is a maximum of 24 h. The need for more frequent biocide dosing and the use of alternate products was emphasised. Although, the minimal biocide dosing poses little risk of biocide resistance development, due to the infrequency of dosing, an increase in regularity of biocide dosing may increase this risk. The efficacy of a biocide dosing regime is site specific

and the proposed Biolog Ecoplate® technique will need to be validated for each site and system.

The results generated by this study highlight that it is essential to follow supplier's directions for optimal biocide dosing concentrations and regularity. Failure to do so will cause inefficient biocide dosage.

In addition, the latest South African *Legionella* Management Standards (SANS 892-1 and -2, 2013) stipulate that biocide efficacy must be proven for the entire cooling water treatment regime. This means that the power stations will have to reconsider the entire cooling water treatment strategy and new monitoring intervals and protocols developed.

This study confirms that more work needs to be conducted on each power plant to develop a base line of their substrate utilisation with and without dosing and at optimal dosing concentrations. In addition monitoring will need to be conducted more regularly than once, 24 h post dosing.

7.2 PROPOSED FUTURE RESEARCH

1. The effect of other commercially available biocides must be evaluated. This includes oxidising and non-oxidising biocides.
2. The effectiveness of the technology must be evaluated on an operational plant where other environmental and system parameters may affect the results.
3. The effect of biocide dosage will be site and biocide specific. Initial optimisation tests will be required for each site.
4. The possible development of VBNC or persister cells, post biocide dosing, must be investigated. This may explain how bacterial communities are able to regenerate as rapidly as they do and why molecular and culture techniques show differing results.
5. Metabolic changes, identified on the Biolog PM plates, should be investigated to determine how bacterial metabolism changes in resistant communities. This may offer a means to control resistant communities.

Appendices

Appendix A

Binary representation of Biolog Ecoplate® substrate utilisation over the duration of the 20 mg/L DBNPA trial period.

		D20 0	D20 15m	D20 30m	D20 1	D20 2	D20 3	D20 4	D20 6	D20 24	D20 48
Water	A1	0	0	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	0	0	0	0	0	0	0	0	0	0
D-Galactonic acid Lactone	A3	1	0	0	0	0	0	0	0	0	1
L Arginine	A4	1	0	0	0	0	0	0	0	0	1
Pyruvic acid Methyl Ester	B1	1	0	0	0	0	1	1	1	1	1
D-Xylose	B2	0	0	0	0	0	0	0	0	0	0
D-Galacturonic acid	B3	0	0	0	0	0	0	0	0	0	1
L-Asparagine	B4	0	0	0	0	0	0	0	0	1	1
Tween 40	C1	1	0	0	0	0	0	1	1	1	1
i-Erythritol	C2	0	0	0	0	0	0	0	0	0	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	0	0	0	0	0	0
L-Phenylalanine	C4	0	0	0	0	0	0	0	0	0	0
Tween 80	D1	1	0	0	0	1	1	1	1	1	1
D- Mannitol	D2	0	0	0	0	0	0	0	1	1	0
4-Hydroxy benzoic acid	D3	1	0	0	0	1	0	1	1	1	0

L-Serine	D4	0	0	0	0	0	0	0	0	0	0
α-Cyclodextrin	E1	1	0	0	0	0	0	1	1	1	1
N-Acetyl-D-Glucosamine	E2	1	0	0	0	0	0	0	0	0	0
γ-Hydroxybutyric acid	E3	0	0	0	0	0	0	0	0	0	0
L-Threonine	E4	0	0	0	0	0	0	0	0	0	0
Glycogen	F1	1	0	0	0	0	0	1	1	1	1
D-Glucosaminic acid	F2	0	0	0	0	0	0	0	0	0	0
Itaconic acid	F3	0	0	0	0	0	0	0	0	1	1
Glycyl-L-glutamic acid	F4	0	0	0	0	0	0	0	0	0	0
D-Cellobiose	G1	0	0	0	0	0	0	0	0	0	0
Glucose-1-phosphate	G2	0	0	0	0	0	0	0	0	0	0
α-Ketobutyric acid	G3	0	0	0	0	0	0	0	0	0	0
Phenyl ethylamine	G4	0	0	0	0	0	0	0	0	0	0
α-D-Lactose	H1	0	0	0	0	0	0	0	0	0	0
D, L - α-Glycerol Phosphate	H2	0	0	0	0	0	0	0	0	0	0
D-Malic acid	H3	1	0	0	0	0	0	0	1	1	1
Putrescine	H4	1	0	0	0	0	0	0	0	1	1

Binary representation of Biolog Ecoplate® substrate utilisation over the duration of the 8 mg/L DBNPA trial.

