APPROACHES TO MONITOR AND CHARACTERIZE THE BIOLOGICAL STABILITY OF DRINKING WATER DISTRIBUTION NETWORKS

Volume I: Establishing Correlations between Flow Cytometry Cell Concentrations, Heterotrophic Plate Counts and Water Quality Data

Report to the
Water Research Commission

by

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This report forms part of a set of three reports. The other reports are:

Approaches to Monitor and Characterize the Biological Stability of Drinking Water Distribution Networks. ***Volume II****: Characterising the Composition of Microbial Communities and Correlations with Baseline FCM and HPC Cell Concentrations* (WRC Report No. 2884/2/23), and

Approaches To Monitor And Characterize The Biological Stability Of Drinking Water Distribution Networks. ***Volume III****: Strategy for the Use of Flow Cytometry for Drinking Water Monitoring in South Africa* (WRC Report No. 2884/3/23).

EXECUTIVE SUMMARY

BACKGROUND

Water utilities use various treatment strategies to ensure that the production of water will not pose a significant health risk to consumers. Water leaving the treatment plant is typically of high quality but conditions within the distribution system can lead to the deterioration of the microbial water quality. Maintaining the biological stability of drinking water is therefore one of the major challenges facing water utilities and local authorities in their endeavours to supply safe drinking water to communities. Where the original focus was on predicting potential changes by controlling parameters such as assimilable organic carbon (AOC) and disinfection residuals, the focus has shifted on the direct assessment of changes in the microbial community within the distribution network using methods such as 16S community profiling, flow cytometry (FCM) and ATP measurements.

Most water utilities in South Africa use heterotrophic plate counts (HPC) to monitor the general microbial quality of treated drinking water and to assess the biostability within the distribution network. The superiority of FCM over HPC has been demonstrated in numerous studies. These studies have shown that FCM is fast (results within 15 min), accurate and reproducible and can even be automated. It has been shown to be the most promising method for the direct assessment of changes in microbial communities in drinking water networks.

RATIONALE

The major difficulty when implementing a direct assessment approach, such as FCM, within the water distribution environment is that there are no clear guidelines as to what constitutes a significant or relevant change in the microbial community. FCM counts have been shown to vary between different systems (chlorinated and chloraminated) and deviations or abnormal changes could only be detected once a proper baseline for both ICC and TCC values had been established for each system. During the study we therefore endeavoured to addressed the following question:

- What are the baseline flow cytometry TCC and ICC values (cell concentrations) for water samples collected from distribution and reticulation networks and how do they correspond with the HPC data (YEA and R2A) for the same samples?

OBJECTIVES AND AIMS

The objective of the project was to provide the necessary foundation for the development of a strategy for the drinking water industry to incorporate FCM when monitoring and managing the biostability of drinking water during distribution as this is a more sensitive and rapid method compared to the HPC currently used.

The project aims addressed in this report were as follows:

1. Adapt the current FCM methodologies for the analysis of disinfected samples with low bacteria levels.
2. Create a baseline FCM (TCC and ICC values), HPC (YEA and R2A) and 16S community profile databases for water samples from chloraminated distribution and reticulation networks.

APPROACH

The main focus of the first part of the project was to investigate the value of flow cytometry as a process indicator when managing water distribution networks. Samples were collected from the a large distribution network at six different sampling locations on a bi-weekly basis over a period of 8 months. For reticulation samples (point of use), water was collected from different residential locations in Tshwane district. Six points were sampled on a bi-weekly basis over the same period of 8 months. Heterotrophic plate counts were performed using yeast extract and Reasoner's 2 agar (R2A) following standard protocols. Flow cytometry

concentrations were determined using SYBR Green I and propidium iodide stains to obtain total and intact cell concentrations. The pH and chlorine concentration of the samples were also determined.

RESULTS AND DICUSSION

Investigating the value of flow cytometry as a process indicator when managing water distribution networks

To address the first two aims of the project, multiple trial runs were conducted to determine the best concentrations and volumes of stains as well as the gating to be used for the FCM analysis. The controls to be included were also confirmed and a final procedure was established which was used throughout the study. Overall, the results showed no strong correlations between the ICC and HPC or chlorine levels. All the points had generally higher R2A concentrations as expected and FCM concentrations were almost 10 times higher than any of the plate counts observed. The results were consistent with what was seen in other studies with weak correlations between the two approaches. HPCs only detect a fraction of the bacteria present in the sample as not all bacteria grow on these general media.

In summary, the flow cytometry results confirmed the presence of a large number of bacteria in the samples. The numbers ranged between $5x10^2$ cells/ml for the production sample to 10^5 cells/ml for some of the point of use samples. It showed that FCM could be used as a reliable process indicator to provide additional information at each sample point but that the results were site specific. FCM could also rapidly detect significant changes or clear trends linked to these communities.

CONCLUSIONS

During this project we demonstrated the use of various analytical tools to study and ultimately manage the biostability of drinking water in distribution and reticulation systems. The analytical methods included culturing (HPC), an approach which has been used by the industry over many years as well as more recent tools such as flow cytometry as an additional process indicator. This project clearly demonstrated that these newer technologies have developed to such a level that they can now easily be incorporated into microbial drinking water quality studies.

RECOMMENDATIONS

Interpretation and integration of the various sets of information and how to apply it when managing large networks remain the main challenge. Implementation of these analyses for routine purposes within the industry should only be considered after a careful cost benefit analysis. The main cost associated with these analyses is not necessarily linked to the direct costs of the analyses or the required infrastructure but often lies with the human resources component. This type of data interpretation requires a highly skilled team of scientists with a detailed understanding of the system, its associated microbiology as well as bioinformatic analyses.

In line with the aims and objectives of the study, the next investigation entails characterising the microbial community of the samples from both sites using 16S profiling to determine how it corresponds to the bacteria isolated using the HPC approach. This portion of work is presented in **Volume II** of this project report.

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ACRONYMS & ABBREVIATIONS

AOC	Assimilable organic carbon
ATP	Adenosine triphosphate
DWDS	Drinking water distribution system
DWTP	Drinking water treatment plant
FCM	Flow cytometry
HPC	Heterotrophic plate counts
ICC	Intact cell counts
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PI	Propidium iodide
SANS	South African National Standard
SG	SYBR Green
SLMB	Schweizer Lebensmittelbuch (Swiss Food Book)
TCC	Total cell counts
WHO	World Health Organisation

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Water utilities use various treatment strategies to ensure that the production of water will not pose a significant health risk to consumers. Water leaving the treatment plant is typically of high quality but conditions within the distribution system can lead to the deterioration of the microbial water quality (Lui *et al*., 2013). Maintaining the biological stability of drinking water is therefore one of the major challenges facing water utilities and local authorities in their endeavours to supply safe drinking water to communities. This challenge is even intensified under conditions of water use restrictions, which is characterised by decreased flows and pressure in the distribution network resulting in stagnation in parts of the system.

There are currently a number of definitions used to define the biological stability of drinking water (Prest *et al*., 2016). The original definition (Rittmann and Snoeyink, 1984) focused primarily on the impact of specific water quality parameters (nutrients for growth support and disinfection residuals) after final treatment. The definition has recently been broadened to focus on the relevant changes within the distribution network: “Biological stability would imply no changes in the concentration and composition of the microbial community in the water during distribution” (Lautenschlager *et al*., 2013). Where the original focus was on predicting potential changes by controlling parameters such as assimilable organic carbon (AOC) and disinfection residuals, the focus has shifted on the direct assessment of changes in the microbial community within the distribution network using methods such as 16S community profiling, flow cytometry (FCM) and ATP measurements (Prest *et al*., 2016).

Most water utilities in South Africa use heterotrophic plate counts (HPC), as captured in SANS 241 (SABS, 2015), to monitor the general microbial quality of treated drinking water and to detect changes within the distribution network. Apart from the fact that results are only available after a minimum of two days, the relevance of HPC has also been questioned (Hammes *et al*., 2008; Gillespie *et al*., 2014) as it only detects a small fraction of the microbial community (i.e. considerably less than 1% of the total community), depending on the culture media and incubation time. Furthermore, HPC does not provide any information on the abundance or actual composition of the bacterial community (van Nevel *et al*., 2017).

Since its first application to detect active bacterial communities in drinking water in 2003 (Hoefel *et al*., 2003), the superiority of FCM over HPC has been demonstrated in numerous studies (van Nevel *et al*., 2017). These studies have shown that FCM is fast (results within 15 min), accurate and reproducible and can even be automated (Besmer *et al*., 2014). As a result, the detection of total cell counts (TCC) has been incorporated as a recommended test method for testing drinking water in Switzerland (SLMB, 2012). Due to the sensitivity of the method and its ability to provide data on cell viability (based on intact cell counts (ICC)), FCM has also been shown to be the most promising method for the direct assessment of changes in microbial communities in drinking water networks.

The major difficulty when implementing a direct assessment approach, such as FCM, within the water distribution environment is that there are no clear guidelines as to what constitutes a significant or relevant change in the microbial community. According to a study by Gillespie *et al*. (2014) FCM counts varied between different systems (chlorinated and chloraminated) and deviations or abnormal changes could only be detected once a proper baseline for both ICC and TCC values had been established for each system. This was supported by van Nevel *et al*. (2017) who suggested that implementation of FCM would require the parallel measurement of FCM and HPC to build a solid databased for the confident interpretation of the FCM results.

Within drinking water distribution networks, community reservoirs have been identified as one of the areas where bacterial regrowth can take place. Stagnation within these reservoirs could occur due to long residence

times, sub-optimal flow dynamics and intermittent water demand. Stagnation is often associated with disinfectant decay (Ling *et al*., 2018) resulting in an increase in bacterial numbers. During a recent WRC study (K5/2469) it was shown that chemoautotrophic bacteria such as *Nitrosomonas* dominated the distribution network. The impact of autotrophic bacteria on the overall deterioration of the microbial quality, especially in these reservoirs, therefore needs to be assessed as they would not be detected then testing for heterotrophic bacteria. Understanding the microbial ecology of these systems will assist in the development of guidelines for the operation of these systems, specifically during the warmers summer months or during periods of water restrictions when a rapid deterioration of the microbial quality could put entire communities at risk.

During the study we addressed the following questions:

- What are the baseline flow cytometry TCC and ICC values (cell concentrations) for water samples collected from distribution and reticulation networks and how do they correspond with the HPC data (YEA and R2A) for the same samples? (**current report – Volume I**)
- What is the composition of the microbial community (based on 16S profiling) of these samples and how does it correspond to the bacteria isolated using the HPC approach? What are the main functions associated with communities that are deviating from the baseline FCM values as determined using a metagenomic approach? What impact does retention time (e.g. flow restrictions) in community reservoirs have on disinfectant residuals, FCM values as well as the community composition? What are the contributions of autotrophic bacteria to biological instability in the distribution system (as measured in reservoirs) and what functional role do they play in this ecosystem? (**Volume II)**
- How can the current FCM methodologies be adapted for disinfected drinking water samples with low bacterial numbers? (**Volume III)**

1.2 MAINTAINING THE BIOLOGICAL STABILITY OF DRINKING WATER IN DISTRIBUTION SYSTEMS

1.2.1 Biological stability

Biological stability has many definitions but is generally referred to as the stability of the microbial population within drinking water from the treatment plant to consumer's taps, where the microbial community's composition and concentration does not change drastically throughout the system over time (Hammes *et al*., 2010; Lautenschlager *et al*., 2013; Nescerecka *et al*., 2014; Prest *et al*., 2016;). The changes in the stability are often the result of various interactions within the microbial community due to environmental factors, nutrient availability (levels of organic and inorganic nutrients) and disinfectant residual concentrations and the presence thereof (Hammes *et al*., 2010; Prest *et al*., 2016). The unpredictability of these events makes it difficult for water utilities to manage the microbial growth (Berry *et al*., 2006).

Maintenance of the biological stability within drinking water is one of the major challenges faced by local municipalities and water utilities because not all factors favouring its constancy can be directly controlled (Nescerecka *et al*., 2014; 2018). Prest *et al*. (2016) suggested that in order to achieve biological stability and provide a high quality of drinking water to consumers, the changes in the bacterial community should be limited as uncontrolled growth leads to high bacterial numbers and the presence of undesirable microorganisms such as pathogens (Prest *et al*., 2016). The limitation of available nutrients and/or maintenance of residual disinfectant concentrations are commonly used to maintain biological stability in most distribution systems (Nescerecka *et al*., 2014).

1.2.2 Factors influencing microbial growth in distribution systems

Growth in the distribution system is determined by general water quality, biological factors and distribution practises (Berger *et al*., 2015; Lu *et al*, 2013; WHO, 2011). The re-growth of microorganisms in DWDSs

(Drinking Water Distribution Systems) largely depends on the available carbon sources and nutrients required for growth as well as the presence and concentration of disinfectant residuals (Berry *et al*., 2006). Higher concentrations of disinfectant residuals typically limit microbial growth within the DWDS and microbial growth therefore has a negative relationship with the concentrations of disinfectant residual (Chowdhury, 2012; Medema *et al*., 2012). Although most methods of disinfection are effective, it is important to keep in mind that some bacteria are quite versatile and can persist in the system even after disinfection (El-Chakhtoura *et al*., 2015), thereby affecting the water quality as well as causing undesirable effects downstream in the DWDS. The issue however may be the loss of disinfectant residuals downstream as loose deposits from biofilms may increase the persistence of bacteria in the system (Nescerecka *et al*., 2014; Prest *et al*., 2016). In most cases, the presence of bacteria in the system does not indicate a health risk as many bacterial species are ubiquitous microorganisms which naturally occur in water (Hanson and Hanson, 1996; Emtiazi *et al*., 2004). The maintenance of disinfection residuals is one of the strategies used within the DWDS is however important in controlling the abundance and occurrence of microorganisms throughout the system as well as inactivating potential pathogens (Lu *et al*., 2013).

Pathogens which form part of the microbial community pose a risk to health and safety of consumers when proper distribution practices are neglected. Pathogenic bacteria form part of the microbial community as one of two types, namely: waterborne or water-based bacterial pathogens. These pathogens such as *Legionella pneumophila* and *Pseudomonas aeruginosa* (van der Wielen and van der Kooij, 2013; King *et al*., 2016) which may potentially occur in DWDSs persist after disinfection and often have different sources of infection and transmission as well as the ability to cause disease through several means. Waterborne pathogens such as *Salmonella* (Mara and Horan, 2003) are typically introduced into the system through contamination events which may occur along the system pipeline. Alternatively, water-based pathogens such as *Legionella pneumophila* (Al-Bahrya *et al*., 2013) usually form part of the aquatic systems microbial community and are well adapted to the conditions within the DWDS (Ashbolt, 2015).

Microbial communities, regardless of the environment, have the tendency to change as a result of external factors, which influence the preferred growth of some microorganisms by giving them a competitive advantage. The unique conditions within DWDSs shape the bacterial community present with regard to its abundance, composition and viability (Mara and Horan, 2003). The bacterial community structure and diversity is influenced by different factors such as the pipe materials used to construct the DWDS, distribution system age and biofilm age in addition to abiotic factors which cannot be directly controlled (Liu *et al*., 2013). Abiotic factors which directly influence bacterial growth in the DWDS include the pH and temperature which influence the preferred growth of some bacteria within the drinking water environment (Lu *et al*., 2013).

1.2.2.1 DWDS Infrastructure

DWDSs consist of a network of pipes constructed from various materials that differ in diameter, age and pipe wall thickness. These pipelines have the function of connecting disinfection booster stations to reservoirs and to the reticulation system which includes building plumbing (Codony *et al*., 2003). The pipe component of the system is constructed from different materials which have the ability to impact the growth of bacteria and other microorganisms in the system. Materials such as copper, iron, epoxy and cement are used to construct or line pipes within various parts of distribution systems. The composition of pipe materials in a distribution system was shown to influence the type of chemicals introduced into the distribution system through processes such as microbial mediated corrosion which additionally influences the biofilm community that occurs within the system (Ingerson-Mahar and Reid, 2013, Douterelo *et al*., 2016; Aggarwal *et al*., 2018). The pipe diameter also affects the biofilm community as the large pipes which form part of the distribution system feed into the smaller pipes in the reticulation system which have a smaller diameter and surface area to volume ratio that promotes biofilm development (Bedard *et al*., 2018).

Studies done by Liu and colleagues on the microbial richness and diversity of the system show that the community is influenced by the materials used to construct pipes in the system (Liu *et al*., 2013). For example, the use of iron to construct pipes provides a suitable environment for the proliferation of iron-oxidizing

bacteria, which form part of biofilms. Prest *et al*. (2016) stated that pipes constructed from PVC and polyethylene-based materials lead to the release of biodegradable organic substances that provide additional nutrient sources for microorganisms to utilize leading to biological instability in the microbial community and proliferation of microbes in the system. Additionally, the presence of nitrifying bacteria was shown to be more commonly associated with pipes constructed from iron, lead and polyethylene materials whereas the copper pipes made it challenging for any bacterial survival (Zhu *et al*., 2014).

1.2.2.2 Nutrients

The availability of nutrients for the growth and survival of microorganisms are limited in DWDSs. The distribution system conditions involve low-oxygen levels, fluctuating temperatures and disinfectant residuals, varying water pressure as well as biogeochemical cycles and interactions between microbes and their environment which play an important part in influencing the nutrient availability for growth (Lu *et al*., 2013; Luo *et al*., 2013). Microorganisms, nevertheless, are versatile and proliferate in any environment considered suitable to their lifestyle if the required nutrients are available.

Crucial nutrients for bacterial growth were recorded as carbon, nitrogen and phosphorus in a 100:10:1 ratio where these nutrients arise from various sources including pipe deposits, dead bacterial cells, mixed carbon sources, lubricating oils, etc. (Chowdhury, 2012). Nutrients present in the distribution system are present as either organic or inorganic compounds. The organic nutrients are those that are mostly obtained from other microorganisms (Nescerecka *et al*., 2018), which are either dead or preyed upon in the system. The nutrients from bacterial sediment consist of carbohydrates, amino acids, fats and vitamins. Organic nutrients contain carbon as a basic component and include assimilable and biodegradable organic carbon. Assimilable organic carbon (AOC) is shown to stimulate the growth of the planktonic microbial community in bulk water as well as the microbial community in biofilm microenvironments and is measured by quantifying selected bacteria in the system (Prest *et al*., 2016). The fraction of dissolved organic carbon (DOC) is measured by the reduction of DOC, which is caused by bacteria naturally growing in the system. A positive relationship between bacterial growth and DOC is usually observed (Liu *et al*., 2013). The inorganic nutrients present in some distribution system are limited, these nutrients include minerals which occur in drinking water as a result of pipe leaching (Cu), natural occurrence (e.g. Ca, Mg, Zn) or intentional addition by water utilities (WHO, 2005, Douterelo *et al*., 2018).

1.2.2.3 Disinfectant residuals

Water utilities often make use of disinfection strategies in order to maintain biological stability within DWDSs and by doing so, the abundance of viable microorganisms is initially reduced. The different treatment strategies used include primary and secondary treatment (i.e. chlorine and chloramine, respectively). Chlorine is widely used as a primary disinfectant and initially has a high reactivity but the residuals are not maintained throughout the system due to increased disinfection decay. Therefore, chloramine is used as a secondary disinfectant and has the same oxidative power as chlorine but less reactivity and residuals are maintained for longer periods in the system (Lu *et al*., 2013; Zhu *et al*., 2014; Prest *et al*., 2016).

The disinfectant residuals in the system are subjected to decay by other external factors in addition to microorganisms in the system through various metabolic processes. Chloramine decay is accelerated by the oxidation of ammonia (introduced in the system through chloramination process) to nitrite which is used in microbial nitrogen metabolism (Mara and Horan, 2003; Zhu *et al*., 2014; Pinto *et al*., 2016). Temperature also plays a role in disinfectant decay by influencing the growth of bacteria and organic carbon concentrations when water is warmer. The result of the latter is low residual concentrations of disinfectants in the system when temperatures are higher resulting in the proliferation of the microbial community leading to biological instability (Prest *et al*., 2016).

Methods of drinking water treatment vary worldwide as the disinfectants used are influenced by the type and quality of source water used (Bitton, 2014; Okaiyeto *et al*., 2016). The treatment processes which make use of

these disinfectants are also important to note. Examples of different types of treatment and disinfection strategies include physical, biological and chemical process namely: coagulation, flocculation and sedimentation which are used in combination with sand filtration to remove large particles; ozonation as well as oxidation are some of the chemical processes involved (Francisque *et al*., 2009; Prest *et al*., 2016). Domingo *et al.* (2015) stated that "Ozonation not only disinfects the water but partly degrades organic contaminants so they are more readily utilized by heterotrophic bacteria in the filter." European countries are known to treat water with ozone prior to granular activated carbon filtration (Zeng *et al*., 2013; Prest *et al*., 2014).

1.2.2.4 Temperature

The optimum temperature at which microorganisms grow drives competition as all bacteria proliferate at their individual optimum temperatures. The resulting temporal changes of drinking water may allow some microbes to out-compete others within the DWDS (Prest *et al*., 2016). The presence of high abundances of indicator coliform bacteria in the community was shown to be the result of an increase in water temperature (Prest *et al*., 2016). The seasonal temperature variations can therefore create problems as a result of the increased microbial growth. This may influence the microbial ecology of the DWDS more dramatically in warmer seasons where more indicator bacteria may be present (Zhang, 2002; Prest *et al*., 2016).

1.2.2.5 pH

According to the guidelines for drinking-water quality by the World Health Organization, the pH of water in the distribution system is controlled by an equilibrium system involving carbon dioxide, bicarbonate and carbonate. Drinking water pH typically falls within the range of 6.5-8.5 (WHO, 2004; McDade, 2008). The pH is sometimes inversely influenced by temperature where a rise in temperature may result in a decreased pH and affecting the microbial community in the system significantly (McDade, 2008). Machado and Bordalo (2014) mentioned that pH together with other external factors in the system as well as the infiltration and percolation of surface water appear to be some of the driving factors which ultimately shape and select the bacterial community and consequently the deterioration of water quality (Machado and Bordalo, 2014).

1.2.3 Management of stability in drinking water distribution systems

The safety of drinking water is essential for human health and water utilities must therefore take the necessary precautions to ensure the highest quality (WHO, 2011; Gillespie *et al*., 2014, Ma *et al.*, 2015). Drinking water is subjected to various treatments from source to tap in order to improve and maintain its safety and quality. The quality of the source water, treatment methods as well as distribution practices all determine the final quality of the water. The chemical and physical properties of drinking water are affected by aspects such as the quality of the system infrastructure as well as the chemicals used in the system during treatment and disinfection. These chemical and physical properties also impact the biological quality of the drinking water by selecting for different types of microorganisms to flourish within the distribution system environment (Mara and Horan, 2003; Lu *et al*, 2013; WHO, 2011).

The main focus of water utilities when managing the microbial quality in the distribution systems is to control the microbial growth as well as to detect the presence of potential pathogens which may persist in the system and could pose a health risk. Indicator organisms are used to help detect any increased faecal matter in a system (WHO, 2011). The presence of these bacterial pathogens increases sporadically within the system when the conditions are favourable and as a result the threat they pose to public health is a safety concern (Kahlisch et al, 2012; Brettar and Hofle, 2008). Ma *et al.* (2015) stated that the need for innovative and efficient development in science, engineering and technology policy will aid in the reduction of potential health risks posed by drinking water.

WHO outlined a preventative management framework to ensure safe drinking water, which target health

concerns, system assessment, monitoring of the system as well as independent surveillance of the system and operational monitoring of the distribution system (Davison *et al*., 2005). Water safety management in DWDSs involves risk assessment planning and risk management, considering all points that water go through before being regarded as safe to consume. Pathogenic microorganisms in water sources, as mentioned previously, are a major concern when planning risk assessment and management (Brettar and Hofle, 2008). A paradigm outlined by the WHO (2004) in the "Guidelines for Drinking-water Quality" stated that risk assessment for pathogen health risks comprised of four important steps. The first step in this model is to identify the hazard and formulate the problem; thereafter an exposure assessment is carried out, after which a dose-response assessment is done and lastly the risk is finally characterized (WHO, 2004). The paradigm is a self-explanatory guideline when assessing water safety. Management of the system involves microbial risk assessment and characterisation prior to acting on the problem (Ashbolt, 2004). It is suggested that molecular methods should be considered as a framework when managing the quality of drinking water (Ashbolt *et al*, 2001), which is mainly affected by microbial growth in addition to chemicals (WHO, 2004).

The approaches to managing pathogens present in drinking water differ between various countries worldwide. Hodgson and Manus (2006) showed that management of the South African drinking water quality is based on a framework focussing on preventative risk management that endeavours to understand the water distribution and supply system as a whole. The framework aims to address four key areas. The first area focused on the roles and responsibilities of institutions such as water service authorities, the Department of Water Affairs and Forestry and the Department of Health to mention a few. These institutions all play a significant role in providing information concerning the monitoring of water and information on disease outbreaks. The second area of focus involves the analysis of the distribution system and aimed to provide effective management strategies and address the problems gathered from the institutions as well as implement preventative measures to ensure safe drinking water. The third area involves support; the training of employees and research development in order to support effective drinking water quality management and lastly continuous evaluation of water quality assessments to make new improvements (Hodgson and Manus, 2006).

Management and monitoring strategies are then implemented to control the microbial growth within the distribution system. Specific bacteria, which form part of the drinking water microbial community, are used as indicator organisms to represent the overall water quality. The use of indicator organisms is a vital part of management to monitor the safety of drinking water (Bartram *et al*., 2003). The relative counts obtained are used to evaluate the overall quality of the drinking water by measuring the concentration of indicator organisms in relation to the guidelines set out by the World Health Organization (2004). Heterotrophic bacteria as well as faecal coliforms form part of these indicators.

1.3 APPROACHES TO MONITOR AND CHARACTERISE BACTERIA IN WATER

Water utilities make use of various methods to monitor the microbial quality of drinking water. Culture-dependent methods, otherwise known as 'traditional' methods are mainly used for the enumeration and detection of indicator organisms and heterotrophic bacteria (Douterelo *et al*., 2014; Tallon *et al*., 2005; Ashbolt *et al*., 2001). The traditional plate count method is however time-consuming and not representative of the microbial community. Newer technology in the drinking water field for cell count monitoring, such as flow cytometry, is now being introduced into drinking water testing laboratories to improve the efficiency and rapid detection of changes in the system so that utilities can act quickly to ensure the quality of drinking water supplied to consumers is satisfactory.

1.3.1 Indicator organisms

Indicator organisms are defined as easily enumerated, rapidly detected, non-pathogenic microorganisms, which show similar survival characteristics to pathogens and may be associated with their presence (Scott *et al*., 2002). In 1891, the concept of bacterial indicators was introduced when water was recognized to be a

source for pathogenic organisms which may threaten human health (Tallon *et al.*, 2005).

Indicators are defined as part of three microbial groups namely: process; faecal, index and model organisms (Ashbolt *et al.*, 2001). Faecal indicators are used to demonstrate the presence of faecal contamination and infer the presence of potential pathogens. These indicators include thermotolerant coliforms and *E. coli* (Ashbolt *et al.*, 2001) and could either indicate faecal contamination of the source water or cross-contamination present in the system (Tallon *et al.*, 2005). Index and model organisms are selected on the basis that they are representative of a group of pathogens in terms of their behaviour under specific conditions. *E. coli* has been used as an index for *Salmonella* (Ashbolt *et al.*, 2001).

Lin and Ganesh (2013) stated that for an organism to be a successful indicator, it must be consistently present. The South African water quality guidelines are centred solely on faecal coliforms; *E. coli* as an indicator of pathogenic pollution and is subject to strict governmental regulations (Lin and Ganesh. 2013, Lee *et al*., 2005). Testing for indicator organisms provides an overview of the bacteriological quality of drinking water in addition to providing information with regard to water treatment impact and possible contamination events (Leclerc and Moreau, 2002; McLellan and Eren, 2014). The use of culture-dependent methods such as plating of faecal indicators is done by drinking water suppliers to perform quality control evaluation of drinking water (Machado and Bordalo, 2014; Van Nevel *et al*., 2017).

Tallon *et al.* defines total coliforms as “all facultative anaerobic, gram-negative, nonspore-forming, oxidase-negative, rod-shaped bacteria”. These organisms are members of the *Enterobacteriaceae* family but are considered unreliable for the indication of faecal contamination due to their ubiquitous nature (Tallon *et al.*, 2005; Lin and Ganesh, 2013; Ashbolt *et al.*, 2001). Sartory (2004) stated that coliforms are no longer acceptable indicators of faecal contamination but serve as general indicators of microbial quality. Coliforms are useful in the detection of changes in the system because of changes of the treatment and disinfection processes (WHO, 2004; Tallon *et al*., 2005; Ashbolt *et al.*, 2001). Coliforms are said to affect the growth of heterotrophic bacteria (Geldreich, 1989; Chowdhury, 2012; Kelly *et al*. 2014; Amanidaz *et al*., 2015). Bitton (2014) stated that “a coliform growth response test, using *Enterobacter cloacae* as the test organism, was developed by the U.S. EPA to measure specifically the growth potential of coliform bacteria in water”.

Examples of some culture-dependent methods for the detection of coliforms include membrane and multiple tube filtration (MF and MTF), plate counts using different media (YEA, NA and R2A) and Colilert®-18 tests (Douterelo *et al.*, 2014; Storey and Kaucner, 2009). These methods are used to monitor water quality worldwide and provide a good overview of the microbiological quality of water. Ashbolt *et al.* (2001) stated that more indicators representative of process efficiency is required over the traditional ‘faecal’ indicator.

