ANTIBIOTIC-RESISTANT BACTERIA AND GENES IN DRINKING WATER

Implications for drinking water production and quality monitoring

Report
to the Water Research Commission

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EXECUTIVE SUMMARY

BACKGROUND

The presence of trace levels of antibiotics and antibiotic-resistant bacteria (ARB) in source water and final drinking water is an emerging quality and health issue. This aspect has been demonstrated in international and local studies. It is widely accepted that antibiotic resistance results from the excessive use of antibiotics and antimicrobial agents. Sub-therapeutic levels of these antibiotics may land in rivers, lakes and wetlands as part of treated sewage or from animal production facilities. The organic and inorganic pollutants could be selecting for antimicrobial-resistant microorganisms (AMRMs). However, the extent to which water sources act as reservoirs for AMRMs and antimicrobial-resistant genes (AMRGs) is relatively undetermined. Another aspect that is receiving more attention is the potential impacts of antimicrobial substances and AMRMs on drinking water production. This contributes to the rapid increase in antibiotic resistance, leading to the failure of treatment of bacterial infections in clinical and veterinary settings. This has been identified as an emerging global health concern. The present study investigated the occurrence of antibiotic-resistant genes (ARGs) and ARB in selected raw water and water after being treated in drinking water production facilities (DWPFs). Such data will inform whether monitoring steps, additional to the current national standards, are required.

AIMS AND OBJECTIVES

The specific objectives of the project were as follows:

- Determine the physicochemical and general microbiological parameters of the different water sources at the time of collecting water for the ARB resistance tests, quantitative polymerase chain reaction (qPCR) and environmental metagenomic analysis.
- Isolate and determine the antibiotic resistance profiles of isolated bacteria for comparison to the next-generation molecular evaluation methodologies.
- Perform qPCR and environmental metagenomic analysis of DNA isolated directly from water and evaluate the analysis processes.
- Evaluate the next-generation molecular method data and determine its implications.
- Use the data to determine whether mitigation strategies are required; if this is positive, evaluate the available options.

METHOD

Sampling and water treatment processes

Water samples of raw water and final drinking water were collected at eight selected drinking water treatment plants. Each of these plants was selected based on the treatment processes used, geographic location and the intensity of catchment activities that could have an impact on the quality of the raw water. The type of water treatment processes used for drinking water production at each of the plants was described. Selected physicochemical parameter data was recorded.

Land cover and threats

For the geospatial analysis, a desktop study was carried out using data from scientific reports and digital databases from the Department of Environmental Affairs (DEA) the Department of Water Affairs and Forestry (DWAF), the National Aeronautics and Space Administration (NASA) and the South African National Biodiversity Institute (SANBI). The data that was the basis for the spatial analysis was the ASTR 90m Digital Elevation Dataset and the 2013/14 South African National Land Cover Dataset. This provided land-use data upstream from the DWTF that may impact on the quality of the raw water.

Isolation and identification of bacterial species

Initially, a dilution series was used to obtain adequate heterotrophic plate count (HPC) bacteria on R2A agar. Heterotrophic plate count bacteria were collected and purified using a successive streak plating approach. These bacteria were identified using Gram staining, morphology and a molecular identification approach. Identification of the isolates provided the opportunity to compare the characteristics of HPC isolates to those isolated in previous studies. The molecular identification briefly entails the following: DNA from isolates was extracted using the Chemagic kit. Endpoint polymerase chain reaction (PCR) was done for the amplification of the 16S rRNA (ribosomal RNA) gene. The 16S rRNA gene was sequenced at Inqaba Biotechnical Industries. The 16S rRNA sequences were analysed using Basic Local Alignment Search Tool (BLAST) software to identify the isolates.

Antibiotic susceptibility test

Antibiotic susceptibility tests were performed on all colonies to determine their antibiotic resistance profiles using the Kirby Bauer disc diffusion method.

Detection of antibiotic-resistant genes

Endpoint PCR was conducted for the detection of various ARGs. These included *ampC, ermB, ermF* and *intl 1*. The selected genes are associated with some of the antibiotic resistance profiles and properties of the isolates.

Pathogenicity

Antibiotic-resistant isolates were streaked onto 5% sheep blood agar for the haemolysin production test, a screening for potential pathogenicity. Beta and alpha haemolytic isolates were subjected to assays that are an indication of the ability to produce extracellular enzymes such as DNase, lipase, gelatinase, lecithinase and proteinase.

Whole-genome sequencing and detection of ARGs, as well as virulence genes

Whole-genome sequencing (WGS) was conducted on the bacilli isolated from raw and drinking water at a selected DWPF. This genus was selected since it was isolated from all compartments of the system (raw water, water after treatment and the distribution system). Paired-end sequencing was done on a MiSeq sequencer (Illumina) using protocols described by Illumina. Sequencing reads were trimmed and assembled using the CLC Genomics Workbench version 9. Subsequent assemblies were then annotated with Rapid Annotation using Subsystem Technology (RAST), which identified genes associated with antimicrobial resistance traits, as well as numerous other genes. The Comprehensive Antibiotic Resistance Database (CARD) was also used to identify antibiotic resistance determinants.

Microbiome analysis

Community environmental DNA was isolated using a product that is used to purify water for hiking, backpacking, camping and the Power Water DNA isolation kit (MoBio, US). The 16S rRNA gene PCR primers for the V3 and V4 region were used for PCR amplification. Amplicons were purified using the AMPure XP beads and procedures of the manufacturer. Nextera indexing primers (N7xx and S5xx) were used in a subsequent PCR. The success of the PCR was determined by agarose gel electrophoresis. Various steps were used to determine the quality of the sequences and filter these to get data that could be used for interpretation. The data was analysed using QIIME software pipelines. Species richness and diversity indices were calculated. Data from this culture-independent analysis could be compared to the culture-dependent (isolation and identification of HPC) analyses.

Predicting metagenomes and antibiotic resistance

Microbial metagenomes were predicted from 16S rRNA gene sequences using the online PICRUSt pipeline (Langille et al., 2013), available at http://galaxy.morganlangille.com/, as described by Zaura et al. (2015). The prevalence of antibiotic-resistant determinants (ARDs) was evaluated by blasting the operational taxonomic units (OTUs) against ARGs downloaded from the Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009). The data from this analysis could be compared to the phenotypic endpoint PCR and WGS data.

Detection of antibiotic residues: LC-MS-MS and ELISA

The detection of selected antibiotics was carried out following the analytical methodology of Ferrer et al. (2010). An ultra-performance liquid chromatography – quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) was used to detect antibiotics. The antibiotics that were selected were those that corresponded to the antibiotics that were used for the susceptibility analysis. An enzyme-linked immunosorbent assay (ELISA) rapid test was conducted using the BIOO Scientific ELISA kits (Austin, Texas, USA), but due to the cost, only three antibiotics (Beta-lactam, Colistin and Trimethoprim) were included. The presence and quantification of antibiotic data could be brought into the context of the antibiotic resistance phenotypes.

Statistical analysis

Where appropriate, Microsoft Excel 2013 was used to calculate averages, standard deviations and student t-test. Canoco for Windows (Version 4.0, GLW-CPRO©) (Ter Braak, 1992) was used to show the effect of environmental variables (physicochemical parameters) on the various sites.

RESULTS AND DISCUSSION

In this study, eight DWPFs were included, ranging from small (2.5 to 14 Mℓ/day) to large (250 to 4 000 Mℓ/day), providing drinking water to populations varying from 36 000 to 12 million. The small plants were named WC-A, NW-C and NC-F; the medium-sized plants were named NW-B, NW-D and NW-E; and the large plants were named NW-G and GT-H. All the plants included in this study generally produced drinking water of a high microbiological and chemical quality, as reflected in the Blue Drop scores for 2012 (DWA, 2012). These systems had at least one filtration step before chlorination. Coagulation/flocculation followed by sedimentation was common. The two larger plants had advanced treatment processes and a single small plant reclaimed sewage directly for potable purposes. The latter plant used high-end advanced processes. Treated source water came from a variety of sources, ranging from surface and ground water to treated wastewater effluent. Land-use activities upstream from the DWPF in all cases included agriculture. In some cases, wastewater treatment plants (WWTPs) and urbanisation (formal and informal) impacted on the water source.

Physical and chemical parameters that mainly impacted on the water in most plants were total dissolved solids (TDS), phosphates and nitrites. In one case, low pH (only in the source water; at WC-F) was an aspect to be considered, but this was corrected with lime before coagulation. Low free chlorine in the drinking water, and in some cases turbidity levels that exceeded the South African National Standard (SANS 241) (SABS, 2015) levels, were also issues. In some cases, nitrites (at NW-C, NW-E and GT-H) were elevated. This was most probably due to elevated levels of this substance in the raw water and microbial activity. In the case of NW-C, the turbidity in the final water and within the distribution systems was at times higher than the source water values. These were, however, all in accordance with World Health Organisation (WHO) standards for human consumption without any health risk or negative impacts of consumption over a lifetime (Hodgson and Manus, 2006).

Several heterotrophic bacteria were isolated from the raw and drinking water, and 16S rRNA gene sequence identification demonstrated some overlaps in bacterial genera between the various compartments. Among these were several *Pseudomonas* spp. and *Bacillus* spp. The genus most consistently isolated from both raw and drinking water in all the plants was *Bacillus* spp. This implies that these Gram positive, spore-forming bacteria survived the treatment processes. It thus made sense to include this in the WGS approach for all the plants. The study did not aim to quantify the culture-based bacterial species. The media used were not specific and thus not selective. Using the 16S microbiome analyses, similar species were identified as were identified with the culture-dependent method. In the former case, *Bacillus* spp. was not as prominent. This could be due to the dominance of other genera. Beta and alpha diversity indices provide a measure of the degree to which samples differ. These did not provide any significant differences between sample types (raw water, water after treatment and drinking water).

Antibiotic resistance phenotype data was obtained. In most cases, the percentage of resistance data indicated that most of the isolates were resistant to some of the antibiotics. What was evident is that resistance to beta-lactam antibiotics and Trimethoprim was the most common resistance phenotype. In some cases, resistance was also towards aminoglycosides. The inhibition zone and resistance or susceptibility data are laborious and challenging to interpret. Multiple antibiotic resistance (MAR) indices for the various sites were thus determined and are easily comparable. This index is an indication of the antibiotic exposure history of the isolates from a specific site. If this value is above 0.2, then most of the isolates would have been exposed to multiple classes of antibiotics. Results for the DWPFs indicated that indices were generally above 0.2. Antibiotics detected in the source water were mainly also detected in drinking water and included beta-lactam antibiotics, Trimethoprim, Colistin and, in several cases, also Ciprofloxacin. These were detected irrespective of the type of land use or water type.

Furthermore, PCR amplification results showed that several genes associated with antibiotic resistance were detected. Genes could be associated with the dominant antibiotic resistance phenotypes observed among the isolates. The most frequently detected genes were the *ermB* and *ermF* genes. These genes are responsible for resistance to a range of antibiotics. *IntI1* and *ampC* were also among the genes detected. The integrase gene (*intI*) is associated with the transfer of genetic material between the same and dissimilar species. This means that the ARB species isolated had means of disseminating the resistance and virulence genes to susceptible non-pathogenic species, rendering them pathogenic. Where whole genomes were sequenced, some similar and additional ARGs and virulence genes were detected. The order of gene abundance in the bacilli isolates, as determined by WGS, was as follows:

Multidrug resistance > Glycopetides > MLS~Bacitracin > beta-lactams~Quinolone~Tetracycline

This pattern was observed in the genomes of bacilli from source water, water after treatment, as well as drinking water. It was similar across the various DWPFs, as well as the metagenomics analysis of the filter beds and reservoir samples. The microbiome data could be used to provide predicted metagenomes. When these were compared to antibiotic resistance databases, similar ARGs were detected as those detected in the WGS and the endpoint PCR. From these results, the genes listed above (*ermB, ermF, IntI1* and *ampC*) were detected using the detection methods used. This implies that these could be used during a monitoring regime that is specifically focused on the detection and quantification of ARGs in water sources in South Africa.

Besides haemolysin, proteinase, DNase and lecithinase, virulence factors were also commonly produced among the isolated bacteria. In some cases, lipase also produced an indication that these isolated bacteria were potentially pathogenic. The WGS also demonstrated that virulence genes were common in the genomes of the bacilli. The various classes of genes could potentially be associated with pathogenic phenotypic characteristics (extracellular enzyme production).

CONCLUSION AND RECOMMENDATIONS

The results presented in this preliminary report are as follows:

- The results from a previous study (Bezuidenhout et al., 2016) are confirmed, i.e. that the quality of the raw water affects the quality of the drinking water and may also impact on the microbial stability and geochemical processes in the drinking water distribution system. Antibiotic-resistant heterotrophic bacteria in drinking water originate from the raw source water, and could survive the drinking water production processes and eventually land in the drinking water distribution system. Similar antibiotics in the raw water is also found in the drinking water, albeit in a very low concentration.
- Various bacterial species were isolated from the raw water and the drinking water, and these had similar antibiotic resistance and virulence phenotypes.
- Genes responsible for antibiotic resistance phenotypes were detected in multiple ARB. This is an indication that the genes are functional and that dissemination of such genes to antibiotic-susceptible, opportunistic pathogens could have detrimental consequences with respect to the treatment of an infection caused by such an ARB.
- *Bacillus* spp. were common in both raw water and drinking water from six of the eight DWPFs. The whole genomes of representatives were determined and data showed that ARGs and virulence determinants were present in representatives from the various water compartments. These findings indicate that these species survive drinking water production barriers. Finding these ARGs and virulence genes in drinking water is a cause for concern as it may affect the infection potential of microbes in the drinking water.
- What was of further concern was that these genomes had genetic elements that are responsible for horizontal gene transfer, enabling the ARGs and virulence genes to be transferred to related and non-related species.
- Hollow fibre membranes are suitable for the isolation of sufficient eDNA from metagenomics studies. A system was developed that would be suitable to harvest eDNA from between 1 000 and 10 000 litres of water in such a manner as to prevent water wastage.

Recommendations

- A considerable body of knowledge is being generated to establish the occurrence of antibiotics, ARB and ARGs in aquatic systems, particularly in drinking water distribution systems. How environmental conditions affect the associated genetic and metabolic changes is not clearly understood. The present study provided some data for examples of drinking water production systems typically in operation in South Africa. However, a coordinated study is needed to obtain baseline data for the various compartments of the environment in order to adequately link it with health.
- Connecting contaminants of emerging concern in aquatic ecosystems to waste and impacts on human health is a theme that is poorly understood and needs to be explored. This is the case, in particular, for antibiotics, ARB and ARGs that are disposed of in water sources, where the latter are used for drinking water production. A systematic review of all the work that has been funded by the WRC and their implications must be undertaken.
- The data gathered in the present study showed that the underlying genetic elements that confer antibiotic resistance may potentially also lead to increased virulence. This is intimately tied to bacterial interactions within communities. A further investigative study is thus necessary to examine the health-related impacts of the bacterial species that have been identified and their associated virulence factors.

- Rapid ELISAs are sensitive and can detect very low antibiotic residues. It is possible to conduct these at DWPFs as part of water safety planning (WSP), particularly where upstream land use involves the use of large quantities of antibiotics in human or animal medicine. The cost for setting up the equipment and analysis is not prohibitively high. It would allow for the quantification of antibiotic residues in water samples and provide trends over time.

- Furthermore, with such substantial data being gathered in the current study, there is a need to link WGS data to inhibition zone analysis data. This will not only give insight into the world of these identified bacterial species, but will also make it possible to trace their lineage and possibly find innovative remediation solutions. The WGS will provide an overview of ARGs associated with target genera.

- It is also important that findings from studies such as this one should be circulated to the relevant stakeholders. Attempts should be made to get this information to those who were not part of this initial study. Such data must also be made available to communities in such a manner that would make it easily understandable to all members.

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ACRONYMS AND ABBREVIATIONS

AJS	Agilent jet stream
Amp	Ampicillin
AMRG	Antimicrobial-resistant gene
AMRM	Antimicrobial-resistant microorganism
ARB	Antibiotic-resistant bacteria
ARD	Antibiotic-resistant determinant
ARG	Antibiotic-resistant genes
ARDB	Antibiotic Resistance Genes Database
AST	Antibiotic susceptibility testing
AT	After treatment
BA	Blood agar
BDC	Blue Drop Certification
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CARD	Comprehensive Antibiotic Resistance Database
cfu	Colony forming units
Chl	Chloramphenicol
CIP	Ciprofloxacin
COD	Chemical oxygen demand
CV	Coefficient of variation
°C	Degree Celsius
DEA	Department of Environmental Affairs
Dis	Distribution system
DNA	Deoxyribonucleic Acid
DWA	Department of Water Affairs
DWAF	Department of Water Affairs and Forestry
DWPF	Drinking water production facility
DWS	Department of Water and Sanitation
EC	Electrical conductivity
ELISA	Enzyme-linked immunosorbent assay
Ery	Erythromycin
ESBL	Extended spectrum beta-lactamases
ESI	Electrospray ionisation
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HLB	Hydrophilic or lipophilic balanced
H_2O_2	Hydrogen peroxide
HPC	Heterotrophic plate count
HT-qPCR	High-Throughput quantitative Polymerase Chain Reaction
IWA	International Water Association

Kan	Kanamycin
Kf	Cephalothin
LC-MS-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAR	Multiple antibiotic resistance
MGE	Mobile genetic elements
Mℓ/day	Megalitre per day
MLS	Macrolide, lincosamide and streptogramin (antibiotics)
mM	Millimole
µg/ℓ	Microgram per litre
MRSA	Methicillin-resistant *Staphylococcus aureus*
µℓ	Nanolitre
NASA	National Aeronautics and Space Administration
Neo	Neomycin
NF	Nanofiltration
ng	Nanogram
ng.ℓ^{-1}	Nanogram per litre
nM	Nanomole
NGS	Next-generation sequencing
NTU	Nephelometric turbidity units
NWRS	National Water Resource Strategy
O_3	Ozone
O-T	Oxy-tetracycline
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
Pen-G	Penicillin G
PNEC	Predicted no-effect concentration
PPCP	Pharmaceutical personal care products
qPCR	Quantitative Polymerase Chain Reaction
RAST	Rapid Annotation using Subsystem Technology
RO	Reverse osmosis
rRNA	Ribosomal RNA
QIMME	Quantitative Insights Into Microbial Ecology
SANBI	South African National Biodiversity Institute
SANS	South African National Standard
SPE	Solid phase extraction
Strep	Streptomycin
TDS	Total dissolved solids
TMP	Trimethoprim

UPLC-QTOF/MS	Ultra-performance liquid chromatography – quatropole time-of-flight mass spectrometry
U/µℓ	Unit per microliter
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	Ultra-violet
Van	Vancomycin
VIR	Virulence gene
WGS	Whole-genome sequencing
WHO	World Health Organisation
WRC	Water Research Commission
WSA	Water Services Authorities
WSI	Water services institutions
WSP	Water Safety planning
WWTP	Wastewater Treatment Plant

GLOSSARY

Term	Meaning
Advanced oxidation	A set of chemical treatment processes designed to remove organic chemicals in water by utilising hydroxyl radicals.
Antibiotic-resistant bacteria	Bacteria that are resistant to antibiotics.
Antimicrobial-resistant genes	Genes that code for one or more antibiotic resistance characteristic.
Antimicrobial-resistant microorganisms	Microbes that are resistant to antimicrobial substances.
Antibiotic susceptibility test	A test conducted to determine whether a bacterium is susceptible to specific drugs at a set clinically relevant concentration.
Blue Drop certification	A certification regulation method introduced to safeguard tap water quality in South Africa.
Coagulation	Process of changing a liquid into a solid or semi-solid state.
Drinking water production facility / Drinking water treatment plant	A facility that uses a series of processes to purify and disinfect water for drinking purposes.
Environmental metagenomic DNA	DNA that is directly isolated from environmental sources and can be intracellular or extracellular.
ELISA	Enzyme-linked immunosorbent assay: it involves a specific enzyme and antibodies to detect specific compounds.
Filtration	Action of filtering.
Flocculation	Process in which small particles aggregate to form lumps.
Green Drop	A certification regulation method introduced in South Africa to ensure that wastewater plant effluent is of satisfactory quality
Land cover	Physical material on the surface of the earth.
Land use	Involves the management and modification of the natural environment
LC-MS-MS	Liquid chromatography mass spectrometry: a chemistry analytical technique for the separation of chemicals in which liquid chromatography is combined with mass spectrophotometric analysis.
Metagenome	The study of all genetic material directly isolated from

	environmental samples.
Microbiome analysis	Study of the microorganisms in a particular environment.
MiSeq sequencing	An integrated process in which clonal amplification, DNA sequencing is combined with various genetic analytic processes.
Next-generation sequencing	A method for sequencing genomes and microbiomes at high speed.
Pathogens	An organism that can cause disease.
Pathogenicity	Ability to cause disease
Polymerase chain reaction	A laboratory method to synthesise multiple copies of a segment of DNA.
Pharmaceutical personal care products	Pharmaceutical products used for personal or cosmetic reasons.
Quantitative polymerase chain reaction	A laboratory method to synthesise multiple copies of a segment of DNA and to measure the amplification (quantity produced) in real-time.
Virulence	Severity of a disease.
Water safety plans	A plan to ensure the safety of drinking water by comprehensively assessing the risk and having mitigation measures in place.
Water services providers	An entity providing water services
Whole-genome sequencing	A laboratory method to determine the sequence of an entire organism.

CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

Antibiotic-resistant bacteria and antibiotic-resistant determinants are major emerging public health threats. Thus, the development and implementation of national and international guidance for risk assessment should be a priority (Pruden, 2014; Bergeron et al., 2015). However, this can only be achieved once sufficient quantitative data is available. In South Africa, the quality of drinking water is regulated according to SANS 241, and water quality requirements for other specific uses are determined according to the South African Water Quality Guidelines, both of which stipulate acceptable concentrations for a variety of substances, such as metals, minerals and selected organic compounds (SABS, 2015; DWAF, 1996). For some time, drinking water has been implicated as a reservoir for ARB and ARGs. A recently published scoping study on the South African scenario provided an overview of the levels of antimicrobials and presence of ARB in selected drinking water treatment systems (Bezuidenhout et al., 2016). The results demonstrated that a cocktail of pharmaceutical personal care products (PPCPs), antimicrobial substances and agrochemicals were present in the source and drinking water. In addition to this, ARB was frequently detected in both water types.

Some studies have also implicated the most commonly used disinfection process – chlorination – as a stressor that can be selected for increased ARB and ARGs in treated water when compared to source water (Bouki et al., 2013; Shi et al., 2013). It is thus important that these parameters (selection and enrichment for ARB and ARGs) are considered in the selection of drinking water treatment processes and for drinking water quality monitoring, as well as in risk assessment models. The WHO and other bodies concerned with health now have these parameters as priorities for inclusion in monitoring programmes as part of WSP (WHO, 2000; Bergeron et al., 2015). There is, however, an ongoing debate on whether these parameters should be regulated. Various studies have shown the presence of multiple antibiotic resistance pathogens or opportunistic pathogen bacterial species in South African source and drinking water habitats (Pavlov et al., 2004; Carstens et al., 2014; Mulamattathil et al., 2000; Mulamattathil et al., 2014a; Mulamattathil et al., 2014b; Mulamattathil et al., 2015; Bezuidenhout, 2013). These studies all used standard culture-dependent methodologies. In addition to this, these studies demonstrated that the isolated antibiotic-resistant species also have pathogenic or virulence features based on the presence of associated genes (Mulamattathil et al., 2014a; Mulamattathil et al., 2014b) or the production of extracellular enzymes causing cytotoxicity (Pavlov et al., 2004; Prinsloo et al., 2013; Prinsloo, 2014; Molale and Bezuidenhout, 2016) or were resistant to amoebas (Carstens et al., 2014). Thus, antibiotic resistance linked to virulence is a real health risk, particularly against the backdrop of the large immune-compromised community that is directly dependent on drinking water provided by the water utilities. Studies to quantify antibiotic resistance and virulence genetic determinants are thus essential.

A considerable body of knowledge is being generated to establish the occurrence of antibiotics, ARB and ARGs in aquatic systems, particularly in raw and drinking water distribution systems (Shi et al., 2013; Bird et al., 2019). In the South African context, such a body of knowledge is insufficient and not well coordinated. It thus called for an intervention such as a coordinated study to obtain baseline data for drinking water production systems typically in operation in South Africa. Such baseline data would be useful for deriving recommendations for drinking water treatment processes and specifically water quality monitoring.

1.2 PROJECT AIMS

The aim of this study was to evaluate the presence of ARB and ARGs in raw and drinking water, as well as the implications for water production and water quality monitoring. The specific objectives were as follows:

- Determine the physicochemical and general microbiological parameters of the different water sources at the time of collecting water for ARB resistance tests, qPCR and environmental metagenomic analysis.
- Isolate and determine the antibiotic resistance profiles of isolated bacteria for comparison to next-generation molecular evaluation methodologies.
- Perform qPCR and the environmental metagenomic analysis of DNA isolated directly from water and evaluate the analysis processes.
- Evaluate the next-generation molecular method data and determine its implications.
- Use the data to determine whether mitigation strategies are required; if this is positive, evaluate the available options.

1.3 SCOPE AND LIMITATIONS

This study included eight drinking water production and distribution systems. Water was sampled before treatment, immediately after treatment, as well as at sites at points of use. The DWPFs ranged from small (2.5 to 14 Mℓ/day) to large (250 to 4 000 Mℓ/day). The small plants were named WC-A, NW-C and WC-F; the medium-sized plants were named NW-B, GT-H. All the systems had at least one filtration step before chlorination. The treated source water was from surface and ground water. Six of the treatment facilities were from the same geographic area. One had limited upstream impacts and one plant was a direct reclamation plant where this reclaimed water was blended with treated ground water. These plants are thus of sizes and inclusive of treatment processes that could be regarded as being fairly representative of South African drinking water production. It is thus possible to apply the findings from the present study to represent the South African scenario with respect to antibiotics and antibiotic resistance in raw and drinking water. There were challenges with respect to the extraction of antibiotics and DNA directly from drinking water. This took longer to complete; hence, the number of repeats and depth of analyses. The planned physical metagenomic sequencing could not be completed. This affected the qPCR analyses. The alternative that could be completed was microbiome analyses for selected plants and WGS of bacilli from these plants. These methods provided data for the distribution of bacterial species, antibiotic resistance and virulence genes. However, metagenomic analyses for one distribution could be made and compared to the microbiome and WGS data, showing overlapping results. Due to the distances between the laboratory and the two Western Cape plants, large financial resources for sampling were required. These could not be sampled as regularly as the other plants.

1.4 REPORT LAYOUT

The report is divided into seven chapters, each dealing with a focused aspect.

- Chapter 1: General introduction and background
- Chapter 2: A review of antibiotic-resistant bacteria and genes in drinking water
- Chapter 3: Study design and methods
- Chapter 4: General description and characterisation of study sites
- Chapter 5: Antibiotic resistance and virulence profiles of bacteria in drinking water
- Chapter 6: Analysis of the drinking water microbiome
- Chapter 7: Conclusions and recommendations

CHAPTER 2: A REVIEW OF ANTIBIOTIC-RESISTANT BACTERIA AND GENES IN DRINKING WATER

2.1 MANAGING RISKS AND ENSURING SAFE DRINKING WATER

In order to provide safe drinking water to consumers, a framework, consisting of health-based targets, WSP and independent surveillance, must be set up and managed (Figure 2.1). Health-based targets are used to provide the basis for the application of guidelines. They provide information against which to evaluate the effectiveness of the treatment process and the quality of the water produced. The various water quality parameters and possible influences are used to define the health-based targets. The WSP team must have adequate experience and expertise to recognise and understand the possible risks and hazards in the system. Each water utility must take responsibility for the design and implementation of the WSP approach. Part of the WSP approach is to identify the responsibilities of other role players and stakeholders. These may include agriculture, forestry, industries, mining houses, transportation, local government and consumers. Some of the role players may not necessary be part of the WSP team, but must be part of the communication network and be aware of the impacts of their contributions (WHO and IWA, 2009).

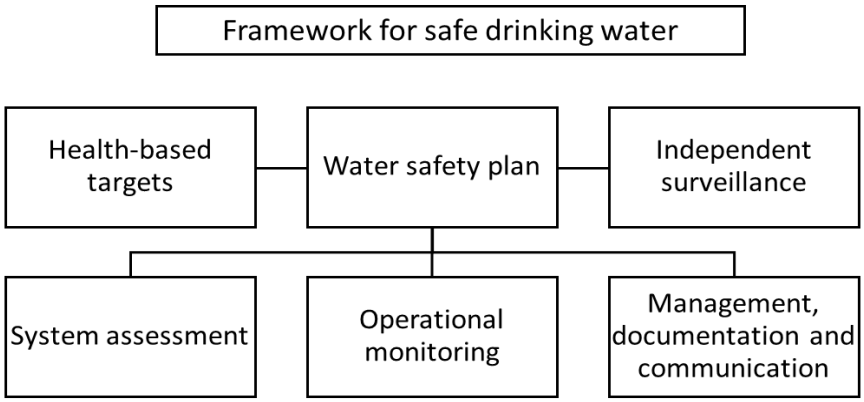


Figure 2.1: A framework that will produce potable and safe drinking water and consists of health-based targets, a water safety plan and independent surveillance (Davidson et al., 2005)

Water safety planning should entail a system assessment, effective operational monitoring, management and communication plans. System assessment is related to the drinking water supply chain and whether the system can deliver drinking water that meets specified health-based targets. Operational monitoring, on the other hand, is a set of routine activities used to determine and monitor specific, identified control measures. These are monitored in a set time course for effective systematic management. They will ensure that any deviations from required performances are rapidly detected and corrective steps immediately implemented. Management, documentation and communication entail actions to be taken during normal and incident conditions. Documentation is essential, and includes the following:

- The description and assessment of the drinking water system
- Programmes to be upgraded to improve water delivery
- Plans for operational monitoring
- Water safety management procedures during normal circumstances, as well as incidents
- Description of supporting programmes

Consumers should have the right information that could affect their health. Procedures should be in place to record, manage and communicate all significant incidents that take place within the system. A summary of such information must be made available to consumers regularly, such as annual reports published in local newspapers and on websites. Mechanisms must be put in place to obtain and actively address complaints from communities (WHO, 2017).

2.2 DRINKING WATER QUALITY MANAGEMENT IN SOUTH AFRICA

2.2.1 Institutional roles and responsibilities

Water delivery is the primary responsibility of local government water services authorities (WSAs) (DWAF, 2005a; Haigh et al., 2010). Furthermore, WSAs have a responsibility to regulate the quality of water supplied by water services providers (DWAF, 2005a). Some local municipalities are both WSAs and water services providers. The Department of Water and Sanitation (DWS) is the sector regulator, who should provide support in a progressive manner. This has resulted in an incentive-based regulation approach (DWAF, 2005b; Hodgson and Manus, 2006). Successful drinking water quality management involves a clear understanding of the entire drinking water supply system (DWAF, 2005a). This includes understanding the hazards and events that can compromise raw and drinking water quality and to put counteractive and preventative measures in place, as well as operational controls.

2.2.2 Drinking water quality framework

South Africa has a drinking water quality framework. This enables the effective management of drinking water quality. The framework is based on a protective approach (Hodgson and Manus, 2006). A key to produce water of a desired quality is to implement multiple barriers, which help to control microbiological pathogens and chemical contaminants that may enter the water supply system (Momba et al., 2009). Such an approach is demonstrated in Figure 2.1. Also important is adopting sound management practices and continually revisiting the source water quality, state of the water treatment and the distribution infrastructure in terms the quality of water produced. Attention should the given to reducing the probability of contaminants entering raw water (DWAF, 2005b). When source or raw water pollution is prevented, treatment cost and potential risks can be reduced. Understanding the identified risks from the catchment to the point of use is thus crucial (DWA, 2013).

2.2.3 Blue Drop Certification Programme

In order to counter poor and non-compliant drinking water that was supplied to rural communities, in particular, the Department of Water Affairs (DWA) introduced an incentive-based programme, the Blue Drop Certification (BDC) Programme, in 2008 (DWA, 2010). It is used to encourage drinking water quality management performance and to provide the public with the correct statistical information on drinking water quality performance (DWAF, 2009; DWA, 2010). The programme consists of the annual assessment of the management and service rendering of WSAs (Nealer and Mtsweni, 2013). This award is granted when 95% compliance of the prescribed criteria is met (DWAF, 2009). Blue Drop status is awarded as an indication of recognising excellence in the approach that the water services institutions (WSI) are using in managing drinking water (DWA, 2013). Independent surveillance such as entailed in the BDC Programme is important to ensure that safe drinking water is consistently produced. As part of BDC, WSPs must be in place at all DWPFs. This is a crucial part of the drinking water production process.

2.2.4 Drinking water quality monitoring

The quality of drinking water in South Africa should comply with microbiological, physical, aesthetic and chemical determinant numeric limits specified in SANS 241 (SABS, 2015). The BDC Programme does not replace SANS 241, but rather enhances the implementation of the standard. Metropolitan areas in South Africa produce water of very high quality that can be consumed directly from the source of supply. However, Momba et al. (2003; 2006) found that, in non-metropolitan areas, water quality could be questionable and not acceptable to world standards.

Most of the physical features (conductivity, pH level and turbidity) of water affect its aesthetic quality (taste, odour and appearance) (WRC, 1998) and do not have a direct public health risk. However, they create perceptions of the acceptability of water (Dietrich, 2006). Turbidity is used to indicate the efficiency of the water treatment process and can be used to determine risks and problems in the infrastructure of the treatment process (Obi et al., 2008; Ramavandi, 2014). Chemical quality is categorised by dissolved substances such as organic substances, salts and metals (WRC, 1998). Some of the chemical substances in water are essentially part of humans' daily required intake, but at maximum levels, they may pose risks to public health. These chemicals may have aesthetic, operational and/or health effects (SABS, 2015). The numerical limits of many of these substances are specified in SANS 241 (SABS, 2015). Examples of chemicals of concern are nitrates, arsenic and fluoride (Feldman et al., 2007; SABS, 2015). Some of these may cause diseases such as cancer, cardiovascular disease, methaemoglobinaemia, neurological disease and miscarriage if they exceed the standards contained in SANS 241 (SABS, 2015).

Safe drinking water is free of pathogenic bacteria, viruses and protozoa. Testing for all known pathogens is costly; thus, tests to ensure the safety of drinking water is based on the absence of faecal indicator bacteria. The dominant health risk from the ingestion of contaminated water may be diarrheal diseases, dysentery and enteric fever (Bain et al., 2014). Should HPCs, such as *Pseudomonas aeruginosa, Aeromonas hydrophila* and *Enterobacter cloacae* occur in water, it could lead to urinary and pulmonary tract infections (Baghal et al., 2013). Immunocompromised patients are most susceptible to contract such infections (Lin et al., 2006; Baghal et al., 2013). Therefore, it is important to monitor the quality of drinking water on a regular basis for faecal indicator bacterial species, but also for bacterial species that are of emerging concern. With modern molecular technologies that are available, it is possible to screen for the presence and levels of such known opportunistic pathogens (Falcone-Dias et al., 2015). Furthermore, ARB and ARGs had not previously been considered as threats in drinking water. These molecular technologies now make it possible to screen for the presence of ARB and ARGs, as well as their levels.

2.3 DRINKING WATER TREATMENT AND QUALITY

2.3.1 Conventional drinking water treatment methods

Water for drinking water production is sourced from rivers, dams and subsurface resources (natural springs or boreholes). The quality of the source water is impacted on by land-use and human activities that occur upstream of the source. Water purification processes are required to produce and ensure that safe drinking water is provided to consumers. Many DWPFs use similar or overlapping basic water purification processes (Hunter Water, 2006), such as demonstrated in Figure 2.2. There are five commonly accepted steps in the treatment process: coagulation, flocculation, sedimentation, filtration and disinfection (Momba et al., 2009). In South Africa, upstream impacts or natural contaminants, population size, the accessibility of resources such as electricity and materials, as well as the level of operators' training skills may be factors that relate to the type of technology or processes employed.

2.3.1.1 Coagulation and flocculation

Coagulants such as aluminium sulphate are added to the water to charge suspended particles, destabilising them and allowing the particles to be attracted. These individual destabilised particles and flocs collide to form larger, heavier flocs (Apostol et al., 2011).

2.3.1.2 Sedimentation

During sedimentation, water and flocs flow slowly into a large sedimentation tank. Slow stirring causes a centrifugal force where flocs sink and settle at the bottom of the tank (Goula et al., 2008). This process is important as it is known to improve the filtration process by removing particulate material (Gregory and Edzwald, 2010). The sludge is pumped out by desludging bridges, followed by its deposition at sludge deposit sites (Rand Water, 2016; Saminu et al., 2013). In some cases, the inorganic and organic load is negligible and these two steps (coagulation-flocculation and sedimentation) are omitted.

2.3.1.3 Filtration

During filtration, water flows through a filter medium to remove particles that were not removed by the previous step of sedimentation. This happens by means of chemical adsorption, where the passage of the contaminants is blocked. The most common medium used is sand. Activated carbon and membranes can also be used. Filtration is a very important step since it enhances the effectiveness of the following step, disinfection (USEPA, 2004).

2.3.1.4 Disinfection

The purpose of disinfection is to eliminate, deactivate or kill pathogenic microorganisms (Achour and Chabbi, 2014). Chlorine is used as a primary disinfectant in water treatment, as well as a disinfectant residual to preserve the water in distribution (Dore et al., 2013; USEPA, 2004). Chlorine is an effective disinfectant, but its effectivity is dependent on concentration, contact time, turbidity, temperature and pH level (LeChevallier and Kwok-Keung, 2004). Secondary disinfection refers to the disinfectant added just before the treated water is distributed. This is to maintain the water quality within the distribution system (USEPA, 2011). However, this step also acts as a final barrier to ensure microbial safety by controlling bacterial regrowth and contamination within the distribution system (Stanfield et al., 2003).

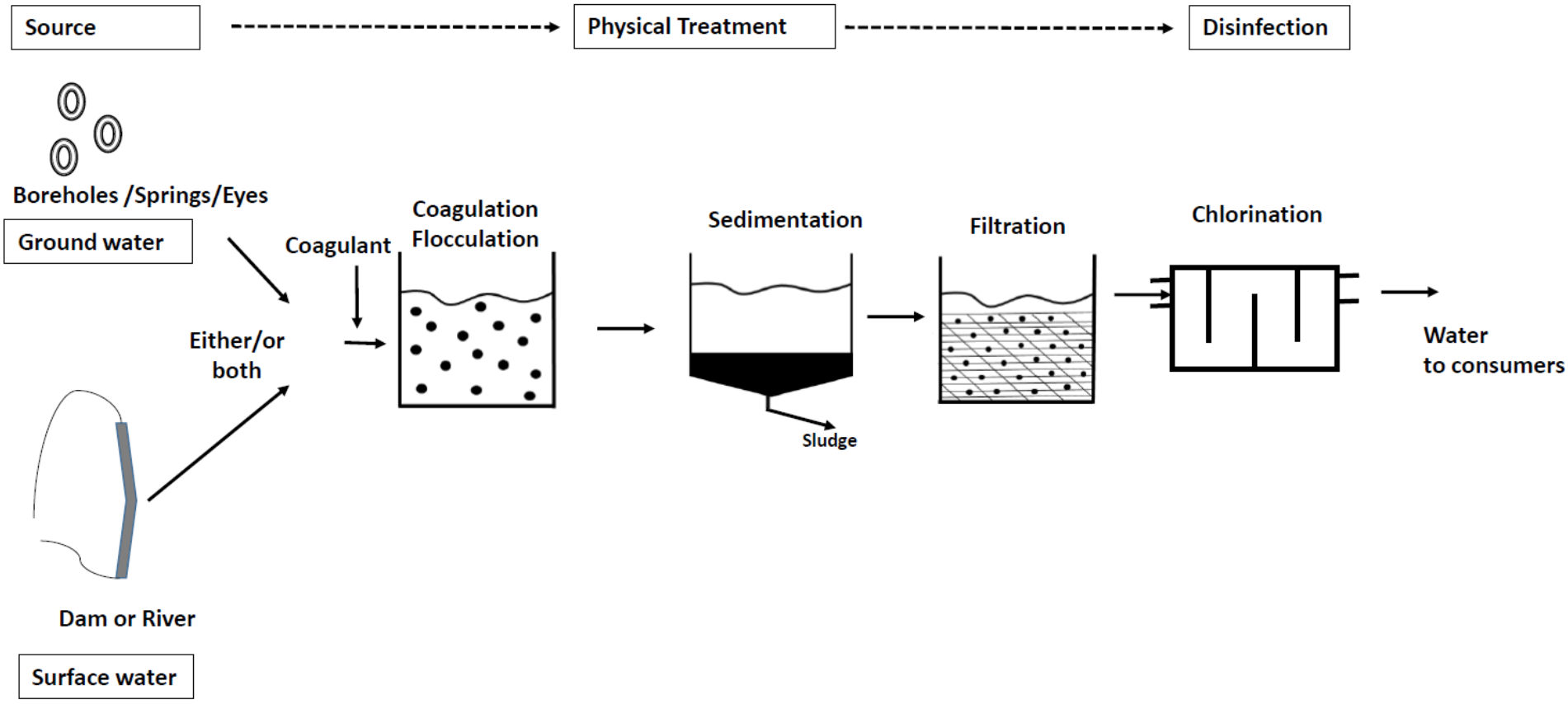


Figure 2.2: Schematic representation of the conventional treatment process

2.3.2 Advanced drinking water treatment processes

When conventional treatment processes are inefficient, particularly when the available source water is of a low quality or when sewage is reclaimed for drinking water production, advanced treatment processes are necessary (DWAF, 2002). The commonly used methods for advanced drinking water treatment include ozonation, desalination, distillation, reverse osmosis (RO) and advanced oxidation (UV + H_2O_2 or O_3) (Schutte, 2006; Maurel, 2006). Ozonation has excellent disinfectant properties, but it is short lived as it interacts with organic and inorganic substances. However, it can inactivate microorganisms such as protozoa, which are very resistant to conventional disinfectants (Van der Walt and Van der Walt, 2009). Membrane processes such as RO and nanofiltration (NF) are alternatives for drinking water treatment, where a high-quality product is desired. The RO and NF membranes successfully remove organic and inorganic compounds, as well as microorganisms (Koyuncu, 2002; Drewes et al., 2003). Advanced methods can overcome many of the problems and are mostly only used when absolutely necessary. However, they have certain limitations, for instance, initial set-up and start-up costs could be very high. High energy consumption is also an issue to be dealt with. The use of membranes is associated with the need for the frequent replacement of filters and membranes. Experts are required to operate and maintain such systems (Wimalawansa, 2013; Bremere et al., 2001). Such processes have been successfully implemented at drinking water treatment plants in South Africa. For example, Midvaal Water Company introduced ozonation (in 1985) and dissolved air flotation (in 1998) to combat issues initially with manganese, and later to increase salinity and organic matter that is difficult to settle (Morrison, 2009; Janse van Rensburg et al., 2016). The Vaalkop DWPF, on the other hand, introduced advanced treatment processes such as pre-chlorination, powdered activated carbon, dissolved air flotation, ozonation, activated carbon, post-chlorination and chloramination to treat water that is affected by cyanobacterial blooms (Swanepoel et al., 2017). Beaufort-West introduced a plant that reclaims sewage effluent for drinking water production. In this process, NF, RO and advanced oxidation (UV and H_20_2) are used, in combination with conventional methods to produce safe drinking water.