		D8 0	D8 15m	D8 30m	D8 1	D8 2	D8 3	D8 4	D8 6	D8 24	D8 48
Water	A1	0	0	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	1	1	1	1	1	1	1	1	1	1
D-Galactonic acid Lactone	A3	1	0	1	0	1	0	1	0	1	1
L Arginine	A4	1	1	1	0	1	1	1	1	1	0
Pyruvic acid Methyl Ester	B1	1	1	1	1	1	1	1	1	1	1
D-Xylose	B2	0	0	0	0	1	0	0	0	0	0
D-Galacturonic acid	B3	0	1	1	1	1	0	0	1	0	0
L-Asparagine	B4	1	1	1	1	1	1	1	1	1	1
Tween 40	C1	1	1	1	1	1	1	1	1	1	1
i-Erythritol	C2	1	1	1	0	1	1	0	1	1	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	1	1	0	1	0	0
L-Phenylalanine	C4	1	1	1	1	1	1	1	1	1	1
Tween 80	D1	1	1	1	1	1	1	1	1	1	1
D- Mannitol	D2	1	1	1	1	1	1	0	1	1	1
4-Hydroxy benzoic acid	D3	1	0	1	1	1	1	1	1	1	0
L-Serine	D4	1	0	1	0	1	1	0	1	0	1

α-Cyclodextrin	E1	1	1	1	1	1	1	1	1	1	1
N-Acetyl-D-Glucosamine	E2	1	1	1	1	1	0	0	0	1	1
γ-Hydroxybutyric acid	E3	0	0	0	0	0	1	0	0	1	0
L-Threonine	E4	0	1	0	0	0	0	0	1	0	0
Glycogen	F1	1	1	1	1	1	1	1	1	1	1
D-Glucosaminic acid	F2	1	0	0	0	0	0	0	0	0	0
Itaconic acid	F3	1	1	1	1	1	1	1	1	1	1
Glycyl-L-glutamic acid	F4	1	0	0	0	0	1	1	0	0	0
D-Cellobiose	G1	1	0	0	0	1	1	0	0	0	0
Glucose-1-phosphate	G2	0	0	0	0	1	1	0	0	0	0
α-Ketobutyric acid	G3	1	0	0	0	1	1	1	1	0	1
Phenyl ethylamine	G4	1	1	1	0	1	1	1	1	1	0
α-D-Lactose	H1	0	0	0	1	1	0	0	1	0	0
D, L - α-Glycerol Phosphate	H2	1	0	0	0	0	1	0	0	1	0
D-Malic acid	H3	1	1	1	1	1	1	1	1	1	0
Putrescine	H4	1	1	1	1	1	1	1	1	1	0

Appendix B

Binary representation of Biolog Ecoplate® substrate utilisation over the duration of the 8mg/L isothiazolone trial period.

		I8 0	I8 1	I8 2	I8 4	I8 6	I8 24	I8 48	I8 72
Water	A1	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	1	1	1	0	1	1	1	1
D-Galactonic acid Lactone	A3	1	0	1	0	0	0	0	0
L Arginine	A4	1	1	1	1	1	1	1	1
Pyruvic acid Methyl Ester	B1	1	1	1	1	1	1	1	1
D-Xylose	B2	0	0	0	0	0	0	0	0
D-Galacturonic acid	B3	1	0	0	1	0	1	0	1
L-Asparagine	B4	1	1	1	1	1	1	1	1
Tween 40	C1	1	1	1	1	1	1	1	1
i-Erythritol	C2	0	0	0	0	0	0	0	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	0	0	0	0
L-Phenylalanine	C4	0	0	0	0	0	0	0	0
Tween 80	D1	1	1	1	1	1	1	1	1
D- Mannitol	D2	1	1	1	1	1	1	1	1
4-Hydroxy benzoic acid	D3	1	0	1	1	1	0	1	0
L-Serine	D4	1	1	1	1	1	1	1	1
α-Cyclodextrin	E1	0	0	0	0	1	0	0	0