1.3.2 Heterotrophic bacteria

The use of heterotrophic plate counts has been applied for many years as part of the analyses of water for effective drinking water quality management (Van Nevel *et al*., 2017) and is still the most common form of testing and monitoring of the bacterial quality of water (Sartory, 2004; Eichler *et al*., 2006). Plate counts are primarily used as a tool to indicate the operational efficiency of water treatment processes instead of a health-based indicator and the correlations between plate counts and newer direct count approaches are limited (Van Nevel *et al*., 2017). Typically, heterotrophic plate counts are applied to verify treatment functionality as well as re-growth potential in DWDSs. Alternative methods such as culture-independent (e.g. flow cytometry) and molecular methods (e.g. DNA-DNA hybridisation) have also been used (Rompre *et al*., 2002; Berry *et al*., 2006; Douterelo *et al*., 2014; Lu *et al*., 2013). As heterotrophic bacteria are not well defined and a worldwide standard procedure for plate counts has not been established, results may vary according to the method employed to perform these counts and correlations between heterotrophic plate counts and culture independent methods are often poor (Ashbolt *et al*., 2001; Sartory, 2004; Greenspan, 2011; Kelly *et al*., 2014).

Amanidaz *et al.* (2015) stated that “heterotrophic bacteria are considered as an accessory indicator of measuring of coliform in water”, they elaborated further by mentioning that an “increase in heterotrophic bacteria could be a sign of trouble in treatment, repair, installation or influence of microbial growth in the distribution system and presence of biofilm.” The bulk of the bacterial community in drinking water consist of heterotrophic bacteria, which draw their energy from the degradation of organic carbon compounds as they require organic nutrients to proliferate. Most heterotrophic bacteria found in DWDSs are ubiquitous and may be cultured on numerous types of media to reflect the nutrient quality of different environments. High nutrient media can be used to reflect high nutrient environments such as biofilms, whereas low nutrient media can be used to reflect low nutrient environments such as the drinking water system bulk water (Allen *et al*., 2004; Bitton, 2014).

The United Kingdom uses heterotrophic plate counts as an operational tool in the analysis of their drinking water, where it was recommended that the plates which were made from YEA agar be incubated at two different temperatures (Sartory, 2004). Incubation at temperatures of 22°C and 37°C represented the general dynamics within the distribution system. Bacteria that occur naturally in drinking water as well as bacteria that could potentially infect and colonize the human gut were shown to reflect potential contamination in the distribution system (Sartory, 2004). Heterotrophic plate count data can be analysed and used in chlorine management as well as the modelling of microbial populations, where the changes observed indicate unexpected growth of bacteria (Sartory, 2004; Francisque *et al*., 2009). The use of heterotrophic plate counts methods is an effective way to test the water quality, however it does not provide a true representation of the microbial community present in drinking water as many bacteria are not cultivable on standard media and the incubation times used for plate counts and waiting period to analyse results are time consuming. Heterotrophic plate counts were shown to only represent a small percent (<1%) of the total diversity in the microbial community present in a drinking water and environmental samples (Douterlo *et al*., 2018).

The concentration and presence of heterotrophic bacteria are dependent on water temperature, organic carbon availability and chlorine residuals (Amanidaz *et al*., 2015). Low concentrations of available organic carbon compounds are significant for limiting the growth of these bacteria and is consequently important for biological stability, the concentrations of heterotrophic bacteria in drinking water vary between 1 CFU/ml and 10 000 CFU/ml (Amanidaz *et al*., 2015; Prest *et al*., 2016). Heterotrophic bacteria have the tendency to grow better at a higher pH (i.e. alkaline conditions) and at warmer temperatures within the drinking water environment thus seasonal fluctuations influence their growth substantially (Sartory, 2004; Francisque *et al*., 2009).

Species of heterotrophic bacteria that have previously been identified in the distribution system and treatment works include but are not limited to gram positive and gram negative microbes namely: *Acinetobacter lwoffi*, *Agrobacterium sp.*, *Aeromonas hydrophila*, *Bacillus brevis*, *Bacillus licheniformis*, *Burkholderia cepacia*, *Bacillus subtilis*, *Corynebacterium sp.*, *Flavobacterium indologenes*, *Flavimonas oryzihabitans*, *Pseudomonas fluorescens*, *Vibrio sp., Proteus, Enterobacter*, *Aeromonas*, *Citrobacter*, *Pseudomonas*, *Klebsiella*, *Flavobacterium*, *Moraxella*, *Alcaligenese*, *Acinetobacter*, *Micrococcus* and *Xanthomonas sp.* (Amanidaz *et al*., 2015; Sartory, 2004) of which some are considered to be pathogenic organisms which could persist in the system after treatment. Sartory *et al*., (2008) also mentioned that only a small fraction of the bacterial community present may be viable and enumerated on various media, however despite knowing this, monitoring of water supplies for heterotrophic bacteria by culturable methods is considered useful for monitoring trends in water quality as well as detecting sudden changes in quality (Amanidaz *et al*., 2015; Allen *et al*., 2004; Sartory, 2004; Sartory *et al*., 2008).

Heterotrophic plate counts have been used in the past and are currently being used as a standard method for the analysis of bacterial growth as well as water quality. Plate counts provide additional information with regards to the distribution system reliability and treatment efficacy. It should be noted that the different types of media used to perform these counts and their specific incubation times and temperatures have a unique effect on the types of organisms that will grow. Standard methods have been used over the years that include both low and high nutrient agars for enumeration. The use of high nutrient media (e.g. yeast extract agar),

incubation at a high temperature for a shorter time (approximately 24 hours), was shown to be useful for picking up pathogenic bacteria. The use of a low nutrient media such as R2A (commonly used in both the United States and United Kingdom) is better known for the enumeration of autochthonous slow-growing bacterial species that inhabit potable water at a low temperature for a longer incubation time (Allen *et al*., 2004; Francisque *et al*., 2009; Douterelo *et al*., 2014; Berger *et al*., 2015). Various methods such as the spread plate, pour plate and membrane filtration methods have also been used in performing these plate counts (Leat, 2011).

The use of culture-dependent methods represents invaluable information for the study of bacterial biology and ecology however, there are limitations to these methods such as the long periods of time until results are available, inability to detect pathogens and diversity as well as selectivity for specifically organisms that can be cultured at specific temperatures (Vaz-Moreira *et al.*, 2013). Douterelo *et al*. (2018) stated that "Culture-dependent methods are convenient diagnostic tools used by water companies given that they are simple to perform and relatively low cost. However, culturing methods are time intensive with companies waiting days for results". The culture-dependent methods provide only an indication of cell counts and viability of culturable bacteria but the inability to differentiate between bulk water bacteria and biofilm particulates are a downfall.

1.3.3 Culture-independent and molecular methods

As a result of the shortcomings of culture-dependent methods mentioned previously, culture-independent and molecular methods have been established. Using these methods, the microbial species present in the system are identified, potential pathogens are detected and their contribution to the microbiome can be evaluated. Some of the culture-independent molecular methods even have the ability to investigate the entire microbial diversity of the water sampled.

Gillespie (2016) outlined some molecular techniques for testing potable water quality which included nucleic acid amplification-based methods (various PCRs), fluorescence-based cytometry methods, fluorescence in situ hybridization (FISH), biosensors and microarrays (Berry *et al*., 2006; Douterelo *et al*., 2014; Lu *et al*., 2013, Rompre´ *et al.*, 2002). These methods were shown to overcome the shortcomings of traditional methods, for instance, microscopic counts and flow cytometry are more rapid ways to monitor water quality by quantification of viable bacterial cells in under 20 minutes as an alternative to heterotrophic plate counts and their long incubation times. Rompre´ *et al*. (2002) stated that immunological methods can be used for the specific detection of coliforms and other quality indicators in drinking water.

In addition to overcoming the limitations of culture-dependent methods with regard to providing an overview of the microbial diversity within the system as a whole, developments in sequencing technologies such as Illumina MiSeq, 454 pyrosequencing and Ion torrent has advanced the understanding of drinking water microbial ecology (Behjati and Tarpey, 2013; Jaszczyszyn *et al*., 2014; Bautista-de los Santos *et al*., 2016). The use of next generation sequencing has allowed for whole community analyses using 16S rRNA gene profiling and metagenomics. These technologies may involve the preparation of libraries where PCR's are performed to amplify specific gene regions from purified environmental DNA samples. The amplification of the 16S rRNA V4 variable gene region for example enables one to perform 16S community profiling (Behjati and Tarpey, 2013; Jaszczyszyn *et al*., 2014; Douterelo *et al*., 2014). The composition and structure of microbial communities can also be assessed using fingerprinting methods (SSCP, T-RFLP) where the changes in the community can be detected over time (Douterelo *et al*., 2014; Eichler *et al*., 2006; Gillespie, 2016; Cimenti *et al*., 2007).

Flow cytometry has been shown to be effective at overcoming the shortcomings of heterotrophic plate counts and can be used as a process parameter for site-specific monitoring. The use of flow cytometry to analyse mammalian cells was developed in the 1960's but more recently it has been adapted to analyse aquatic microorganisms (Props *et al*., 2018; De Roy *et al.*, 2011). It was shown that measuring the total cell concentration using flow cytometry compensates for standard operational tools like heterotrophic plate counts

because of its sensitivity, ease, and descriptiveness with regard to cell counts when analysing drinking water (Hammes *et al*., 2008).

1.4 USING FLOW CYTOMETRY TO MONITOR MICROBIAL COMMUNITIES

1.4.1 Overview

The flow cytometry technology is used for the analysis of multiple parameters of individual cells within heterogeneous populations. The basic principle of flow cytometry is to enumerate cells based on their fluorescence. The data is captured by passing thousands of stained cells through a laser beam which captures the light that emerges from each cell which passes through. The cells are focused into a narrow stream and carried in a fluid sheath pass a certain number of lasers depending on the machine used. The fluorescence (present in the dyes/stains used for the cells) are detected by a series of photomultipliers. Forward and side scattering of the light can be used to detect cell size and morphology (Gillespie, 2016; Douterelo *et al*., 2014; Selliah *et al*., 2019; Rockey *et al*., 2019; Van Nevel *et al*., 2017; Hammes *et al*., 2010; 2012; Prest *et al*., 2013). Props *et al.* (2016) simply stated that "Flow cytometry exploits the hydrodynamic focusing technology to guide individual cells through a laser beam. In this process, the interaction of the incident light with each individual bacterial cell is registered. Most applications focus on the quantification of cell densities, despite the multitude of interesting single cell aspects that can be measured in one analysis, such as cell size and density as well as nucleic acid content and viability markers".

The threshold limit used in the flow cytometer can be adjusted to accommodate for either large eukaryotic cells (high threshold, e.g. 80 000) or smaller prokaryotic cells (low threshold, e.g. 500-1000) (Besmer *et al*., 2014; 2016; 2017; Nescerecka *et al*., 2018).

De Roy *et al.* (2011) outlined that "the total cell number is a good indicator of changes in water quality during various treatments" and filtration processes. Furthermore, Hammes *et al*. (2008) made use of flow cytometry counts together with nucleic acid stains like SYBR Green I and propidium iodide to enumerate total and intact bacterial cell concentrations in drinking water samples as a descriptive process parameter to reflect the viable cells present before and after treatment.

1.4.2 Flow cytometry applications in microbial water quality assessment

Literature has shown several applications of the use of flow cytometry as a tool for drinking water quality monitoring and analyses. The use of this technology has provided a means of gaining better insight into the drinking water environment and potential future applications of the technology. By using flow cytometry, several water utilities have implemented and successfully adapted the use of multi-parameter analyses to better understand the changes required to the water systems as well as how to effectively maintain the biological stability.

In a study by Props *et al.* (2016), flow cytometry was used for measuring the biodiversity of microbial communities in freshwater ecosystems. The study demonstrated the use of flow cytometry for "sensitive single-cell measurements of phenotypic attributes". The study involved the development of a cytometry-based method used to track the biodiversity of microbial communities with high temporal resolution using computational methods. Methods involved the microbial communities' DNA content being stained with SYBR Green I (100x concentrate in 0.2 um-filtered dimethyl sulfoxide) and incubated for 20 minutes at 37°C (final concentration of 1x concentrate) and then measured using the C6 Accuri flow cytometer using virtual gating. The stain separated the community into different populations based on the concentration of nucleic acid present. Flow cytometry data was analysed using the Flowcore package (v1.34.3) in R (v3.2.1) and phenotypic fingerprints were compared. The results showed that using advanced flow cytometry analysis of

controlled ecosystem data represents similar results to that of benchmark amplicon sequencing methods such as 16S rRNA profiling. (Props *et al*., 2016)

Besmer *et al*. (2016) applied fully automated online flow cytometry to study a groundwater system located in the Frenke Valley in Northwestern Switzerland to monitor the microbial dynamics. Detailed measurements of chemical, physical and biological characteristics which could influence the community were also determined. The study involved the measurement of total bacterial cell concentrations by staining water samples with SYBR Green I ([Life Technologies, Eugene OR, USA] final concentration 1:10 000) and recording measurements after 10 minutes incubation at 37°C over a period of 2 weeks, every 15 minutes daily to investigate irregular and regular dynamics which could not be done before. The Accuri C6 flow cytometer (BD Accuri, San Jose CA, USA) was used and the data was exported as fcs files with custom software where the high (HNA) and low (LNA) nucleic acid content of the bacteria were distinguished using fixed gates which cut out background signals. Results from the study showed that the majority of the changes in the system occurred at night. The changes and measurements identified in the system were used to specifically test and access the impact of these changes on the water quality. The study suggested that baseline datasets for dynamic systems be established and interpreted for future measurements of any system. It was also highlighted that "Major remaining challenges are automated and meaningful data processing, as well as developing online systems that are financially feasible for small/medium scale water utilities as well" (Besmer *et al*., 2016).

Van Nevel *et al*. (2016) conducted a study and proposed the combination of flow cytometric bacterial cell counts with flow cytometry fingerprinting as a fast and sensitive method for the monitoring of drinking water networks. Three cases, based on full-scale system, were evaluated after they were emptied for maintenance purposes and thereafter flushed with clean drinking water before being put into operation again. Samples of water were collected after flushing and chlorine dosing and stored and stained with SYBR Green I (SG, 10000x diluted from stock, Invitrogen) for total cell concentrations or SG combined with propidium iodide (PI, final concentration 4 uM, Invitrogen) for intact cell concentration before flow cytometry measurements. Reference samples before flushing and maintenance were also analysed and compared to samples after flushing. The Accuri C6 flow cytometer was used and cell counts were measured using fixed gating for the green and red fluorescence. Fingerprinting was done using custom-made package flowFDA in R. Each case in the study were interpreted individually where two cases showed a drop-in cell concentration after flushing which stabilized within 3 hours when compared to the reference sample and the third case showed that the flushing sample cell concentration did not stabilize until 6 hours, which was caused by the large level of stagnation in the pipe. The fingerprinting results for each case showed vast variations where some were larger than others. The overall feedback on the combination of these methods stated that "While cell concentrations give you clear and straightforward information, the fingerprinting takes all data into account. Since fingerprinting focusses on both background and bacteria, the sensitivity of the bacterial measurement is lowered. Therefore, this should be combined with cell concentration measurements". Overall conclusions of this study were that flow cytometry is able to produce fast and reliable information on pipe flushing events and should be done in conjunction with hygienic water quality bacterial indicators to help drinking water suppliers provide safe and high-quality water to consumers.

A review in 2019 by Safford and Bischel on the application of flow cytometry in water treatment, distribution and reuse reviewed over 300 studies published over a period of 18 years. It described the benefits and challenges of flow cytometry for "assessing source-water quality and impacts of treatment-plant discharge on receiving waters, wastewater treatment, drinking water treatment, and drinking water distribution." From the review, a section highlighted the combination and comparison of flow cytometry with other indicators namely: heterotrophic plate count; epifluorescence microscopy; molecular techniques such as PCR and DNA sequencing; adenosine triphosphate (ATP) and assimilable organic carbon (AOC) assays. These additional indicators added their own value to the specific water quality study. Various stains mentioned included fluorescent probes, propidium iodide (PI), SYBR stains, EtBr, etc. where each stain has its own application for the specific questions asked. SYBR Green I and propidium iodide were shown to be most popular for drinking water analyses. The research needs outlined, included flow virometry (viral detection), specific pathogen

detection, automation, computational tools for FCM (Flow cytometry) data analysis and lastly standardization. The value of using flow cytometry was emphasised and the variety of applications validated provide a promising approach for routine microbial water quality assessment. Future development also show promise for the advancement of the technology for water quality management (Safford and Bischel, 2019).

Flow cytometric fingerprinting is a statistical analysis method (Van Nevel *et al*., 2017), which when used in combination with flow cytometry, may provide a powerful approach in detecting and quantifying disturbances in drinking water system microbial communities. The fingerprints provide better insight into the microbial dynamics that occur during sporadic changes detected in the system (Props *et al.*, 2018). In addition to the generation of flow cytometry data, fluorescence fingerprints unique to each sample point are generated, which, Prest *et al.* (2014) stated are dependent on the DNA content and bacterial community composition which are not obtained from counting only the cells. This data is valuable in increasing the information provided by flow cytometry and aiding in the rapid and quantitative detection of bacterial communities in the system (Prest *et al*., 2014, Props *et al*., 2018).

Results from previous studies by Rogers *et al*. (2008); Prest *et al.* (2014); Van Nevel *et al.* (2017) and Props *et al*. (2018) shared similar findings with the interpretation, analyses and information gathered from flow cytometry fingerprinting. In the study by Props *et al.* (2018) the fingerprints were shown to correlate strongly between conventional flow cytometry metrics (total cell concentration and high nucleic acid bacteria) and fingerprinting metrics (phenotypic diversity index and phenotypic fingerprinting metrics). From the results where, synthetic data was generated in the study by Rogers *et al.* (2008), the objective was to detect rare events using custom software in the program R and small changes were detected and identified against the background distribution which overlapped these changes but it was said to do so in “the presence of significant biological variability”. The studies by Prest *et al.* (2014) and Van Nevel *et al.* (2017) reported similar effects of the flow cytometric fingerprint where, when the statistical software was used, it is ideal for monitoring the stability of the community and drinking water quality and the concentrations of the bacterial cells was related to changes in the flow cytometry fingerprints and bacterial community composition (Rogers *et al*., 2008; Prest *et al.*; 2014; Van Nevel *et al.*, 2017; Props *et al*., 2018).

Drinking water treatment and distribution systems are recognised as complex aquatic environments offering multiple unique habitats that support microbial growth through the different stages of treatment and distribution. Drinking water leaving the treatment plant may be of high biological quality however, the treated water is subjected to various conditions within the distribution system, which may ultimately lead to the deterioration of water quality at the consumers’ tap. Maintaining the biologically stability of the drinking water is one of the biggest obstacles facing drinking water utilities. Lautenschlager *et al,* (2013) and Prest *et al*, (2016), described biological stability as the condition where the microbial community within a drinking water distribution system (DWDS) does not change in its composition and concentration over time and space, i.e. from the treatment plant, throughout the distribution system and to the consumer’s tap. This is often not possible to achieve as the microbial community can change dramatically from treatment to tap. Drinking water at the tap can contain up to 10^6-10^8 microbial cells per litre (Hammes *et al*., 2008), comprising of a highly diverse microbial community, including bacteria, archaea, eukaryota and viruses (You *et al*., 2009; Thomas and Ashbolt, 2011; Siqueira and Lima, 2013; Lui *et al.,* 2013a; Pinto *et al*., 2014; Gall *et al*., 2015).

Biological instability, i.e. changes in the bacterial community, can only be managed based on a clear understanding of the microbial ecology within the treatment and distribution system. The microbial ecology of DWDSs is governed by multiple environmental and engineering factors as well as operational conditions that influence the composition and structure of microbial communities present in the bulk water, biofilms, and sediments (Wang *et al*., 2014; El-Chakhtoura *et al*., 2015). The study of microbial ecology aims to determine what mechanisms govern the assembly of microbial communities, and what drives the changes in community composition and structure along different spatial and temporal scales (Hanson *et al*., 2012, Nemergut *et al*., 2013). Understanding the microbial ecology will help answer questions about the community’s origin, stability and the interactions that restrict them or keep them stable (Gülay *et al*., 2016).

Insight into the composition and structure of microbial communities of different environmental settings has led to the better understanding of species abundance, distribution and biogeographical patterns. The mechanisms that govern species abundance and biogeographical patterns within microbial communities is not uniform across different environments. However, microbial community assemblages may be governed by four ecological processes, i.e. drift, dispersal, speciation, and selection. These ecological processes rarely operate in isolation, although the relative importance of each process may vary across different taxonomic groups and environmental settings (Nemergut *et al*., 2013). Speciation and dispersal may introduce new species into communities, whereas drift and selection affect change in the presence, absence and relative abundance of species within communities (Roughgarden, 2009; Vellend, 2010).

Microbial communities are not limited to a single taxonomic group but are typically highly diverse with a high level of species present at low relative abundances and fewer species present at high relative abundances (Ferrenberg *et al*., 2013). Although these low-abundance species may increase the diversity of the community, they may not have a significant impact on the dynamics of the community as a whole in an environmental setting. However, they have been shown to be important indicator of environmental disturbances (Szabó *et al*., 2007; Hubert *et al*., 2009; Gülay *et al*., 2016). In microbial ecology, diversity is typically defined in terms of alpha and beta diversity. The inventory or alpha diversity, describes the diversity found within an environment on the smallest spatial scale (within a sampling point). In contrast, beta diversity, also referred to as differentiation diversity, measures the diversity between environments (amongst sampling point) (Nemergut *et al*., 2013). Both alpha and beta diversity is defined by species richness and species abundance information (Little *et al*., 2012) using different diversity metrics and indices.

Many alpha and beta diversity studies have focussed on the high-abundance species constituting the core microbial community, which are often linked to microbial-mediated processes (Richardson and Simpson, 2011; Albers *et al*., 2015; Gülay *et al*., 2016). Microbial-mediated processes within drinking water treatment and distribution systems not only play a beneficial role through biofiltration, but they may also have a negative impact on water quality as they may contribute to microbially induced corrosion of the DWS infrastructure and cause nitrification (Pinto *et al*., 2012; Camper, 2013; Roeselers *et al*., 2015). Furthermore, microbial communities may also harbour potential pathogens, specifically those communities associated with biofilms. Therefore, some of the major challenges facing water utilities is to minimise these negative aspects and supply microbially safe drinking water to communities. The responsibility of drinking water treatment plant (DWTP) and the drinking water distribution system (DWDS) is to limit microbial growth and to maintain and deliver microbially stable drinking water to consumers. It is important that potential water-borne pathogens are removed and the control of water quality is maintained throughout the distribution system ensuring water at the tap is safe.

Supplying drinking water that is both chemically and microbially safe as well as acceptable in terms of taste, odour and appearance is vital to public health and economic growth (Geldreich, 1996; Hunter *et al.,* 2010). Therefore, understanding the microbial ecology, that is, the abundance and distribution of drinking water microbial communities as well as their interactions with the environment and each other will improve microbial management strategies for drinking water industries (Bautista-de los Santos *et al*., 2016). If the ecology of the drinking water microbiome is fully understood, potential predictive modelling can be formulated and implemented. This will enable potential manipulation of conditions within the DWDS to obtain desired results (Little *et al*., 2012; Liu *et al*., 2013a; Zarraonaindia *et al*., 2013).

1.5 SUMMARY

Drinking water utilities aim to produce water that is of the highest quality to their consumers. This implies the delivery of water that is aesthetically acceptable, free from pathogens and operationally within the limits set by South African drinking water standards. Based on the information available, treatment strategies used by various utilities are monitored using heterotrophic plate counts which have many shortcomings. The efficacy of treatment processes employed by treatment plants are managed after analyses of these plate count results

which is time-consuming and not reflective of the total bacterial community composition. The ability of water treatment plants to rapidly detect, manage and monitor changes in the system can be done using flow cytometry technology. The treatment operations used, play an essential role in the improvement of water quality as well as the composition of the microbial community which, if not monitored and managed effectively, may be carried through into the distribution system. Studies done on the use of flow cytometry as a tool to monitor bacterial counts and sporadic changes in the system have proven to be successful in its rapidity and effectivity. The correlations between the current operational tools, heterotrophic plate counts with flow cytometry data have been shown to be very poor in numerous studies. The use of flow cytometry as a process parameter as a means of monitoring the drinking water system have already been implemented successfully in European countries and the implementation in South African drinking water systems will prove to be beneficial. Understanding the site-specific changes that occur in South Africa's drinking water pipeline networks, with regard to the microbial community present, can aid in the elimination of potential health risks thereby improving water management. The strategy for the implementation of flow cytometry to monitor cell counts may help in the upgrade of water quality monitoring methods and as a result, effective management of a sustainable system.

CHAPTER 2: STUDY DESIGN AND METHODS

2.1 SCOPE AND LIMITATIONS

The main focus of this portion of the study was to establish flow cytometry baseline cell concentration (TCC and ICC) values, and correlations with heterotrophic plate counts as well as other wate quality parameters in treated (disinfected) drinking water samples collected from distribution networks from two different systems, operated by a large water utility as well as reticulation samples collected from different residential locations at the point of use, within the Gauteng province of South Africa. Although all these systems were from the Gauteng area, it would be possible to apply the main findings of the project to other drinking water utilities in South Africa as the treatment as well as distribution conditions and management practices are fairly representative of South African systems.

2.2 PROJECT OBJECTIVES AND AIMS

The objective of the project was to provide the necessary foundation for the development of a strategy for the drinking water industry to incorporate FCM when monitoring and managing the biostability of drinking water during distribution as this is a more sensitive and rapid method compared to the HPC currently used. The project also focused on the impact of community reservoirs on the microbial quality of drinking water supplied to consumers. The project aims were to:

- Adapt the current FCM methodologies for the analysis of disinfected samples with low bacteria levels.
- Create a baseline FCM (TCC and ICC values and fluorescent fingerprints), HPC (YEA and R2A) and 16S community profile databases for water samples from chloraminated distribution and reticulation networks.
- Investigate the main biological functions associated with communities that deviate from the baseline FCM values.
- Develop a strategy for the drinking water industry and regulatory authorities for the incorporation of FCM as a technique when managing the biological stability of drinking water during distribution.
- Investigate the contribution of autotrophic bacteria to biological instability in distribution system (as measured in reservoirs) and establish the functional role of these bacteria in the ecosystem.
- Investigate the impact of increased retention time in community reservoirs on disinfectant residuals, FCM values and community composition in order to assist the develop procedural guidelines for the management of these reservoirs.

2.3 SAMPLING

Samples for this purpose were collected at six different sampling locations of a large water distribution network on a bi-weekly basis over a period of 8 months. For reticulation samples (point of use), water was collected from different residential locations in Tshwane district. Six points were sampled on a bi-weekly basis over the same period of 8 months.

2.3.1 Study site 1

Samples were collected from a large distribution network at six different sampling locations on a bi-weekly basis over a minimum period of 6 months. The six sites were randomly chosen based on an initial set of 20 samples collected at various locations along the distribution system. The samples included reservoirs with potentially high bacterial counts and points along the distribution network. The six sample points were: MB2 (production), Ga-Luka (GL), P4PL, Rust-P6 (RP6), Brak-RS3-IN (BR3) and Ben-Res2-IN (BR2). MB2

represented a production sample taken directly after the final treatment and was included as control and for comparative purposes. For all samples, the source water was a canal from the Vaal dam and treatment and purification consisted of seven stages namely: coagulation; flocculation; sedimentation; stabilization; filtration; disinfection and chloramination. Samples MB2 and P4-PL were collected from completely different sections of the distribution network compared to RP6, GL, BR2, and BR3 which belonged to the same section of the distribution network. Samples were collected in sterile 8 L Large Narrow Mouth Nalgene polycarbonate bottles (Thermo Scientific™, South Africa). The samples were collected from the end of July 2018 to March 2019.

2.3.2 Study site 2

For reticulation samples, water was collected from different residential locations at the point of use in the Pretoria area (Figure 2.1). Six points were sampled on a bi-weekly basis over a minimum period of 6 months. The six sites were randomly chosen from an initial set of 10 samples based on the results of FCM and plate concentrations to include both high and low concentrations. The six sample points were: Waverly (WAV), Silverton (SIL), Groenkloof (GK), Valhalla (VAL), Natural Sciences building 2 (NS2) and Agricultural building (AB) (both NS2 and AB were in Hatfield on the main campus of the University of Pretoria). Of the six samples, four were obtained from household taps and two were obtained from taps in buildings on the University of Pretoria Hatfield campus. These two, AgricBuild and NS2 were from taps linked to storage tanks on the building's rooftop. The samples obtained from household taps were obtained from locations within the range of 10-30 km apart. Samples were collected in sterile 8 L Large Narrow Mouth Nalgene polycarbonate bottles (Thermo Scientific™, South Africa).