2.4 ANTIBIOTIC-RESISTANT BACTERIA AND GENES IN WATER

2.4.1 Sources of ARB and ARGs in aquatic systems

Antimicrobial-resistant microorganisms may originate from waste, including human and animal faeces (Burgmann et al., 2018). This is generally due to the fact that the microbes from these organisms had, at some stage, been exposed to antibiotics, either for therapeutic purposes (infection control or as a prophylactic) or as growth promoters in the case of animal-rearing practices (Burgmann et al., 2018). Antibiotics that are used for any purpose are not completely degraded and find their way into excretion products such as urine and faeces. This eventually lands in wastewater treatment systems and, if not treated adequately, could enter the freshwater environment (including rivers and lakes) (Yang et al., 2018). Okoh et al. (2007) focused on WWTPs in the Eastern Cape and demonstrated that these systems are pollution sources of pathogens and AMRGs. This aspect was demonstrated and summarised in several studies from developing and developed countries (Tong and Wei, 2012; Bouki et al., 2013). From literature, it is also evident that the presence of PPCPs, biocides, metals and agrochemicals may all contribute to the development of antibiotic resistance among bacteria (Li and Webster, 2018). In many WWTPs, a mixture of these chemicals may be present, creating an evolutionary pressure for the selection and development of antibiotic resistance.

Antibiotics, biocides and PPCPs have long been recognised as emerging pollutants with environmental concentrations ranging from <1 ng.ℓ^{-1} to several hundred ng.ℓ^{-1} (Bengtsson-Palme and Larsson, 2016). According to Pruden et al. (2006), ARGs should also be regarded as emerging pollutants. As such, minimum levels of no risk could be determined, and regulations put in place to enforce such limits.

Antibiotic resistance has, for an extended period (since the initial introduction of antibiotics in the 1940s), been recognised as a potential risk that should be managed. In his Nobel Prize acceptance speech for discovering penicillin, Alexander Fleming referred to this risk (Fleming, 1945). Until recently, antibiotic resistance was mainly the domain of clinical microbiologists, and medical and veterinarian practitioners. However, the rapid increase in antibiotic resistance on a global scale, and finding these antimicrobial residues and resistance microbes in environmental settings, particularly aquatic systems such as rivers and lakes, has had a profound effect on the view that the environmental dimensions and dynamics of antibiotic resistance are important (Burgmann et al., 2018). Connecting these contaminants of emerging concern in aquatic ecosystems to waste and impacts on human health, through a "one health" approach, needs to be explored.

Human exposure to pathogenic organisms that are also resistant to antimicrobials (e.g. Methicillin-resistant *Staphylococcus aureus* (MRSA)) is considered a real risk, and in clinical settings, monitoring and preventative procedures are normally put in place. In environmental waters, such procedures are not considered necessary or practical. However, aquatic systems such as rivers and dams are the major receptacles of antibiotics, ARB and ARGs. Due to the prevailing hydrological conditions, these aquatic ecosystems may remain the major pools for antibiotic residues, ARB and ARGs (Biyela et al., 2004). Various studies have considered the effects that antibiotics may have on the population dynamics of aquatic microorganisms, as well as biogeographical processes within such communities (see Yang et al., 2018, for an overview). The distribution, adsorption and degradation potential has also been studied in aquatic systems. Due to hydraulic differences between rivers and lakes (including dams), assumptions made in one system cannot directly be extrapolated to the other. Residence times in lakes and dams are much lower than in rivers, and the accumulation and impact potential of antibiotic residues, ARGs and ARB are thus greater.

South Africa is a water-scarce country with specific rainy seasons is various parts of the country. Dams and lakes provide important storage facilities for freshwater that is used for drinking water production. The same water sources are also used in agriculture, which is the largest consumer of fresh water (DWA, 2017). Biyela et al. (2004) reported that an aquatic system in KwaZulu-Natal could be regarded as a reservoir of ARB and ARGs. This was the first study of its kind demonstrating this scenario in South Africa. Furthermore, Bezuidenhout (2013), Carstens et al. (2014) Prinsloo et al. (2013) and Prinsloo (2014) have demonstrated a similar scenario for groundwater. Thus, when studies on antibiotic residues, ARB and ARGs are considered in the context of their potential impact on drinking water or food production, surface water sources (rivers, dams or lakes) and ground water sources should be considered.

2.4.2 Heterotrophic plate counts, ARGs and virulence genes: a threat of emerging concern in drinking water?

Microbial-safe drinking water is based on test results that conclusively demonstrate that there is no faecal indicator bacteria present. Such a result eliminates the potential of the faeco-oral transmission of pathogens, particularly diarrheal pathogens (Pruden, 2014; Bergeron et al., 2015). This is a practice that is easily implementable and cost effective (DWA, 2013). It has protected communities for more than a century (Ramírez-Castillo et al., 2015). On the other hand, drinking water distribution systems are not isolated, sterile environments. Conditions in the systems may allow "injured" bacteria (viable but non-culturable) with an opportunity for regrowth and, due to the large size of these systems, their age and nature, contamination from environmental sources frequently occur (Fakruddin et al. 2013). Bacteria from these events form biofilms that are mainly occupied by bacterial species that can be enumerated as aerobic HPC. The permissible HPC limit is 100 colony forming units (cfu) per mℓ, but up to 1 000 cfu/mℓ could be regarded as acceptable, as long as only a small percentage of samples has higher values.

When measured, these values are mainly used to determine the efficiency of the drinking water production processes and sanitary quality of the distribution system (Allen et al., 2004; WHO, 2003). The general health risk implications of these species have been questioned since insufficient data was available at the time (WHO, 2003).

A WHO report (WHO, 2002) recognised that the development and application of molecular techniques to study HPCs may provide additional information for future revision. With the development of molecular methods, the standardisation of techniques, as well as new data that has emerged, it is perhaps time to re-evaluate these conclusions. A recent body of work edited by LeChevallier (2015) focused on water pathogens. These studies and reviews show that, besides the normal faecal indicator organisms, opportunistic pathogens may also be associated with the ability to grow in amoebae, are resistant to disinfectants and are prone to the formation of biofilms. Carstens et al. (2014) demonstrated that opportunistic bacterial pathogens that are resistant to amoebae and multiple antibiotics are present in the groundwater sources of North West. Mulamattathil et al. (2015) highlighted the fact that many inland municipalities, particularly in North West, are dependent on groundwater sources for the production of drinking water.

Furthermore, Mulamattathil et al. (2014a; 2014b) also demonstrated that bulk water and biofilms in a distribution system, where the raw water originated from both ground and surface water, contained various bacterial species with antibiotic-resistant and virulence phenotypes. Prinsloo et al. (2013) and Prinsloo (2014) demonstrated the cytotoxic effects of exudates of antibiotic-resistant HPC bacteria from ground and drinking water on intestinal cell lines. Studies such as these suggest that the quality of the raw water that is used for the production of drinking water needs to be carefully monitored for all risk factors. Where there are challenges to the microbial quality, appropriate technologies must be put in place to ensure that the water is risk free and suitable for consumption by all sectors of the community, including the immunocompromised. In addition to this, and against sustainable water re-use strategies, in a scenario of limited surface water resources, the direct re-use of treated sewage effluent is considered an option (Pruden, 2014). Such effluent should be of a high standard, and should comply with or be of a better quality than the required standards (DWA, 2012). Of concern is that wastewater plants had been demonstrated as hotspots for ARB and ARGs (Okoh et al., 2007; Pruden, 2014). When upstream WWTPs are not effectively managed and operated, the opportunities of such antimicrobial residues and genes, pathogens and opportunistic pathogens to be present in drinking water at elevated levels after processing are enhanced (Bouki et al., 2013).

In the northern, inland provinces of South Africa many WWTPs are not working efficiently and, in some cases, are not fully operational, creating such opportunities for wastewater to pollute environmental water (DWA, 2012). Water sources receiving poorly or untreated sewage are then used to produce drinking water. Many of the water provision systems in these northern provinces are based on open systems where water is only used once by a town or city before being discarded through the WWTPs. However, several of these towns are downstream from neighbouring towns, agricultural production systems and industrial or mining areas, and are thus indirect re-users. Mulamattathil et al. (2014a; 2014b) demonstrated that, among the bacteria in bulk water and biofilms, pathogenic *Pseudomonas* sp. and *Aeromonas* sp. were present in a South African drinking water distribution system. The isolates from these species could also be positively associated with virulence genes and were mostly resistant to multiple antibiotics. Studies by Bai et al. (2015) and Xu et al. (2016) demonstrated that chlorine and chloramine disinfection, and even the use of a biologically activated carbon filtration system, increased the incidence of ARB and ARGs, which could be detected in consumers' tap water. This further demonstrated that the distribution system could be an important resevoir for ARB and ARGs.

The notion that HPC bacteria was harmless to mankind had its merits (WHO, 2003; Allen et al., 2004; Vaz-Moreira et al., 2014). However, Pavlov et al. (2004), Mulamatthil et al. (2014a; 2014b) and Horn et al. (2016) demonstrated that HPC from drinking water systems produce various virulence factors that would make them opportunistic pathogens. There are several extracellular enzymes (virulence factors) that can be used as indicators for the pathogenic potential of HPCs, including haemolysin, DNase, proteinase, lecithinase and lipase. The presence of these enzymes can be easily demonstrated by a culture-based spot inoculation using appropriate media (Horn et al., 2016). When two or more extracellular enzymes are produced, the isolates could be classified as potentially pathogenic (Pavlov et al., 2004). Once HPC bacteria produce these extracellular enzymes, they have the potential to be invasive, which makes them more prone to be or become pathogenic (Horn et al., 2016). In the immune compromised (young children, the elderly, those with underdeveloped immune systems, transplant and chemotherapy patients) the risk of HPC infections is real (Pavlov et al., 2004).

In a study by Carstens et al. (2014), it was demonstrated that many HPC bacteria isolated from ground water were resistant to amoeba species. Such amoeba feed on bacteria by phagocytosis; thus, for the bacteria to survive, certain resistance mechanisms were developed. Such mechanisms include the resistance of microbicidal effectors in the phagocytes, the ability to replicate in the intracellular environment, or the secretion of toxins that kill the amoebae. The phagocytosis mechanism of amoeba is similar to that of human macrophages. Thus, amoeba-resistant bacteria may possess an increased ability to resist phagocytosis by macrophages in the human immune system. These factors may contribute to the pathogenicity of ARB to humans (see Carstens et al., 2014, for more details).

Many of these antibiotic- and macrophage-resistant, as well as virulence factors, are coded by genetic elements and are thus subjected to normal genetic exchange-uptake processes such as conjugation, transformation and transduction. These could thus be disseminated among bacterial populations. Finding large concentrations of bacteria with these features in source and drinking water should thus be of concern. During wastewater treatment and drinking water production, efforts are made to remove, reduce or kill harmful bacteria by processes that lead to lysis. The intracellular content (including the genome) of the bacteria thus land in the water. Such DNA could thus be taken up by competent non-antibiotic-resistant, non-pathogenic bacteria, rendering them resistant to antibiotics (Wang et al., 2016). It would thus be logical to argue that insufficient information is available on the potential pathogenic characteristics of ARB and ARGs in drinking water in South Africa. This present study thus provides some baseline data.

2.5 NEED FOR A MICROBIOME ANALYSIS OF DRINKING WATER

2.5.1 Overview

Modern molecular technologies such as qPCR and NGS, have contributed tremendously towards understanding that safe, high-quality drinking water has a unique biodiversity that is impacted on by the quality of the source water, purification process, materials used in the distribution system, and physical forces in the system (Liao et al., 2014; Bruno et al., 2018). It is now understood that the microbial ecosystems in such systems are complex and that these interact at networks, expanding several levels. These species can impact on the quality of the distribution system (Bruno et al., 2018; Vosloo et al., 2018). It is known that bacterial growth in the system is positively impacted on by elevated water temperatures, low residual chlorine and nutrients (carbon, phosphorus, nitrogen, and iron) (Bruno et al., 2018). Pinto et al. (2014) demonstrate how the layout of a distribution system could impact on microbial diversity. They also showed that microbial communities are also further impacted on by temporal trends. Their work formed the basis for recommending the gathering of long-term datasets that would be useful in predictive modelling (Pinto et al., 2014). A recently completed WRC project (K5/2469/1/18) has generated some NGS data for one of the main distribution systems in South Africa (Vosloo et al., 2018).

Data obtained by 16S rRNA gene profiles are informative at the population and community level, and can be processed into various ecological diversity indices (Pinto et al., 2014; Liao et al., 2014; Bruno et al., 2018). However, Bowman and Ducklow (2015) described a useful method (pipeline) to explore using such phylogenetic datasets to extrapolate metabolic and ecosystem functioning. The microbial metagenomes can be predicted from 16S rRNA gene sequences using the online PICRUSt pipeline (Langille et al., 2013; Zaura et al., 2015). Recently, Mukherjee et al. (2017) reported how they effectively used this approach to demonstrate which taxa associated with contaminated environments, in particular, would potentially be useful for remediation purposes. The prevalence of ARDs could also be evaluated by blasting OTUs against ARGs downloaded from appropriate ARG databases, such as the ARDB (Liu and Pop, 2009).

2.5.2 Molecular methods for the detection of ARB and ARGs in water

2.5.2.1 Polymerase chain reaction

Conventional PCR is commonly used to detect bacteria and ARGs in complex DNA mixtures because it is easy, quick and inexpensive (Hongbao, 2005). It allows for the detection of ARGs' encoding resistance to antibiotics like tetracycline, βlactams and aminoglycosides in pure and mixed environmental samples (Zhang et al., 2009). Furthermore, previous studies detected ARGs by PCR directed to genes such as *amp*C, *Int*I, *erm*B, *erm*C, *erm*F, *tet*O and *tet*W (Schwartz et al., 2003; Beukers et al., 2018; Selvaraj et al., 2018) in aquatic environments.

2.5.2.2 Quantitative real-time PCR

The real-time quantitative PCR (qPCR) is a technique used to quantify the amount of target gene copies in a specific sample (Zhang and Fang, 2006). This technique is used because of its accuracy, precision, sensitivity and high-throughput capacity (Yu et al., 2005). Fluorescent dyes have made it possible to monitor the amplification process in qPCR, allowing relative quantification of the initial amount of target gene copies (Lievens et al., 2011). Real-time qPCR is used to compare the levels of ARGs present in an environment over time and in comparison to other environments. This technique has delivered quantitative answers to questions concerning the effect of antibiotics on the development and spread of antibiotic resistance in the environment (Walsh et al., 2011). Studies have applied qPCR to quantify and study the effects of environmental factors or treatment processes on the removal of the following ARGs: *sul*, *tet, erm, amp*C and *bla*TEM genes (Zhang et al., 2009; Xi et al., 2009; Shi et al., 2013). Waseem et al. (2019) reviewed high-throughput qualitative PCR (HT-qPCR) to investigate the diversity, abundance and distribution of ARGs and mobile genetic elements (MGEs) in the environment. The use of HT-qPCR is becoming a popular tool to rapidly obtain information on ARGs. An et al. (2018) used this method to track the antibiotic resistome during wastewater treatment.

2.5.2.3 Whole-genome sequencing

The sequencing of whole genomes of organisms by using next-generation sequencing (NGS) and modern computational methods had become a valuable tool in clinical and public health microbiology (Kwong et al., 2016). Kwong et al. (2016) predicted that this technology could potentially replace standard strain characterisation by traditional typing methods, as well as resistance-gene detection. A subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) investigated the role of WGS in the antibiotic susceptibility testing (AST) of bacteria. They reviewed over 200 publications (published by 2015/16) and concluded that the traditional AST methodologies could be performed in any microbiological laboratory. They reflected on more than just the presence of genetic elements. However, they concluded that too little data is currently available to consider a drastic step to make a definitive recommendation to include WGS in AST.

The cost of sequencing and the computational skills required are some of the factors preventing greater participation. However, the methodology is gaining traction and is a rapid method for gaining insights into novel metabolic genes, ARGs and virulence genes (Kwong et al., 2016). Gupta et al. (2018) briefly featured how WGS in genome-wide association studies, in combination with machine learning methodologies, was able to uncover novel antibiotic-associated genetic elements in *Mycobacterium tuberculosis*. They also featured a study in which machine learning and WGS could predict the minimum inhibitory concentration of *Streptococci pneumoniae* to six beta-lactam antibiotics.

2.5.2.4 Metagenomics

Metagenomic analysis entails the high-throughput sequencing of all DNA directly extracted from an environmental sample. This is a promising tool to study ARG diversity and abundance in such environments (Zhang et al., 2011). This method has been applied to drinking and raw water compartments in the recent past (Shi et al., 2013). Shi et al. (2013) demonstrated that chlorination-concentrated ARGs, plasmids, as well as MGEs, were involved in horizontal gene transfer.