N-Acetyl-D-Glucosamine	E2	1	1	1	1	1	1	1	1
γ-Hydroxybutyric acid	E3	0	0	0	0	0	0	0	0
L-Threonine	E4	1	1	1	1	1	1	0	1
Glycogen	F1	1	1	1	1	1	1	1	1
D-Glucosaminic acid	F2	0	0	0	0	0	0	0	0
Itaconic acid	F3	0	1	0	0	1	1	1	1
Glycyl-L-glutamic acid	F4	0	0	0	0	0	0	0	0
D-Cellobiose	G1	1	0	0	0	0	1	0	0
Glucose-1-phosphate	G2	1	0	0	0	0	0	0	0
α-Ketobutyric acid	G3	1	1	1	1	1	1	1	1
Phenyl ethylamine	G4	0	0	0	0	0	0	0	0
α-D-Lactose	H1	0	0	0	0	0	0	0	0
D, L - α-Glycerol Phosphate	H2	1	0	0	0	0	1	1	1
D-Malic acid	H3	0	0	0	0	0	1	0	0
Putrescine	H4	1	0	0	1	1	1	1	1

Binary representation of Biolog Ecoplate® substrate utilisation over the duration of the 20mg/L isothiazolone trial period.

		I20 0	I20 1	I20 2	I20 4	I20 6	I20 24	I20 48	I20 72
Water	A1	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	0	0	0	0	0	0	0	0
D-Galactonic acid Lactone	A3	0	0	0	0	0	0	0	1
L Arginine	A4	1	1	1	1	1	0	0	0
Pyruvic acid Methyl Ester	B1	0	1	1	1	1	0	1	1
D-Xylose	B2	0	0	0	0	0	0	0	0
D-Galacturonic acid	B3	1	1	1	1	0	0	1	0
L-Asparagine	B4	1	1	1	1	1	1	1	1
Tween 40	C1	1	1	1	1	0	0	1	1
i-Erythritol	C2	0	0	1	0	0	0	0	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	0	0	0	0
L-Phenylalanine	C4	0	0	0	1	0	0	0	0
Tween 80	D1	1	1	1	1	1	1	1	1
D- Mannitol	D2	0	0	1	0	1	0	0	0
4-Hydroxy benzoic acid	D3	1	1	0	1	1	0	0	0
L-Serine	D4	1	1	1	1	0	0	0	0
α-Cyclodextrin	E1	0	0	0	0	0	0	0	0
N-Acetyl-D-Glucosamine	E2	0	0	0	0	0	0	0	0
γ-Hydroxybutyric acid	E3	0	0	0	1	1	0	1	0

L-Threonine	E4	1	1	1	1	1	0	0	1
Glycogen	F1	0	0	1	1	1	0	1	1
D-Glucosaminic acid	F2	0	0	1	1	1	0	0	0
Itaconic acid	F3	1	1	1	0	0	0	0	1
Glycyl-L-glutamic acid	F4	0	0	1	1	1	0	1	0
D-Cellobiose	G1	1	1	1	1	0	0	0	0
Glucose-1-phosphate	G2	0	1	0	1	1	0	0	0
α-Ketobutyric acid	G3	0	0	0	0	0	0	0	0
Phenyl ethylamine	G4	0	0	1	0	0	0	0	0
α-D-Lactose	H1	0	1	1	1	1	0	0	0
D, L - α-Glycerol Phosphate	H2	1	1	1	1	1	0	0	0
D-Malic acid	H3	1	1	1	1	1	0	0	0
Putrescine	H4	0	1	1	1	0	0	1	0

Binary representation of Biolog Ecoplate® substrate utilisation over the duration of the 50mg/L isothiazolone trial period.

		I50 0	I50 1	I50 2	I50 4	I50p 6	I50 24	I50 48	I50 72
Water	A1	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	0	0	0	0	0	0	0	0
D-Galactonic acid Lactone	A3	0	0	0	0	0	0	1	0
L Arginine	A4	1	0	0	0	0	0	1	1
Pyruvic acid Methyl Ester	B1	1	0	0	0	0	0	1	1
D-Xylose	B2	0	0	0	0	0	0	0	0
D-Galacturonic acid	B3	0	0	0	0	0	0	1	1
L-Asparagine	B4	1	1	0	0	0	0	1	1
Tween 40	C1	1	0	0	0	1	1	1	1
i-Erythritol	C2	0	0	0	0	0	0	0	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	0	0	0	0
L-Phenylalanine	C4	0	0	0	0	0	0	0	0
Tween 80	D1	1	0	0	0	0	1	1	1
D- Mannitol	D2	0	0	0	0	1	0	1	1
4-Hydroxy benzoic acid	D3	0	0	1	0	0	0	0	0
L-Serine	D4	0	0	0	0	0	0	0	0
α-Cyclodextrin	E1	0	0	0	0	0	0	0	0
N-Acetyl-D-Glucosamine	E2	0	0	0	0	0	0	0	0
γ-Hydroxybutyric acid	E3	1	0	0	0	0	0	0	0