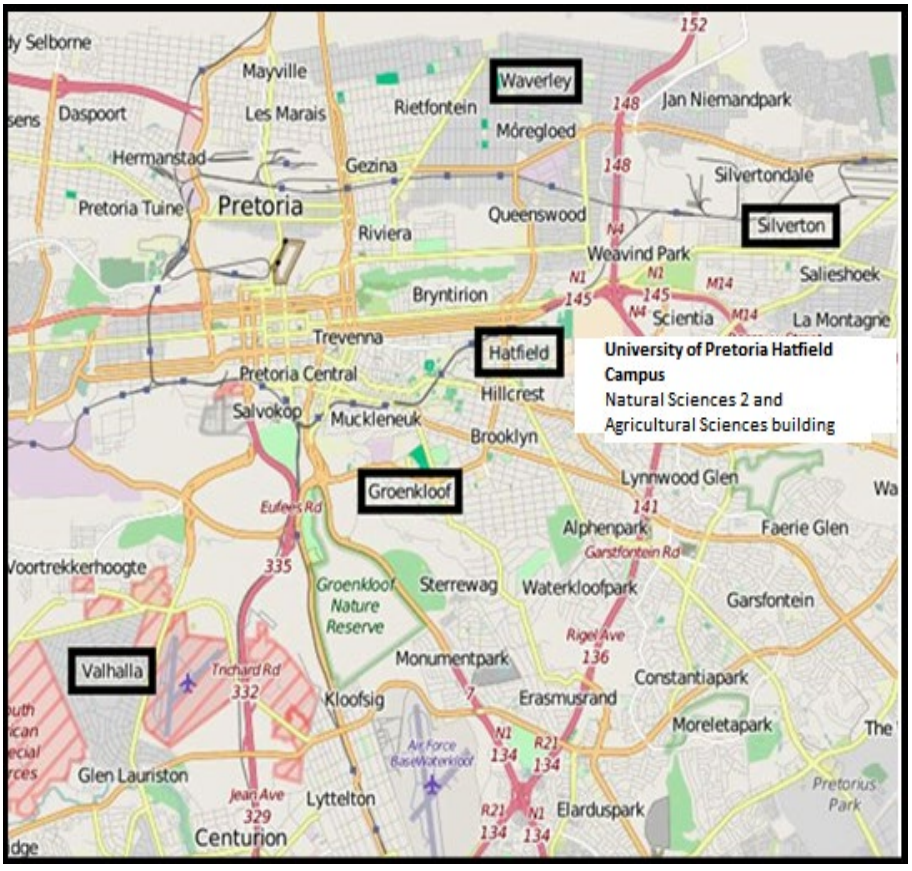

Figure 2.1: Geographical layout of the reticulation sampling locations.

2.4 SAMPLE ANALYSIS

On each of these samples, Flow Cytometry, Heterotrophic Plate Count (HPC), Chlorine and pH analyses were conducted according to methods described below.

2.4.1 Flow cytometry analysis

Flow cytometry concentrations using SYBR Green I and propidium iodide stains were used to establish total and intact cell concentrations per sampling point and represented the culture-independent methods. The nucleic acid stains used for the quantification of intact cells (ICC, intact cells) and total cells (TCC) were Propidium iodide combined with SYBR Green I (SGPI) and SYBR Green I (SG) respectively. The stains were used because of their specific membrane permeability properties and nucleic acid binding ability. SYBR Green I was used for the detection of the total cell concentration (TCC) in a sample. This dye excites in the blue light range of the electromagnetic spectrum at wavelength of 488 nm and emits in the green light range of the electromagnetic spectrum at wavelength of 520 nm. The propidium iodide stain was used for the detection of the intact cell concentrations (ICC) as it fluoresces red when entering the cells which have damaged cytoplasmic membranes and are not intact anymore. The dye excites in the blue light range of the electromagnetic spectrum at wavelength of 488 nm and emits in the red-light range of the electromagnetic spectrum at wavelength of 636 nm.

The dye combination was initially compared to the LIVE/DEAD® Bac*Light*™ Bacterial Viability and Counting Kit (L34856), 2004 in an experiment to determine the combination best suited for use in this study. Multiple trial runs were conducted using the stains at various concentrations and volumes and thereafter trial runs were conducted on the final stains used (SYBR Green I and propidium iodide) to establish the gating to be used for each sample set. The standard operating procedure described in Nescerecka *et al*. (2016) was followed as it was found to be reproducible and adjustments of the PI concentration as well as control samples were confirmed after the trial FCM readings were gathered.

For the final FCM analyses a volume of 500 µl of each sample was prepared in duplicate. The samples were pre-warmed for 3 minutes in a heating block at 37°C to allow for the efficient binding of the stains. One of the samples in the set was stained with 5 µl SYBR Green I (SG) while the other sample was stained with 5 µl of SYBR Green I and Propidium iodide (SGPI) at a concentration of 6 µm and briefly vortexed. The samples were thereafter incubated in the dark for 10 minutes at a temperature of 37°C in a heating block. Control samples comprising of 500 µl aliquots of nuclease-free water filtered using a 0.22 µm syringe filter, a staining control using the nuclease-free water; bottled water and bottled water disinfected with 5 µl of bleach (1.33 mg/l free chlorine) and thereafter quenched with 0.5 µl of sodium thiosulphate were included. All control samples were analysed prior to analysing the water samples. The Accuri C6 flow cytometer (BD Biosciences) with the correct settings was used for analysis. A volume of 100 µl on the machine was analysed using a threshold of 500 on the FL1 (FITC) laser with a fluidic rate of 66 µl/min corresponding to the “fast” rate on the machine. A gate in the P3 region zoomed into the fingerprint was also drawn to avoid the spill-over of damaged cells into the intact cell region on the plots. (Van Nevel *et al*., 2016). As the samples were chlorinated and chloraminated, the results obtained were analysed with caution.

2.4.2 Heterotrophic plate counts (HPC)

HPCs on yeast extract and Reasoner’s 2 agar (R2A) representative of the traditional way of monitoring water quality was used to enumerate bacteria following standard protocols used by drinking water utilities. The YEA pour plate method as outlined by SANS 313 which is identical to the ISO 6222:1999 was used in this study (SABS, 2004). YEA medium (Oxoid) was prepared according to the recommended instructions prior to sample processing and kept at a temperature of ± 52°C. For the analysis, a volume of 1 ml of the sample was placed in a sterile 90 mm Petri dish and thereafter YEA medium was poured into the petri dish (± 20-25 ml) and mixed with the sample by swirling. Inoculated plates were incubated at 35°C for a period of ± 44 hours (SABS,

2004; Rand Water Analytical Services, 2017 (a); 2017 (b)). The number of colonies observed on the plates after the set incubation period was used to determine the colony-forming units per mL (CFU/ ml). All single colonies on the plates were recorded. Analyses were done in triplicate. No dilution of any sample point was done to keep the analysis consistent across all sample points.

For the R2A spread plate method, the protocol described by Standard Methods 1995, HPC (9215) for Microbiological examination (9000) was followed. Prior to inoculation, R2A plates were pre-prepared and stored at 4°C. A volume of 0.1 ml of the respective sample was spread on R2A plates using a sterile spreader under sterile conditions. The R2A agar is regarded as being a low nutrient medium and therefore, the inoculated plates were incubated for ± 3-5 days at a temperature of 22°C which favours the growth of most water microorganisms. Counting and analysis methods were done according to those followed by Rand Water (Rand Water Analytical Services, 2017 (b)) described previously.

2.4.3 Chlorine and pH determination

Chlorine analyses were done with the HACH Test Kit pocket colorimeter™ II Cat. No. 58700-00. The total and free chlorine of each sample was analysed using Permachem® DPD Total and Free Chlorine reagents for a 10 ml sample. The low range (0.02 to 2.00 mg/l Cl_2) chlorine analysis was performed based on the USEPA DPD method. Blanks were prepared prior to each sample batch using dH_20. For the total chlorine measurements, one 10 ml DPD Total chlorine reagent powder pillow was added, and the sample cell was inverted to dissolve the reagent. The undissolved powder did not affect the accuracy of the measurement and the sample colour turned pink if chlorine was present. Total chlorine was measured after letting the sample reaction take place at room temperature. Samples were analysed after 3 minutes but within 6 minutes after adding the reagent. For the free chlorine measurements, one 10 ml DPD Free chlorine reagent powder pillow was added and the sample cell was inverted to dissolve the reagent for 20 seconds. The undissolved powder did not affect the accuracy of the measurement and the sample colour turned pink if chlorine was present. Free chlorine was measured within 1 minute after adding the reagent. All results were recorded in mg/l Cl_2. The pH of each sample (approximately 30 mL) without the addition of sodium thiosulphate was measured using a Beckman pH meter (Beckman Instruments, Inc. Fullerton, CA 92634-3100).

CHAPTER 3: RESULTS

3.1 INTRODUCTION

The study was designed to investigate the use of flow cytometry (FCM) methodologies to monitor microbial water quality in disinfected drinking water samples with low bacterial numbers. The aim was to establish a baseline database for the use of the culture-independent flow cytometry method as a process parameter in the monitoring of water quality to complement the traditional methods. This was done by first establishing the flow cytometry baseline cell concentration values, Total Cell Count (TCC) and Intact Cell Counts (ICC) and comparing with Heterotrophic Plate Count (HPC) data. Thereafter, the correlation between FCM baseline values and HPC data was investigated. Furthermore, trends and correlations between the ICC data and total and free chlorine residuals present in the samples were investigated. Although all these systems were from the Gauteng area, it would be possible to apply the main findings of the project to other drinking water utilities in South Africa as the treatment as well as distribution conditions and management practices are fairly representative of South African systems.

3.2 RESULTS DATA

To gain insight as to where the flow-cytometry data comes from, raw data FCM plots representing the highest concentrations per sample point over the sampling period are included. The plots represent the total cell concentrations (Sample names followed by SG) and intact cell concentrations (Sample names followed by PI). The plots (Figure 3.1 and 3.2) typically show a movement of cells out of the gated area between the SG (SYBR Green I) and PI (Propidium iodide) stains reflecting the decrease in cell numbers between total and intact cells. Reticulation network samples' electronic gating was established to fit all samples in the data set and distribution samples had one fixed gated area across all samples in the data set. The distribution samples (Figure 3.1) were lower in overall concentrations and had smaller clusters present in the dot plots. Ben-Res2-IN (Figure 3.1A) had a clear high nucleic acid and low nucleic acid cluster overlapping. Ga-Luka (Figure 3.1C) had the highest concentrations and a small high nucleic acid cluster present but the concentrations were so high that the dots are not distinguishable from one another. Across the local sampling points (Figure 3.2), various clusters were observed at each sample point. AgricBuild (Figure 3.2A), Groenkloof (Figure 3.2B) and Silverton (Figure 3.2D) had clear high nucleic acid cell clusters and also a lot of background present outside of the gated region. Some of the plots had the background/damaged cells cutting through the intact cell region.

Note 1: Supplementary data and results

For supplementary results and raw data obtained from the analysis of samples collected from the large distribution network (site 1) and reticulation sampling points (site 2) refer to Figures A1-A30 and Table A1 in **Appendix A** of this report.

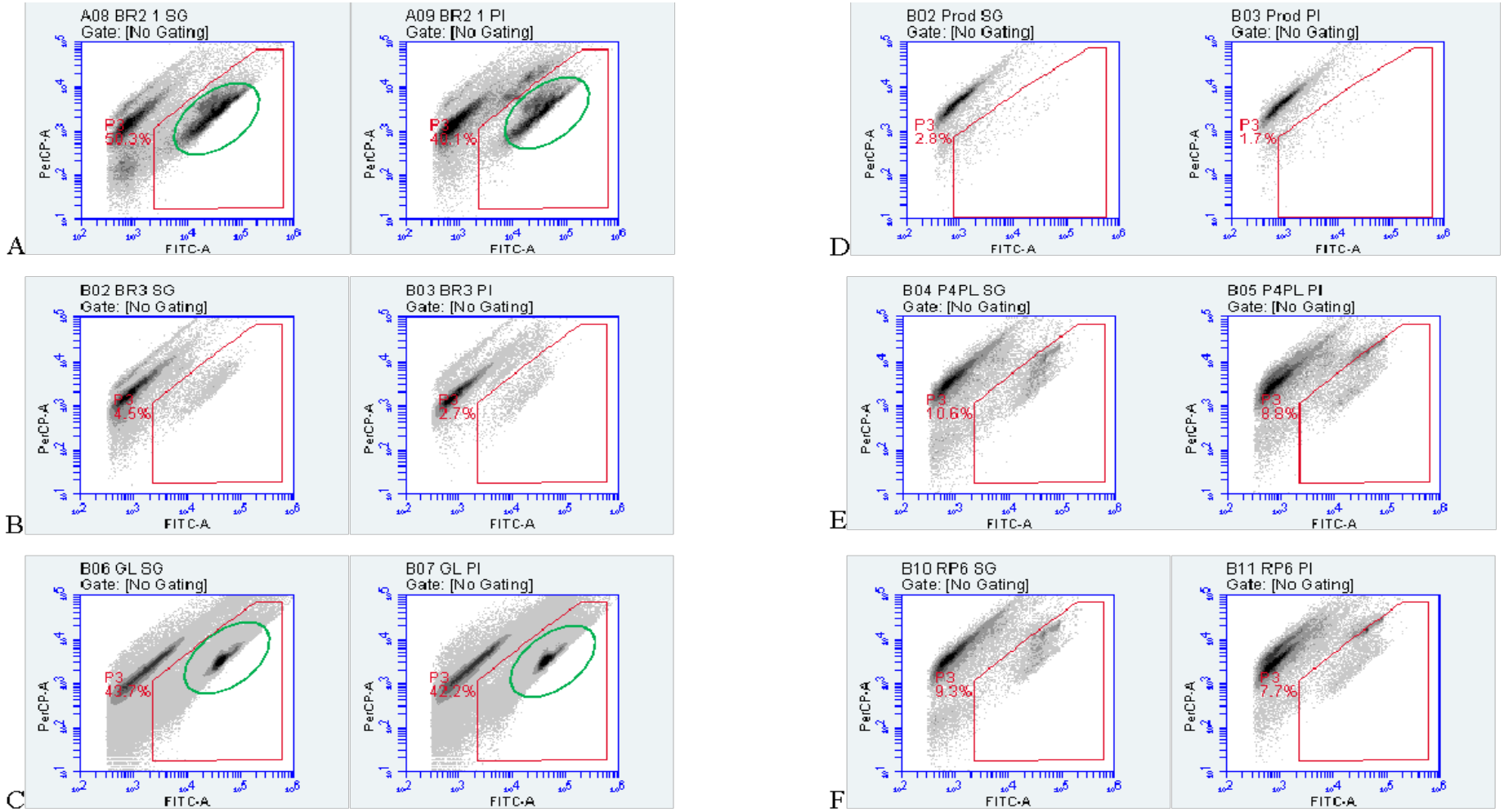

Figure 3.1: Dot plots representing the raw flow cytometry plots for the highest cell concentrations for Site 1 samples. PerCP-A (FL3) indicates the red fluorescence (670 nm) and FITC-A (FL1) indicates the green fluorescence (533 nm). Electronic gates were used to separate the bacterial cell cluster from the background and damaged cells. (A) Represents Ben-Res2-IN, (B) Brak-RS3-IN, (C) Ga-Luka, (D) MB2, (E) P4-PL and (F) Rust-P6. Sample names followed by SG represents the total cell concentration plots and PI represents the intact cell concentration plots. The regions circled in green represent the clear high nucleic acid cells (HNA).

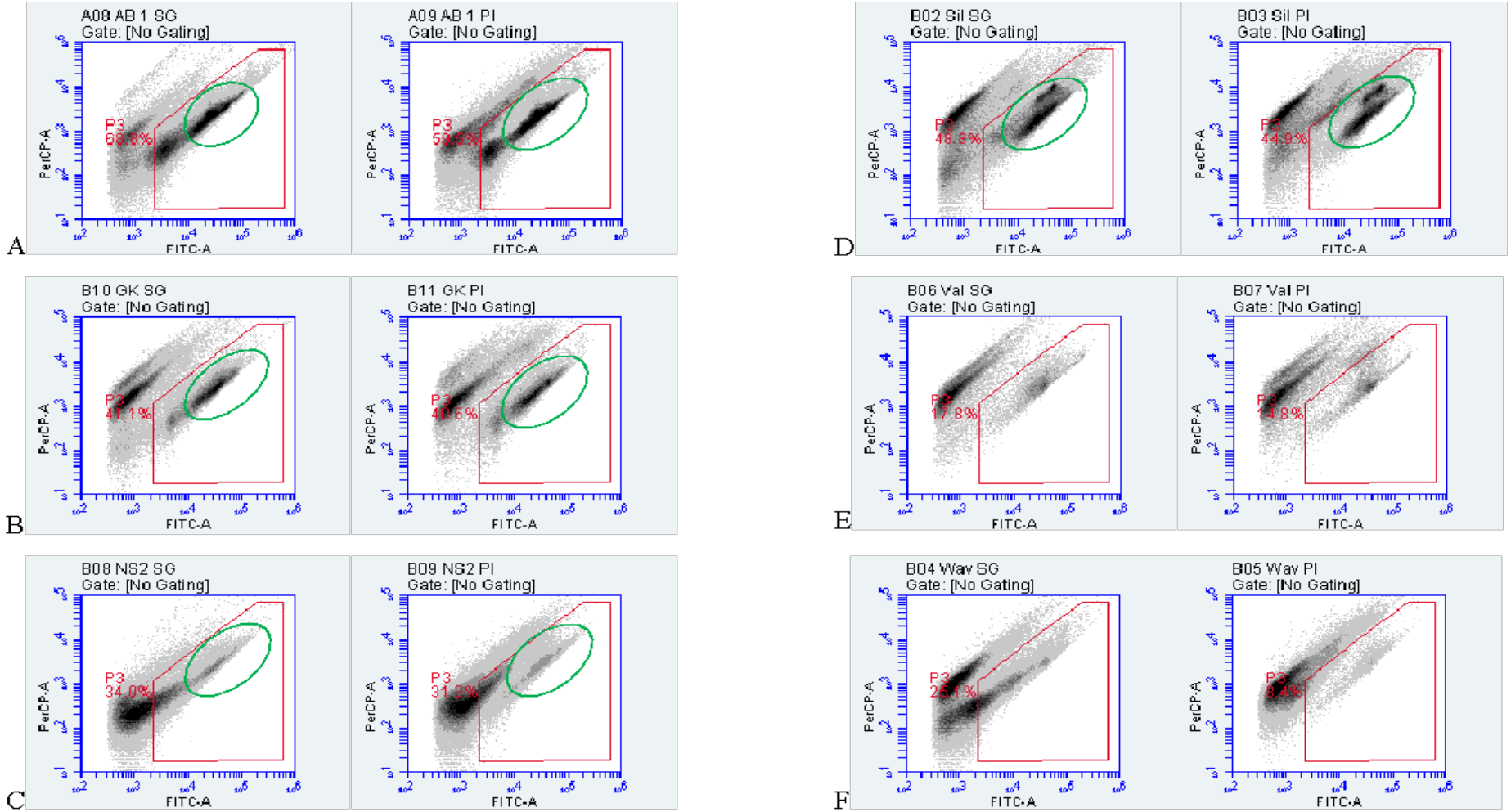

Figure 3.2: Dot plots representing the raw flow cytometry plots for the highest cell concentrations for the local samples. PerCP-A (FL3) indicates the red fluorescence (670 nm) and FITC-A (FL1) indicates the green fluorescence (533 nm). Electronic gates were used to separate the bacterial cell cluster from the background and damaged cells. (A) Represents AgricBuild, (B) Groenkloof, (C) NS2, (D) Silverton, (E) Valhalla and (F) Waverley. Sample names followed by SG represents the total cell concentration plots and PI represents the intact cell concentration plots. The regions circled in green represent the clear high nucleic acid cells (HNA).

3.3 ESTABLISHING CORRELATIONS BETWEEN BASELINE BACTERIAL CELL CONCENTRATION VALUES AND WATER QUALITY DATA

3.3.1 Site 1 – water samples from a large drinking water distribution network

The heterotrophic plate concentration data using two different media (the Reasoner's 2 agar (R2A) and yeast extract agar (YEA)) for the distribution points showed variation across the sampling location points and the results of a few sample points stood out. The average plate count for YEA ranged from 4-137 CFU/ml and 24-2276 CFU/ml for R2A. Lower overall plate concentrations were observed in the samples collected in distribution system sample points. The sampling points Ben-Res2-IN (Figure 3.3) and Brak-RS3-IN (Figure A2) had low plate concentrations that clustered together. The average concentrations were 4 CFU/ml (YEA) and 398 CFU/ml (R2A) for Ben-Res2-IN and 6 CFU/ml (YEA) and 84 CFU/ml (R2A) for Brak-RS3-IN. Ben-Res2-IN had visibly higher overall R2A concentrations compared to YEA concentrations.

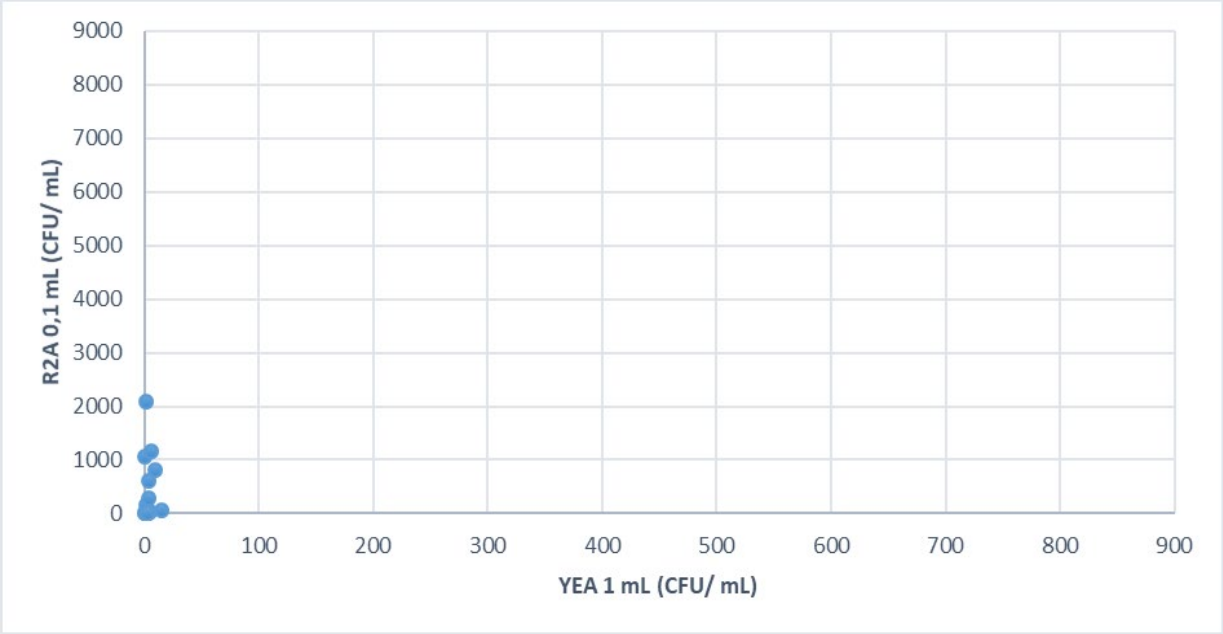

Figure 3.3: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for distribution network point Ben-Res2-IN.

Ga-Luka (Figure 3.4) had an outlier point and showed a scattered pattern in addition to a wide variation in plate concentration numbers across the sampling period and high plate concentrations were observed with average concentrations of 137 CFU/ml (YEA) and 2276 CFU/ml (R2A). This point had the most noticeable variation of all twelve sampling locations in plate concentrations and included an outlier point which had an R2A plate concentration of 9000 CFU/ml. Other points on the plot were also high and the R2A had higher numbers of bacteria compared to YEA.

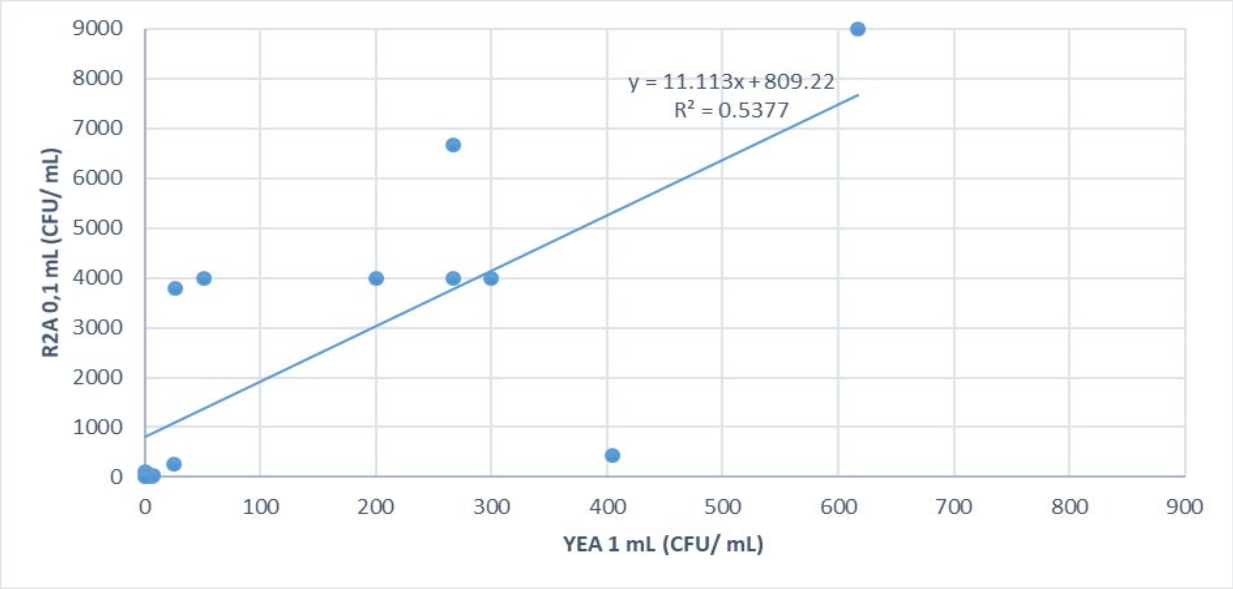

Figure 3.4: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for distribution network point Ga-Luka.

MB2 (Figure A2, Appendix A) point collected post-treatment, and the concentrations observed were extremely low on the plates which reflected the efficacy of the treatment used. The average plate counts were 4 CFU/ml (YEA) and 28 CFU/ml (R2A) for MB2. Low plate concentrations were also observed at P4-PL (Figure A3, Appendix A) and Rust-P6 (Figure A4, Appendix A) which were points collected along the distribution line. The average concentrations for both these points were also low, 5 CFU/ml (YEA) and 25 CFU/ml (R2A).

The distribution network samples showed sample-specific trends when the log values of flow cytometry concentrations were plotted over time (Figures A8-A11, Appendix A). Log values of flow cytometry concentrations for samples collected at the points: Ben-Res2-IN (Figure A8, Appendix A9) and Brak-RS3-IN (Figure 3.5), as well as Ga-Luka (Figure 3.6), showed high cell concentrations in comparison to other samples ranging between 10000-300000 cells/ml. Brak-RS3-IN (Figure 3.5) showed irregular peaks in the cell concentrations from the fourth month of the sampling period. The zig-zag peak pattern was interesting. The intact cell concentrations detected were low. It was noticeable that Brak-RS3-IN had low intact cell concentrations in comparison to the total cell concentrations. The Ga-Luka (Figure 3.6) total and intact cell concentrations were fairly stable and were not distinguishable from each other. This sampling location had the highest FCM concentrations.

Point MB2 (Figures A9, Appendix A) had the lowest concentrations of all distribution samples which was expected because this point was collected directly after treatment and final chlorination. This sample had the most variation in the sample set with varying concentrations throughout the sampling period, but the concentrations were generally quite low which was expected for a post-treatment water sample. P4-PL (Figures A10, Appendix A) and Rust-P6 (Figures A11, Appendix A) samples which were collected along the distribution line showed steady cell concentration numbers with similar concentrations. Both sampling locations had a sporadic increase towards the end of the sampling period during the warmer months. P4-PL, however, showed higher total cell concentrations in the last half of the sampling period. When plate concentrations were plotted against flow cytometry intact cell concentrations different distributions of values were observed.