CHAPTER 3: STUDY DESIGN AND METHODS

3.1 MATERIALS

Acetonitrile	(Sigma, USA)
Agar	(Oxoid, UK)
Antibiotics	(Oxoid Ltd, UK)
Blood agar (BA) plates	(Thermo Fisher Scientific, USA)
BIOO Scientific ELISA kits	(Austin, Texas, USA)
Brain heart infusion broth	(Oxoid, UK)
Chemagic Viral DNA/RNA kit	(PerkinElmer®, USA)
Chemical kits for chemical oxygen demand (method 8000), free chlorine (method 8021), nitrates (method 8039), nitrites (method 8153), phosphates (method 8178), sulphide (method 8131) and sulphates (method 8051)	(Hach, USA).
Dream Taq PCR master mix	(Thermo Fisher Scientific, USA)
1 kb O'Gene Ruler	(Thermo Scientific, US)
DNase agar	(Merck, Germany)
Egg yolk mix	(Merck, Germany)
Ethidium bromide	(Bio-Rad, UK)
Formic acid	(Sigma, USA)
Gelatine powder	(Merck, Germany)
HLB 47 mm extraction disks	(Horizon Technology)
McClung-Toabe agar	(Difco, France)
Meat extract agar	(Lab M Ltd., UK)
Methanol	(Sigma, USA)
Mueller-Hinton agar	(Lab M Ltd., UK)
Orange loading dye	(Thermo Fisher Scientific, USA)
Peptone	(Merck, Germany)
R2A agar	(Lab M Ltd., UK)
Skimmed milk agar	(Oxoid, UK)
Toluidine blue	(Sigma, USA)
Trypticase soy agar	(Merck, Germany)
Tween 80	(Sigma, USA)

3.2 SAMPLING

3.3 STRATEGY AND METHODS

3.3.1 Sampling locations

Written permission was obtained from the municipality or water services provider. The participating organisations operated a DWPF that uses one of the following water sources and processes:

- A direct re-use/reclamation plant, supplementing treated dam and borehole water: WC-A
- A groundwater and semi-direct re-use system with a WWTP and subsistence agriculture: NW-B and NW-D
- A system that uses groundwater source water with impacts from agriculture: NW-C
- A conventional system with upstream impacts from mining, agriculture and urbanisation: NW-E

- A conventional system with minimal upstream impacts: WC-F
- A system that uses ozone in the drinking water production process: NW-G
- A system that uses conventional purification and a combination of chlorination and monochloramine as disinfection: GT-H

Details for the plant treatment trains are provided in Chapter 4.

3.3.2 Sample collection points and frequency

At each of the selected plants, at least two sampling rounds were done at the following points:

- Raw water
- Final water – after before being sent to distribution
- Two places in the distribution system (one close to the DWPF and one a considerable distance from the DWPF)

3.3.3 Onsite and laboratory analysis of physical and chemical properties

The temperature, pH level, TDS or electrical conductivity and salinity of the water samples were measured on site by using an Oakton PCSTestr 35 waterproof pH/conductivity/TDS/salinity tester (Thermo Fisher Scientific, USA). The following parameters were measured in the laboratory: turbidity, chemical oxygen demand, free chlorine, phosphates, nitrates, nitrites, sulphide and sulphates. The turbidity of the water samples was measured using the HACH 2100P portable turbidity meter (Hach, US) following the manufacturer's instructions. Chemical oxygen demand (method 8000), free chlorine (method 8021), nitrates (method 8039), nitrites (method 8153), phosphates (method 8178), sulphide (method 8131) and sulphates (method 8051) in the water samples were measured using the Hach DR 2800 spectrophotometer (Hach, USA) following the manufacturer's instructions.

3.4 SCREENING AND QUANTIFICATION OF ANTIBIOTICS IN WATER

3.4.1 Screening for selected antibiotics

Screening of the selected antibiotics and antimicrobials were carried out for samples collected following the analytical methodology of the analytical facility at the North-West University. Glass containers (1 ℓ) were used for sampling. Contaminants from previously used glassware were removed using the USEPA (2007) method.

3.4.1.1 Extraction

The extraction of target compounds from water was based on methods used by Ferrer et al. (2010) for pharmaceuticals. Target compounds were concentrated 2 000 times by automated solid phase extraction (SPE) using the SPE-DEX system (Horizon Technology, Salem, New Hampshire, USA). Oasis hydrophilic or lipophilic balanced (HLB) disks were used as they are efficient at extracting analytes with various polarities and acid or base characteristics at different pH levels (Pedrouzo et al., 2011). HLB-L was the best fit for the purpose of the study as the research team had a low organic sample type. The US EPA method 1694 for PPCP analysis makes use of these disks. The HLB extraction disks (47 mm, Horizon Technology) were used according to the application note. The eluent was concentrated to near dryness using a gentle stream of nitrogen gas. The samples were reconstituted in methanol and subjected to UPLC-QTOF/MS for analysis.

3.4.1.2 Screening using ultra-performance liquid chromatography

The UPLC system used consisted of an Agilent 1290 Infinity binary pump (G4220A), a 1290 Infinity autosampler (G4226A) and a 1290 Infinity thermostatted column compartment (G1316C) coupled to an Agilent 6540 accurate mass QTOF/MS) G6540A (Agilent Technologies, Santa Clara, California, USA) (Table 3.1). The desolvation and ionisation of samples were achieved by positive and negative electrospray ionisation (ESI) enhanced with Agilent jet stream (AJS) technology. The QTOF was set to scan from 50 to 950 m/z and the instrument was set to an extended dynamic range (2 GHz). The software used was MassHunter Data Acquisition (version B.05.00), MassHunter Qualitative Analysis (version B.05.00) and Quantitative Analysis for QTOF (version B.05.01). Mass axis calibration of QTOF was performed daily for positive and negative ionisation with tuning mixes (G1969-85000, Agilent). A reference solution with masses of 121.050873 [M+H] and 922.009798 [M+H] were constantly infused as accurate mass references.

Table 3.1: Analysis method on the UPLC-QTOF instrument

Parameters	**Positive ionisation**	
Injection volume	1 µℓ	
Column	Poroshell 120 Bonus-RP column (Agilent, 2.1 x 100 mm, 2.7 µm)	
Column temperature	25 ˚C	
Flow rate	0.6 mℓ/min	
Mobile phase A	Water + 0.05% formic acid	
Mobile phase B	Acetonitrile methanol + 0.05% formic acid	
Gradient (min)	A (%)	B (%)
0	90	10
8.5	90	10
8.6	50	50
13	50	50
13.3	0	100
14.3	0	100
15	90	10
Post run-time	2 minutes	
Total run-time	17 minutes	
Drying gas temperature	275 °C	
Drying gas flow	10 ℓ/min	
Nebuliser pressure	45 psi	
Sheath gas temperature	400 °C	
Sheath gas flow	10 ℓ/min	
VCap	3 000 V	
Nozzle voltage	0 V	
Fragmentor	130 V	
Skimmer	48 V	
OCT RF Vpp	750 V	

3.4.2 Quantification of beta-lactam antibiotics, Trimethoprim and Colistin using ELISA

3.4.2.1 Extraction

The method used to extract antibiotics from water was based on Ferrer et al. (2010) for pharmaceuticals. Target compounds were concentrated 2 000 times by automated SPE using the SPE-DEX system (Horizon Technology, Salem, New Hampshire, USA). The extraction was done according to the application note. The eluent was concentrated to near dryness using a gentle stream of nitrogen gas. The samples were reconstituted in methanol and subjected to UPLC-QTOF/MS for analysis. However, these extracts were diluted to perform the ELISA. The extracts were diluted 200 times, which means that the ELISA plates received samples that were concentrated 10 times.

3.4.2.2 Detection and quantification using ELISA

Recently, ELISA has demonstrated results comparable with liquid chromatographic or gas chromatographic methods. These assays are a reliable and good substitute for the quantification of levels of contaminants in water and other sources (depending on the type of kit used). Beta-lactams were quantified using a BIOO scientific ELISA kit (Cat # 1065) (Austin, Texas, USA). The method was performed according to the manufacturer's instructions. A six-point calibration curve of Penicillin G ranged from 0 to 1.2 µg/ℓ. The ELISA kit for Colistin was from the same supplier as the beta-lactams (Cat # 1095-01B). The instructions enclosed in the kit were used and the calibration curve ranged from 0 to 50 µg/ℓ. Trimethoprim was also determined using an ELISA kit from BIOO Scientific (Cat # 1099). The calibration concentration curve was from 0 to 1.08 µg/ℓ. In short, the samples, blanks and standards were added to the 96-well plates, and after various steps, absorbance was measured at 450 nm.

3.4.2.3 Quality control of the ELISA

All samples were quantified in triplicate for each specific antibiotic. The mean absorbance values were calculated and the coefficient of variation (CV) should be <20%. The limit of detection (LOD) and limit of quantification (LOQ) were determined using a regression analysis of the calibration curves where LOD = $3S_b/b$ and LOQ = $10S_b/b$ with S_b = slope uncertainty and b = slope. The concentrations for beta-lactams, Colistin and Trimethoprim were determined against the linear regression line of the calibration curve, with an R^2 as close as possible to 1. The responses from the ELISA were back-calculated to account for the 10 times concentration. The final concentration in the sample is reported in the results section. Each water sample was subjected to ELISA plates in triplicate along with blanks and standards to obtain calibration curves. The CV calculated for each sample, across the three different ELISA plates, was deemed acceptable with good precision <20%. The LOD and LOQ were determined for each target compound from the various ELISA plate tests (Table 3.2).

Table 3.2: The LOD, LOQ and R^2 values for each ELISA kit (before the back-calculation).

	Trimethoprim	Colistin	Beta-lactams
LOD (µg/ℓ)	11	25	31
LOQ (µg/ℓ)	38	83	103
R^2	0.99	0.96	0.98

3.5 METHODS FOR THE ISOLATION AND CHARACTERISATION OF BACTERIA

3.5.1 Isolation of HPC bacteria

To isolate HPC, a bacteria dilution series was prepared up to 10^{-5}. A hundred microliters of the serial dilution were spread on R2A agar (Lab M Ltd., UK). Spread plates were incubated for six days at room temperature. After incubation, the colonies were counted, and forming units per mℓ were determined. Single colonies were selected based on morphology and streaked out on R2A agar and incubated for six days at room temperature. To ensure purity, colonies were streaked out multiple times. The Gram staining method was performed as described by Claus (1992). This was done to determine if the isolates were pure, and to classify the isolates as Gram positive or negative to determine the range of antibiotics used.

3.5.2 Identification and characterisation of HPC bacteria

3.5.2.1 DNA isolation

Pure colonies were inoculated on R2A broth and incubated at 28 ºC for 24 hours. After incubation, the Chemagic Viral DNA/RNA kit (PerkinElmer®, USA) was used to isolate the nucleic acid. The nucleic acid of bacterial species was isolated as indicated on the manufacturer's protocol. A DNA gel electrophoresis was conducted to determine whether the nucleic acid had been successfully isolated. Only successfully isolated nucleic acid was subjected to the nanodrop™ 1000 Spectrophotometer v 3.5.2 (Thermo Fisher Scientific, USA) to determine its purity (A_{260nm}:A_{280nm}) ratio and concentration (ng/µℓ).

3.5.2.2 PCR amplification of 16S rRNA gene sequences

Purified DNA samples were subjected to amplification of the 16S rRNA gene using universal primers 27 F (AGA GTT TGA TCM TGG CTC AG) and 1492 R (GG TTA CCT TGT TAC GAC TT). These primers amplify the V3 and V4 region of the 16S rRNA gene (Manaka et al., 2017; Jordaan and Bezuidenhout, 2013). The total volume of the PCR reactions was 25 µℓ [12,5 µℓ Dream Taq PCR master mix (5 U/µℓ Taq DNA polymerase in reaction buffer, 2 mM $MgCl_2$, 0.2 nM of each dNTP) (Thermo Fisher Scientific, USA), 1 µℓ of forward primer, 1 µℓ of reverse primer, 1 µℓ of 20 ng DNA template and 9.5 µℓ of nuclease-free water]. Reaction mixtures for positive and negative controls were also prepared. The Techne™ PCRmax Alpha Cycler 1 PCR (Thermo Fisher Scientific, USA) was used to amplify the nucleic acid products using the following thermal cycling conditions: initial denaturation at 95 °C for five minutes, 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 30 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes.

3.5.2.3 Gel electrophoresis

After the completion of the PCR run, products were subjected to gel electrophoresis carried out on 1.5% agarose gel (w/w) in 1 x TAE buffer (20 mM acetic acid, 40 mM Tris and 1 mM EDTA at pH 8.0). Two microlitres of 6 x orange loading dye (Thermo Fisher Scientific, USA) was premixed with 3 µℓ of the PCR product, and 5 µℓ was loaded into the pores of the gel. A 1 kb DNA was also loaded (O'Gene Ruler, Thermo Scientific, USA) on the gel to measure the size of the DNA product in base pair (bp). The gel electrophoresis was allowed to run for 45 minutes at 80 V. The gel was stained with 10 µℓ of ethidium bromide (Bio-Rad, UK). A ChemiDoc MP imaging system (Bio-Rad, UK) was used to generate and capture the image of the gel electrophoresis for the analysis of PCR products. Successful amplicons with approximately 1 500 bp were subjected to sequencing.

3.5.2.4 Sequencing

Amplicons were sequenced at North-West University's Microbiology Sequencing Facility or sent to Inqaba Biotechnology Industries (Pty) Ltd in Pretoria for sequencing. Bacterial sequences were analysed using Finch TV (Version 1.4.0). Nucleic acid sequences obtained from BLAST software were exported to EzTaxon software to identify bacterial species.

3.5.2.5 Endpoint PCR for the detection of ARGs

This study focused on the detection of various ARGs (Table 3.3). The protocols used for detecting each of these genes are covered below. The total volume of the reaction mixture for each gene was also 25 µℓ. Reaction mixtures consisted of 12.5 µℓ Dream Taq PCR master mix (5 U/µℓ Taq DNA polymerase in reaction buffer, 2 mM $MgCl_2$, 0.2 nM of each dNTP) (Thermo Fisher Scientific, USA), 0.4 µℓ forward and reverse primer (Inqaba Biotec, RSA), 1 µℓ of 20 ng DNA template and 9.5 µℓ nuclease-free water. The success of the PCR products was determined using agarose gel electrophoresis as described in Section 3.4.2.3.

Table 3.3: Oligonucleotide primers for PCR amplification of 16S rDNA, *erm*F, *intI1, erm*B and *ampC* genes; F – Forward primer and R – Reverse primer

Target gene	Name	Sequence (5'…..3')	Size (bp)	Reference
16S rDNA	27F	AGA GTT TGA TCM TGG CTC AG	1 465	Jiang et al., 2006
	1492R	GG TTA CCT TGT TAC GAC TT		
bla$_{TEM}$	TEM-F	ATT CTT GAA GAC GAA AGG GC	1 150	Costa et al., 2007
	TEM-R	ACG CTC AGT GGA ACG AAA AC		
*erm*F	ermF1	CGG GTC AGC ACT TTA CTA TTG	466	Chung et al., 1999
	ermF2	GGA CCT ACC TCA TAG ACA AG		
*erm*B	ermB-F	GAA AAG GTA CTC AAC CAA ATA	638	Tran et al., 2013
	ermB-R	AGT AAC GGT ACT TAA ATT GTT TAC'		
IntI1	HS463A	CTG GAT TTC GAT CAC GGC ACG	473	Labbate et al., 2008
	HS464	ACA TGC GTG TAA ATC ATC GTC G		
ampC	AmpC-F	TTC TAT CAA MAC TGG CAR CC	550	Coertze and Bezuidenhout, 2018
	AmpC- R	CCY TTT TAT GTA CCC AYG A		

(a) *ermF* and *ermB* ARGs

The PCR protocol for the amplification of these genes (*ermF* and *ermB*) was performed as described by Chung et al. (1999) and Fourie (2017). The PCR cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 seconds, annealing (*ermF* at 50 °C for 30 seconds and *ermB* at 48 °C for 1 minute), elongation at 72 °C for 2 minutes and final extension at 72 °C for 10 minutes.

(b) *IntI1* ARG

The PCR conditions for the detection of *IntI1* genes were as follows: initial denaturation at 95 °C for 5 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 64 °C for 30 seconds, elongation at 72 °C for 60 seconds and final extension at 72 °C for 5 minutes. Successful amplicons have a length product of 473 bp (Labbate et al., 2008; Coertze and Bezuidenhout, 2018).

(c) *ampC* ARG

The following thermal cycling conditions were used: initial denaturation at 94 ºC for 5 minutes, 33 cycles of denaturation at 94 °C for 30 seconds, annealing at 49 °C for 30 seconds, elongation at 72 °C for 60 seconds and final extension at 72 °C for 7 minutes. Successful amplicons have a length product of 550 bp (Coertze and Bezuidenhout, 2018; Schwartz et al., 2003).

3.5.3 Antibiotic susceptibility of HPC

Antibiotic susceptibility testing was done using the disc diffusion method described by Bauer et al. (1966). Spread plates of each isolate were made on Mueller-Hinton agar (Lab M Ltd., UK). Commercially prepared disks, each of which were pre-impregnated with a standard concentration of a particular antibiotic, were placed on the agar. Plates were incubated for 24 hours at 37 ˚C. The antibiotics used and their concentrations were as follows: 10 mcg Ampicillin, 30 mcg Cephalothin, 30 mcg Chloramphenicol, 5 mcg Ciprofloxacin, 15 mcg Erythromycin, 30 mcg Kanamycin, 30 mcg Neomycin, 30 mcg Oxytetracycline/tetracycline, 10 iu Penicillin G (Gram positive only), 25 mcg Streptomycin, 5 mcg Trimethoprim and 30 mcg Vancomycin (Gram positive only; Oxoid Ltd., UK). After incubation, the inhibition zones present on the agar were measured in mm. The antibiotic susceptibility profiles of the isolates were determined using the Performance Standards for Antimicrobial Susceptibility Testing provided by the Clinical and Laboratory Standards Institute (CLSI, 2014).

3.5.4 Multiple Antibiotic Resistance Index

The method used to determine MAR values per sampling site is provided below. Such an index provides an overview of the historical antibiotic exposure of the isolates from a particular area (Guan et al., 2002)

MAR Index = *a / (b x c)* per sample **Equation 1**

where:
a = total amount of resistance to antibiotics
b = amount of antibiotics used
c = number of isolates in sample

3.5.5 Resistance to Colistin

The R2A media supplemented with Colistin was to be used for the detection of resistance to this last-resort antibiotic available for therapy in cases where other classes of antibiotics are ineffective. A spot inoculation method in which multiple isolates are inoculated on a single plate will be used. None of the isolates from the plants were able to grow on the media. This was not considered any further.

3.5.6 Determination of virulence factors

3.5.6.1 Haemolysis

Blood agar plates (Thermo Fisher Scientific, USA), supplemented with 5% sheep blood, were used for the haemolysis test. Isolates were streaked on a BA plate and incubated for 24 hours at 37 °C. The following results were taken after incubation: the beta-hemolytic (β) isolates completely break down the red blood cells (represented by a clear zone), the alpha-hemolytic (α) isolates partially break down the red blood cells (represented by a partially clear zone) and gamma haemolytic isolates do not break down the red blood cells (Russell et al., 2006).

3.5.6.2 DNase

The DNase medium consisted of DNase agar (Merck, Germany) prepared according to the manufacturer's instructions. The medium was supplemented with 0.01% toluidine blue (Sigma, USA). Toluidine blue acts as a dye and substrate by binding to the hydrolysed DNA (Prinsloo, 2014). Plates were inoculated and incubated at 37 °C for 24 to 48 hours. Bacterial species that hydrolyse DNA are represented by a clear zone or colour change around the colony (Pavlov et al., 2004).

3.5.6.3 Lipase

The lipase medium consisted of Trypticase soy agar (Merck, Germany) prepared according to the manufacturer's instructions. The medium was supplemented with 1% Tween 80 (Sigma, USA). Tween 80 acts as a substrate. Isolates were inoculated and incubated at 37 °C for 72 hours. Bacterial species that hydrolyse lipids were indicated by a turbid halo around the colonies (Prinsloo, 2014).

3.5.6.4 Gelatinase

The gelatinase medium consisted of gelatine powder (Merck, Germany) prepared according to the manufacturer's instructions, 3 g/ℓ meat extract (Lab M Ltd., UK) and 5 g/ℓ peptone (Merck, Germany). The pH level of the medium was adjusted to 6.8 and autoclaved. Isolates were inoculated on slants and incubated at 37 °C for 24 to 96 hours. After incubation, the medium was put in a 4 °C fridge for 10 to 15 minutes. Positive isolates were represented by a liquefied media (Pavlov et al., 2004).

3.5.6.5 Proteinases

The proteinase medium was composed of 3% (v/v) skimmed milk agar and brain heart infusion broth prepared according to the manufacturer's instructions (Oxoid, UK) with the addition of 15 g/ℓ agar. Ingredients were prepared and autoclaved separately. Isolates were inoculated and incubated at 37 °C for 48 hours. Bacterial species that hydrolyse protein were indicated by a clear zone around the colonies (Prinsloo, 2014).

3.5.6.6 Lecithinase

The secretion of lecithinase by bacteria was determined using McClung-Toabe egg yolk agar (Steffen and Hentges, 1981). Briefly, McClung-Toabe agar (Difco, France) was prepared according to the manufacturer's instructions and sterilised. After cooling, one part of the 50% egg yolk mix (Merck, Germany) was added to nine parts of agar and plates were prepared. Plates were examined for evidence of egg yolk degradation after 24 hours of incubation at 28 °C to 30 °C. A distinct zone of opacity around or beneath the inoculum spot on the egg yolk agar indicated the production of lecithinase (Jula et al., 2011).