L-Threonine	E4	0	0	0	0	0	0	0	0
Glycogen	F1	0	0	0	0	0	0	0	1
D-Glucosaminic acid	F2	0	0	0	0	0	0	1	0
Itaconic acid	F3	0	0	0	0	0	0	1	0
Glycyl-L-glutamic acid	F4	0	0	0	0	0	0	0	0
D-Cellobiose	G1	0	0	0	0	0	0	0	0
Glucose-1-phosphate	G2	0	0	0	0	0	0	0	0
α-Ketobutyric acid	G3	0	0	0	0	0	0	0	0
Phenyl ethylamine	G4	0	0	0	0	0	0	0	0
α-D-Lactose	H1	0	0	0	0	0	0	0	0
D, L - α-Glycerol Phosphate	H2	0	0	0	0	0	0	0	0
D-Malic acid	H3	0	0	0	0	0	0	0	0
Putrescine	H4	0	0	0	0	0	1	1	1

Appendix C

Binary representation of Biolog Ecoplate® substrate utilisation over the duration of the isothiazolone resistance trial period.

		IR 10	IR 16	IR 30	IR36	IR 50	IR56	IR80	IR86	IR 90	IR 91	IR 92	IR 94	IR 96	IR 924	IR 948	IR 972
Water	A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
D-Galactonic acid Lactone	A3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L Arginine	A4	0	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1
Pyruvic acid Methyl Ester	B1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D-Xylose	B2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Galacturonic acid	B3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
L-Asparagine	B4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Tween 40	C1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
i-Erythritol	C2	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-Phenylalanine	C4	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
Tween 80	D1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
D- Mannitol	D2	1	0	1	0	1	1	0	0	1	1	1	1	1	0	0	0
4-Hydroxy benzoic acid	D3	0	0	0	0	1	1	0	0	1	1	1	1	0	1	1	0
L-Serine	D4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

α-Cyclodextrin	E1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-Acetyl-D-Glucosamine	E2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
γ-Hydroxybutyric acid	E3	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	1
L-Threonine	E4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glycogen	F1	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0
D-Glucosaminic acid	F2	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Itaconic acid	F3	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Glycyl-L-glutamic acid	F4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Cellobiose	G1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Glucose-1-phosphate	G2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
α-Ketobutyric acid	G3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phenyl ethylamine	G4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-D-Lactose	H1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
D, L - α-Glycerol Phosphate	H2	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0
D-Malic acid	H3	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0
Putrescine	H4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Binary representation of Biolog Ecoplate® substrate utilisation for the duration of the isothiazolone resistance with alternate DBNPA trial.

		IR2_10p	IR2_16p	IR2_30p	IR2_36p	IR2_50p	IR2_56p	IR2_80p	IR2_86p	IR2_90p	IR2_91p	IR2_92p	IR2_94p	IR2_96p	IR2_924p	IR2_948p	IR2_120p	IR2_1215mp	IR2_1230mp	IR2_121p	IR2_122p	IR2_123p	IR2_124p	IR2_126p	IR2_1224p	IR2_1248p
Water	A1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B-methyl-D-Glucoside	A2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Galactonic acid Lactone	A3	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	0	0	1	1
L Arginine	A4	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	1
Pyruvic acid Methyl Ester	B1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	1
D-Xylose	B2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Galacturonic acid	B3	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1
L-Asparagine	B4	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	1	0	0	0	0	0	0	0	1	1
Tween 40	C1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1
i-Erythritol	C2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2-Hydroxy Benzoic Acid	C3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-Phenylalanine	C4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Tween 80	D1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	0	1	0	0	0	0	0	0	0	1	1

D- Mannitol	D2	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1
4-Hydroxy benzoic acid	D3	1	1	1	0	0	1	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1
L-Serine	D4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	1
α-Cyclodextrin	E1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
N-Acetyl-D-Glucosamine	E2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
γ-Hydroxybutyric acid	E3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
L-Threonine	E4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Glycogen	F1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
D-Glucosaminic acid	F2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Itaconic acid	F3	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Glycyl-L-glutamic acid	F4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
D-Cellobiose	G1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glucose-1-phosphate	G2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-Ketobutyric acid	G3	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Phenyl ethylamine	G4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
α-D-Lactose	H1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D, L - α-Glycerol	H2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Phosphate																									
D-Malic acid	H3	1	1	0	1	0	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1
Putrescine	H4	1	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1