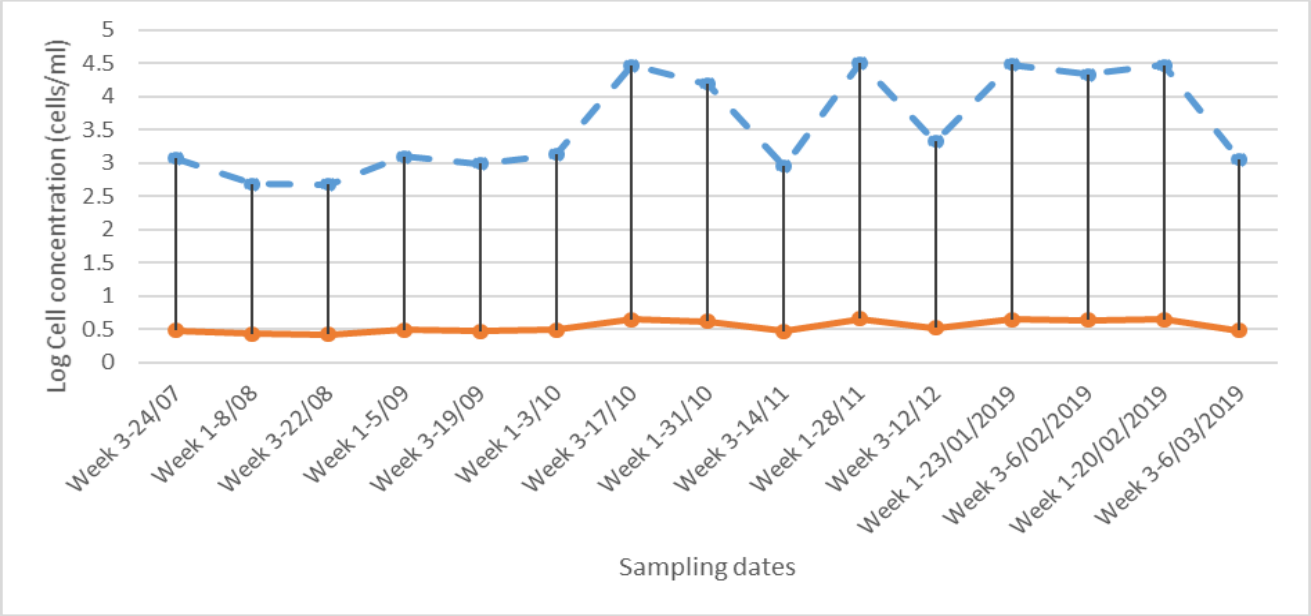

Figure 3.5: Graph representing the log value of raw total and intact cell concentrations for distribution point Brak-RS3-IN over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

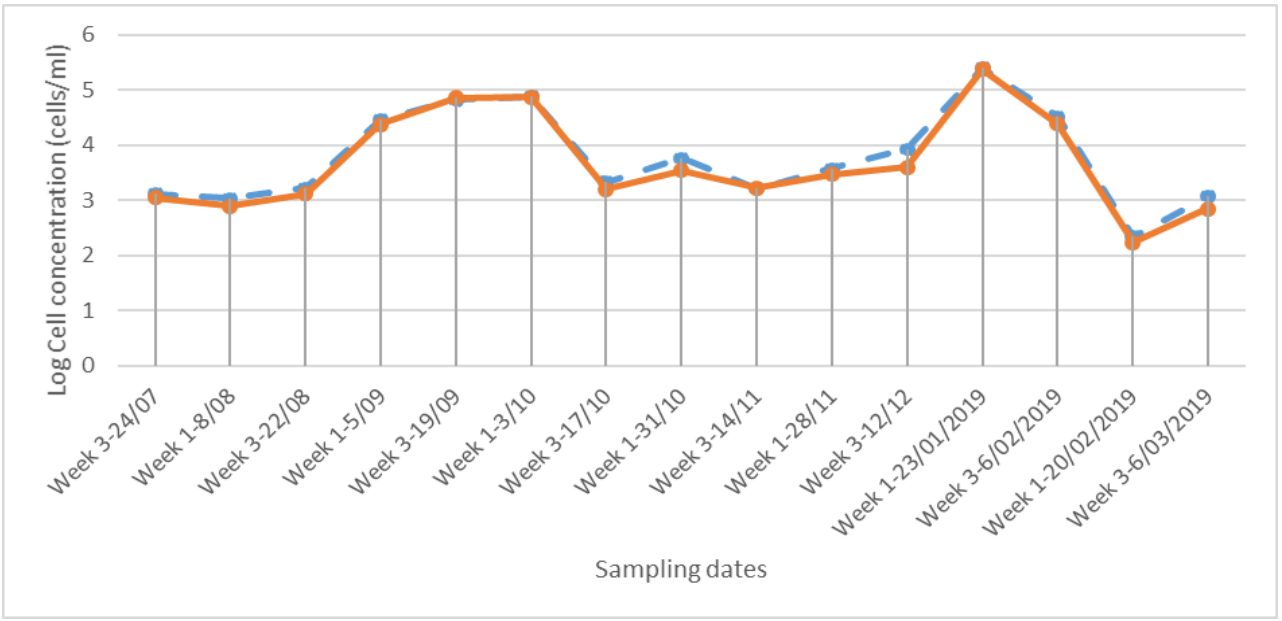

Figure 3.6: Graph representing the log value of raw total and intact cell concentrations for distribution point Ga-Luka over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

Ben-Res2-IN (Figure 3.7) showed higher R2A concentrations linked to some of the higher intact cell concentrations. Brak-RS3-IN (Figure 3.7) had very low plate concentrations but a range of intact cell concentrations were observed. Ga-Luka (Figure 3.9) had a wider distribution of points with higher R2A values often corresponding to higher intact cell concentrations.

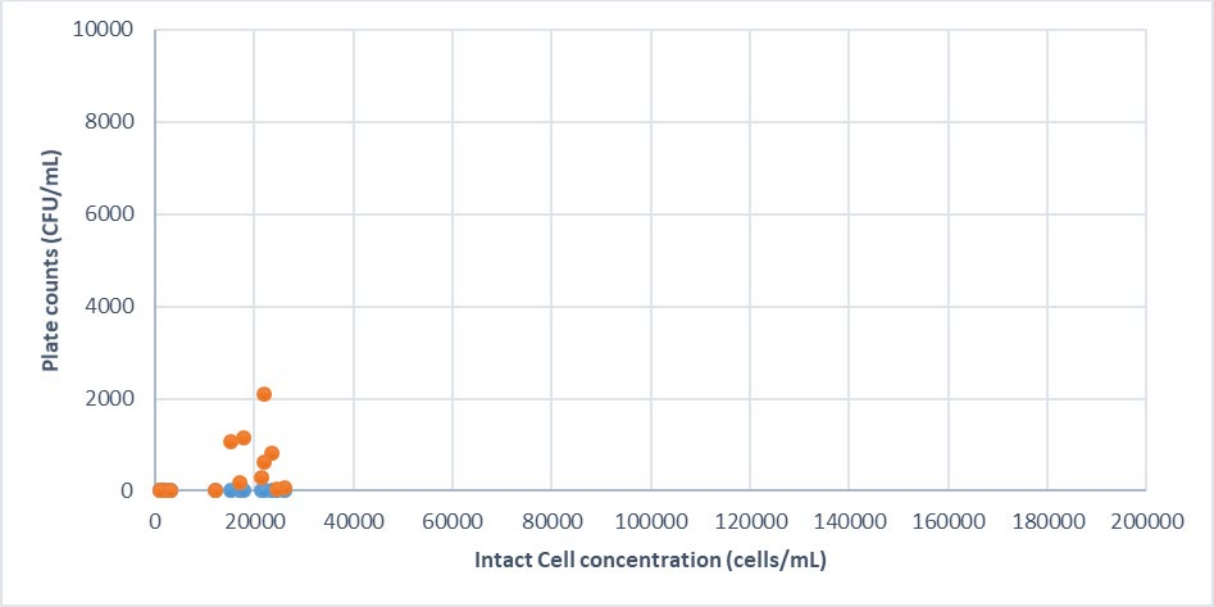

Figure 3.7: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for distribution point Ben-Res2-IN for all samples.

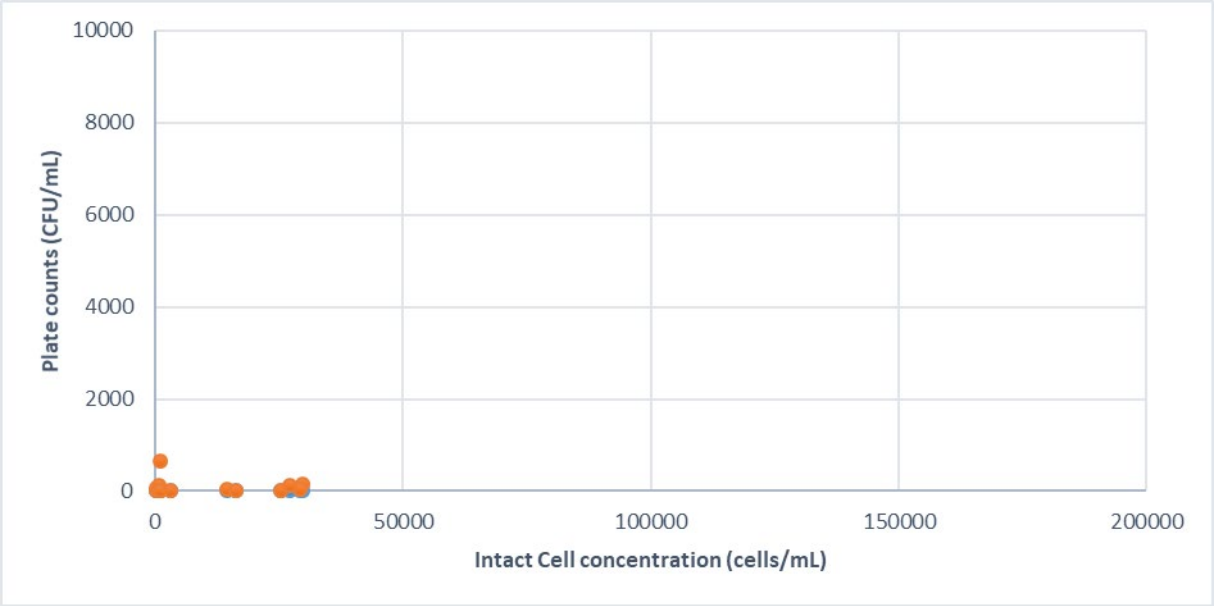

Figure 3.8: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for distribution point Brak-RS3-IN over the sampling period.

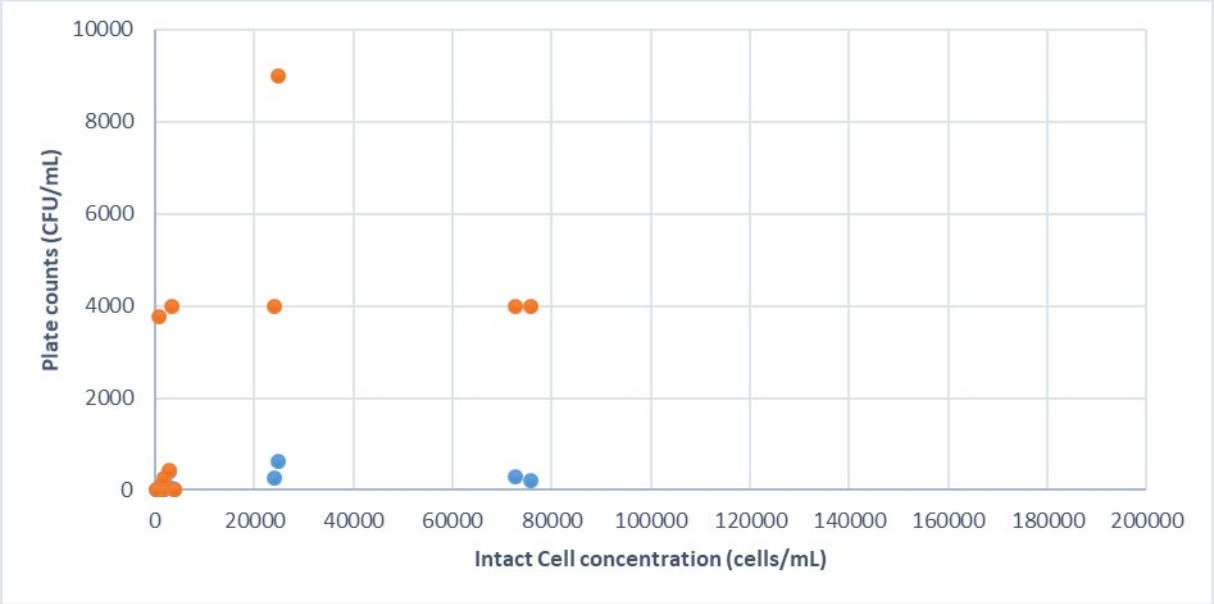

Figure 3.9: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for distribution point Ga-Luka over the sampling period.

MB2 (Figures A5, Appendix A), P4-PL (Figures A6, Appendix A) and Rust-P6 (Figures A7, Appendix A) showed both low plate concentrations and intact cell concentrations for all samples. These distribution points were expected to have higher levels of chlorine residuals as these are along the distribution line but the concentrations were within that of SANS 241 (SABS, 2015) which is ≤5 mg/L for free chlorine. MB2 (Figure 3.10), the production sample had a wide spread of variation across all the points with no overall correlations observed. The chlorine residuals were however quite low (<1.00 mg/L) across the sampling point and total and free chlorine residuals overlapped with each other.

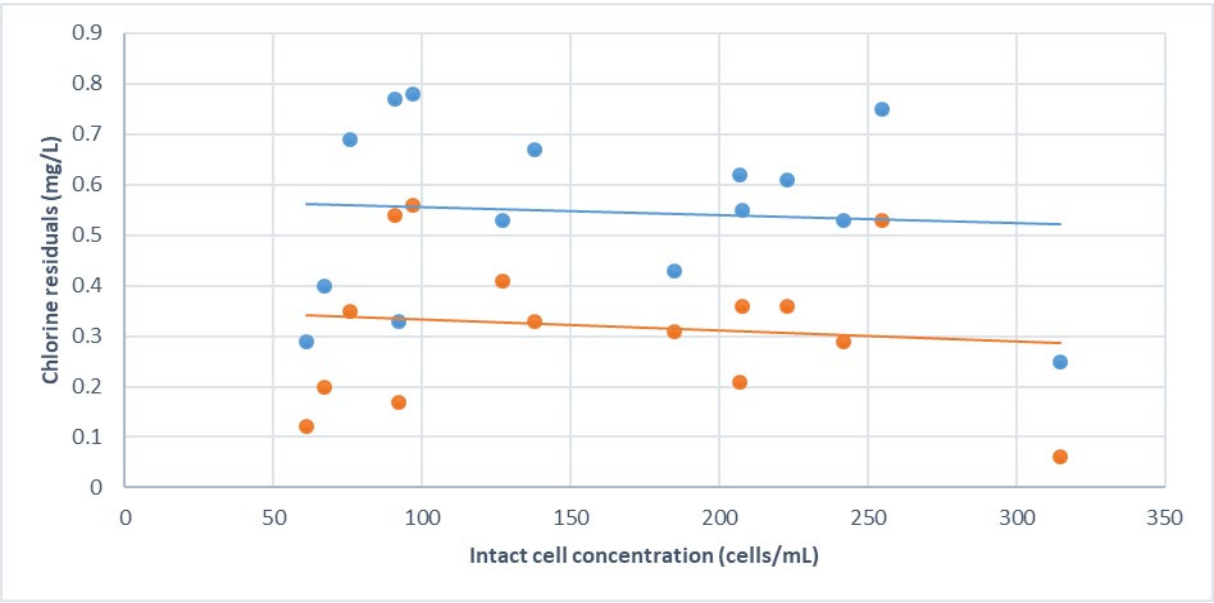

Figure 3.10: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for distribution point MB2 over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

The overall total chlorine residuals were higher for five of the six locations with MB2 as the outlier sample location having significantly higher free chlorine than the other points. P4-PL (Figure 3.11) had a spread of chlorine residual values and the total and free residual points formed two clusters. Two free chlorine residual points grouped with the total chlorine residuals and one outlier was present corresponding to a total chlorine outlier and the highest intact cell concentration of the sample point. A clear correlation was observed where the higher total chlorine residuals corresponded to lower intact cell concentrations for the sample. Rust-P6 (Figure A13, Appendix A) also had two clear clusters with the total and free chlorine residual points grouping together. There was one outlier for each type of residual where the free chlorine residual was quite low and the total chlorine residual was the highest at this point and both corresponded to high intact cell concentrations.

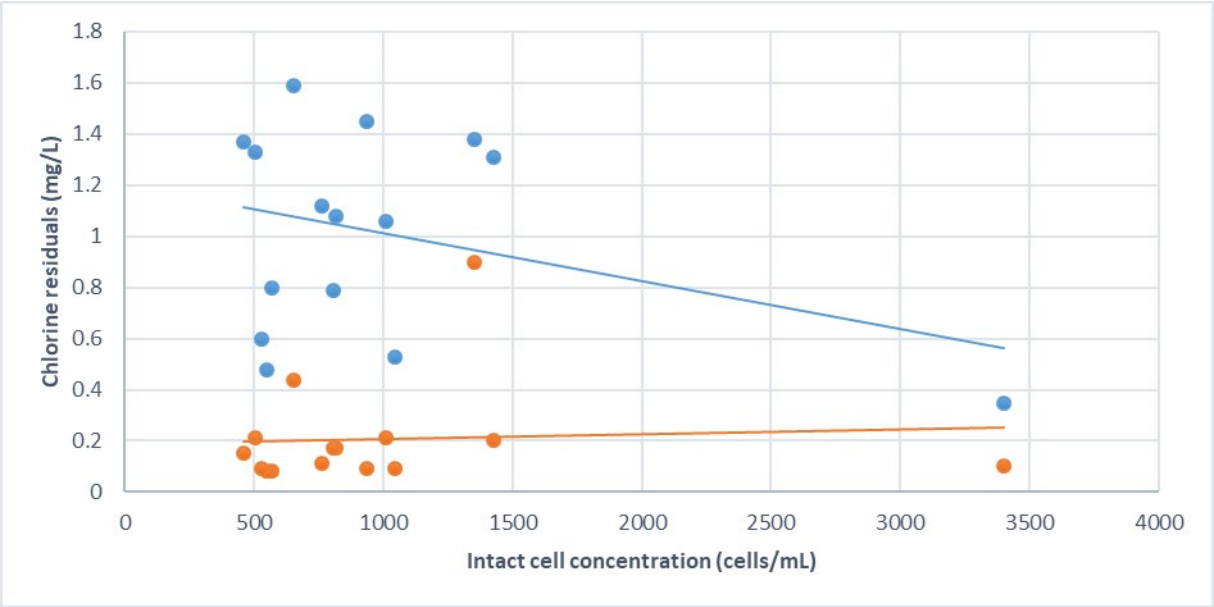

Figure 3.11: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for the distribution point P4-PL over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

Ben-Res2-IN (Figures A12, Appendix A) had a correlation with higher total chlorine residuals having lower corresponding intact cell concentrations. The free chlorine residuals were overall very low.

Brak-RS3-IN (Figure 3.12) had a variety of different values observed for both free and total chlorine levels. A few outliers across all points were present but the results per sample point were interesting. Five of the total chlorine residual points were at the lower end and had higher corresponding intact cell concentrations and the majority of the other points were high with lower corresponding intact cell concentrations. One point was a clear outlier with higher total chlorine residual and high intact cell concentration. The free chlorine residuals were generally quite low with two outliers higher than the other points.

Ga-Luka (Figure 3.13) had a very interesting spread of the points where a large majority of the samples clustered at the lowest range of the intact cell concentrations and all corresponding chlorine residual points, total and free, were on the y-axis. There were three clear groups of outliers for total and free chlorine residuals with the outliers being quite low values of the outliers corresponding to higher intact cell concentration concentrations.

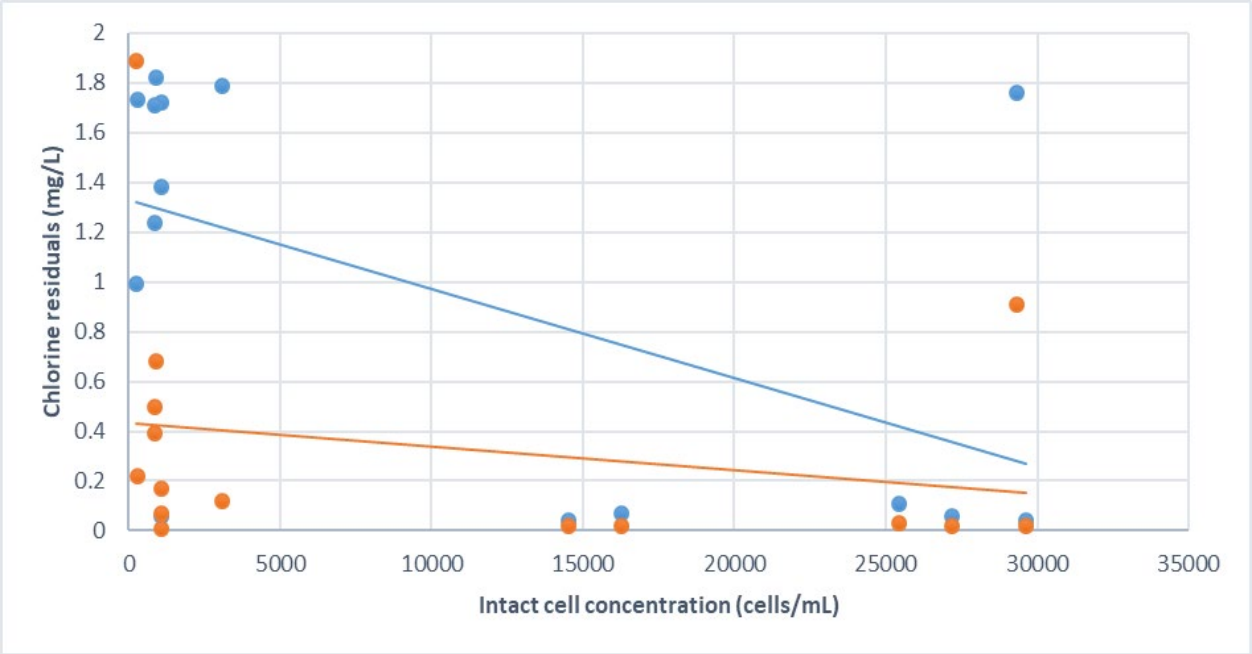

Figure 3.12: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for distribution point Brak-RS3-IN over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

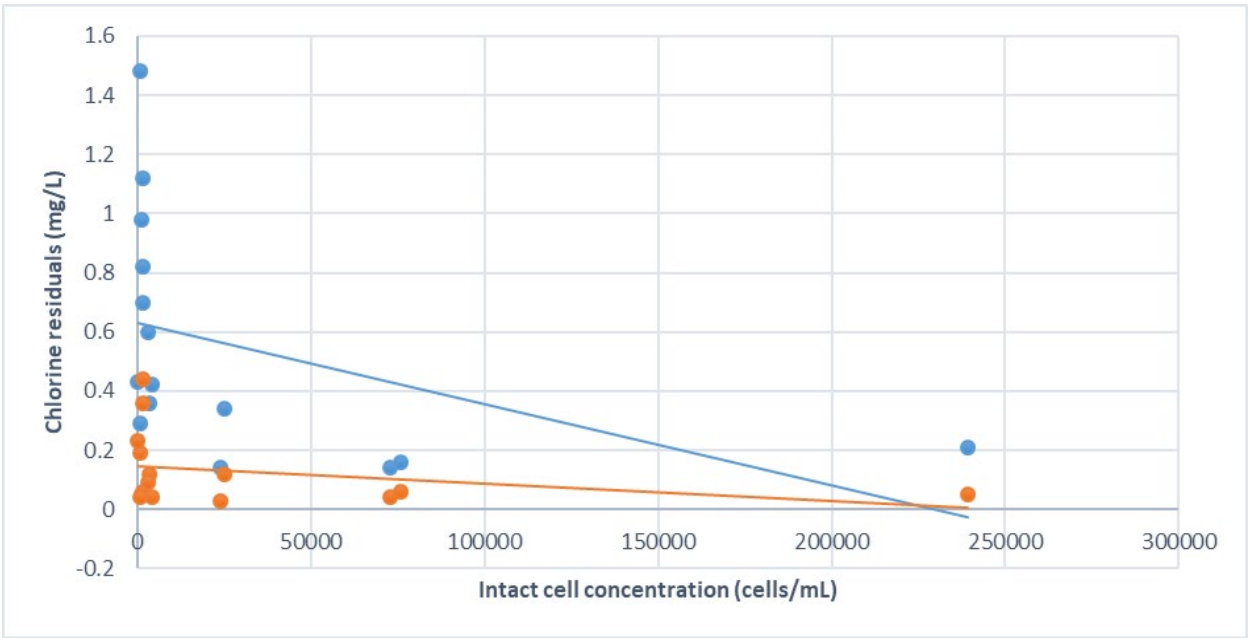

Figure 3.13: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for distribution point Ga-Luka over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

3.3.2 Site 2 – water samples from household taps and catchment/storage tanks

The six local sample points collected from household taps and catchment/storage tanks showed variation in the plate concentrations on different media. Two of the six local reticulation points contained outliers. AgricBuild (Figure 3.14), which is water collected from a lab tap with the source from a storage tank on the roof of the building generally had high plate concentrations with an average of 83 CFU/ml (YEA) but higher R2A values with an average of 1593 CFU/ml.

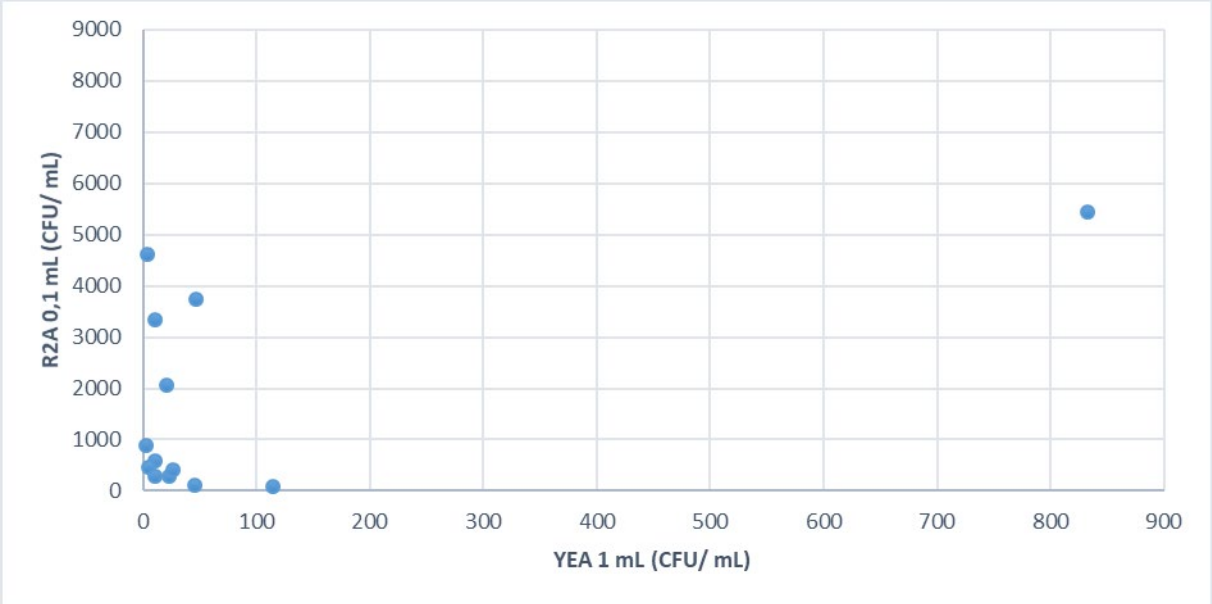

Figure 3.14: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for local point AgricBuild.

The Groenkloof (Figure A14, Appendix A) sample location points were quite clustered and a general low concentration was observed with average concentrations of 22 CFU/ml (YEA) and 161 CFU/ml (R2A). There was one clear outlier observed at this point.

NS2 (Figure A15, Appendix A) had one outlier with higher R2A concentrations (an average of 504 CFU/ml) and a low corresponding YEA concentration (an average of 17 CFU/ml) when compared to other times sampled. The water at this point was collected from a tap in a building that had the source water from a storage tank on the roof of the building.

Silverton (Figure 3.15) had the most variation in plate concentrations out of the local sampling points and generally higher concentrations at different sampling times were observed. The average concentrations at this point were 54 CFU/ml (YEA) and 417 CFU/ml (R2A).

Valhalla (Figure A16, Appendix A) and Waverley (Figure A17, Appendix A) had consistently low concentrations across the sampling period and no clear outliers. The average concentrations at these points ranged between 20-34 CFU/ml (YEA) and 117-241 CFU/ml (R2A) at these two points.

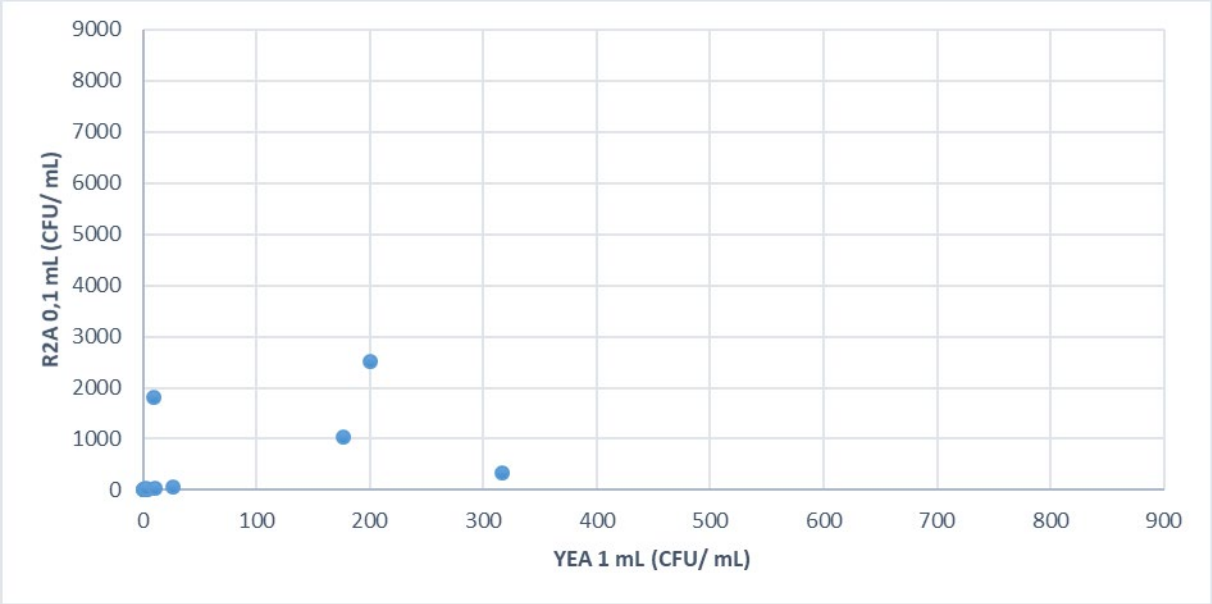

Figure 3.15: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for local point Silverton.

For the local sample points, a clustering of the plate concentration numbers was observed at five of the six sampling points when the plate concentrations were plotted against the intact cell concentrations. AgricBuild was the one point that (Figure 3.16) had little clustering and a clear outlier was observed with high intact concentrations and a lower plate concentration. The R2A plate concentrations, however, were quite high, with the highest concentration reaching >5000 CFU/mL. Despite the intact cell concentrations being high, this point showed a range of values over the sampling period.