3.5.7 Whole-genome sequencing

In this study, the research team performed WGS on isolates belonging to the genus *Bacillus.* This was done according to the procedure shown in Figure 3.1. These were identified by 16S rRNA gene sequencing. The WGS was concluded for one plant and the data analysed. Paired-end sequencing was performed on a MiSeq sequencer (Illumina) using protocols as described by Illumina. Sequencing reads were trimmed and assembled using the CLC Genomics Workbench Version 9. Subsequent assemblies were then annotated using RAST, which identified genes associated with antimicrobial resistance traits, as well as numerous other genes. CARD (The Comprehensive Antibiotic Resistance Database) was also used to identify ARGs.

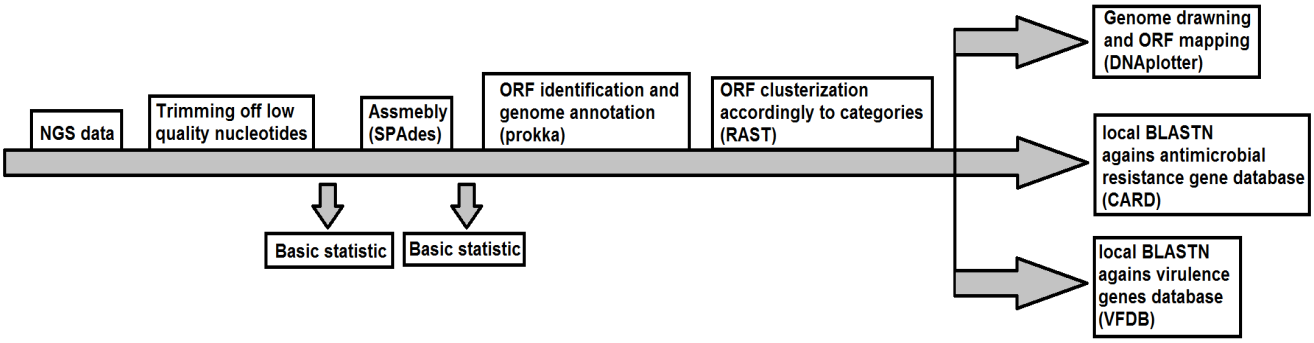


Figure 3.1: Schematic presentation of the sequence analysis

3.5.8 Microbiome sequencing analyses

3.5.8.1 Isolation of eDNA for microbiome sequencing

A product that is used to purify water during activities such as hiking, backpacking and camping (according to the product's description) was used to filter up to 1 000 ℓ of water (see Figure 3.2). It has a hollow fibre membrane through which water is filtered. By adding a few adapters, as seen below, water is filtered directly after chlorination at a DWPF or from any tap that can fit to the connecting adapter. The water leaving the filter can be recycled back into the raw untreated water so that it can go through the DWPF's treatment processes again. This filtering system can also be used at home where it is connected to an outside tap, and water can be used as usual. This system was adapted to be linked to a submersible pump for the filtering of raw water.

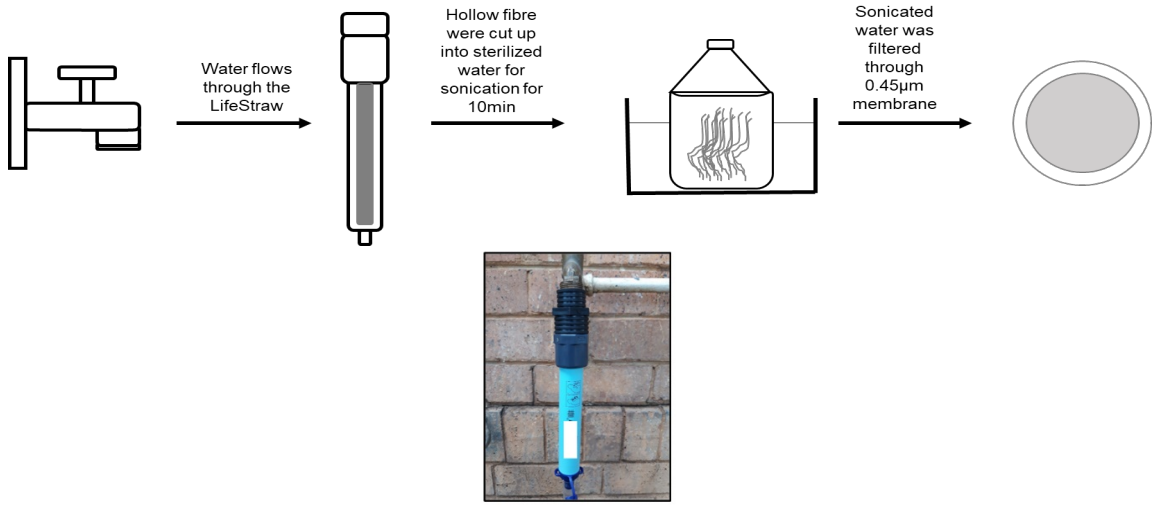


Figure 3.2: Diagrammatic and photographic illustrations of the free-floating microorganism and eDNA capturing, small volume (1 000 to 4 000 ℓ) system

3.5.8.2 Sequencing

The microbial communities were studied using a MiSeq (Illumina Inc., California, USA) at the Microbiology Sequencing Facility of North-West University. The bacterial barcode genes (16S rRNA gene) were amplified using universal primers 341F and 805R (Klindworth et al., 2013) modified with Illumina forward and reverse adapters (Illumina Inc., California, USA), respectively.

Library preparation of the 16S rRNA genes was performed exactly as described in the MiSeq 16S library preparation workflow of Illumina (Illumina Inc., California, USA). The workflow included amplification using primer pair 341F/805R, amplicon clean-ups, indexing, normalisation, pooling and denaturation. Thereafter, a 2 x 300 bp paired-end sequencing was performed using the MiSeq v3 reagent kit on the Illumina MiSeq sequencer (Illumina Inc. California, USA). Sequence reads were quality checked using FastQC (Babraham Bioinformatics, UK). These were trimmed using Trimmomatic software (Bolger et al., 2014), and forward and reverse reads were merged and filtered for ambiguous bases ("N") and read length using PANDAseq (Masella et al., 2012). Merged quality-filtered reads were then clustered into operational taxonomic units (OTU) at 97% 16S rRNA gene similarity ("closed reference picking") using Usearch61 (Edgar, 2010; Edgar et al., 2011) against the SILVA reference Database (Release 128) (Quast et al., 2013) in QIIME 2 software (Caporaso et al., 2010). The OTU count table was exported and the online MicrobiomeAnalyst software used to analyse the data for beta and alpha diversity based on the relative abundance of OTUs in all the samples.

3.5.8.3 Predictive functional profiling of microbial communities

Microbial metagenomes were predicted from 16S rRNA gene sequences using the online PICRUSt pipeline (Langille et al., 2013; Zaura et al., 2015). The prevalence of ARDs was evaluated as previously described by blasting OTUs against ARGs downloaded from the ARDB (Liu and Pop, 2009). Raw data (250 bp) obtained for the metagenomics analysis of selected samples from one distribution system (GT-H) was provided by the University of Pretoria. These were quality checked using FastQC software. Only samples showing satisfactory parameters were used. Sequences were analysed using ad hoc bioinformatics pipelines. Sequences were annotated to functional categories against varying database such as BLASTX, SEED subsystems hierarchy and MEGARes (antimicrobial resistance databases)

3.6 STATISTICAL ANALYSES

Where appropriate, Microsoft Excel 2013 was used to calculate averages, standard deviations and student t-tests. Canoco for Windows (Version 4.0, GLW-CPRO©) (Ter Braak, 1992) was used to show the effect of environmental variables (physicochemical parameters) on the various sites.

CHAPTER 4: GENERAL DESCRIPTION AND CHARACTERISATION OF STUDY SITES

4.1 INTRODUCTION

Written permission was obtained from the municipality or water company. The participating organisations operated a DWPF that uses one of the following water sources and processes:

- A direct reuse/reclamation plant, supplementing treated dam and borehole water: WC-A
- A conventional system with minimal upstream impacts: WC-F
- A groundwater and semi-direct re-use system with a WWTP and subsistence agriculture: NW-B and NW-D
- A system that uses groundwater source water with impacts from agriculture: NW-C
- A conventional system with upstream impacts from mining, agriculture and urbanisation: NW-E
- A system that uses ozone in the drinking water production process: NW-G
- A system that uses conventional purification and a combination of chlorination and monochloramine as disinfection: GT-H

Generalised land-cover or land-use data was collated for the areas in which the participating DWPFs are located. Some of the systems use water from a major river that passes through South Africa and there are multiple impacts.

4.2 WC-A: A DIRECT POTABLE WATER REUSE OR RECLAMATION PLANT

4.2.1 Description of the plant

Plant WC-A is a direct potable water reuse plant with a production capacity of 2.5 Mℓ/day, but currently only produces 1.1 Mℓ/d of drinking water (Grimmer and Tuner, 2013). On the other hand, the boreholes and the dam were the main suppliers of water (70%) to the inhabitants. Thus, 30% of reclaimed water is blended with treated water from a dam or borehole (Grimmer and Tuner, 2013). Figure 4.1 indicates the processes involved in the drinking water production processes followed at WC-A. In one scenario, effluent from the WWTP is reclaimed for potable water preparation (Figure 4.1A). Surface and ground water are also treated (Figure 4.1B).

Activities upstream of the dam mainly include agriculture (Figure 4.2), which mainly involves sheep farming, as well as game farming. There are no large industries or mining and other activities that could impact on the water quality. The dam is rain-fed, but due to a severe drought during the study period, it had completely dried up by November 2017. In 2016, the population size was 51 080 and the number of households was 14 935. In this municipality, 77.9% of households had piped water inside the dwelling (Municipalities of South Africa, 2018).

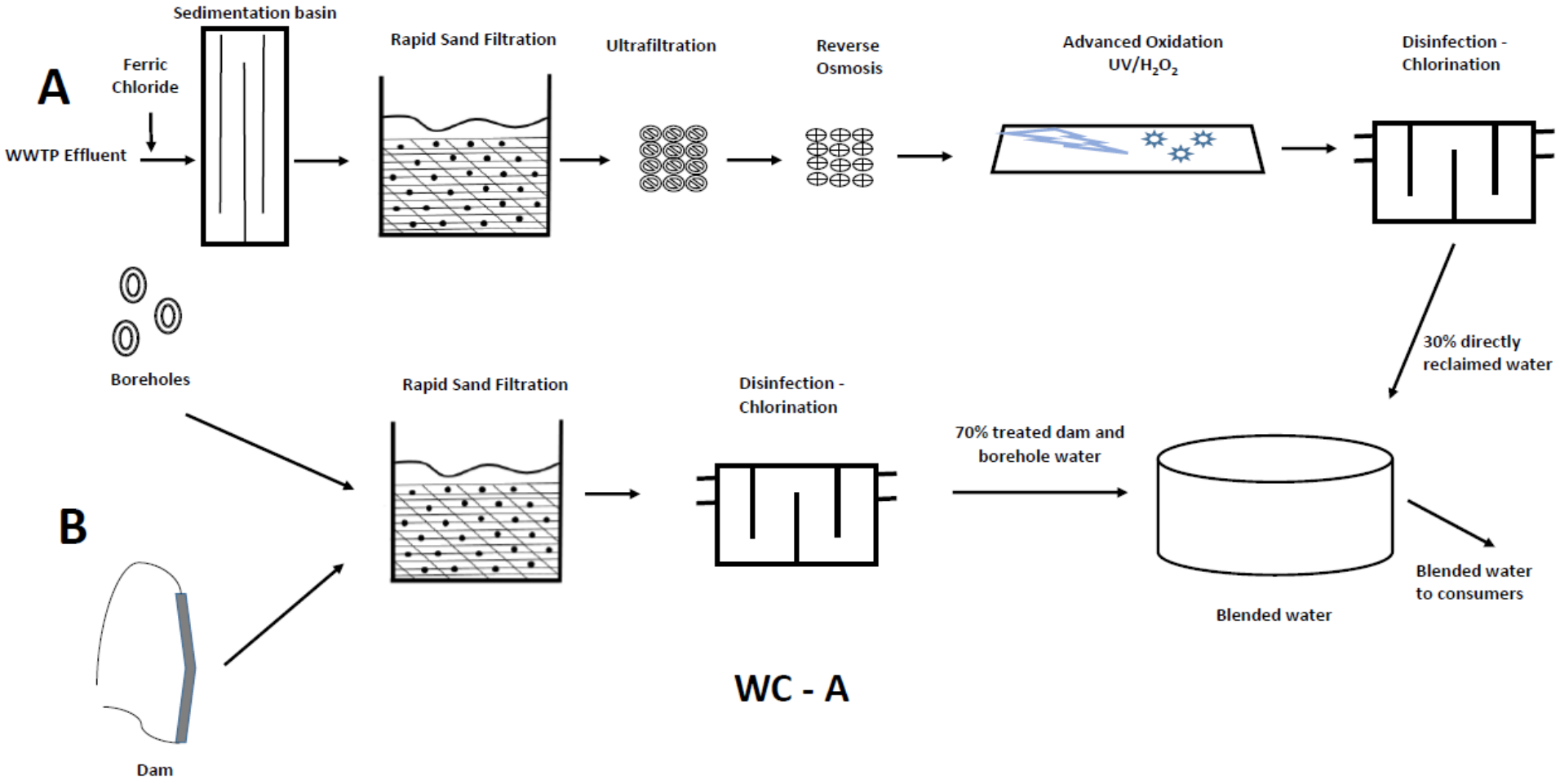


Figure 4.1: Drinking water production facility WC-A

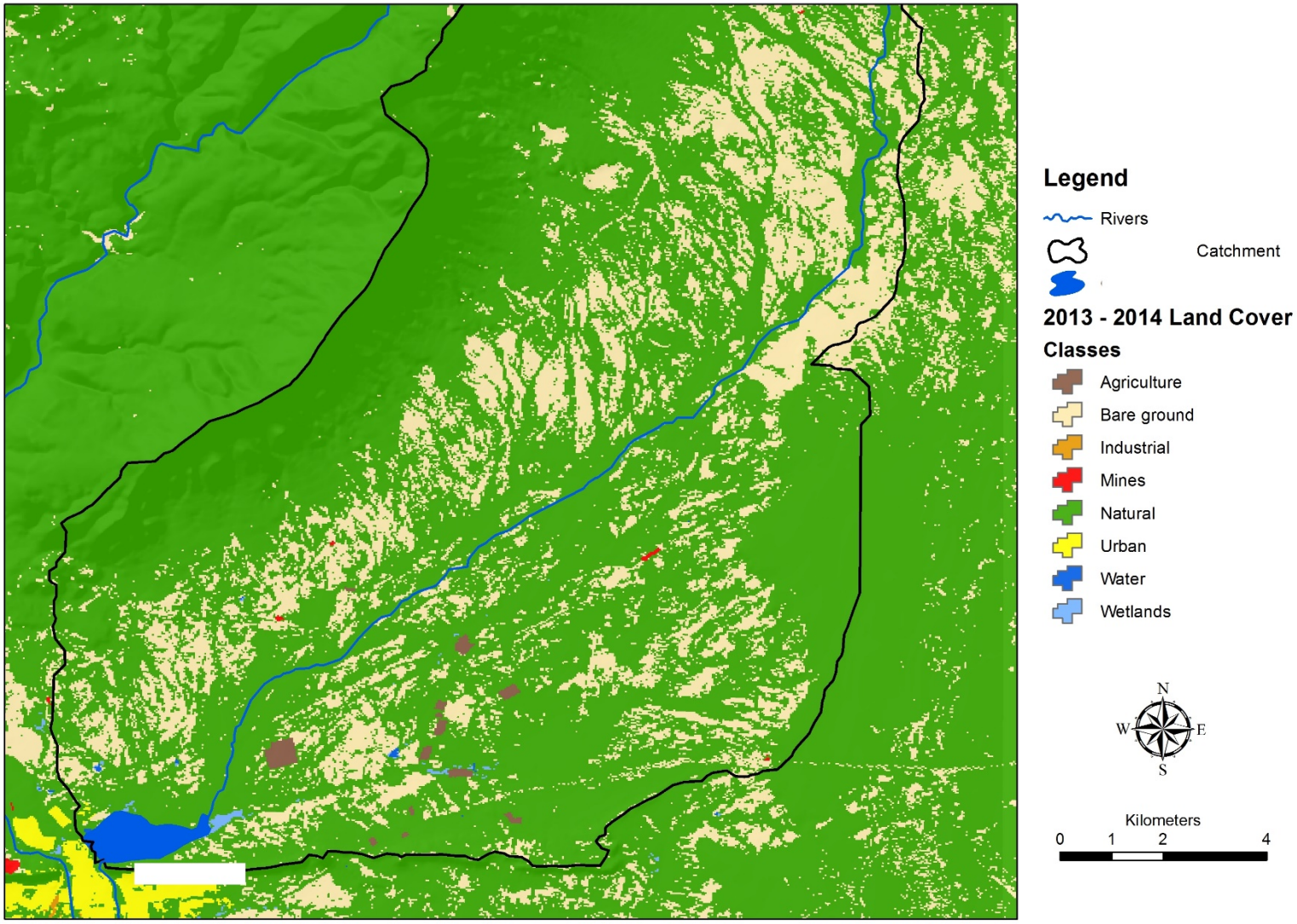


Figure 4.2: Land cover and upstream activities at WC-A's dam

4.2.2 Physicochemical parameters of the drinking water

The overall Blue Drop score for this plant was 95.00% in 2010, and improved to 96.27% in 2012. There were improvements in various categories, including WSPs, compliance with national standards and asset management (DWA, 2012).

Table 4.1 represents the average of the physical and chemical parameters for the June and November 2017 sampling run from plant WC-A. Raw 1 water samples (water from the WWTP) had elevated physicochemical parameters. The physical parameters of treated water from the direct potable water reuse plant represented by RO were generally low for both sampling runs. A trend was observed in which the physical parameters were elevated after treatment at the plant treating borehole and dam water. This had an impact on the values of the blended water throughout the distribution network.

Table 4.1: Selected physical parameters of the drinking water at WC-A

Sampling period	Sample location	Temperature (°C)	pH	TDS (mg/ℓ)	Turbidity (NTU)
June 2017	**Raw 1**	12.3	7.55	956	4.39
	Raw 2	18.5	7.74	924	0.55
	Raw 3	15.2	8.26	356	9.58
	RO	12.6	6.37	144	0.08
	Final	16.6	7.75	729	0.47
	Dis	15.8	7.80	714	0.57
November 2017	**Raw 1**	17.0	8.14	987	4.62
	Raw 2	19.1	7.70	950	0.21
	RO	17.2	7.47	218	0.26
	Final	21.0	7.98	850	0.37
	Dis	17.5	7.93	906	0.47

Sampling period	Sample location	Free chlorine (mg/ℓ)	Phosphates (mg/ℓ)	Nitrates (mg/ℓ)	Nitrites (mg/ℓ)
June 2017	**Raw 1**	0.05	4.00	9.45	0.57
	Raw 2	0.05	4.74	0.47	0.03
	Raw 3	0.13	5.28	0.00	0.01
	RO	0.04	4.11	2.37	0.18
	AT	0.03	3.34	1.13	0.00
	Dis	0.07	3.36	0.77	0.11
November 2017	**Raw 1**	0.03	3.50	4.60	0.01
	Raw 2	0.07	4.45	1.13	0.01
	RO	0.33	2.49	2.30	0.07
	AT	0.03	3.6	0.97	0.00
	Dis	0.04	3.74	1.27	0.10

Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 – Dam water; TDS – Total dissolved solids; AT – After treatment; Dis – Distribution system, NTU – Nephelometric turbidity units

All the water samples (before and after treatment) had elevated phosphate levels. The raw water levels varied (June 2017: 4.00 to 5.28 mg/ℓ; November 2017: 3.50 to 4.5 mg/ℓ). In drinking water, the levels were not much lower (June 2017: 3.34 to 4.11 mg/ℓ; November 2017: 2.49 to 3.9 mg/ℓ). Nitrates were not detected in the dam water samples (Raw 3) for June 2017.