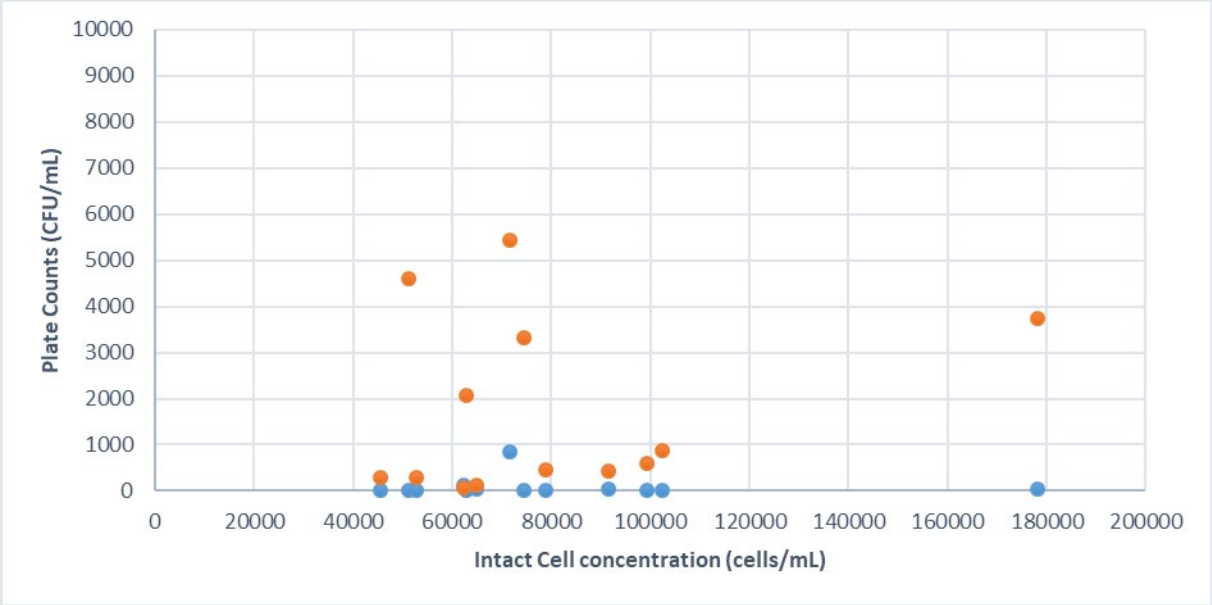

Figure 3.16: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for local point AgricBuild over the sampling period.

Groenkloof (Figure A18, Appendix A) had points clustered together because of low concentrations in comparison with the majority of the values from other sampling points. NS2 (Figure A19, Appendix A), Silverton (Figure A20, Appendix A) and Valhalla (Figure A21, Appendix A) had outliers that were scattered at either higher intact cell concentrations or higher R2A plate concentrations and the other samples clustered together. Waverley (Figure A22, Appendix A) also had points clustered together because of the low concentrations in comparison with the bulk of the values from other sampling points.

The local samples had high overall flow cytometry cell concentrations for each point. Two of the six points showed a sporadic increase in the cell concentration over the sampling period while four points had higher concentrations toward the end of the sampling period in the warmer months. The overall concentration numbers varied per sampling point. AgricBuild (Figure 3.17) had relatively high concentrations with a sporadic increase in the intact cell concentration at Week 4 – 29/08/2018 and an increase in the total cell concentration at Week 2-30/01/2019. AgricBuild had the highest concentrations observed.

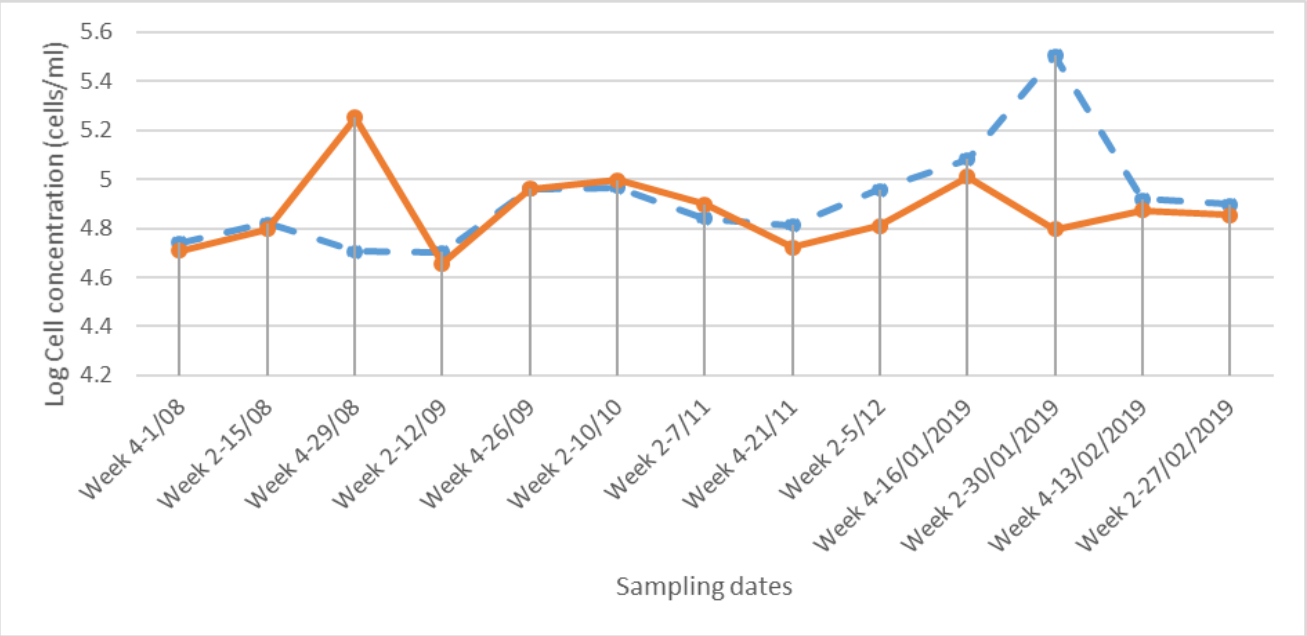

Figure 3.17: Graph representing the log value of raw total and intact cell concentrations for local point AgricBuild over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

Figures A23-A25 (Appendix A), showing Groenkloof, NS2 and Silverton, respectively, depict an increase in the cell concentrations during the warmer months from the middle of the sampling period. Valhalla (Figure A26, Appendix A) had irregular trends in the cell concentrations over the sampling period with the total cell concentrations always being consistently higher than the intact cell concentrations which is what is the expected result and sporadic increases in the overall concentrations. Waverley (Figure A27, Appendix A) was also one of the sampling points that increased in cell concentration towards the end of the sampling period. The local sampling points generally show little change over time when log scaled. AgricBuild points had fairly consistent log values over time apart from the two outliers for intact and total concentrations. Groenkloof, NS2, Silverton, Valhalla and Waverley displayed some changes over the sampling period and indicated the variation in the intact cell concentrations.

The chlorine residual concentrations of the local samples varied between 0.03-0.97 mg/L free chlorine and 0.09-2.2 mg/L total chlorine. AgricBuild (Figure 3.18) had overall very low chlorine residual levels and there were no correlations. Two outliers were observed at this point where the total chlorine residuals were considered high for this specific point in relation to the rest of the points. All points at this sample had a chlorine residual level <0.5 mg/L.

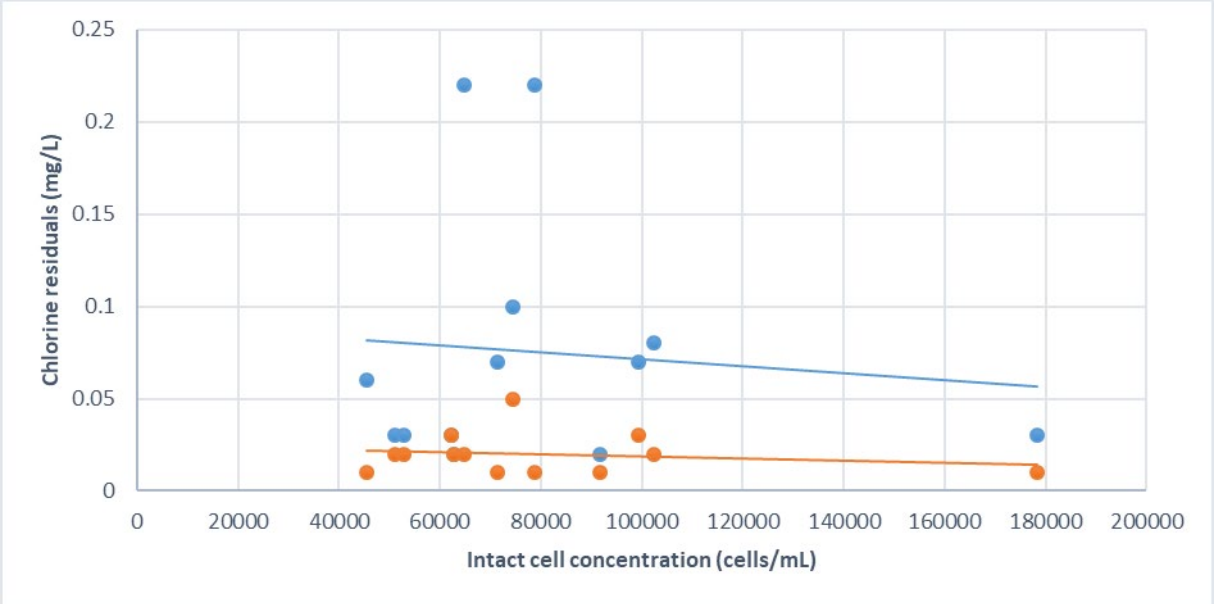

Figure 3.18: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for local point AgricBuild over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

Groenkloof (Figure A28, Appendix A) had the outlier which had the highest total chlorine residual concentration across all the points. The free chlorine residuals were <0.5 mg/L and total chlorine residual concentrations varied between 0.36-1.05 mg/L. Three outliers were present with low chlorine residual concentration and higher corresponding intact cell concentration values indicating the relationship between the two factors.

NS2 (Figure A29, Appendix A) points were evenly split between the total and free chlorine residuals across the intact cell concentrations and only one point seen as an outlier for the free chlorine level.

Silverton (Figure 3.19) had higher levels for the total chlorine corresponding to fewer intact cell concentrations. Four of the points were outliers with lower levels of chlorine residuals corresponding to higher concentrations of intact cell concentrations and one point was at a lower chlorine level at a lower intact concentration.

Valhalla (Figure A30, Appendix A) points were scattered across the plot with two free chlorine residual points which were quite high and were in the region with the total chlorine residual points. There were three total chlorine outlier points.

Waverley (Figure A31, Appendix A) had a clear clustering of high total chlorine residual points corresponding to low intact cell concentrations and five points were outliers. The overall free chlorine residual was quite low across all the points.

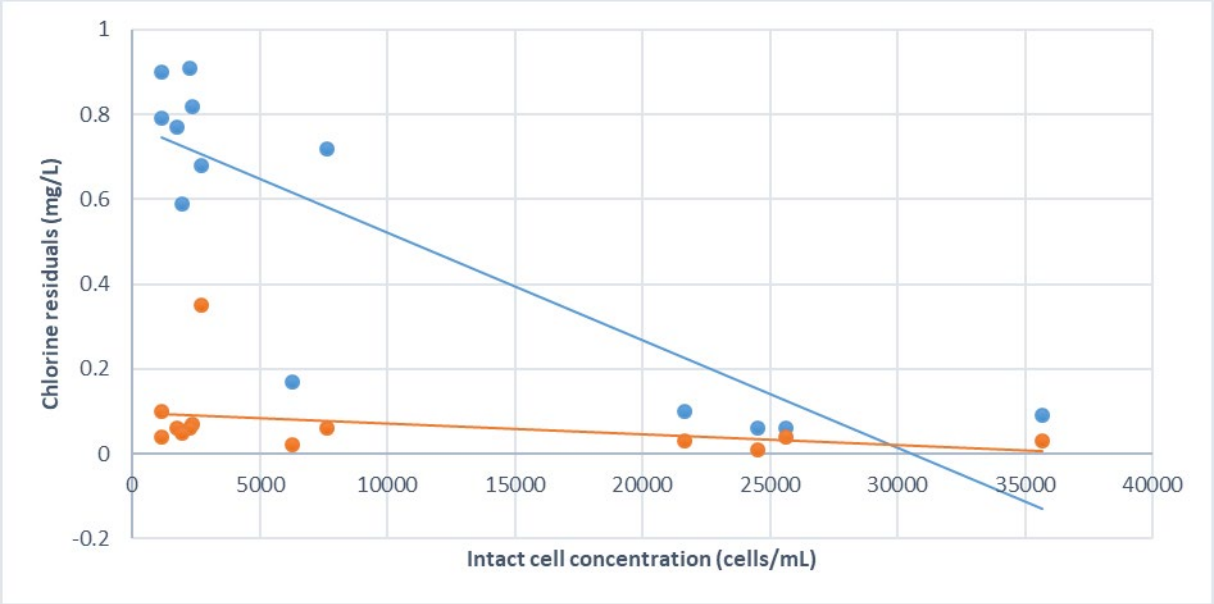

Figure 3.19: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for local point Silverton over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

3.4 SUMMARY

A poor correlation was observed between the plate concentrations on the two different media when plots were drawn to observe any trends. The results show sample point specific trends and higher concentrations are observed on R2A media than YEA media. The large distribution network sample points collected from reservoirs points and a point post-treatment displayed a more clustered pattern. Five of the six sampling locations had no apparent correlations between the media.

The plate concentrations and intact cell concentrations had no clear correlation, in some cases, a high plate concentration value corresponded to a high intact cell concentration while some values were both low which is what would be expected for a positive correlation between the datasets. Sample points AgricBuild; NS2; Silverton; Valhalla; Ben-Res2-IN and Ga-Luka had points that were spread out with no apparent correlations in the data. The sporadic trends as seen with the total and intact cell concentration trends over time can be seen in the flow cytometry concentration graphs.

The chlorine residual concentrations were overall quite low in free chlorine across all twelve points. The correlations observed were low in most of the samples and trends showing any correlations were sample-specific. The large distribution network samples had higher total chlorine residual concentrations and there were no clear correlations. The point of use sampling locations was overall low in free chlorine levels, some points that had a high chlorine residual concentration corresponded to low intact cell concentrations. No real correlation was observed but lower chlorine residual concentrations correlated to higher intact cell concentrations at some sampling location points.

CHAPTER 4: SUMMARY AND CONCLUSIONS

4.1 BASELINE FLOW CYTOMETRY BACTERIAL CELL CONCENTRATIONS AND HETETROPHIC PLATE COUNT DATA

4.1.1 Flow cytometry

FCM data showed higher concentrations, more than tenfold per point, because HPC cannot culture all the bacteria present in the sample. In this study we were interested in the intact cell concentrations over time and not those damaged by treatment processes, hence the total cell concentrations were not used as their use originate from countries which do not use chlorine to treat their water (Van Nevel *et al*., 2013; 2016; 2017). Intact cell concentrations reflect the undamaged cells present in the sample and therefore the decrease in cell viability. For most of the sample points the TCC and ICC values were very similar indicating that most of the bacteria detected were viable. The only sample where a large difference between the TCC and ICC values were observed was for Brak-RS3 IN. The drop in ICC counts could be the effect of the recent introduction of chloramine to the system.

The ICC values ranged on average between $5x10^2$ cells/ml for the production sample up to 10^4 cells/ml for samples taken within the distribution network. For the reticulation samples the ICC values varied on average around 10^4 cells/ml. The only sampling point that had higher values was the AgricBuild with around 10^5 cells/ml. These slightly higher values could be linked to the fact that these samples were collected from a multi-storey building with a water holding tank on the roof. These tanks are known to impact negatively on the microbial quality of the water. AgricBuild also showed inconsistencies with the concentrations and in some instances the intact cell concentrations were higher than the total cell concentrations. The reason for the intact concentrations being higher may also be due to some interferences with the sample staining.

The flow cytometry results confirmed the presence of diverse communities of culturable and uncultivable bacteria. FCM can be used as a valuable process indicator to provide additional information at each sample point. FCM can also rapidly detect significant changes or clear trends linked to these communities as was demonstrated for the Ga-Luka sampling site. A gradual increase could be noticed between August and October with the onset of warmer temperatures as well as a spike of nearly two log values as seen in Jan 2019.

Most of the trends observed in the absolute values of total and intact cell concentrations were sample point specific which is in line with what is reported in the literature. The sample-specific trends indicate that the establishment of a baseline database for each point is necessary to monitor any significant changes in the cell concentrations that may occur. Using standard criteria for detecting the flow cytometry concentrations across all locations would not be feasible as a result of location-specific changes and trends. Seven of the samples showed an increase in overall concentrations toward the end of the sampling period in the summer months. Warmer temperatures are generally noted to favour the growth of bacteria.

The flow cytometry concentrations were higher than the plate concentrations and showed a more realistic representation of the bacterial community abundances at the sampling locations. This is expected as the plates do not culture all bacteria present in the community and not all viable cells are culturable. When looking at the concentrations from a water quality perspective, the concentrations are reflective of the normal values and their variation. Any abnormal differences observed would be indicative of a problem at a location. Flow cytometry is said to be characteristic of the treatment processes, disinfectants used and viability of cells (Hammes *et al*., 2008, Van Nevel *et al*., 2017).

4.1.2 Heterotrophic plate concentrations

Drinking water utilities worldwide use HPCs or colony concentrations as an operational tool for management of water quality and monitoring treatment processes and microbial growth in the distribution system (Sartory, 2004). In literature, the concentrations were typically used to show trends and changes in water quality and aided in detection of issues in the system. The community detected through these concentrations are either non-hazardous microbes or indication of a source of contamination. The different temperatures and incubation times favour the growth of selected organisms.

The overall plate concentrations observed across all twelve sampling points were between 0-9000 CFU/mL on both media. The alert values stated was 5000 CFU/ml which was later re-evaluated and updated to <1000 CFU/ml in 2015 (SABS, 2015). The plate count media and method of inoculation, however, is not standardized across the world (Sartory, 2004; Safford and Bischel, 2019) and differences detected in drinking water systems are interpreted according to the local water utility guidelines and a “no abnormal change observed” approach (Sartory, 2004; Van Nevel *et al*., 2017). The SANS 241 also does not outline a specific media or method and following with that of Rand Water, it is assumed that SANS 241 was based on YEA HPCs. The high plate concentration values indicate that there was a reasonably large culturable bacterial community present in the system.

All YEA HPC concentrations were below the SANS 241. The highest concentrations, found at the distribution sample point Ga-Luka, were above the SANS 241 alert value recommended, but found on the R2A plates (SABS, 2015). This point was also interesting as the concentrations fluctuated drastically over time. These observations highlight that there might be a potential problem at this sample location. High plate concentrations may indicate disinfection and treatment failures or sporadic contamination events, which all affect the quality of drinking water at end-point (Allen *et al*., 2004; Sartory, 2004; Francisque *et al*., 2009). Of the other eleven sampling locations, AgricBuild, NS2, Silverton and Ben-Res2-IN had points with plate concentrations above the <1000 CFU/ml recommended and the water quality at specific points at these locations are not consistent in quality. The inconsistencies in the water quality may arise from the source water. Two of these locations were sampled from tap water which was sourced from the water storage tanks on the roof of the buildings and the seasonal variation in temperature as well as tank exposure to the environment, localised growth in plumbing systems and fixtures may have affected the high plate concentrations.

HPCs however only detect a fraction of the bacteria present in the sample because not all viable cells can be cultured on these general growth media (Allen *et al*., 2004; Hammes *et al*., 2008). The heterotrophic community is a heterogeneous bacterial community that can be cultured on different media. Hammes *et al*. (2008) stated that most media used for plate concentrations contain excessive amounts of nutrients. In contrast R2A media represents a low nutrient and carbon concentration environment compared to YEA and conditions are similar to that in drinking water (Allen *et al*., 2004; Reasoner *et al*., 1985). This medium is suitable for the growth of aquatic bacteria that grow in lower nutrient conditions. R2A is currently used by drinking water utilities in regions such as Europe, North America and Asia (Williams *et al*., 2004; Noble *et al*., 1991; Ng, 2018; Reasoner, 2004; Gensberger *et al*., 2015). For this reason, R2A was included in this study in addition to yeast extract agar. As expected, the R2A concentrations generally represented higher concentrations when compared to yeast extract agar concentrations across all sampling points. The R2A media used had higher plate concentrations than the YEA media by tenfold and therefore was consistent with literature and favoured the growth of bacteria in drinking water.

4.2 CORRELATIONS BETWEEN FLOW CYTOMETRY, HETEROTROPHIC PLATE COUNT AND WATER QUALITY DATA

To correlate plate concentrations to FCM concentrations, the intact cell concentrations were plotted against corresponding values of plate concentrations on R2A and YEA media in a scatter plot. Overall, the results showed no strong correlations of the concentrations. All the points had generally higher R2A concentrations as mentioned previously but FCM concentrations were almost 10 times higher than any of the plate concentrations observed. The results are consistent with what was seen in other studies with weak correlations between the two methods (Hoefel *et al*., 2003; Siebel *et al*., 2008; Nescerecka *et al*., 2014; Van Nevel *et al*., 2017, Cheswick *et al*., 2019). Hammes *et al*. (2008) stated that “From a practical perspective, HPCs results had a standard error of >30% compared with FCM results with a standard error of <5%. From a statistical perspective, the HPC method detects between 30 and 300 events to obtain a result, while FCM analysis detects between 40 and 20,000 events to obtain a result without any dilution required”. The viability of cells, however, should be considered. In this study, biochemical parameters such as the adenosine triphosphate concentrations and available carbon for bacterial proliferation were not collected due to the small scale of the study.

Gillespie *et al*. (2014) stated that “Chloramine tended to result in lower proportions of intact cells than chlorine over a wider residual range”. The locations studied were part of a chloramine treated network with high free chlorine concentrations and the intact cell concentrations are therefore relatively low. With respect to the water quality, the additional water quality parameters such as chlorine, pH and plate concentrations are a complement to ICC and TCC and deviations from baseline values established are helpful for indicating changes in the source water or contamination events (Gillespie *et al*., 2014). The expected trend was that the intact cell concentrations would decrease with an increase in the chlorine residual concentration and vice versa. The WHO recommends “a residual concentration of free chlorine of greater than or equal to 0.5 mg/litre after at least 30 minutes contact time at pH less than 8.0.” The alert values stated for residual chlorine levels according to SANS 241 lie between 0.2-0.5 mg/L. The levels of chlorine residuals considered appropriate the point of use samples is 0.5 mg/L and for the distribution line 0.2 mg/L, falling within the low range (SABS, 2015).

The CDC SWS Project suggests that “A free chlorine level of 0.5 mg/L can maintain the quality of water through a distribution network, but is not optimal to maintain the quality of the water when it is stored in the home in a bucket or jerry can for 24 hours.” After looking at the correlations between plate concentrations and corresponding intact cell concentrations, the correlation between the chlorine residuals and intact cell concentration was investigated to establish the relationship and understand how the levels of chlorine residuals affect the intact cell concentrations. The total chlorine residuals measured are a representation of the chlorine that has remained in the water after the chlorine demand of the water has been met. The free chlorine residuals represent the concentration of chlorine still available for further disinfection as the water travels through the system network. The local samples of chlorine residual concentrations were generally quite low.

In a chloraminated system, it was shown that the chlorine residual, which is measured, changes as the chlorine dose to the system increases under a constant ammonia concentration where chlorine is added to ammonia in a 5:1 ratio (Ward, 2013). The disinfectant residuals were shown to play an important role in the HPC numbers and these concentrations were shown to be the same in chlorinated and chloraminated distribution systems in a study done at The University of North Carolina (Zhang and DiGiano, 2002). Chloraminated systems may suffer from the loss chloramine residual caused by bacteria which oxidize ammonia (Van Nevel *et al*., 2017).

In this study, the correlation between plate concentrations on two different media and FCM intact cell concentrations at twelve different sampling point's part of either a distribution or reticulation network was investigated. A baseline database for these points was established in order to aid in the successful implementation of FCM as a valuable process parameter to monitor drinking water quality in South Africa. Additionally, the pH and chlorine residual data were collected to gain insight on the chemical properties of drinking water which influence the bacterial concentrations that affect the water quality. Each sampling point had site-specific trends associated with all the data collected and temporal trends were common across more than one sampling point.

4.3 CONCLUSIONS

The sampling points used to set a baseline database to help further study of the South African drinking water networks, and establish standardized protocols for successful implementation of FCM as a means of water quality monitoring as a ***process parameter***. The raw data plots of the highest cell concentrations detected across all sampling points showed clear background problems that are consistent along with all samples which need further work to refine. The findings are however sufficient going forward to help perfect the staining protocols used for chlorinated and chloraminated systems.

The main conclusions are that FCM data showed higher concentrations per point because HPC cannot culture all bacteria present in the sample. The trends observed in the data are sample point-specific. FCM can be used as a valuable ***process indicator*** coupled with HPC to provide more information per sample point and detect changes over time. It would be a good approach to make use of the R2A media using the 1 ml pour plate method as the preferred medium for HPC to analyse drinking water quality in South Africa. The sampling dataset needs to be expanded over a long time to make more definitive conclusions but the sample-specific trends may also be looked at individually and studied further to establish protocols that are better suited for producing clear FCM plots to carry out fingerprinting.

4.4 NEXT STEPS

In line with the aims and objectives of the study, the next investigation entails characterising the microbial community of the samples from both sites using 16S profiling to determine how it corresponds to the bacteria isolated using the HPC approach. This portion of work is presented in Volume II of this report.

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APPENDIX A: SUPPLEMENTARY DATA FOR MONITORING THE MICROBIAL COMMUNITY AT SELECTED DISTRIBUTION AND RETICULATION DRINKING WATER SAMPLE POINTS

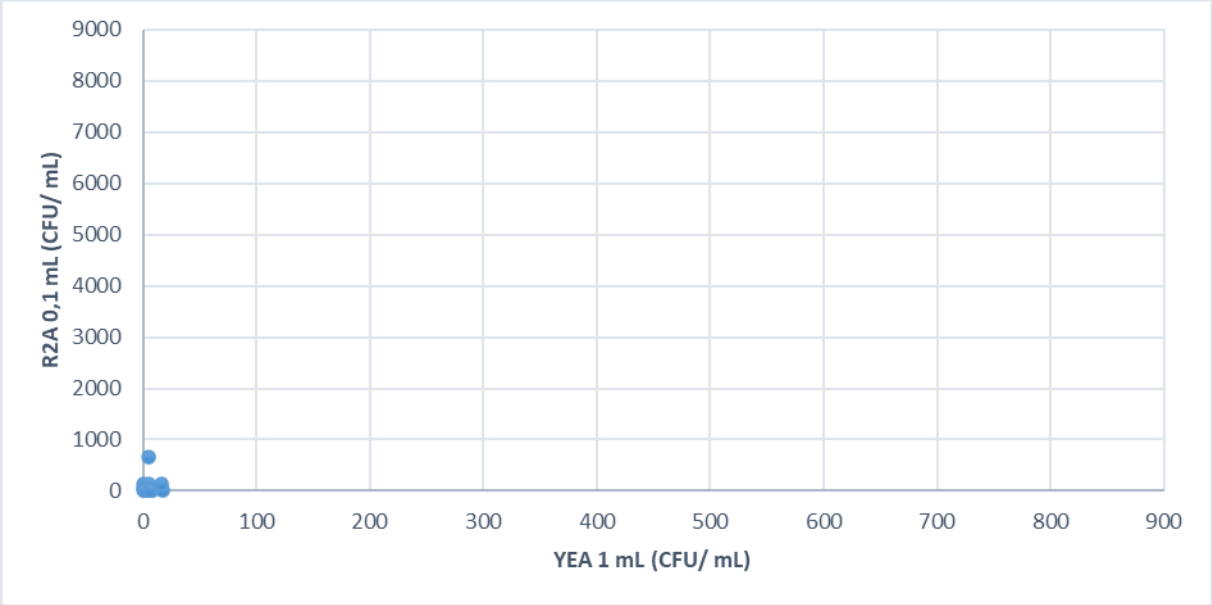

Figure A1: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for distribution point Brak-RS3-IN.

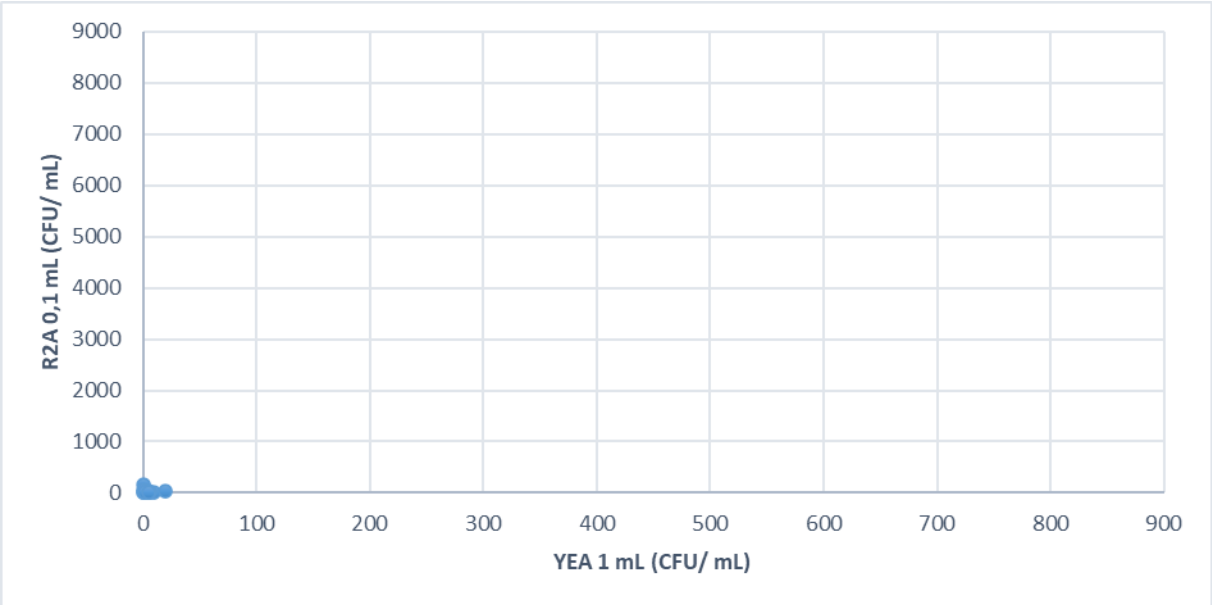

Figure A2: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for distribution point MB2.