The nitrate levels for the raw water were considerably elevated in the borehole water (4.60 to 9.45 mg/ℓ for June 2017: 4.00 to 5.28 mg/ℓ; November 2017: 2.49 to 4.5 mg/ℓ). In the drinking water, it was very low (0.9 to 2.37 mg/ℓ for June 2017: 4.00 to 5.28 mg/ℓ; November 2017: 2.49 to 4.5 mg/ℓ). Turbidity for the borehole water was very high (4.39 to 4.62 NTU), but was successfully reduced to below 1 NTU. The elevated levels of phosphates (and even if the nitrate and nitrite levels are low) could contribute to microbial growth, including biofilm development.

4.3 WC-F: A CONVENTIONAL DRINKING WATER TREATMENT PLANT

4.3.1 Description of the plant

The system design capacity of the WC-F plant is 8 Mℓ/d and the operational capacity is 52.50% (DWA, 2014). The purification process is demonstrated in Figure 4.3 and consists of surface and groundwater that was kept in a holding dam undergoing flocculation and settling, followed by sand filtration and chlorination. There is a storage dam situated about 5 km out of town (Figure 4.4). It is situated in the mountains and there are no activities around its drainage. The groundwater is obtained from a local farm and agricultural activities could impact on water quality. There are, however, no industries. The ground and surface water are mixed and stored in a retention dam in town. Storm water runoff may impact on the quality of this mixed water. The 2016 statistics indicated that the population was 36 000 and consisted of 11 321 households. Indwelling piped water was provided to 84.5% of households (Municipalities of South Africa, 2018).

4.3.2 Physicochemical parameters of the drinking water

The overall Blue Drop score for this plant was 78.13% in 2010 and improved to 91.23% in 2012. The improvements include WSP, compliance with national standards and asset management. Microbial and chemical compliance was 99.9% and 99.0%, respectively (DWA, 2012).

Table 4.2 indicates the physicochemical parameters of the drinking water at WC-F. The pH level of the raw water was low (5.2 and 5.6). Total dissolved solids ranged between 221 and 455 mg/ℓ. Free chlorine in the drinking water was very low. Phosphate levels were 2.28 and 3.81 mg/ℓ. Nitrites were not detected and nitrate levels were also very low.

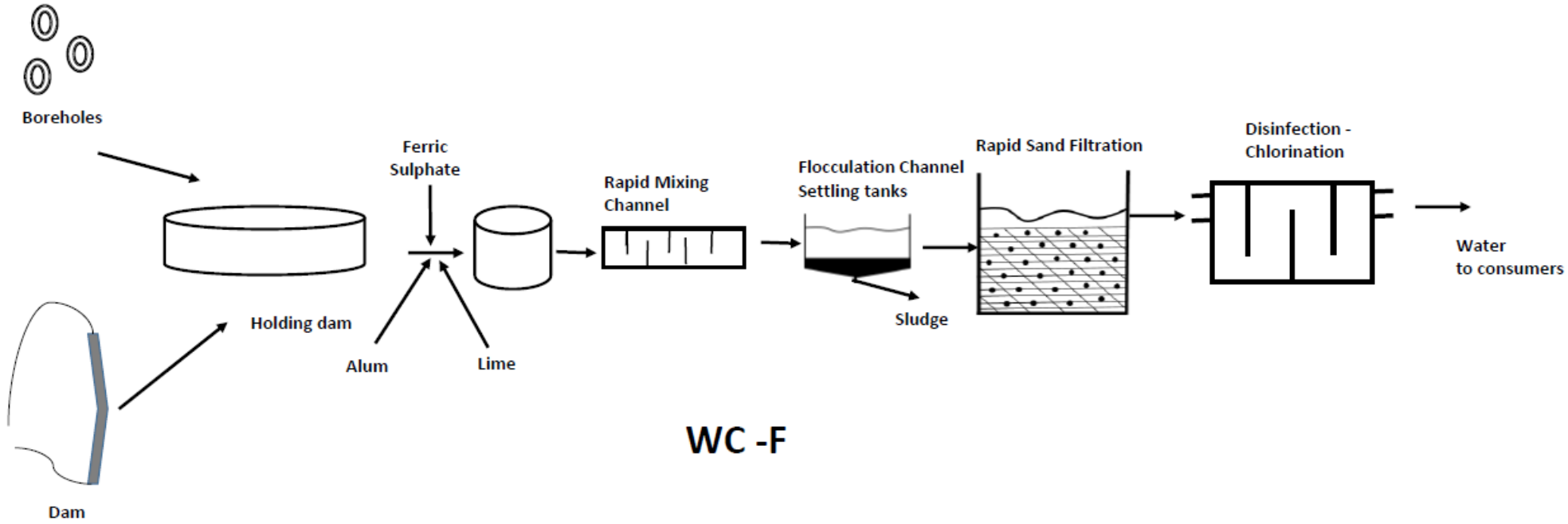


Figure 4.3: Drinking water production facility WC-F

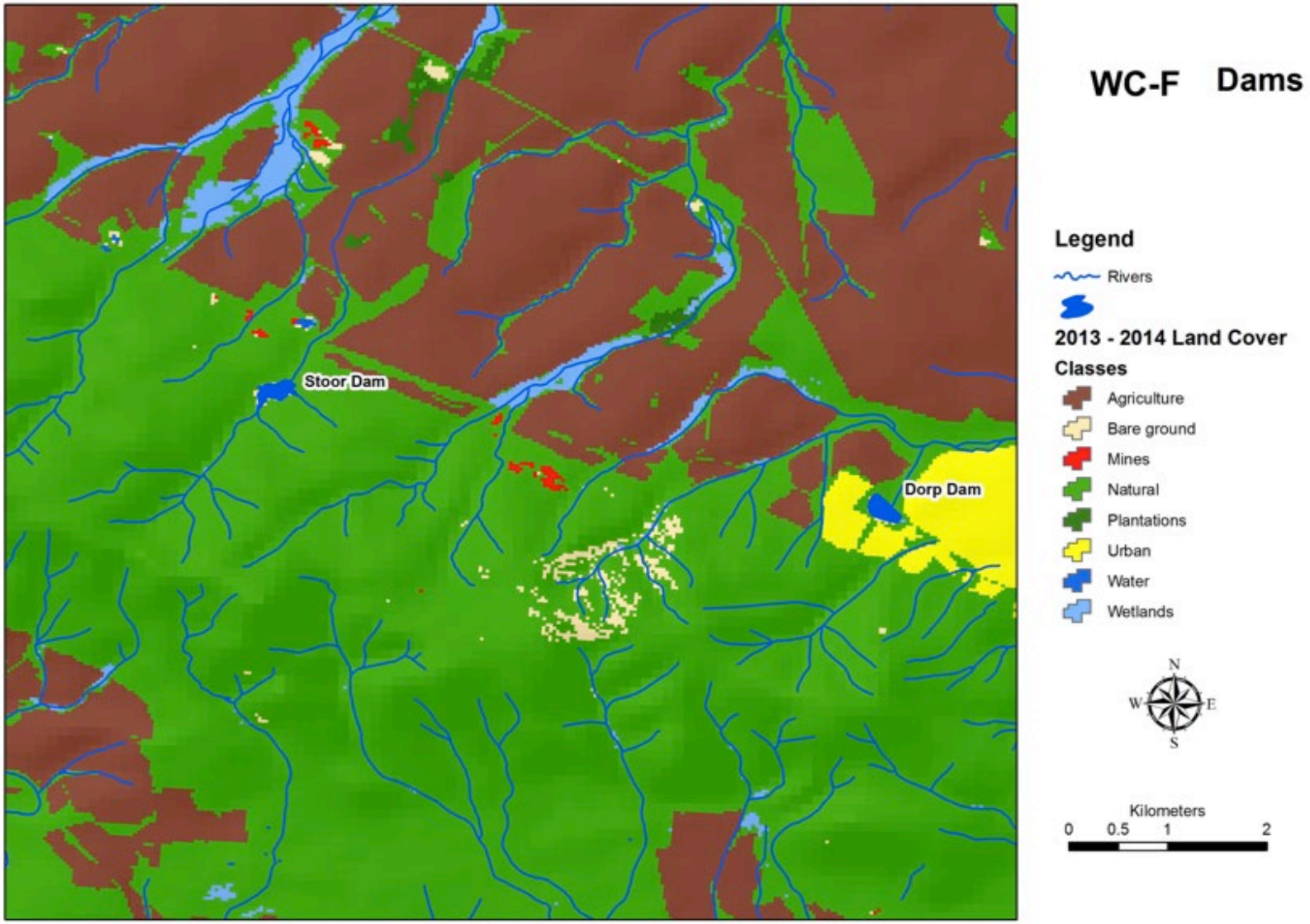


Figure 4.4: Land cover and upstream activities at WC-F's holding dams

Table 4.2: Selected physical parameters of the drinking water at WC-F

Sampling period	Sample location	Temperature (°C)	pH	TDS (mg/ℓ)	Turbidity (NTU)
June 2016	**Raw 1**	20.5	5.2	455	N/D
	Raw 2	18.5	5.5	307	N/D
	Raw 3	17.1	5.4	221	N/D
	AT	16.8	6.5	385	N/D
	Dis 1	15.6	7.1	388	N/D
	Dis 3	16.4	7.3	385	N/D
June 2017	**Raw 1**	21.3	5.64	422	3.13
	Raw 2	13.1	7.16	371	9.99
	AT	13.0	7.23	391	0.21
	Dis	15.8	7.55	394	0.31

Sampling period	Sample location	Free chlorine (mg/ℓ)	Phosphates (mg/ℓ)	Nitrates (mg/ℓ)	Nitrites (mg/ℓ)
June 2017	**Raw 1**	0.07	3.00	0.37	0.00
	Raw 2	0.00	3.07	0.10	0.00
	AT	0.01	3.81	0.33	0.00
	D	0.04	2.28	0.17	0.00

Raw 1 – borehole water; Raw 2 – Mixed raw water; AT – After treatment; Dis – Distribution system

4.4 NW-B: DRINKING WATER PRODUCTION USING A SURFACE WATER SUPPLY SYSTEM

4.4.1 Description of the plant

The plant is a surface water supply system with a design capacity of 20 Mℓ/d (Mulamattathil, 2015). The process followed by this plant is sedimentation, floatation, filtration, softening, absorption and disinfection (Figure 4.5). Source water is obtained from a dam that has two WWTPs upstream from the abstraction point (Mulamattathil, 2015). According to Figure 4.6, the quality of the raw water may be affected by at least one WWTP, formal and subsistence agriculture, informal urbanisation and formal urban and industrial areas.

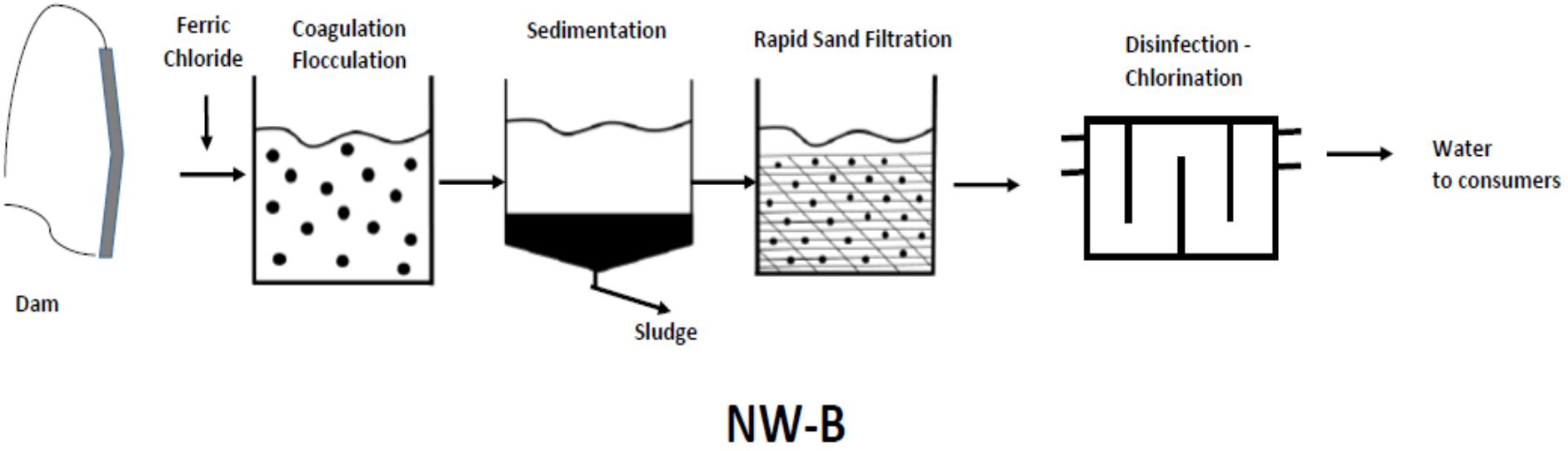


Figure 4.5: Drinking water production facility NW-B

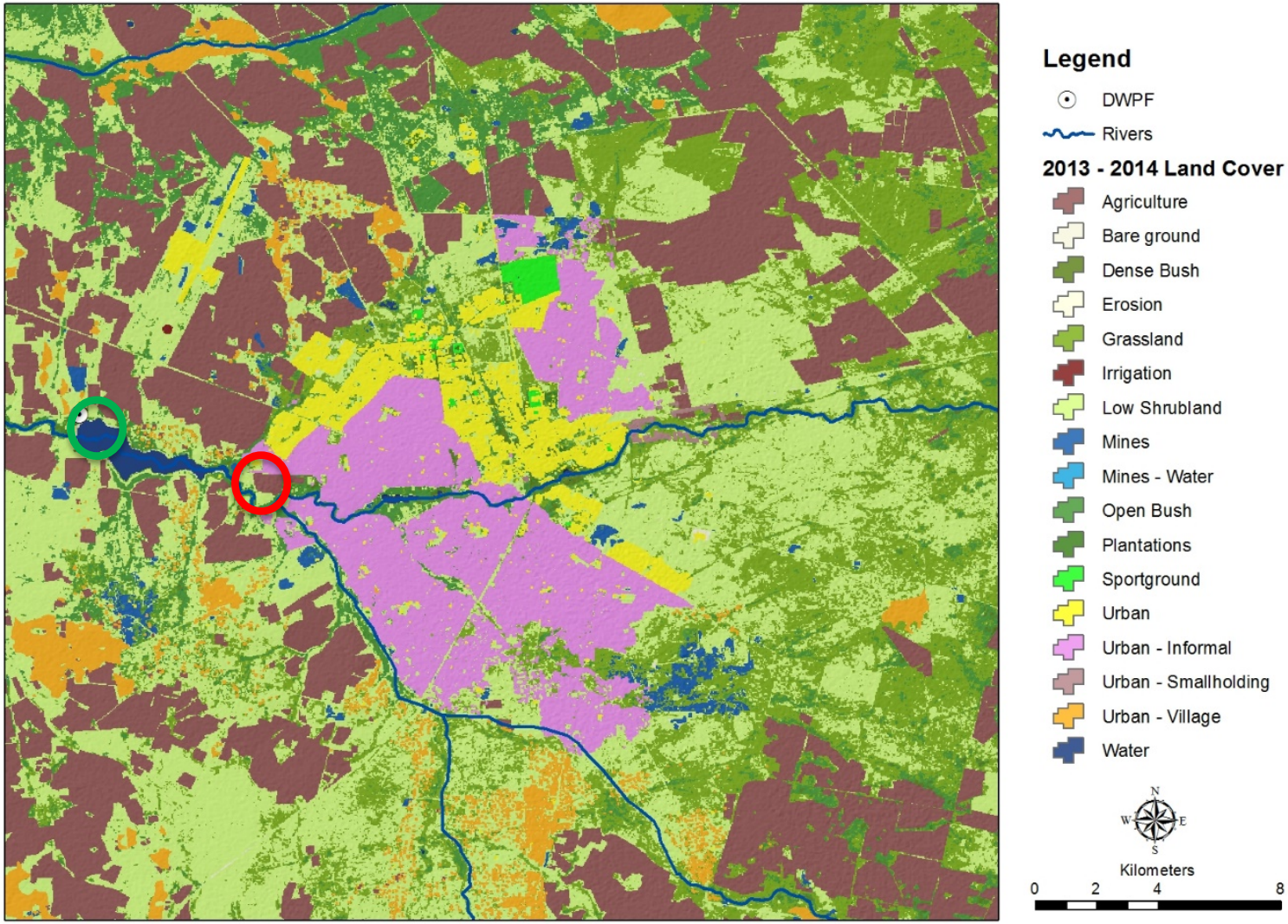


Figure 4.6: Land cover and use map showing the proximity of the WWTP (red circle) and the drinking water abstraction (green circle) for NW-B

4.4.2 Physicochemical parameters of the drinking water

Temperature values reflect the seasonality of the sampling (Table 4.3). The source water for NW-B is surface water. The pH level varied between 7.37 and 9.94. Total dissolved solids were generally between 413 and 816 mg/ℓ, except in March 2017 when these values were between 152 and 559 mg/ℓ.

Table 4.3: Selected physical parameters of the drinking water at NW-B

Sampling date	Sampling site	Temperature (˚C)	pH	TDS (mg/ℓ)
August 2016	**Raw**	18.7	9.53	508
	Inlet	16.5	9.40	489
	AT	18.5	8.94	533
	D1	21.0	9.17	473
	D2	19.6	9.62	413
November 2016	**Raw**	25.7	9.94	481
	Inlet	23.9	9.93	487
	AT	24.8	9.47	487
	D1	27.0	9.17	443
	D2	28.3	8.37	426
March 2017	**Raw**	28.5	7.42	181
	Inlet	22.5	7.37	152
	AT	22.3	7.47	164
	D1	27.8	7.34	183
	D2	26.4	7.37	559
May 2017	**Raw**	23.5	8.86	765
	Inlet	20.9	8.58	764
	AT	21.1	8.28	811
	D1	22.4	9.03	816
	D2	21.8	8.66	789

Raw – dam; Inlet – abstracted water from catchment prior to treatment; AT – After treatment; D1 – Random household tap water from treatment plant distribution system; D2 – Second random household tap water from treatment plant distribution system

4.5 NW-C: A SYSTEM THAT USES GROUNDWATER AS SOURCE WATER

4.5.1 Description of the plant

Ground water obtained from a natural spring is used to produce drinking water. The drinking water production capacity is currently at 14 Mℓ/day (Municipalities of South Africa, 2018). The source water (natural spring) originates 6 km from the treatment plant and has constant levels, even during the dry seasons (Diedericks, 2013). The water is treated according to the processes shown in Figure 4.7.

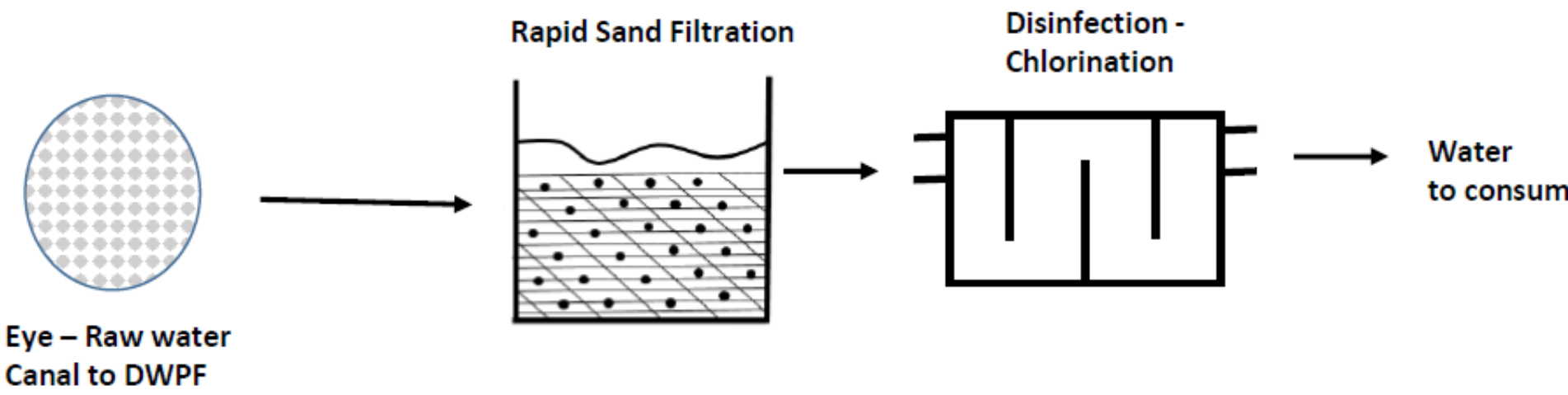


Figure 4.7: Drinking water production facility NW-C

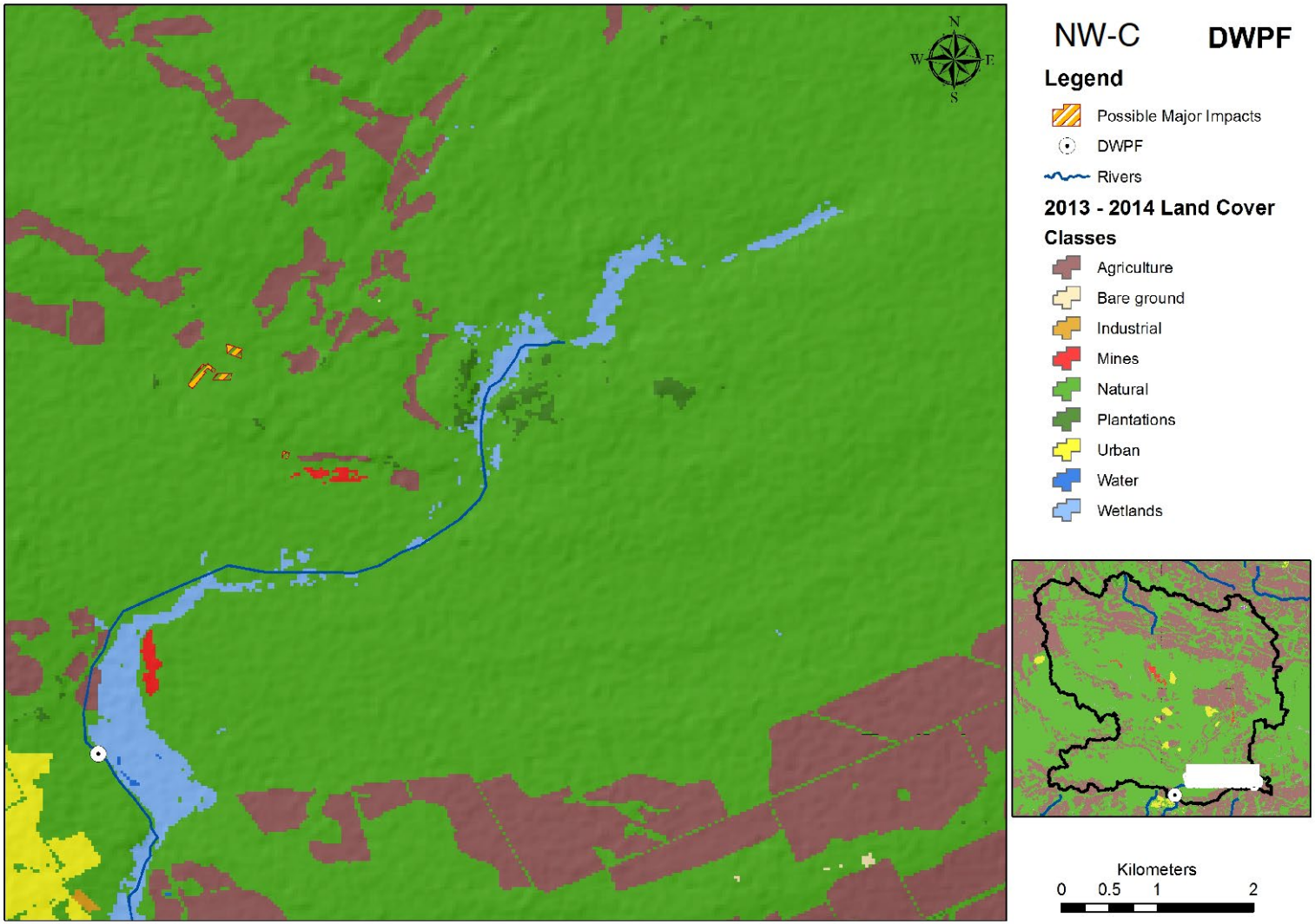


Figure 4.8: Land cover and activities upstream of NW-C

From Figure 4.8, it evident that there are minimal upstream activities that could directly impact on the water quality at NW-C. The actual catchment is huge with a variety of activities, ranging from agriculture to mining, taking place overall. NW-C is situated in an area with limited urban development and no large industries that can pollute the environment. Fresh water is abstracted for drinking water. The local municipality has a population of approximately 56 702 people and 14 562 households However, piped water inside dwellings is a challenging 20% (Municipalities of South Africa, 2018).

4.5.2 Physicochemical parameters of the drinking water

The overall Blue Drop score for this plant was 19.25% in 2010 and improved to 55.98% in 2012. This improvement demonstrates a commitment from the local municipality and a concerted effort to improve participation in all categories. Microbial and chemical compliance was at 98.1% and 99.9%, respectively in 2012 (DWA, 2012).

The physicochemical parameters for the drinking water are summarised in Table 4.4 and Table 4.5. The turbidity data for the system indicates that the final water had higher values than the raw water. This could be due to processes during purification that should be addressed. The TDS were below 400 mg/ℓ and the pH levels were elevated (8.5 to 8.75). The turbidity levels were, in most cases, higher after treatment compared to the raw water. The managers and operators at NW-C are aware of this issue. The free chlorine for the drinking water was between 0.3 and 1.6 mg/ℓ. The phosphate levels in the drinking water were between 0.22 and 3.17 mg/ℓ. Nitrites varied between 0.67 and 5.00 mg/ℓ and the level was only lower than 0.9 mg/ℓ in one case. The nitrate levels were very low (1.15 to 2.75 mg/ℓ). The balance between the nitrites and the nitrates was probably due to microbial activities. Temperatures ranging between 15 and 23 °C, sufficient carbon (chemical oxygen demand (COD)) and nutrients (nitrate/nitrites, phosphates and sulphates) provide ideal conditions for bacterial regrowth and biofilm development.

Table 4.4A: Selected physicochemical parameters of the drinking water at NW-C

Sampling date	Sampling site	Temperature (˚C)	pH	TDS (mg/ℓ)	Turbidity (NTU)
March 2016	**Raw**	18.4	8.56	374.0	0.20
	AT	17.5	8.54	373.0	2.32
	Dis	23.0	8.50	374.7	1.50
	Raw	15.7	8.74	382.0	1.74
May 2016	**AT**	15.9	8.60	384.0	0.34
	Dis	17.4	8.60	380.0	1.90
Sampling date	**Sampling site**	**Temperature (˚C)**	**pH**	**TDS (mg/ℓ)**	**Turbidity (NTU)**
August 2016	**Raw**	15.9	8.62	367.0	0.29
	AT	16.2	8.50	366.0	1.90
	Dis	15.8	8.70	372.7	3.30
May 2017	**Raw**	16.2	8.80	381.7	0.31
	AT	15.5	8.70	385.7	0.89
	Dis	18.6	8.70	380.3	0.69
November 2017	**Raw**	20.5	8.60	332.7	0.39
	AT	18.7	8.54	334.0	0.59
	Dis	23.2	8.50	331.1	0.55

TDS – Total dissolved solids; EC – Electrical conductivity; SANS – South African National Standard; AT – After treatment; Dis – Distribution system

Table 4.5B: Selected physicochemical parameters of the drinking water at NW-C

Sampling date	Site	Free chlorine (mg/ℓ)	Phosphorus (mg/ℓ)	Nitrites (mg/ℓ)	Nitrates (mg/ℓ)	Sulphides (mg/ℓ)	Sulphates (mg/ℓ)	COD (mg/ℓ)
March 2016	Raw	-	0.00	3.00	1.15	12	0.00	0
	AT	-	0.50	1.00	1.55	14	0.00	0
	Dis	-	0.28	1.67	1.57	14	0.00	11
May 2016	Raw	0.03	0.55	5.00	2.00	11	4.00	0
	AT	0.99	1.10	5.00	2.30	23	0.00	63
	Dis	0.41	1.33	4.50	2.03	21	0.00	6
August 2016	Raw	0.05	0.13	3.50	2.75	50	1.00	0
	AT	0.02	0.22	3.50	1.35	58	2.00	32
	Dis	0.60	0.15	5.00	2.27	46	1.00	19
May 2017	Raw	0.06	0.22	3.33	1.77	5	6.00	0
	AT	1.60	0.23	0,67	1.90	0	0.00	3
	Dis	0.19	0.21	1,56	1.79	1	0.00	1
November 2017	Raw	0.01	2.48	3,33	3.63	13	0.33	0
	AT	1.05	3.17	4,00	2.97	12	0.33	0
	Dis	0.35	0.66	2,89	2.50	16	0.33	0

TDS – Total dissolved solids; EC – Electrical conductivity; AT – After treatment; Dis – Distribution system

4.6 NW-D: A DRINKING WATER PLANT USING A MIXTURE OF RAW SOURCES

4.6.1 Description of the plant

The system receives water from a borehole and a natural spring currently used for recreation (diving). Once the water reaches the DWPF, it is filtered (sand filtration), chlorinated and supplied to a section of the community (Figure 4.9 and Figure 4.10). The capacity of this plant is 45 Mℓ/d. A large portion of water from this plant is also mixed with water from NW-B (Mulamattathil, 2013). Land use around the area includes agriculture, plantations, bushes and houses. The 2016 population of this town was just over 314 000, consisting of 103 000 households, most of which were not connected to the main sewage system, but used septic tanks for wastewater collection. The provision of piped water services inside dwellings was at 23.1%, which implied that a large proportion of the population had either piped water in the yard or at a facility nearby (Municipalities of South Africa, 2018).

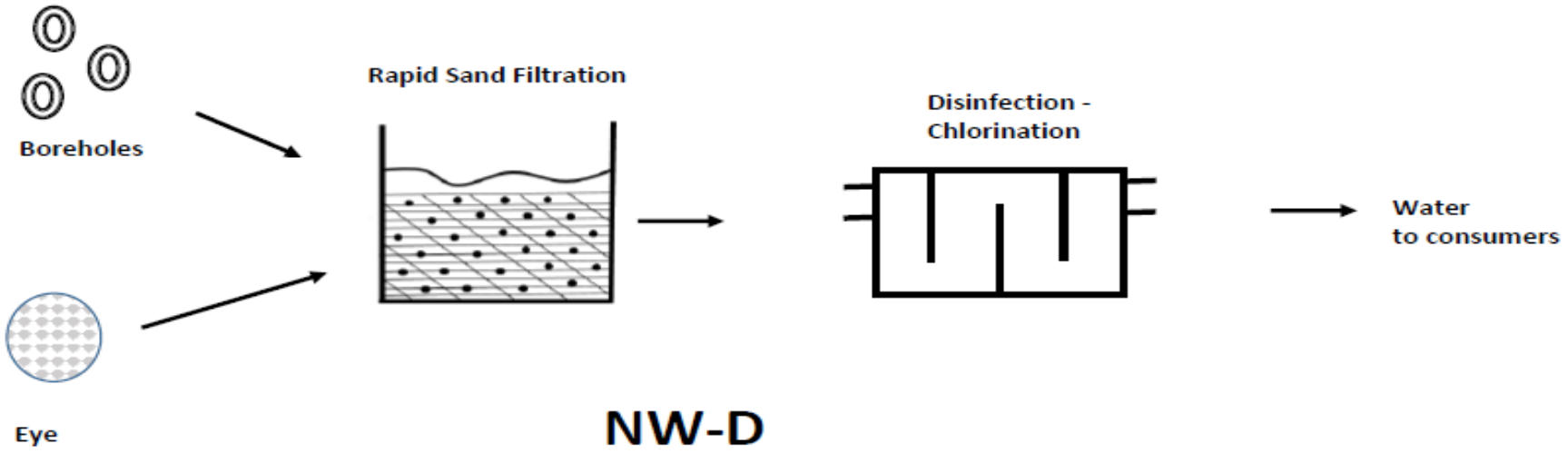


Figure 4.9: Drinking water production facility NW-D

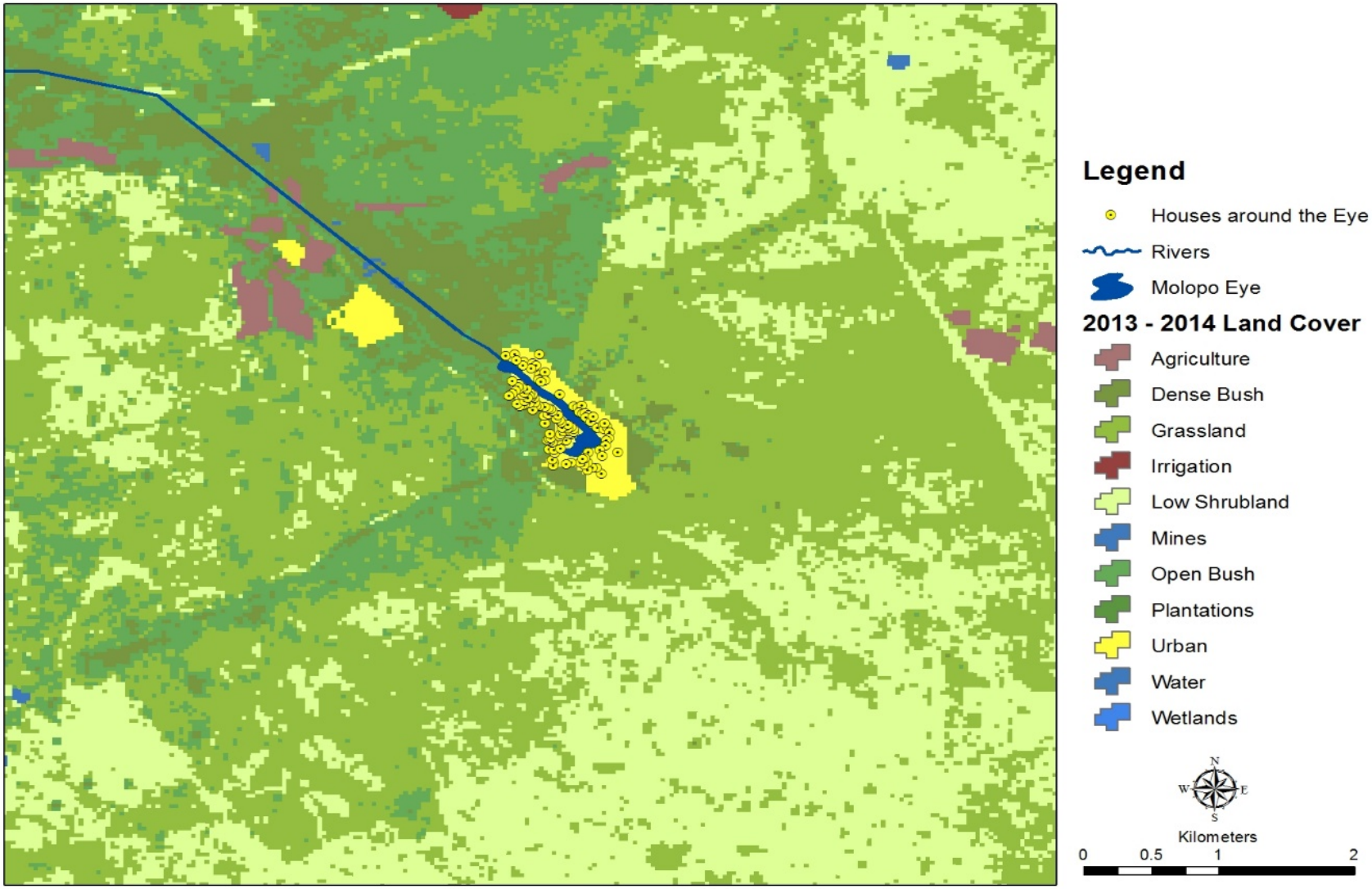


Figure 4.10: Land use around the natural spring supplying water to NW-D

4.6.2 Physicochemical parameters of the drinking water

At NW-D, similar trends were observed as at NW-B (Table 4.6). The temperature was generally between 20 °C and 30 °C, except for the raw water in August 2016 and May 2017 when the temperatures were below 20 °C. Total dissolved solids varied between 167 mg/ℓ and 827 mg/ℓ. Low TDS values (<180 mg/ℓ) were obtained for March 2017 and >690 mg/ℓ for May 2017. The pH values varied between 7.60 and 9.56.

Table 4.6: Selected physicochemical parameters of the drinking water at NW-D

Sampling date	Sampling site	Temperature (˚C)	pH	TDS (mg/ℓ)
August 2016	**Raw**	10.4	7.76	827
	Inlet	21.0	8.55	182
	AT	21.2	8.52	195
	D1	22.2	7.73	507
	D2	27.0	8.17	193
November 2016	**Raw**	24.6	8.96	246
	Inlet	26.5	8.79	192
	AT	24.5	8.85	202
	D1	31.1	8.56	202
	D2	26.5	8.78	192
March 2017	**Raw**	24.5	7.84	167
	Inlet	25.2	7.64	169
	AT	24.8	7.60	172
	D1	26.8	7.64	180
	D2	29.2	7.61	183
May 2017	**Raw**	17.6	9.56	747
	Inlet	20.0	8.65	717
	AT	21.0	8.43	727
	D1	24.6	8.88	760
	D2	25.6	8.62	690

Raw – Natural spring; Inlet – Abstracted water from catchment prior to treatment; AT – After treatment; D1 – Random household tap water from treatment plant distribution system; D2 – Second random household tap water from treatment plant distribution system

4.7 NW-E: A CONVENTIONAL DRINKING WATER TREATMENT SYSTEM

4.7.1 Description of the plant

The DWPF is in North West. Potable water in this facility is produced from raw water obtained from surface and groundwater. There are two dams that store raw water. The water from one of the dams is transported to the water purification plant in a 12-km long uncovered cement canal (Figure 4.12 (Annandale and Nealer, 2011). It then undergoes several water production processes (illustrated in Figure 4.11) to ensure that the water meets SANS 241 (SABS, 2015) before it is distributed to consumers. The processes involved are coagulation-flocculation, sedimentation, rapid sand filtration, activated carbon filtration and chlorination. This DWPF has a capacity to produce 33.6 Mℓ/day. From Figure 4.12, it is evident that the water quality in this catchment is affected by agricultural fields and pivots, feedlots and poultry farms, mines and digging, and urban and industrial areas. The population in 2011 was 162 762. More than 52 000 households were provided with piped water. Piped water inside the dwelling was, however, only at 56.9% (Municipalities of South Africa, 2018).