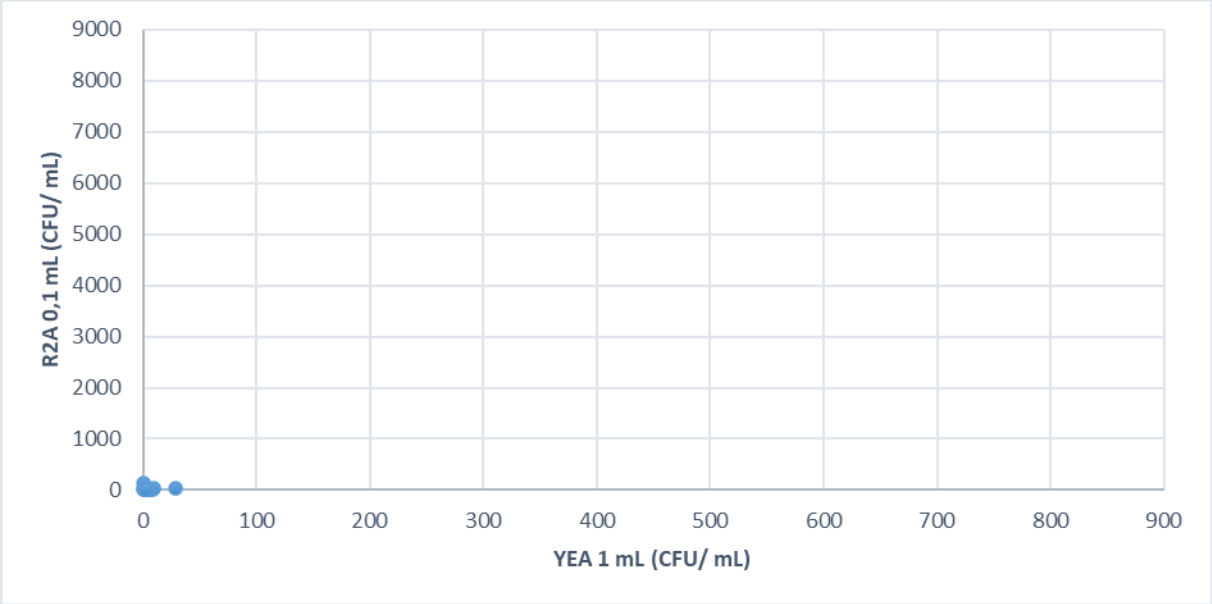

Figure A3: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for distribution point P4-PL.

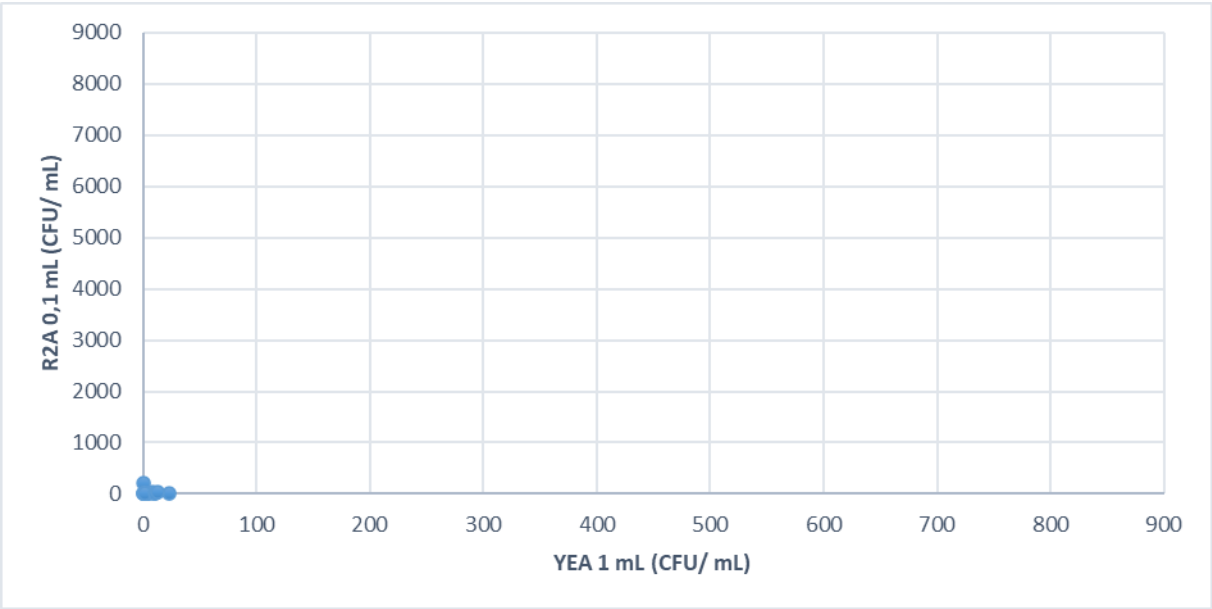

Figure A4: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for distribution point Rust-P6.

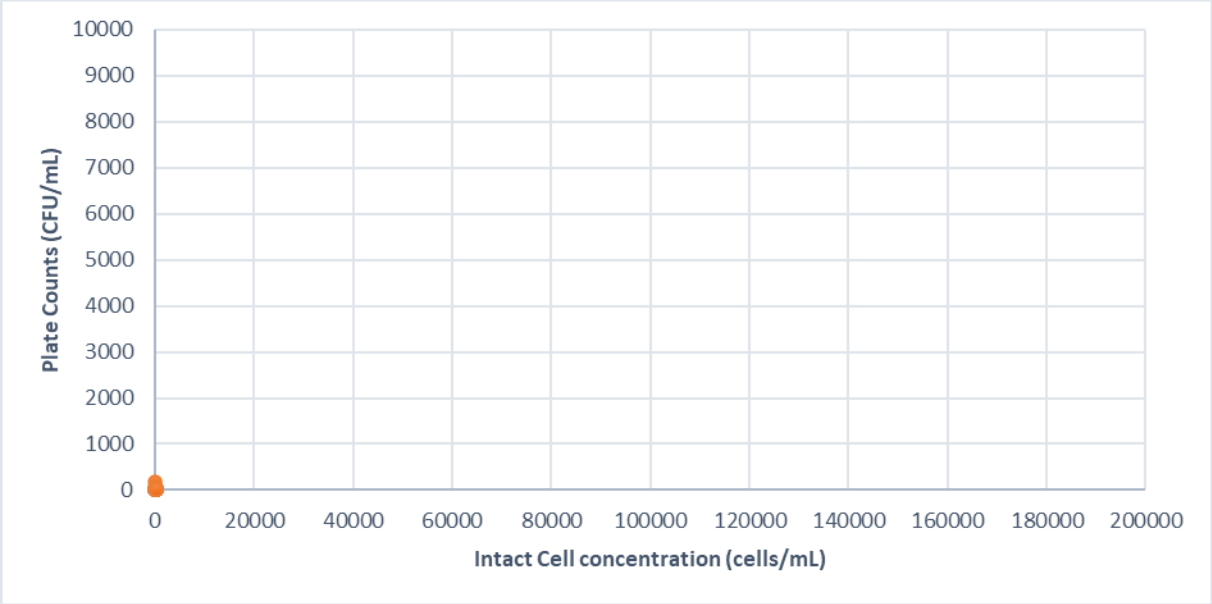

Figure A5: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for distribution point MB2 over the sampling period.

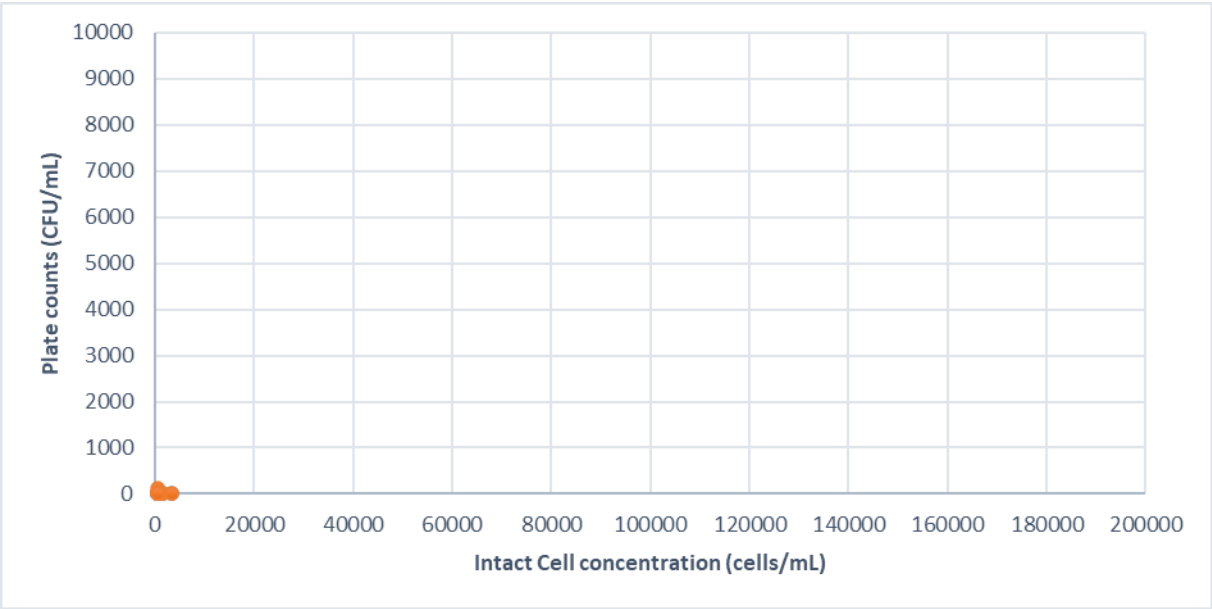

Figure A6: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for distribution point P4-PL over the sampling period.

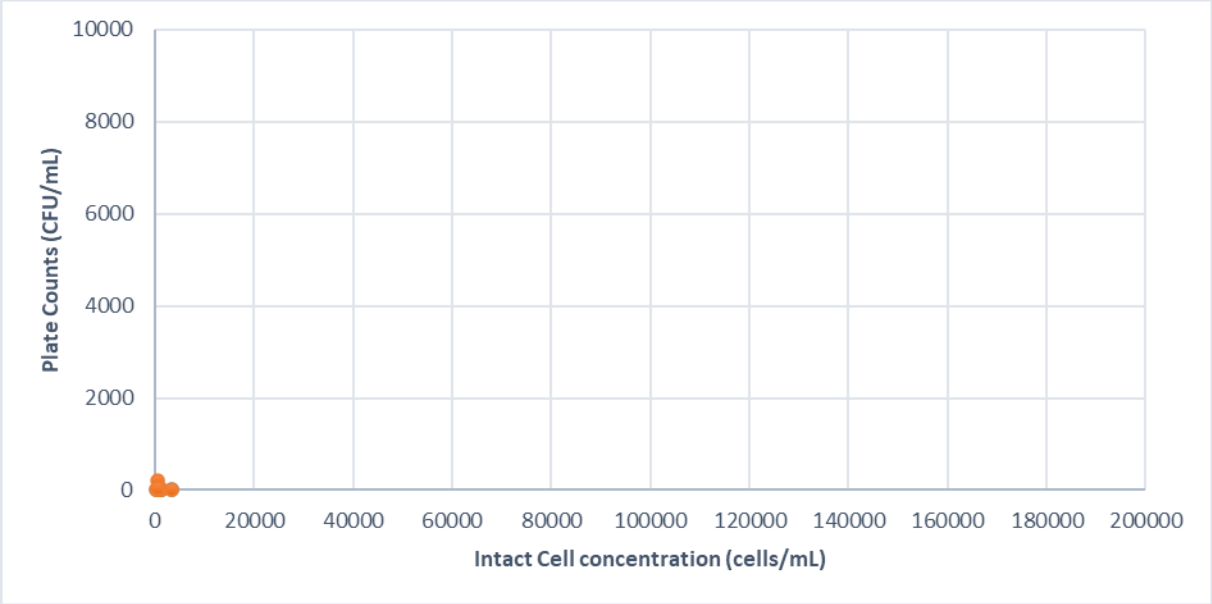

Figure A7: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for distribution point Rust-P6 over the sampling period.

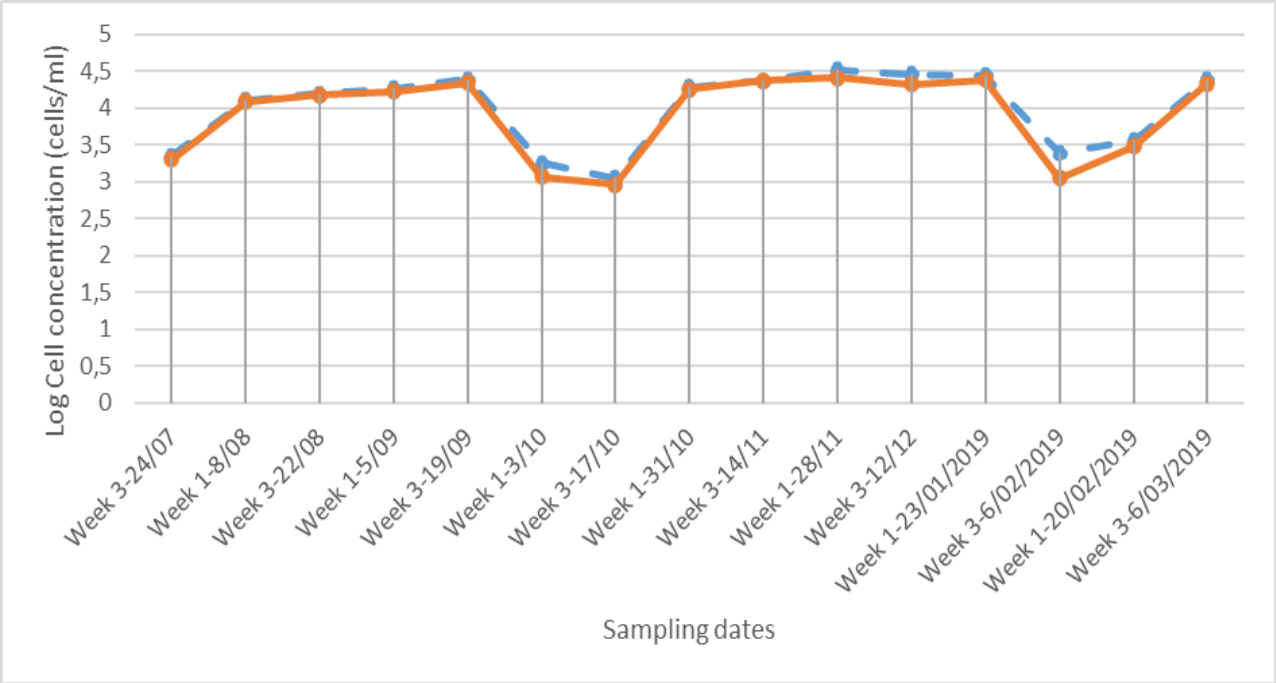

Figure A8: Graph representing the log value of raw total and intact cell concentrations for distribution point Ben-Res2-IN over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

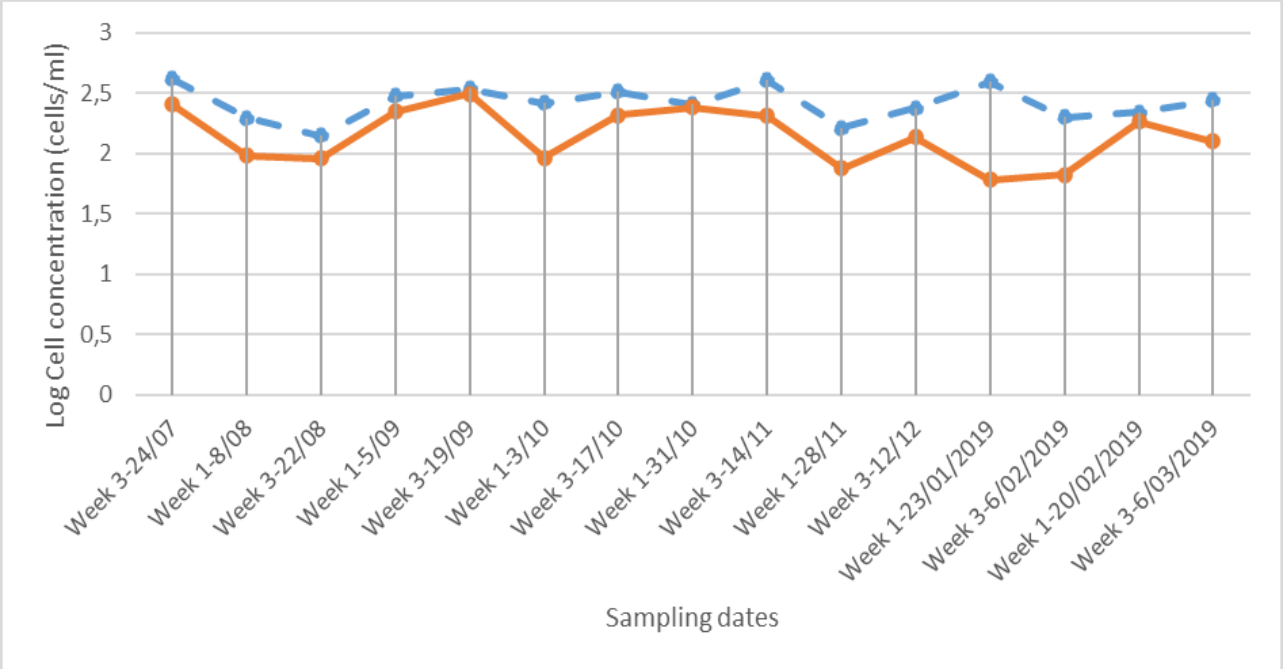

Figure A9: Graph representing the log value of raw total and intact cell concentrations for distribution point MB2 over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

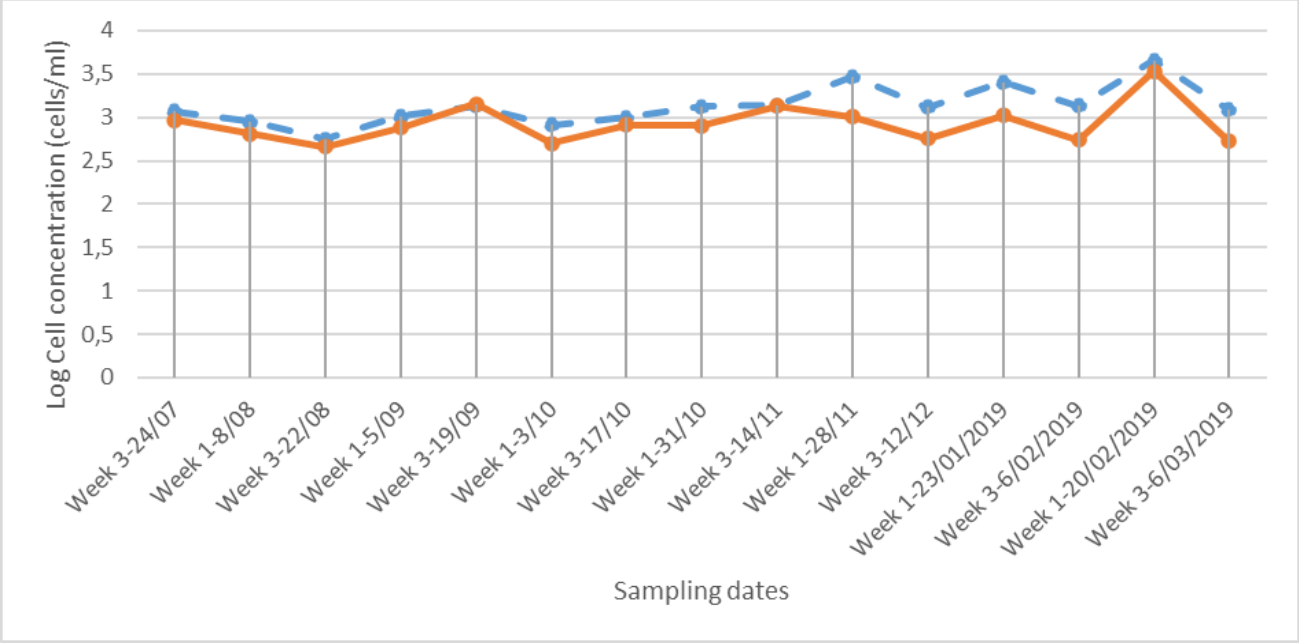

Figure A10: Graph representing the log value of raw total and intact cell concentrations for distribution point P4-PL over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

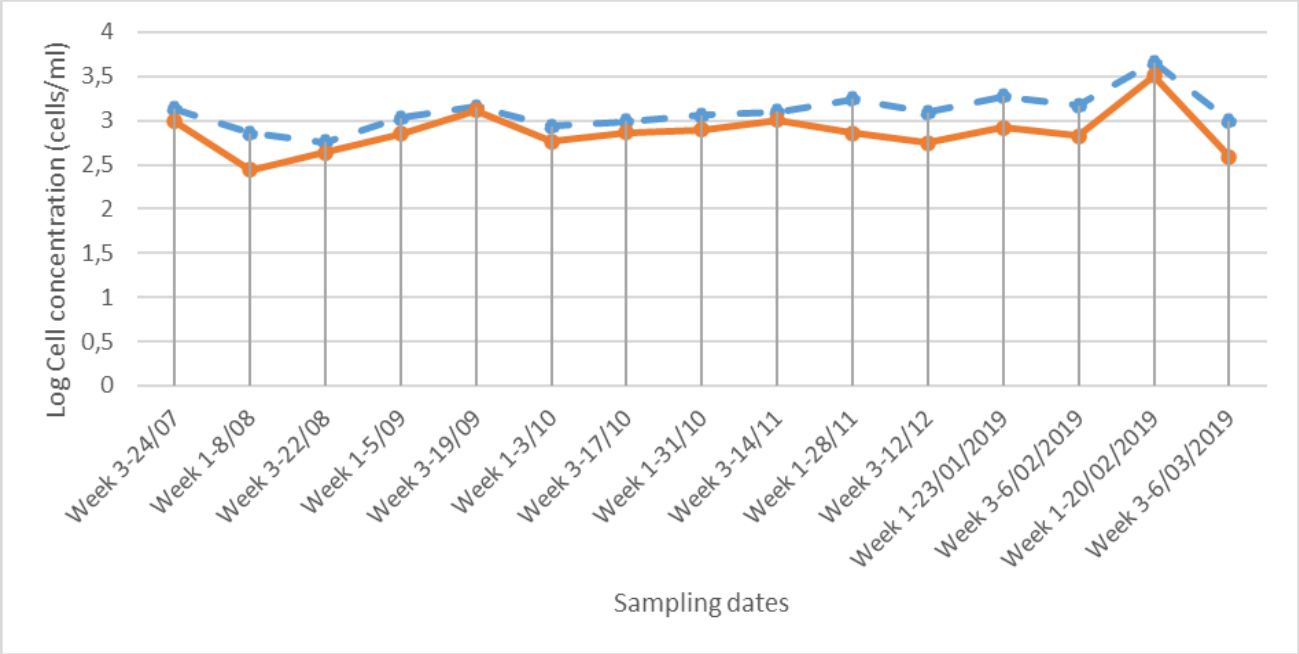

Figure A11: Graph representing the log value of raw total and intact cell concentrations for distribution point Rust-P6 over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

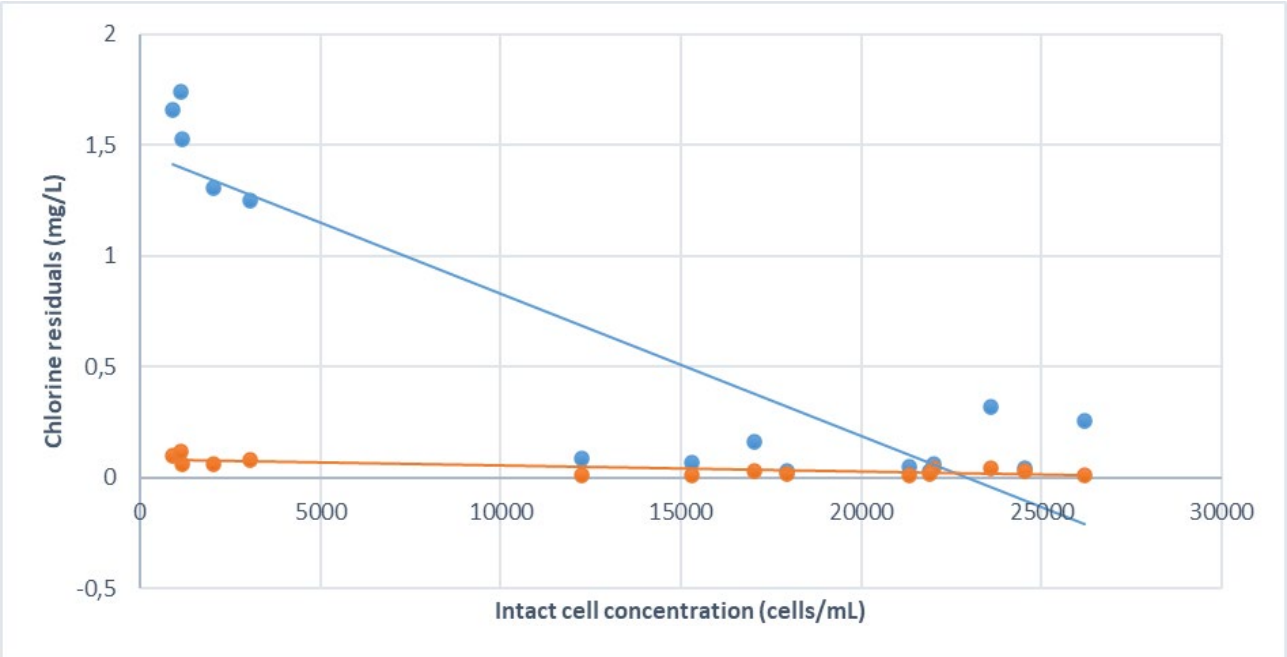

Figure A12: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for distribution point Ben-Res2-IN over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

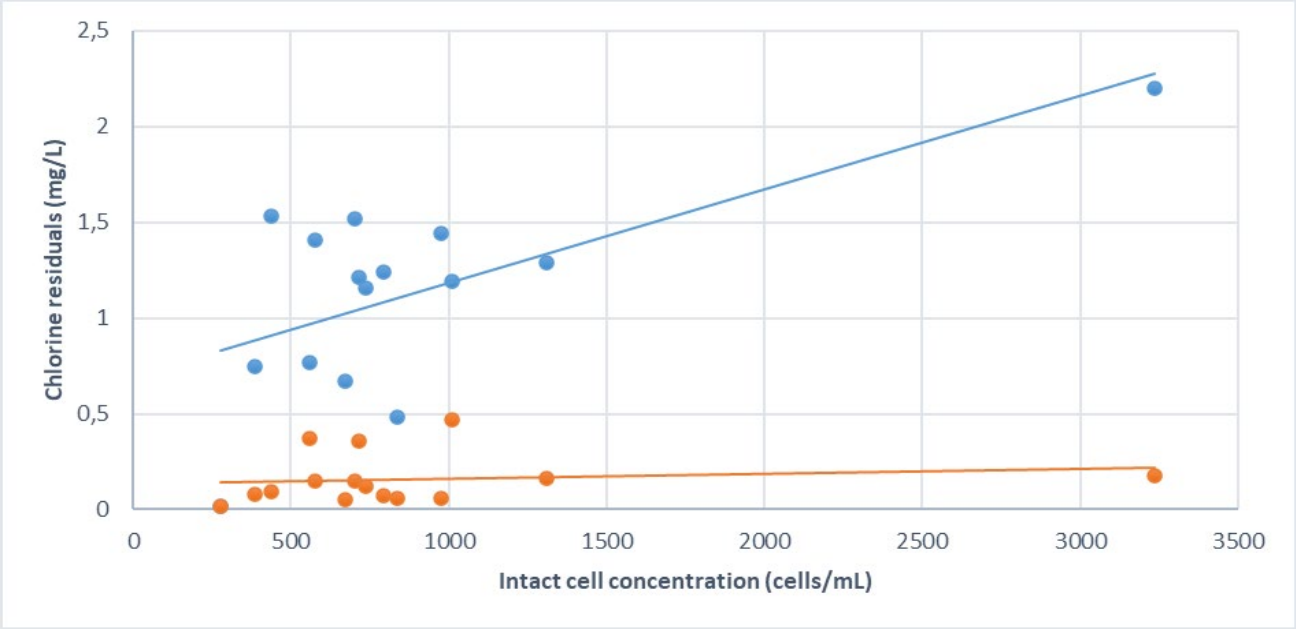

Figure A13: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for distribution point Rust-P6 over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

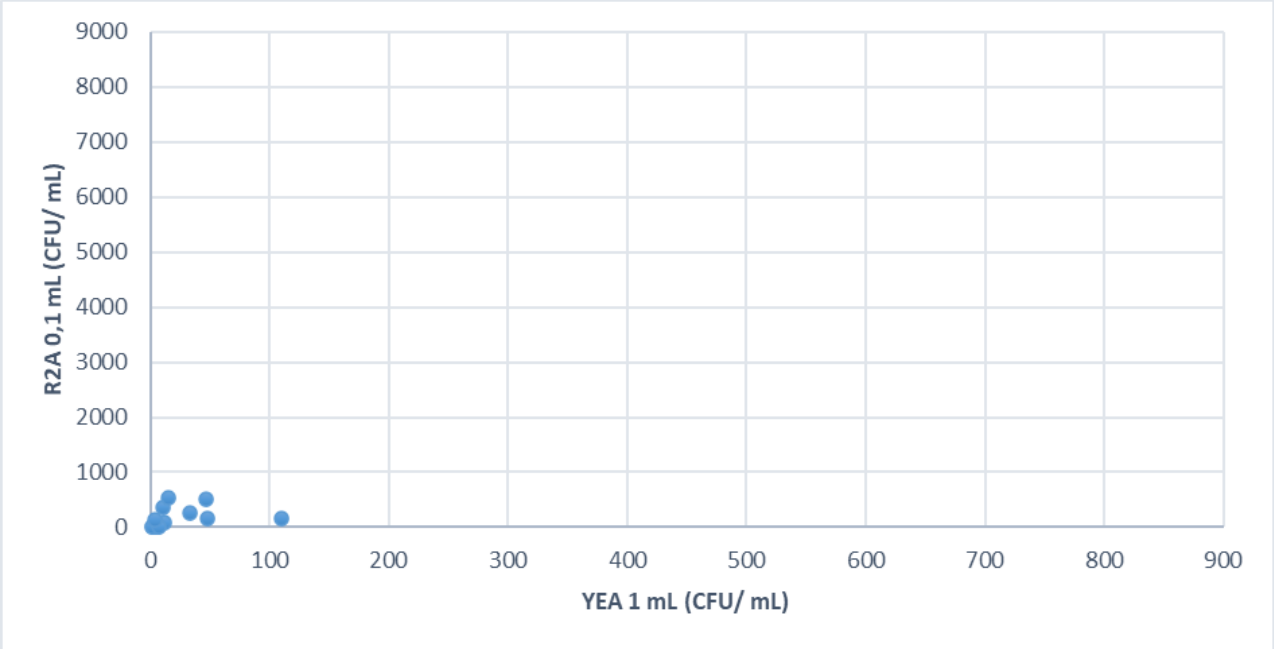

Figure A14: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for local point Groenkloof.

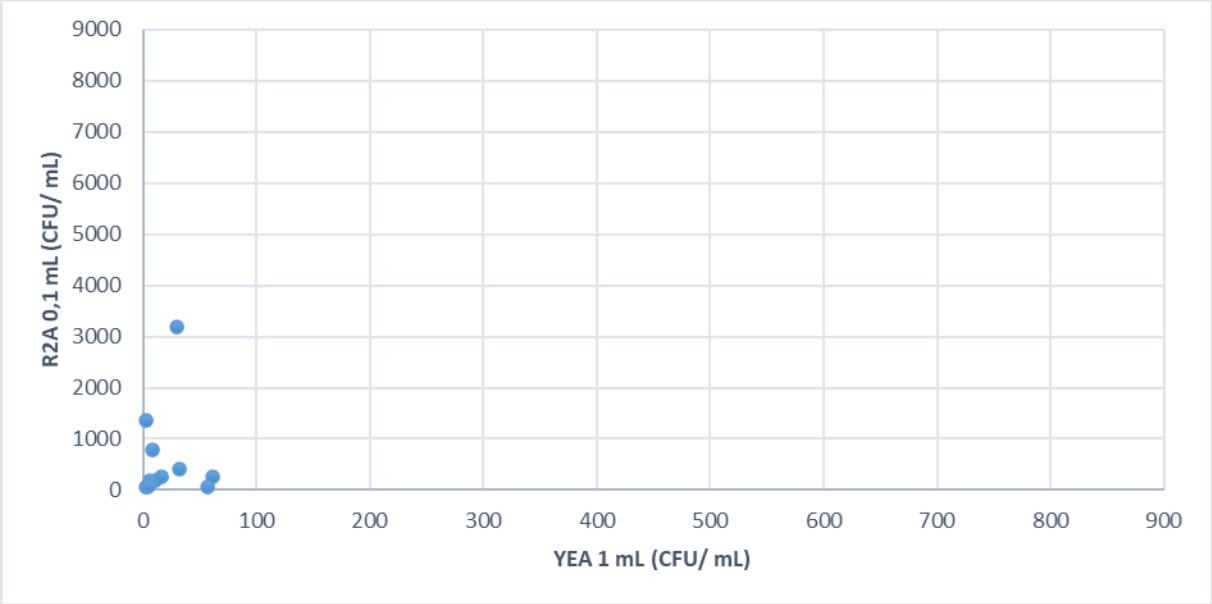

Figure A15: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for local point NS2.

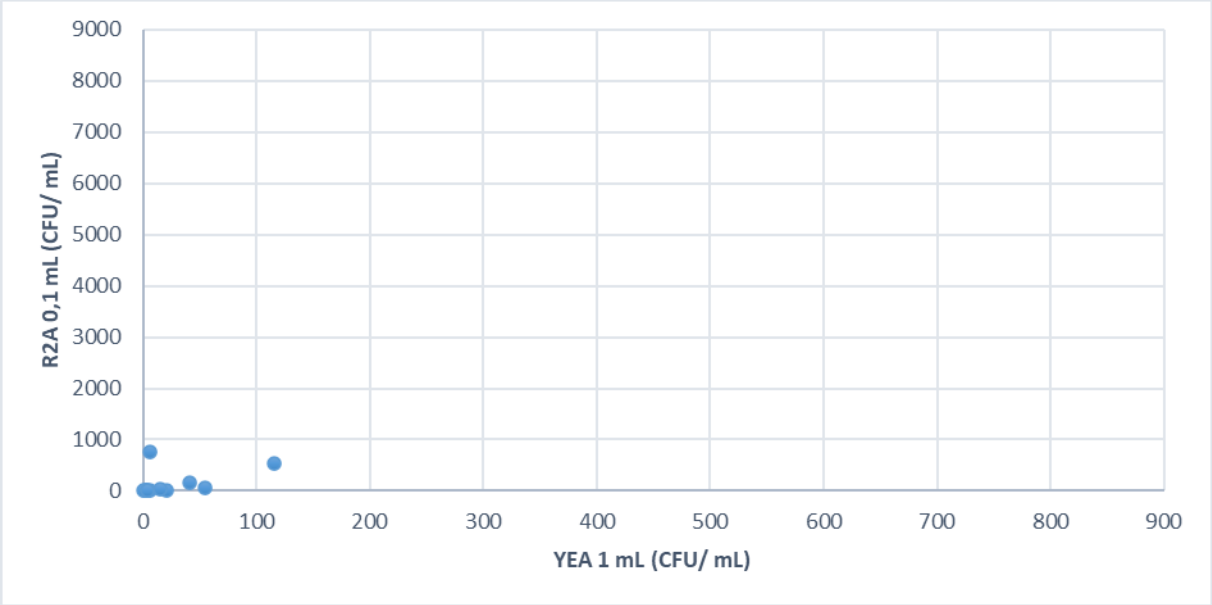

Figure A16: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for local point Valhalla.

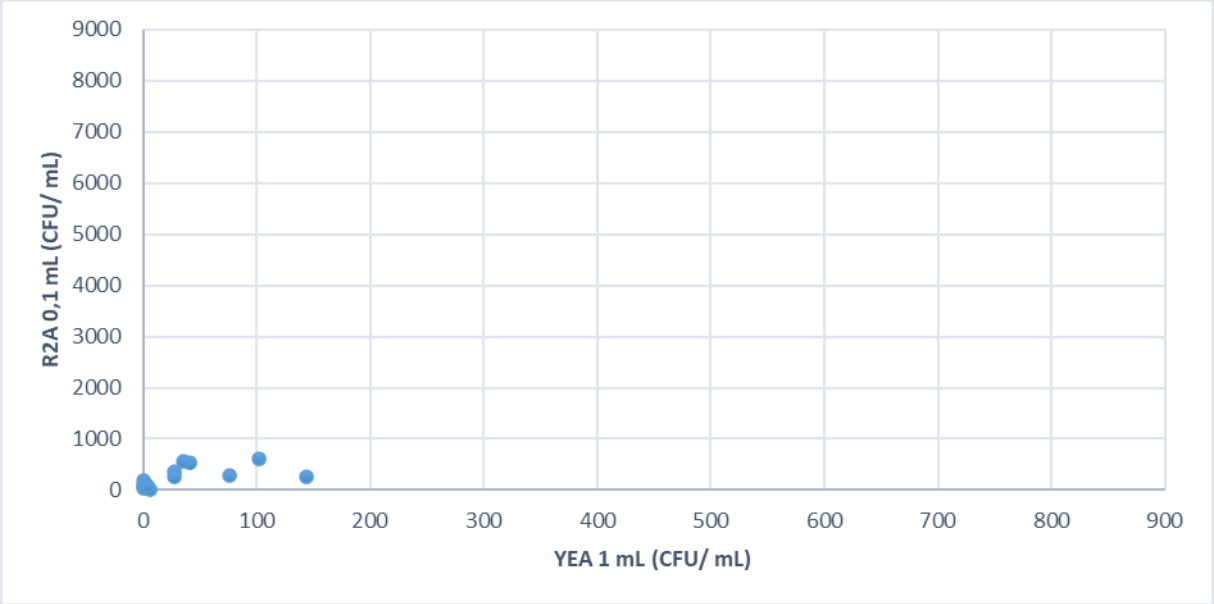

Figure A17: Scatter plot correlating plate concentrations (colony forming units (CFU) per ml) on Reasoner's 2 agar (R2A) and yeast extract agar (YEA) across the sampling period for local point Waverley.

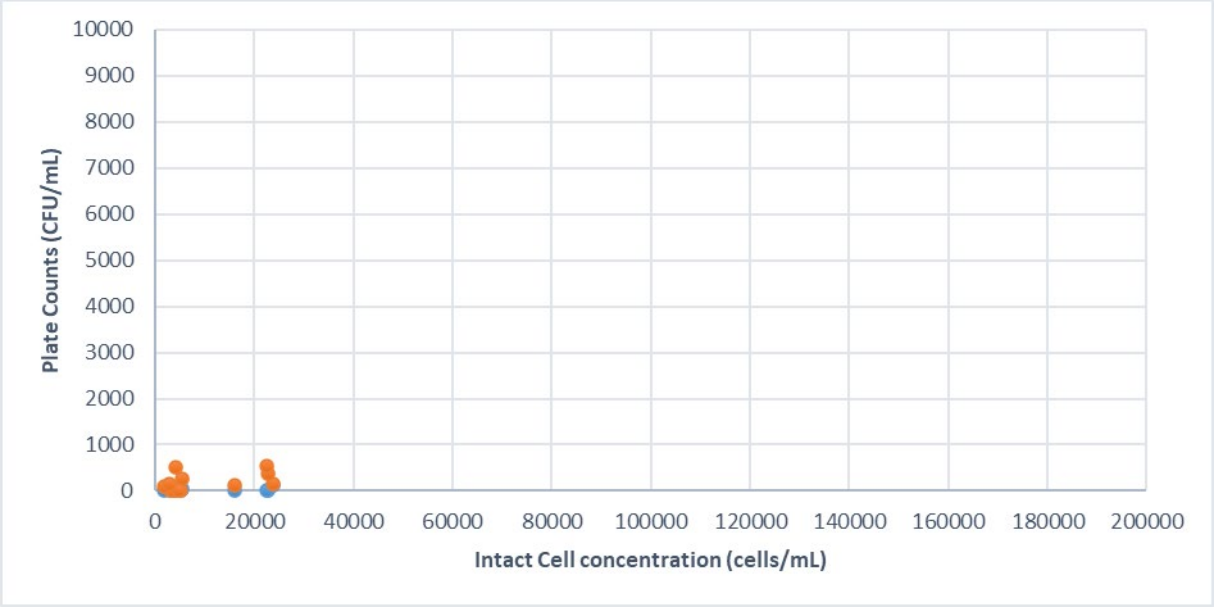

Figure A18: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for local point Groenkloof over the sampling period.

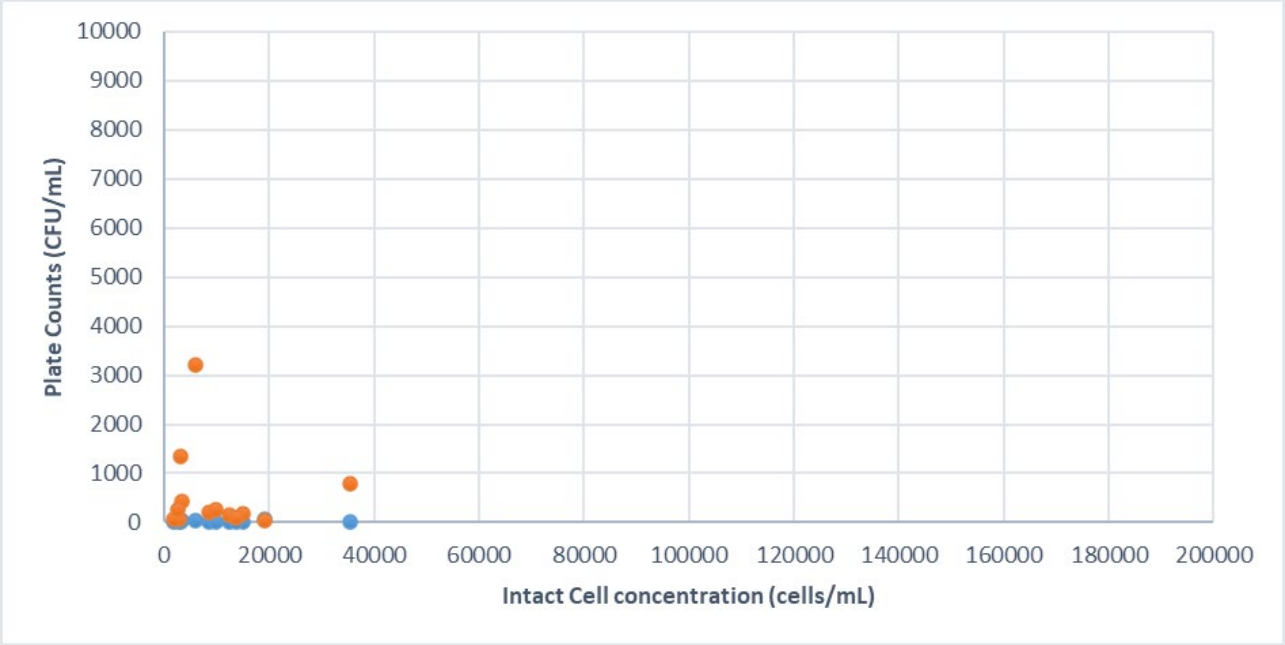

Figure A19: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for local point NS2 over the sampling period.

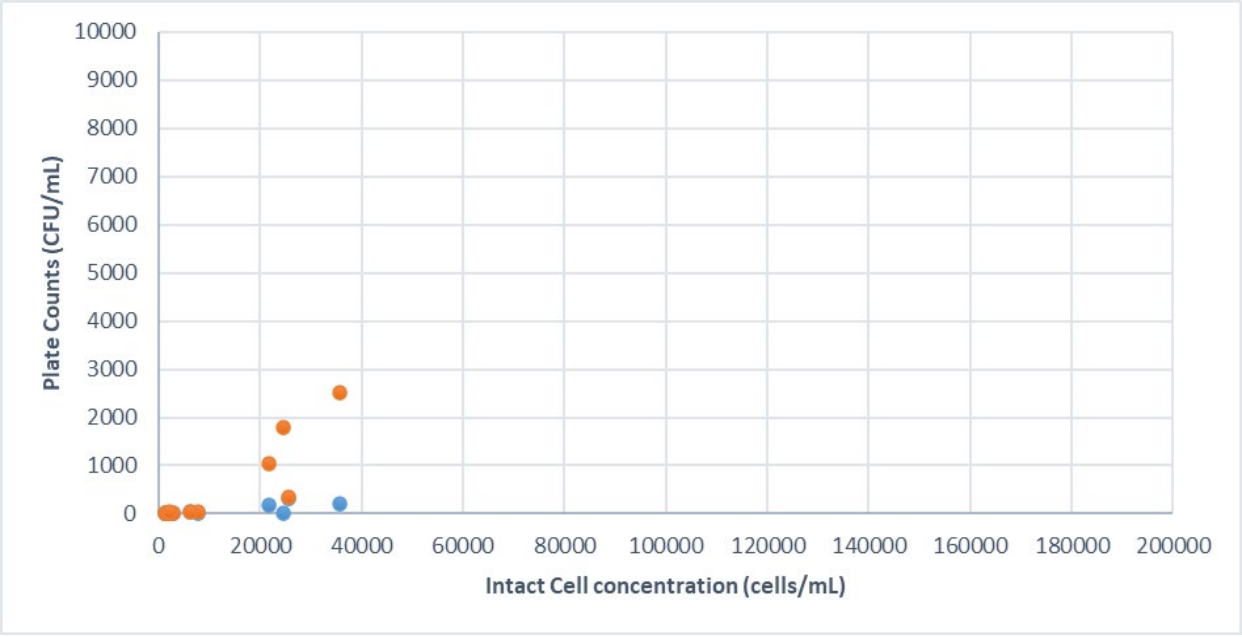

Figure A20: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for local point Silverton over the sampling period.

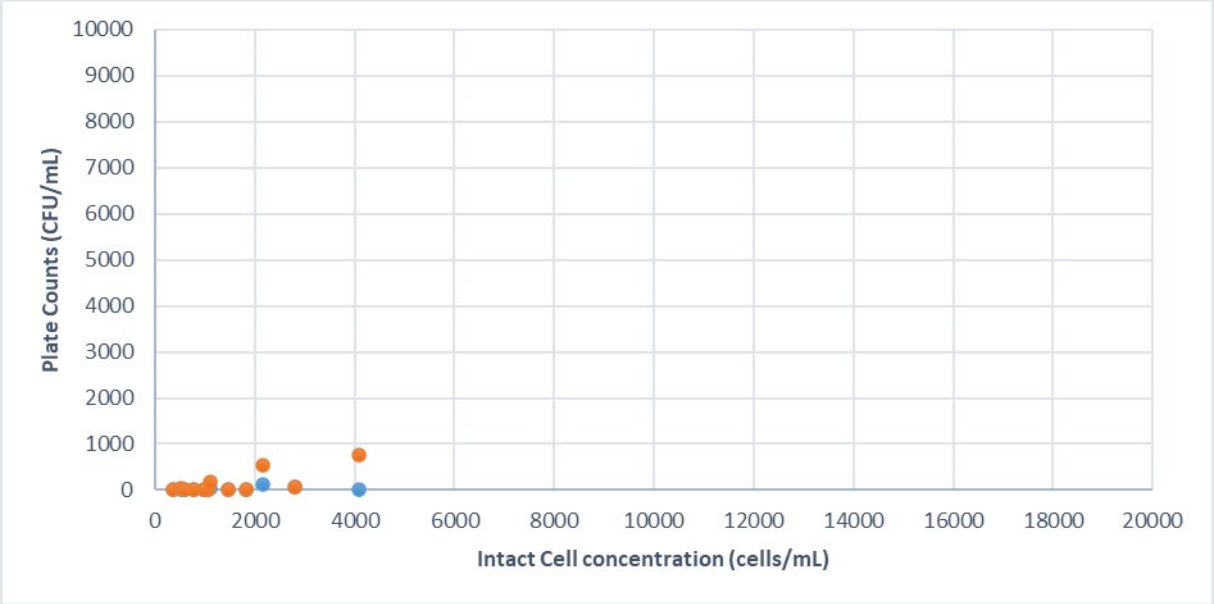

Figure A21: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for local point Valhalla over the sampling period.

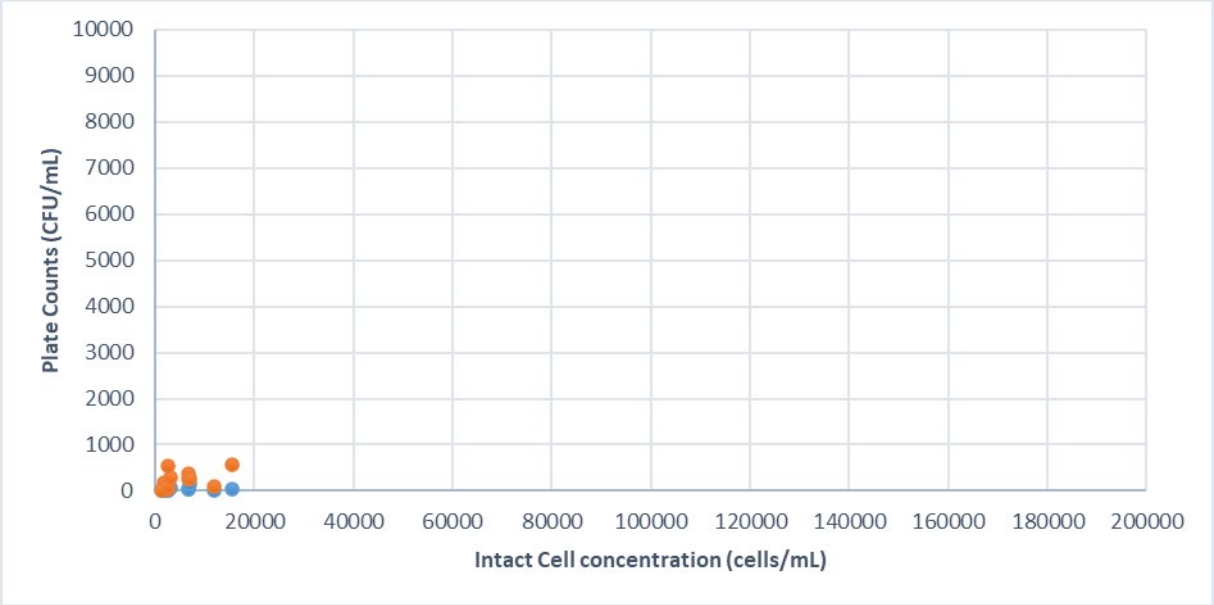

Figure A22: Scatter plot representing CFU/mL on YEA (Blue) or R2A (Orange) media against intact cell concentrations for local point Waverley over the sampling period.

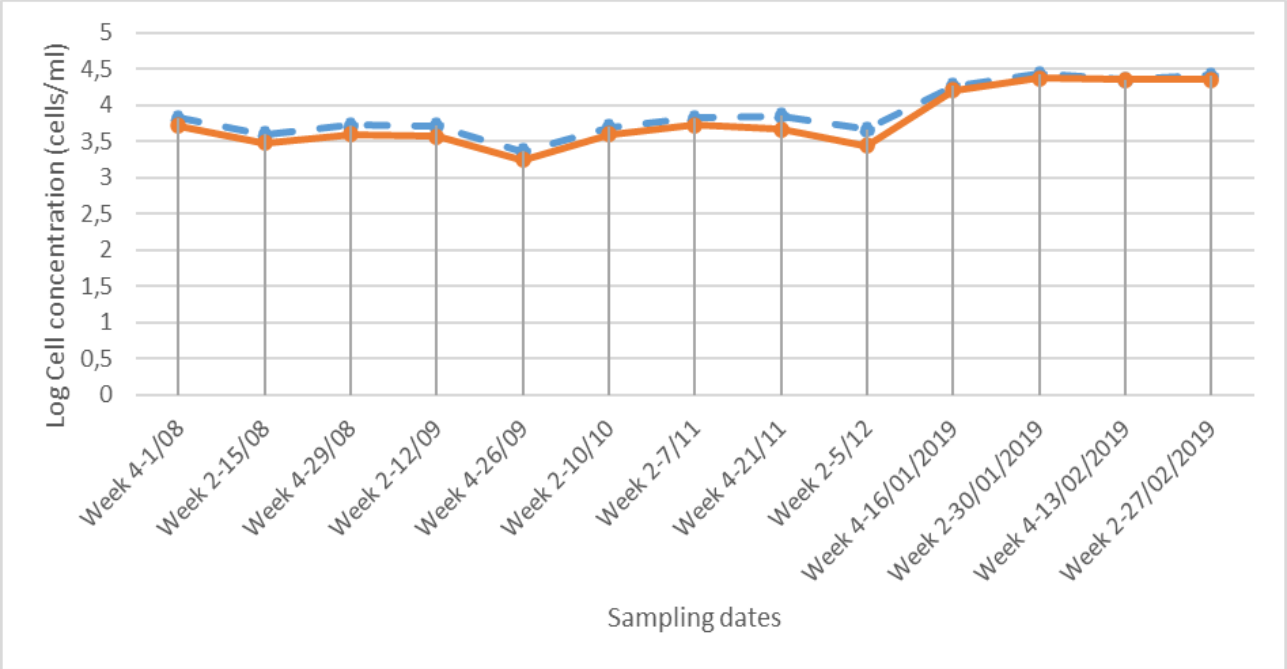

Figure 23: Graph representing the log value of raw total and intact cell concentrations for local point Groenkloof over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

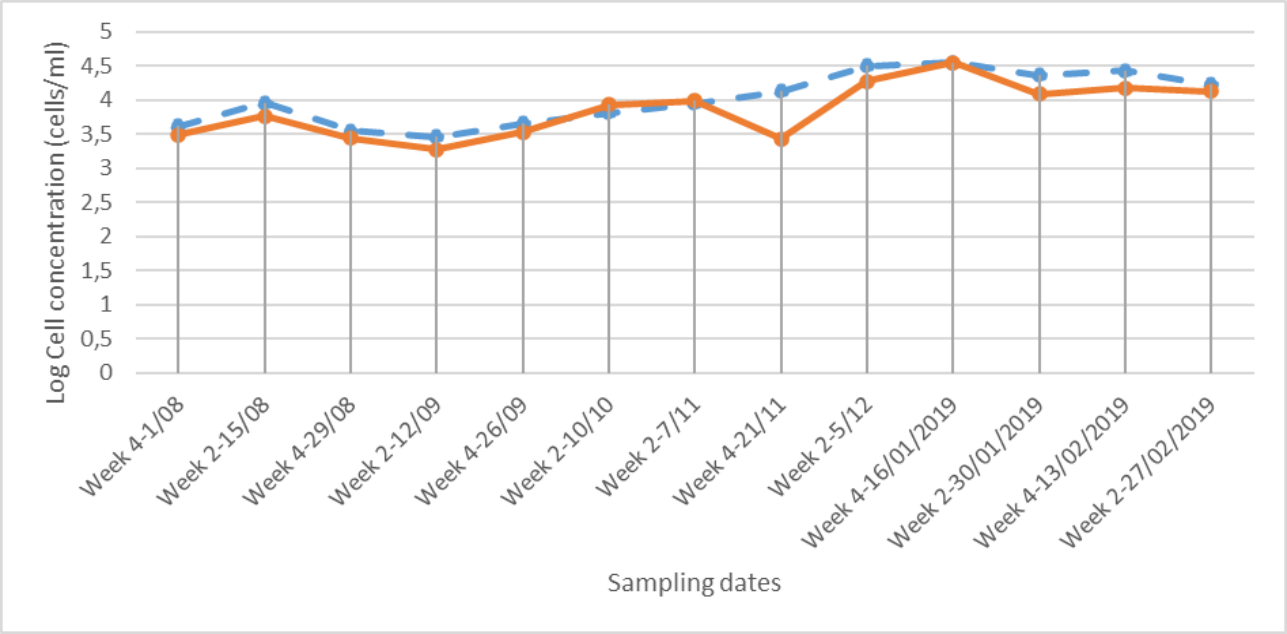

Figure A24: Graph representing the log value of raw total and intact cell concentrations for local point NS2 over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

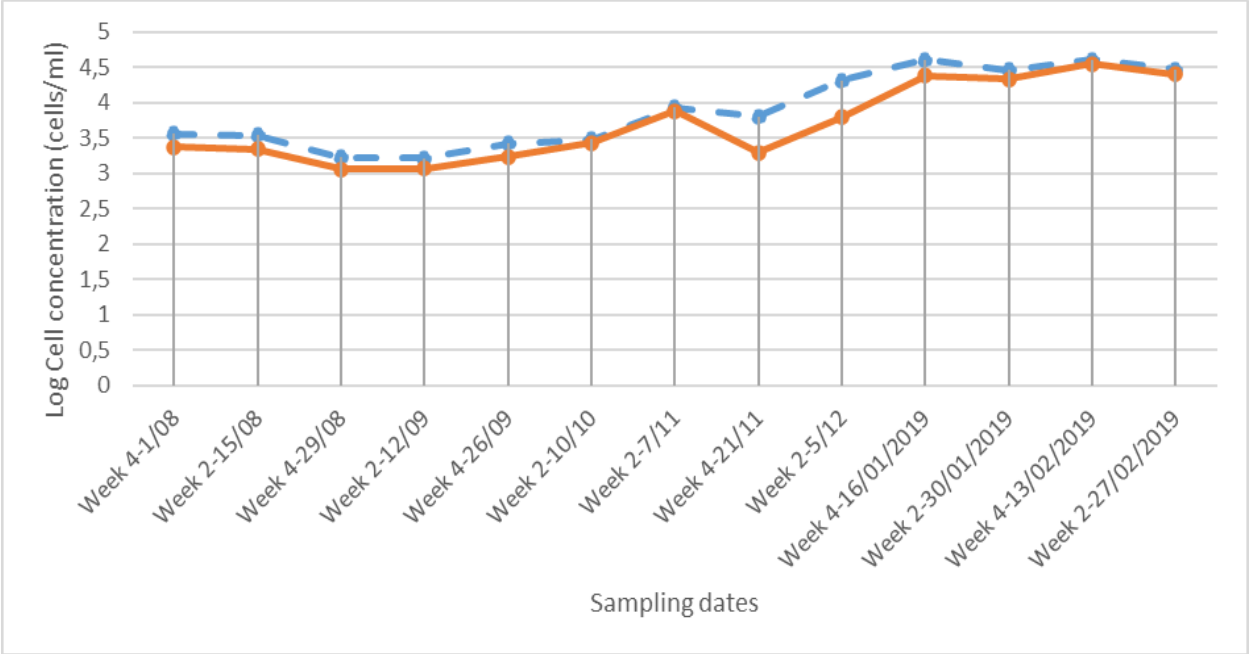

Figure A25: Graph representing the log value of raw total and intact cell concentrations for local point Silverton over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

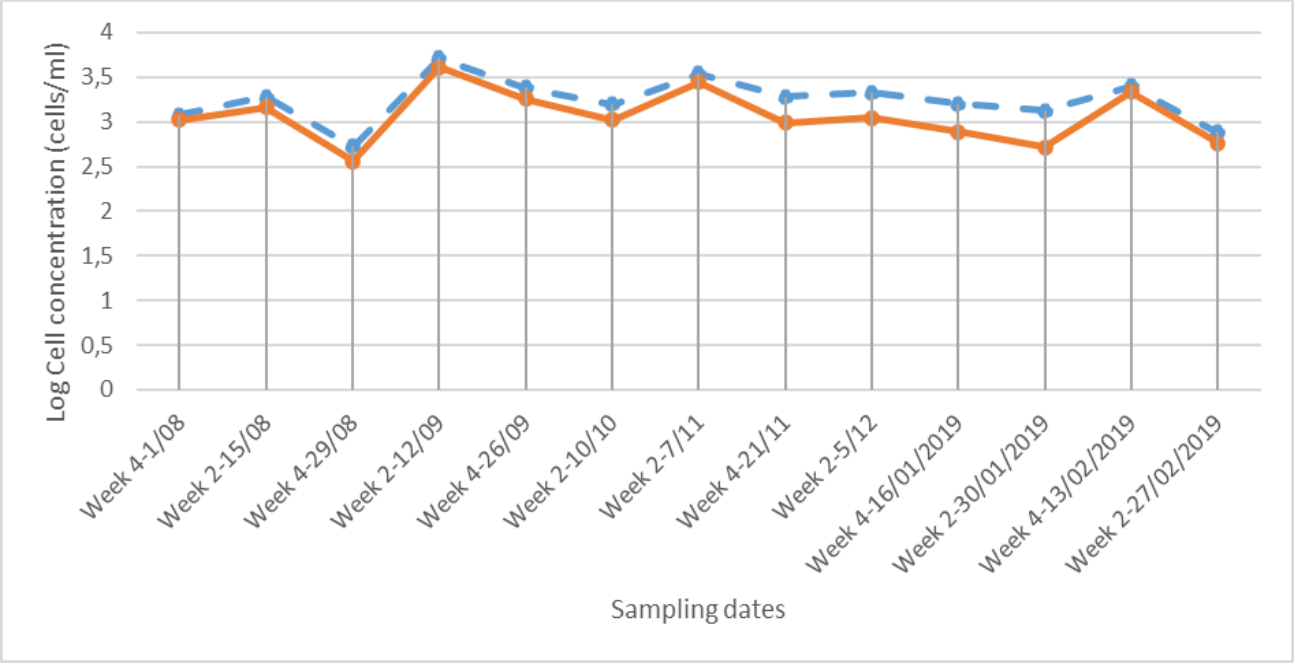

Figure A26: Graph representing the log value of raw total and intact cell concentrations for local point Valhalla over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

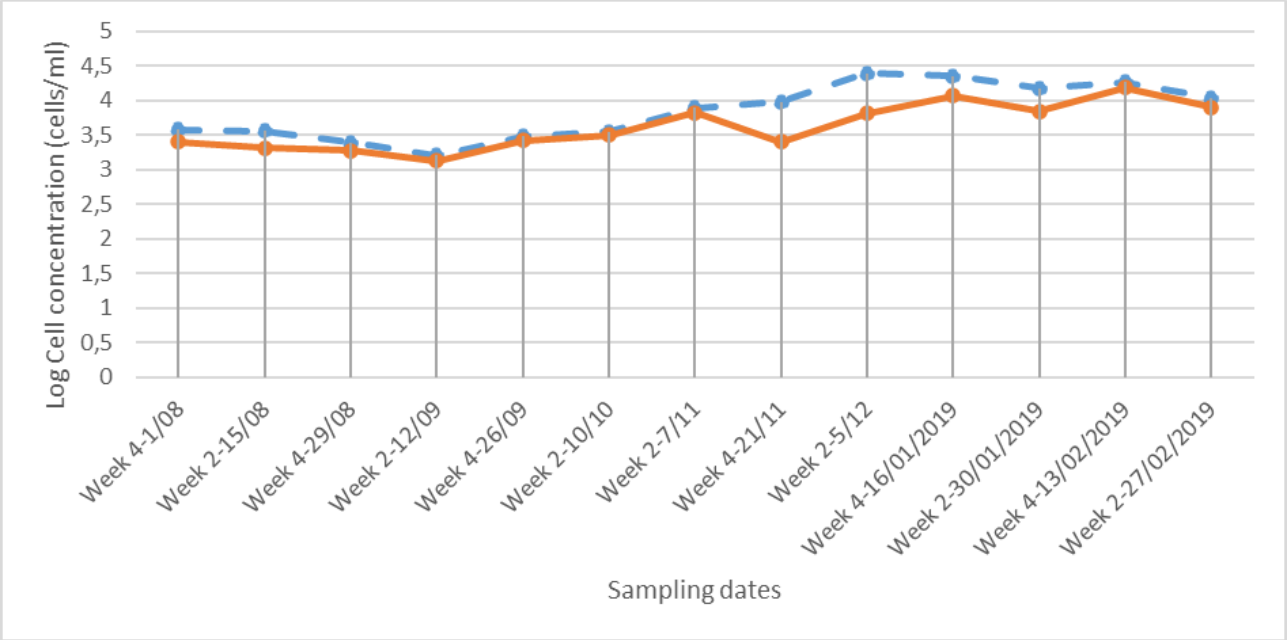

Figure A27: Graph representing the log value of raw total and intact cell concentrations for local point Waverley over the sampling period. Total cell concentrations detected by SYBR Green I represented by a dashed line and Intact cell concentrations detected by SYBR Green I and propidium iodide represented by a solid line.

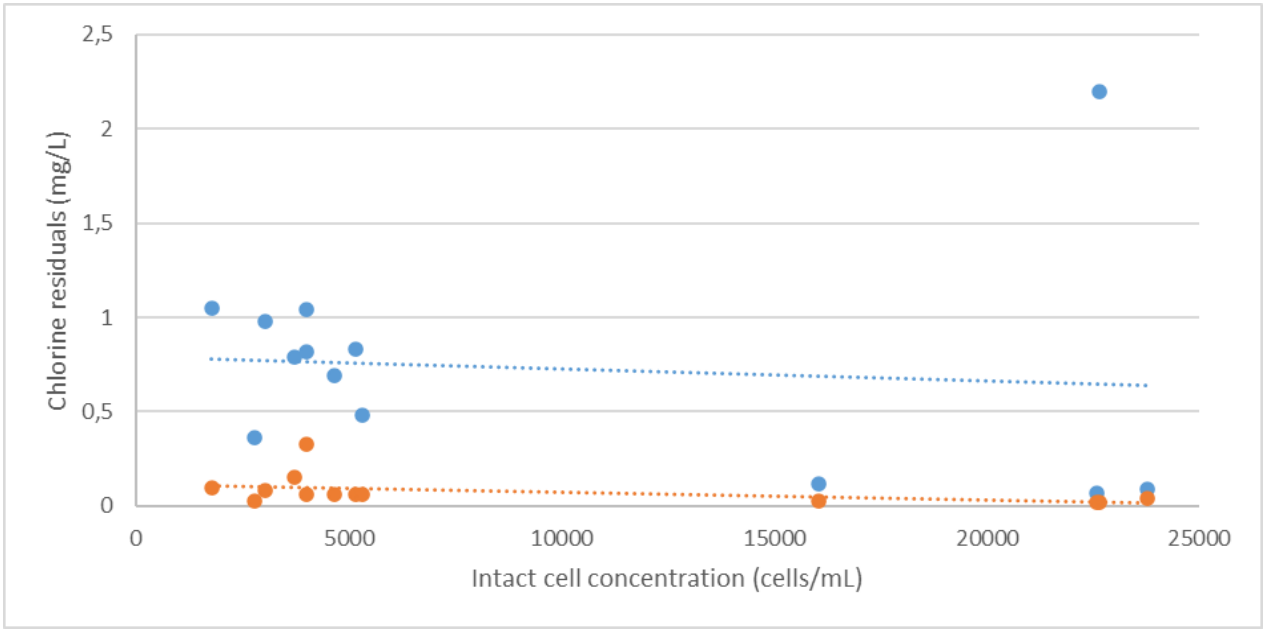

Figure A28: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for local point Groenkloof over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

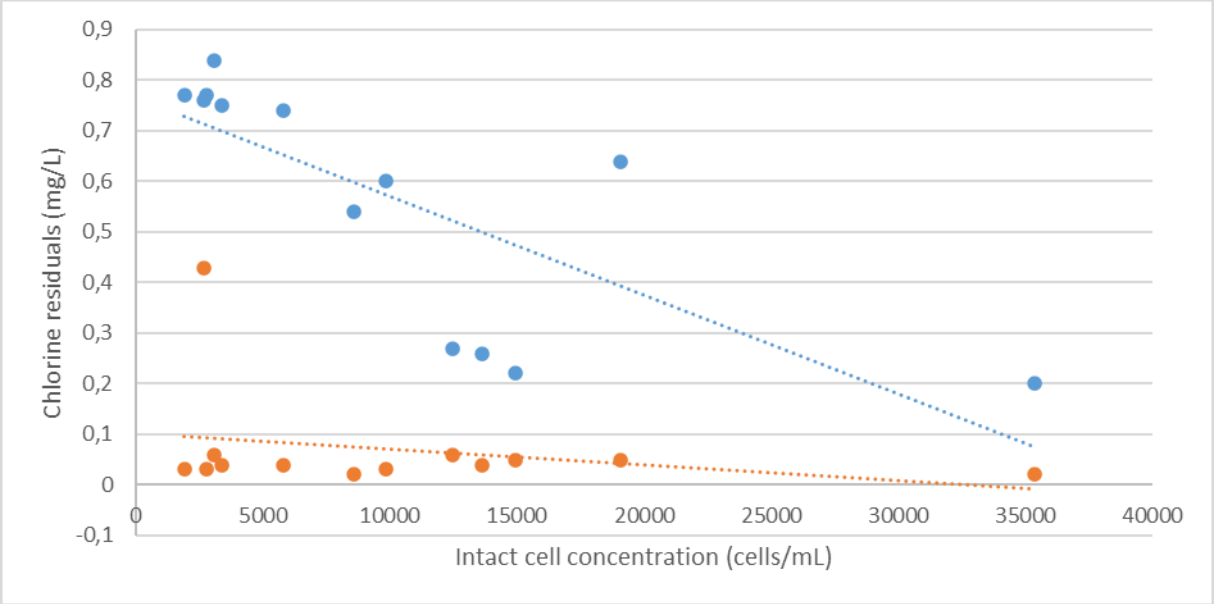

Figure A29: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for local point NS2 over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

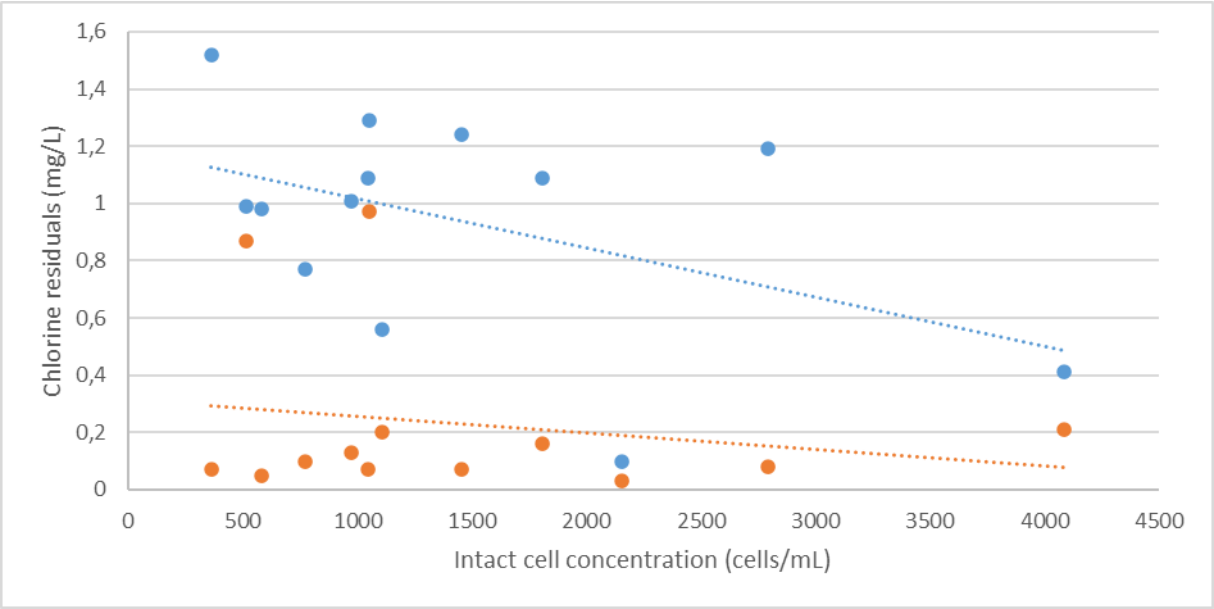

Figure A30: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for local point Valhalla over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

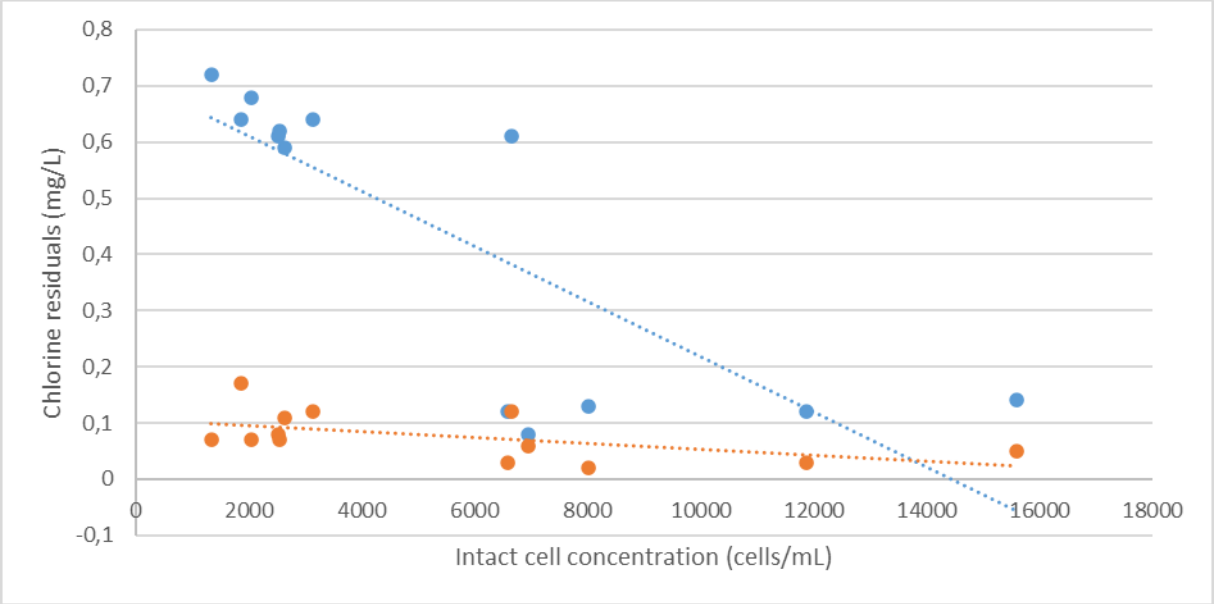

Figure A31: Scatter plot representing intact cell concentrations (cells/mL) for DPD total (blue) and free (orange) chlorine residual concentrations (mg/L) values for local point Waverley over the sampling period with simple regression analysis equations and R^2 values in the corresponding blocks.

Table A1: Raw data for chlorine residuals and pH results per sample over the sampling period

SAMPLE					
BEN-RES2-IN		**Cl analysis**			**pH**
		total	**free**	**combined chlorine**	
Month 1	Week 1-11/07	0,1	0,02	0,08	
	Week 3-24/07	1,31	0,06	1,25	8,78
Month 2	Week 1-8/08	0,09	0,01	0,08	7,26
	Week 3-22/08	0,07	0,01	0,06	8
Month 3	Week 1-5/09	0,16	0,03	0,13	8,14
	Week 3-19/09	0,06	0,04	0,02	7,88
Month 4	Week 1-3/10	1,53	0,06	1,47	8,12
	Week 3-17/10	1,66	0,1	1,56	8,18
Month 5	Week 1-31/10	0,03	0,02	0,01	7,77
	Week 3-14/11	0,32	0,04	0,28	8,02
Month 6	Week 1-28/11	0,26	0,01	0,25	7,91
	Week 3-12/12	0,05	0,01	0,04	7,89
Month 7	Week 1-23/01/2019	0,04	0,03	0,01	8,16
	Week 3-6/02/2019	1,74	0,12	1,62	8,25
Month 8	Week 1-20/02/2019	1,25	0,08	1,17	8,51
	Week 3-6/03/2019	0,03	0,02	0,01	8,28
BRAK-RS3-IN					
Month 1	Week 1-11/07	0,12	0,03	0,09	
	Week 3-24/07	1,72	0,07	1,65	8,64
Month 2	Week 1-8/08	0,99	1,89	-0,9	8,03
	Week 3-22/08	1,73	0,22	1,51	8,35
Month 3	Week 1-5/09	1,24	0,39	0,85	8,27
	Week 3-19/09	1,71	0,5	1,21	8,27
Month 4	Week 1-3/10	1,38	0,17	1,21	8,47
	Week 3-17/10	0,04	0,02	0,02	7,83
Month 5	Week 1-31/10	0,04	0,02	0,02	7,72
	Week 3-14/11	1,82	0,68	1,14	8,45
Month 6	Week 1-28/11	1,76	0,91	0,85	8,26
	Week 3-12/12	0,06	0,01	0,05	7,84
Month 7	Week 1-23/01/2019	0,11	0,03	0,08	8,34
	Week 3-6/02/2019	0,07	0,02	0,05	7,91
Month 8	Week 1-20/02/2019	0,06	0,02	0,04	8,28
	Week 3-6/03/2019	1,79	0,12	1,67	8,72
GA-LUKA					
Month 1	Week 1-11/07	0,21	0,03	0,18	
	Week 3-24/07	0,98	0,05	0,93	8,11
Month 2	Week 1-8/08	1,48	0,19	1,29	8,31
	Week 3-22/08	1,12	0,36	0,76	8,2

Month 3	Week 1-5/09	0,14	0,03	0,11	8,23
	Week 3-19/09	0,14	0,04	0,1	7,86
Month 4	Week 1-3/10	0,16	0,06	0,1	7,58
	Week 3-17/10	0,82	0,44	0,38	7,67
Month 5	Week 1-31/10	0,36	0,12	0,24	7,81
	Week 3-14/11	0,7	0,06	0,64	7,83
Month 6	Week 1-28/11	0,6	0,09	0,51	7,76
	Week 3-12/12	0,42	0,04	0,38	7,7
Month 7	Week 1-23/01/2019	0,21	0,05	0,16	7,95
	Week 3-6/02/2019	0,34	0,12	0,22	7,18
Month 8	Week 1-20/02/2019	0,43	0,23	0,2	7,98
	Week 3-6/03/2019	0,29	0,04	0,25	8,13
P4-PL					
Month 1	Week 1-11/07	0,08	0,01	0,07	
	Week 3-24/07	1,45	0,09	1,36	8,42
Month 2	Week 1-8/08	1,59	0,44	1,15	8,61
	Week 3-22/08	1,37	0,15	1,22	8,03
Month 3	Week 1-5/09	1,12	0,11	1,01	8,45
	Week 3-19/09	1,31	0,2	1,11	8,35
Month 4	Week 1-3/10	1,33	0,21	1,12	8,3
	Week 3-17/10	1,08	0,17	0,91	8,32
Month 5	Week 1-31/10	0,79	0,17	0,62	8,39
	Week 3-14/11	1,38	0,9	0,48	8,49
Month 6	Week 1-28/11	1,06	0,21	0,85	8,45
	Week 3-12/12	0,8	0,08	0,72	8,43
Month 7	Week 1-23/01/2019	0,53	0,09	0,44	8,69
	Week 3-6/02/2019	0,48	0,08	0,4	8,44
Month 8	Week 1-20/02/2019	0,35	0,1	0,25	8,57
	Week 3-6/03/2019	0,6	0,09	0,51	8,77
RUST-P6					
Month 1	Week 1-11/07	0,1	0,01	0,09	
	Week 3-24/07	1,44	0,06	1,38	8,48
Month 2	Week 1-8/08	0,02	0,02	0	8,71
	Week 3-22/08	1,53	0,09	1,44	8,47
Month 3	Week 1-5/09	1,52	0,15	1,37	8,5
	Week 3-19/09	1,29	0,16	1,13	8,19
Month 4	Week 1-3/10	1,41	0,15	1,26	8,16
	Week 3-17/10	1,16	0,12	1,04	8,35
Month 5	Week 1-31/10	1,24	0,07	1,17	8,32
	Week 3-14/11	1,19	0,47	0,72	8,47
Month 6	Week 1-28/11	1,21	0,36	0,85	8,42
	Week 3-12/12	0,77	0,37	0,4	8,36
Month 7	Week 1-23/01/2019	0,48	0,06	0,42	8,64

	Week 3-6/02/2019	0,67	0,05	0,62	8,32
Month 8	Week 1-20/02/2019	2,2	0,18	2,02	8,51
	Week 3-6/03/2019	0,75	0,08	0,67	8,65
MB2					
Month 1	Week 1-11/07	0,15	0,02	0,13	
	Week 3-24/07	0,75	0,53	0,22	8,63
Month 2	Week 1-8/08	0,78	0,56	0,22	8,57
	Week 3-22/08	0,77	0,54	0,23	8,45
Month 3	Week 1-5/09	0,61	0,36	0,25	8,62
	Week 3-19/09	0,25	0,06	0,19	8,15
Month 4	Week 1-3/10	0,33	0,17	0,16	8,39
	Week 3-17/10	0,55	0,36	0,19	8,45
Month 5	Week 1-31/10	0,53	0,29	0,24	8,36
	Week 3-14/11	0,62	0,21	0,41	8,47
Month 6	Week 1-28/11	0,69	0,35	0,34	8,43
	Week 3-12/12	0,67	0,33	0,34	8,54
Month 7	Week 1-23/01/2019	0,29	0,12	0,17	8,86
	Week 3-6/02/2019	0,4	0,2	0,2	8,52
Month 8	Week 1-20/02/2019	0,43	0,31	0,12	8,6
	Week 3-6/03/2019	0,53	0,41	0,12	8,91
NS2					
Month 1	Week 2-18/07	0,56	0,04	0,52	
	Week 4-1/08	0,84	0,06	0,78	8,42
Month 2	Week 2-15/08	0,74	0,04	0,7	8,06
	Week 4-29/08	0,77	0,03	0,74	8,2
Month 3	Week 2-12/09	0,77	0,03	0,74	8,22
	Week 4-26/09	0,75	0,04	0,71	8,23
Month 4	Week 2-10/10	0,54	0,02	0,52	8,22
Month 5	Week 2-7/11	0,6	0,03	0,57	7,94
	Week 4-21/11	0,76	0,43	0,33	8,48
Month 6	Week 2-5/12	0,64	0,05	0,59	8,05
	Week 4-16/01/2019	0,2	0,02	0,18	7,93
Month 7	Week 2-30/01/2019	0,27	0,06	0,21	8,02
	Week 4-13/02/2019	0,22	0,05	0,17	8,16
Month 8	Week 2-27/02/2019	0,26	0,04	0,22	8,4
VALHALLA					
Month 1	Week 2-18/07	0,93	0,07	0,86	
	Week 4-1/08	1,09	0,07	1,02	8,39
Month 2	Week 2-15/08	1,24	0,07	1,17	8,09
	Week 4-29/08	1,52	0,07	1,45	8,27
Month 3	Week 2-12/09	0,41	0,21	0,2	8,26
	Week 4-26/09	1,09	0,16	0,93	8,17
Month 4	Week 2-10/10	1,29	0,97	0,32	8,14

Month 5	Week 2-7/11	1,19	0,08	1,11	8,15
	Week 4-21/11	1,01	0,13	0,88	8,54
Month 6	Week 2-5/12	0,56	0,2	0,36	8,13
	Week 4-16/01/2019	0,77	0,1	0,67	8,08
Month 7	Week 2-30/01/2019	0,99	0,87	0,12	8,13
	Week 4-13/02/2019	0,1	0,03	0,07	8,22
Month 8	Week 2-27/02/2019	0,98	0,05	0,93	8,52
AGRICBUILD					
Month 1	Week 2-18/07	0,05	0,01	0,04	
	Week 4-1/08	0,03	0,02	0,01	7,81
Month 2	Week 2-15/08	0,02	0,02	0	7,67
	Week 4-29/08	0,03	0,01	0,02	7,84
Month 3	Week 2-12/09	0,06	0,01	0,05	7,86
	Week 4-26/09	0,02	0,01	0,01	7,55
Month 4	Week 2-10/10	0,07	0,03	0,04	7,8
Month 5	Week 2-7/11	0,22	0,01	0,21	7,7
	Week 4-21/11	0,03	0,02	0,01	8,05
Month 6	Week 2-5/12	0,22	0,02	0,2	7,76
	Week 4-16/01/2019	0,08	0,02	0,06	7,83
Month 7	Week 2-30/01/2019	0,03	0,03	0	7,8
	Week 4-13/02/2019	0,1	0,05	0,05	8,07
Month 8	Week 2-27/02/2019	0,07	0,01	0,06	8,27
GROENKLOOF					
Month 1	Week 2-18/07	0,94	0,06	0,88	
	Week 4-1/08	0,83	0,06	0,77	8,25
Month 2	Week 2-15/08	0,98	0,08	0,9	8,08
	Week 4-29/08	1,04	0,06	0,98	8,15
Month 3	Week 2-12/09	0,79	0,15	0,64	8,01
	Week 4-26/09	1,05	0,1	0,95	8,12
Month 4	Week 2-10/10	0,82	0,33	0,49	7,95
Month 5	Week 2-7/11	0,48	0,06	0,42	7,94
	Week 4-21/11	0,69	0,06	0,63	8,39
Month 6	Week 2-5/12	0,36	0,03	0,33	7,97
	Week 4-16/01/2019	0,12	0,03	0,09	7,94
Month 7	Week 2-30/01/2019	0,09	0,04	0,05	7,89
	Week 4-13/02/2019	2,2	0,02	2,18	8,07
Month 8	Week 2-27/02/2019	0,07	0,02	0,05	8,42
SILVERTON					
Month 1	Week 2-18/07	0,94	0,06	0,88	
	Week 4-1/08	0,82	0,07	0,75	7,97
Month 2	Week 2-15/08	0,91	0,06	0,85	7,8
	Week 4-29/08	0,9	0,04	0,86	8,3
Month 3	Week 2-12/09	0,79	0,1	0,69	8,18

	Week 4-26/09	0,77	0,06	0,71	8,11
Month 4	Week 2-10/10	0,68	0,35	0,33	8,12
Month 5	Week 2-7/11	0,72	0,06	0,66	8,12
	Week 4-21/11	0,59	0,05	0,54	8,48
Month 6	Week 2-5/12	0,17	0,02	0,15	8,08
	Week 4-16/01/2019	0,06	0,01	0,05	8,1
Month 7	Week 2-30/01/2019	0,1	0,03	0,07	8,05
	Week 4-13/02/2019	0,09	0,03	0,06	8,15
Month 8	Week 2-27/02/2019	0,06	0,04	0,02	8,43
WAVERLEY					
Month 1	Week 2-18/07	0,53	0,03	0,5	
	Week 4-1/08	0,62	0,07	0,55	8,61
Month 2	Week 2-15/08	0,68	0,07	0,61	7,82
	Week 4-29/08	0,64	0,17	0,47	8,36
Month 3	Week 2-12/09	0,72	0,07	0,65	8,43
	Week 4-26/09	0,59	0,11	0,48	8,09
Month 4	Week 2-10/10	0,64	0,12	0,52	8,09
Month 5	Week 2-7/11	0,61	0,12	0,49	8,2
	Week 4-21/11	0,61	0,08	0,53	8,47
Month 6	Week 2-5/12	0,12	0,03	0,09	8,16
	Week 4-16/01/2019	0,12	0,03	0,09	8,19
Month 7	Week 2-30/01/2019	0,08	0,06	0,02	8,21
	Week 4-13/02/2019	0,14	0,05	0,09	8,35
Month 8	Week 2-27/02/2019	0,13	0,02	0,11	8,46