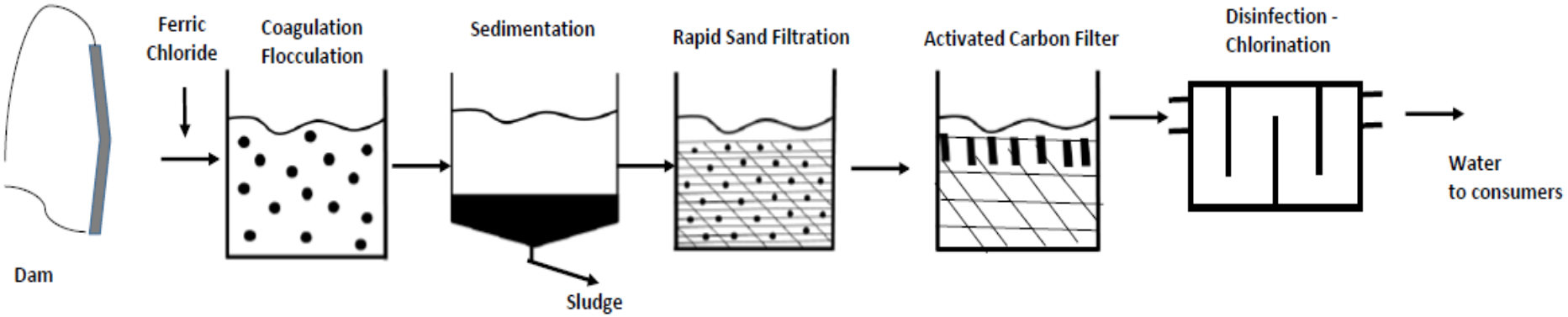


Figure 4.11: Drinking water production facility NW-E

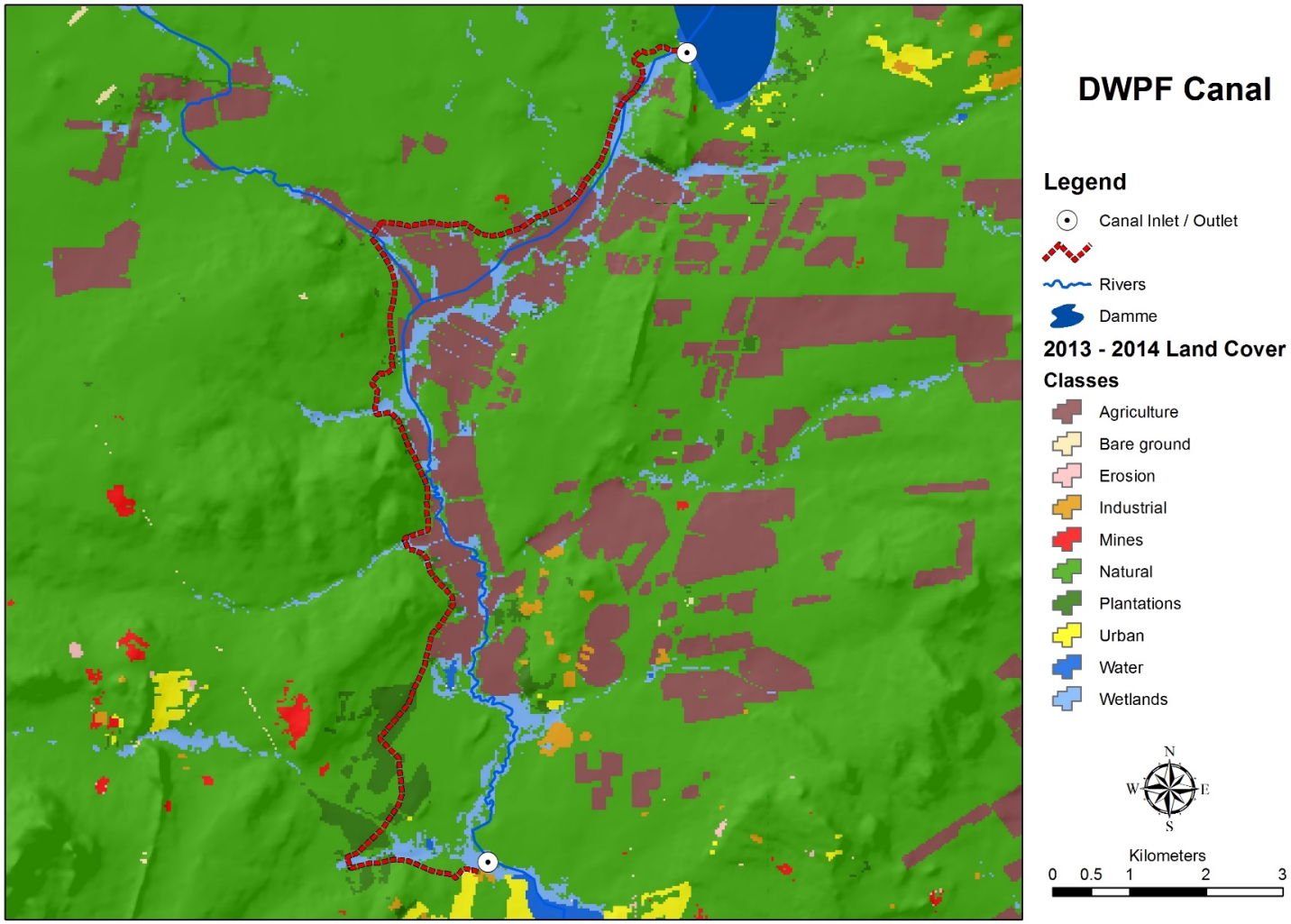


Figure 4.12: Land cover and activities upstream of NW-E

4.7.2 Physicochemical parameters of the drinking water

The overall Blue Drop score for this plant was 95.11% in 2010 and improved to 98.45% in 2012. There had been improvements in various categories, including compliance to WSP, national standards and improved asset management (DWA, 2012).

Physical parameters were mostly within the limits of SANS 241 (SABS, 2015) within the distribution system (Table 4.7 and Table 4.8). However, turbidity levels in drinking water were elevated during four sampling periods. The pH level ranged between 7.24 and 8.86. Total dissolved solids were elevated (425 mg/ℓ to 552 mg/ℓ). Phosphorous levels in the raw water ranged between 0.36 mg/ℓ and 5.04 mg/ℓ and drinking water levels ranged between 0.02 mg/ℓ and 4.95 mg/ℓ. The elevated levels were recorded in both the after-treatment samples and within the distribution system. The COD, phosphate and nitrate/nitrite levels, as well as the water temperatures, were such that HPC bacterial growth was possible.

Table 4.7: Selected physicochemical parameters of the drinking water at NW-E

Sampling date and site	Turbidity (NTU)			pH level			Temperature (°C)			TDS (mg/ℓ)		
	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis
March 2016	3.87	1.63	0.52	7.90	7.40	7.62	22.5	23.5	23.6	495	499	494
May 2016	3.65	2.24	0.47	8.86	8.24	7.24	17.5	19.0	19.2	518	533	515
August 2016	2.39	2.11	0.44	8.22	8.52	8.70	11.9	12.6	14.2	543	552	542
May 2017	2.33	0.42	0.23	8.46	8.12	8.01	16.5	15.5	18.6	520	515	514
October 2017	0.67	0.60	1.47	8.63	8.30	8.36	18.6	17.4	16.7	436	444	425

Raw – Raw water; AT – After treatment; Dis – Distribution system

Table 4.8: Selected chemical parameters of the drinking water at NW-E

	Phosphorus (mg/ℓ)			Nitrite (mg/ℓ)			Free chlorine (mg/ℓ)		
Sampling site	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**
March 2016	0.68	0.70	1.23	11.5	1.00	1.70	1.23		1.49
May 2016	1.11	1.40	1.88	1.8	1.26	1.53	1.26		1.59
August 2016	1.01	0.02	1.80	1.00	0.10	2.75	0.10		0.23
May 2017	0.38	0.71	1.10	1.72	0.33	0.89	0.10	0.05	0.07
October 2017	5.04	4.95	2.33	0.03	0.00	0.00	0.01	0.07	0.05
	COD (mg/ℓ)			**Nitrate (mg/ℓ)**		
Sampling site	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**
March 2016	1.00	ND	4.00	0	0.05	0.67
May 2016	5.00	4.00	6.00	2.90	1.25	1.72
August 2016	ND	7.00	3.00	2.10	0.5	0.89
May 2017	3.33	2.33	1.38	0.30	0.71	1.10
October 2017	2.67	3.67	2.50	2.1	0.57	0.89

Raw – Raw water; AT – After treatment; Dis – Distribution system

4.8 NW-G: A SYSTEM THAT USES OZONE IN THE DRINKING WATER PRODUCTION PROCESS

4.8.1 Description of the plant

Water is abstracted directly from one of the major rivers in South Africa. NW-G purchases raw and untreated water from DWAF and has abstraction rights for 238 Mℓ of raw water per day. It has a capacity to supply and distribute 250 Mℓ of potable treated water per day and has an installed capacity of 320 Mℓ of water per day. The process design is provided in Figure 4.12 and includes coagulation, dissolved air flotation, ozonation, sedimentation, sand filtration and disinfection. The local municipality that serves as the WSA for NW-G is situated in North West. The population in 2016 stood at 417 282 and there were 135 894 households. Piped water inside dwellings stood at 48.7%. The catchment of the DWPF is enormous (Figure 4.13) and a large number of human activities could impact on the water quality in the raw water. There are a number of WWTPs that decant effluent into the river. Some of the deepest gold mines are situated in the proximity of the river. There are large agricultural and manufacturing industries along this river. Impacts on the water quality could thus come from a variety of sources.

4.8.2 Physicochemical parameters of the drinking water

The overall Blue Drop score for this municipality was 59.63% in 2010 and improved to 95.35% in 2012. There had been improvements in various categories, including WSP, compliance with national standards and asset management (DWA, 2012).

Data for NW-G is provided in Table 4.9 and Table 4.10. The pH range for NW-G was between 7.97 and 9.7 and the TDS ranged between 450 mg/ℓ and 530 mg/ℓ. The turbidity of the raw water was generally very high (+10 NTU), but was mostly reduced by the treatment processes. The chemical parameter levels in the raw water were generally very high (Table 4.10). All these values were greatly reduced by the purification processes. The nitrite levels were similar to or higher than the nitrate levels. This could also be due to microorganism activity. In two cases, the sulphate levels in the drinking water exceeded the 250 mg/ℓ levels. Free chlorine levels were mostly sufficient for maintaining hygienic standards and suppressing regrowth.

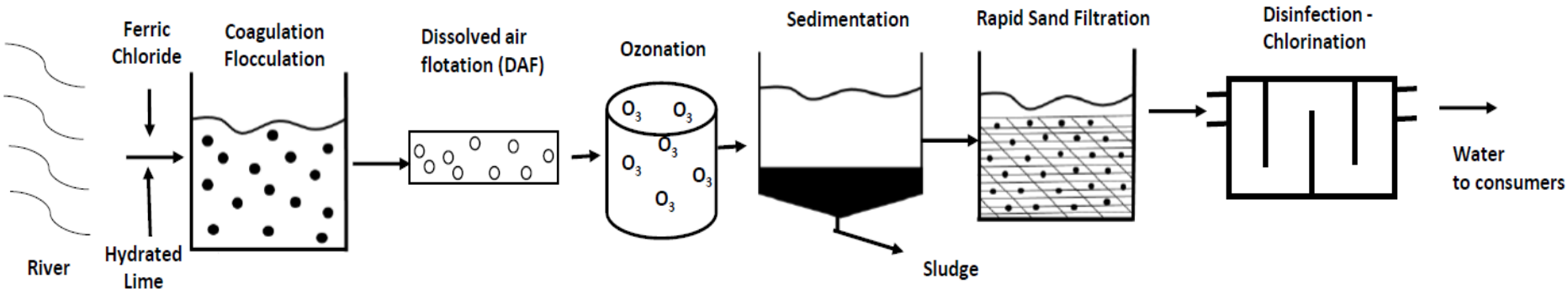


Figure 4.13: Drinking water production facility NW-G

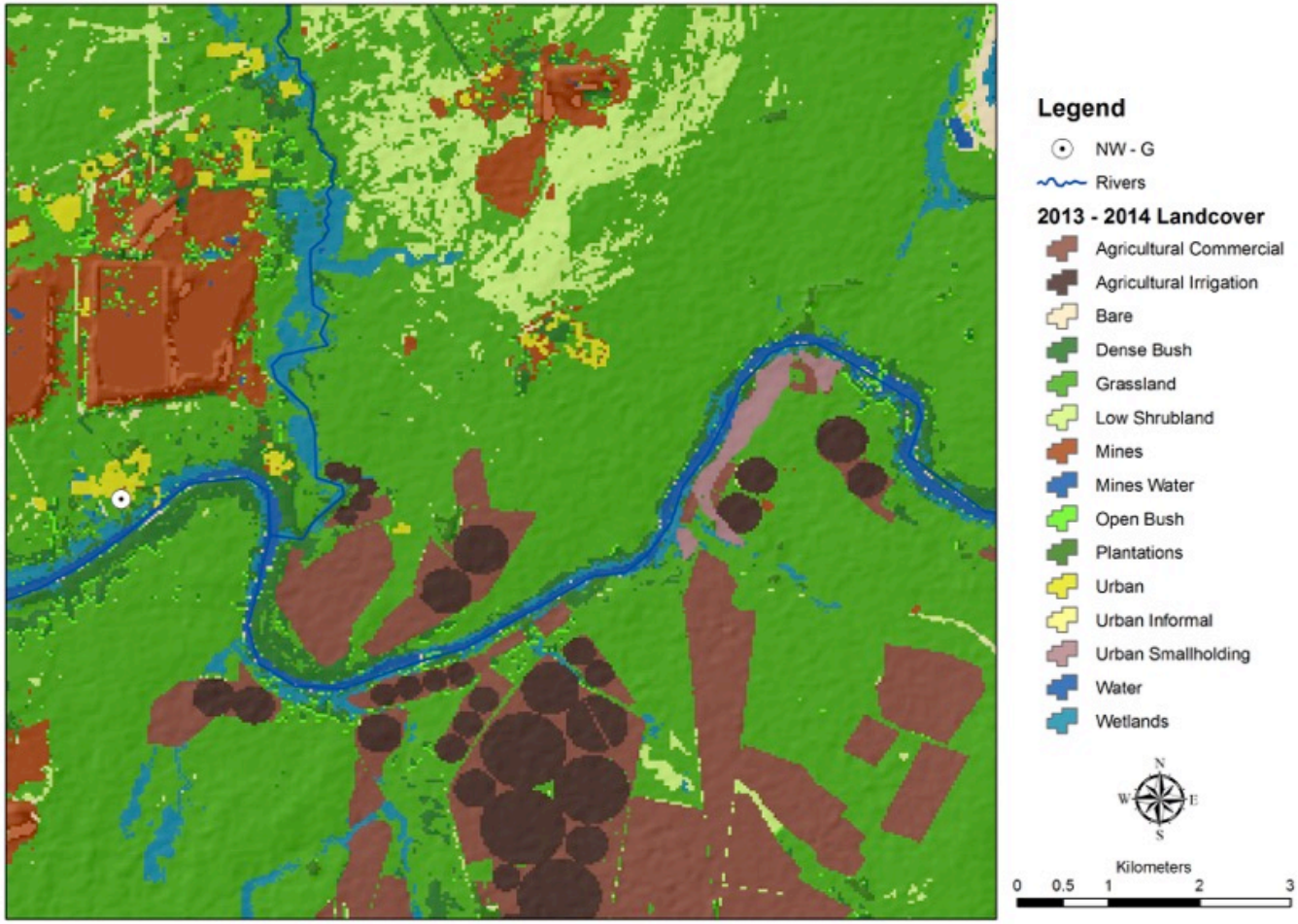


Figure 4.14: Land cover and activities upstream from NW-G

Table 4.9: Selected physicochemical parameters of the drinking water at NW-G

		Temperature (°C)	pH	TDS (mg/ℓ)	Turbidity (NTU)
March 2016	**Raw**	23.3	8.40	452.0	17.50
	AT	23.2	8.45	453.0	0.31
	Dis	26.5	8.40	479.3	0.30
May 2016	**Raw**	15.9	9.30	456.0	11.70
	AT	16.3	8.51	468.0	0.33
	Dis	18.9	8.28	452.0	0.24
August 2016	**Raw**	12.2	9.70	447.0	14.20
	AT	11.3	8.34	511.0	3.85
	Dis	16.1	8.14	527.0	3.27
May 2017	**Raw**	13.0	9.20	465.7	18.40
	AT	14.5	8.70	464.3	0.83
	Dis	15.4	8.90	537.0	0.41
November 2017	**Raw**	22.4	9.53	563.0	19.00
	AT	24.4	8.17	583.0	0.43
	Dis	23.8	8.30	580.1	1.00

TDS – Total dissolved solids; SANS – South African National Standard; AT – After treatment; Dis – Distribution system

Table 4.10: Selected chemical parameters of the drinking water at NW-G

		COD (mg/ℓ)	Free chlorine (mg/ℓ)	Phosphates (mg/ℓ)	Nitrates (mg/ℓ	Nitrites (mg/ℓ)	Sulphide (mg/ℓ)	Sulphate (mg/ℓ)
March 2016	**Raw**	25	ND	1.02	0.25	2.00	47	615
	AT	12	ND	0.85	3.75	4.00	14	195
	Dis	10	ND	1.06	2.20	2.17	16	330
May 2016	**Raw**	51	ND	0.31	0.30	36.00	41	115
	AT	7.5	ND	0.58	1.35	4.50	208	120
	Dis	16	ND	0.44	1.30	10.50	161	133
August 2016	**Raw**	63	0.05	6.70	1.15	10.50	82	135
	AT	58	0.03	2.70	2.00	5.00	51	465
	Dis	28	0.40	2.72	1.98	9.50	45	145
May 2017	**Raw**	19	0.11	4.23	0.97	1.33	45	93
	AT	8	0.80	0.28	4.27	7.67	36	90
	Dis	19	0.24	0.22	2.22	3.78	9	95
November 2016	**Raw**	33	0.15	3.62	0.00	1.33	17	92
	AT	19	0.59	3.09	2.30	6.33	41	102
	Dis	19	0.07	1.34	1.86	4.22	43	103

COD – Chemical oxygen demand; SANS – South African National Standard; Raw – Raw water; AT – After treatment; Dis – Distribution system

4.9 GT-H: A DRINKING WATER SYSTEM THAT USES A COMBINATION OF CHLORINATION AND MONOCHLORAMINE FOR DISINFECTION

4.9.1 Description of the plant

The DWPF supplies an average of 3 200 million litres of water to more than 12 million people on a daily basis. It achieved BDC status with compliance of 95.48% to 97.22% from 2011 to 2014 (DWA, 2014). The facility receives its raw water from a dam through a canal and a gravity pipeline. The water must go through the purification processes demonstrated in Figure 4.15. The water purification plant uses coagulation/flocculation, sedimentation, sand filtration, granular activated carbon treatment, UV irradiation and chlorination to treat the source water (Figure 4.14). A multi-barrier approach is used to achieve the water quality target in accordance with SANS 241 (SABS, 2015). The UV plant was installed to ensure the efficient removal of protozoan pathogens (WHO, 2010). The physical attributes and chemical composition of the water are continually monitored so that corrective action can be taken to prevent the water quality from differing from the prescribed limits (Nel and Haarhof, 2011). Once the water is purified, it is pumped to several reservoirs (55) located in the area of supply. From Figure 4.16, it is evident that various land-use activities could potentially affect the raw water quality at GT-H. These include various mining activities, particularly coal and gold mining. Diverse agricultural activity and industries also impact on the water quality.

4.9.2 Physicochemical parameters of the drinking water

Physicochemical data for the drinking water at this plant is shown in Table 4.11 and Table 4.12. The turbidity of the raw water was high (3.33 to 9.99 NTU), but was reduced during treatment. The pH level of the raw and drinking water was above 8 and TDS were elevated (above 450 mg/ℓ). Phosphate levels of raw water were between 0.38 and 2.0. The levels in the drinking water varied between 0.71 mg/ℓ and 1.5 mg/ℓ. The free chlorine was lower in June 2017 compared to November 2017. The nitrite levels in the drinking water were higher in June 2017, with 1.00 mg/ℓ in the final distribution system. In November the levels in the raw water and thus throughout the system were below 0.25 mg/ℓ. Nitrate levels were low in June (0.27 mg/ℓ to 0.67 mg/ℓ) and high in November (4.12 mg/ℓ to 6.09 mg/ℓ). These nitrite-nitrate dynamics could potentially be due to microbial activity in the bulk water or biofilms.

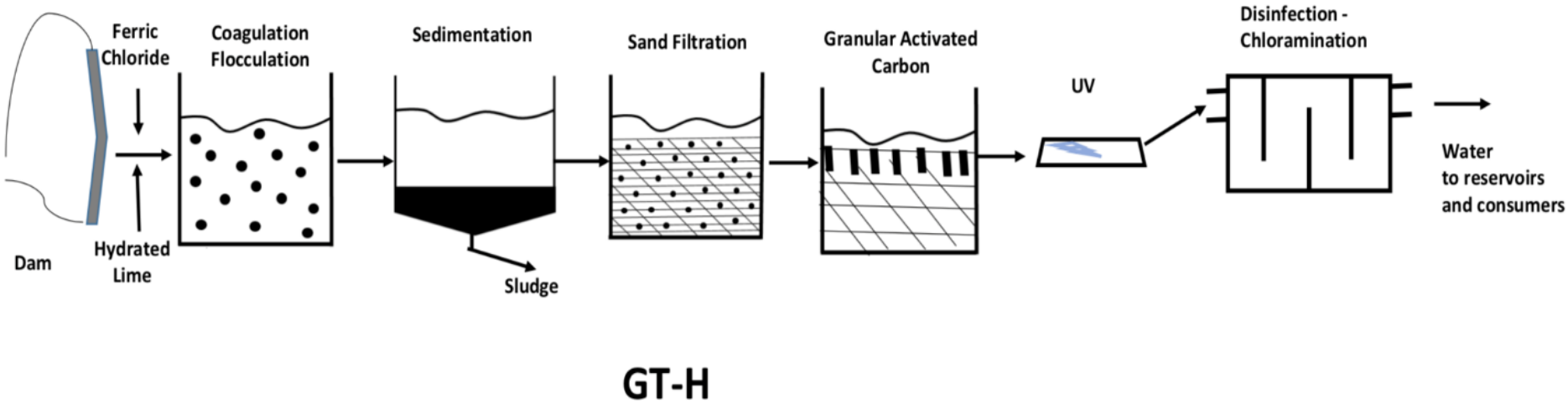


Figure 4.15: Diagram for drinking water production processes at GT-H

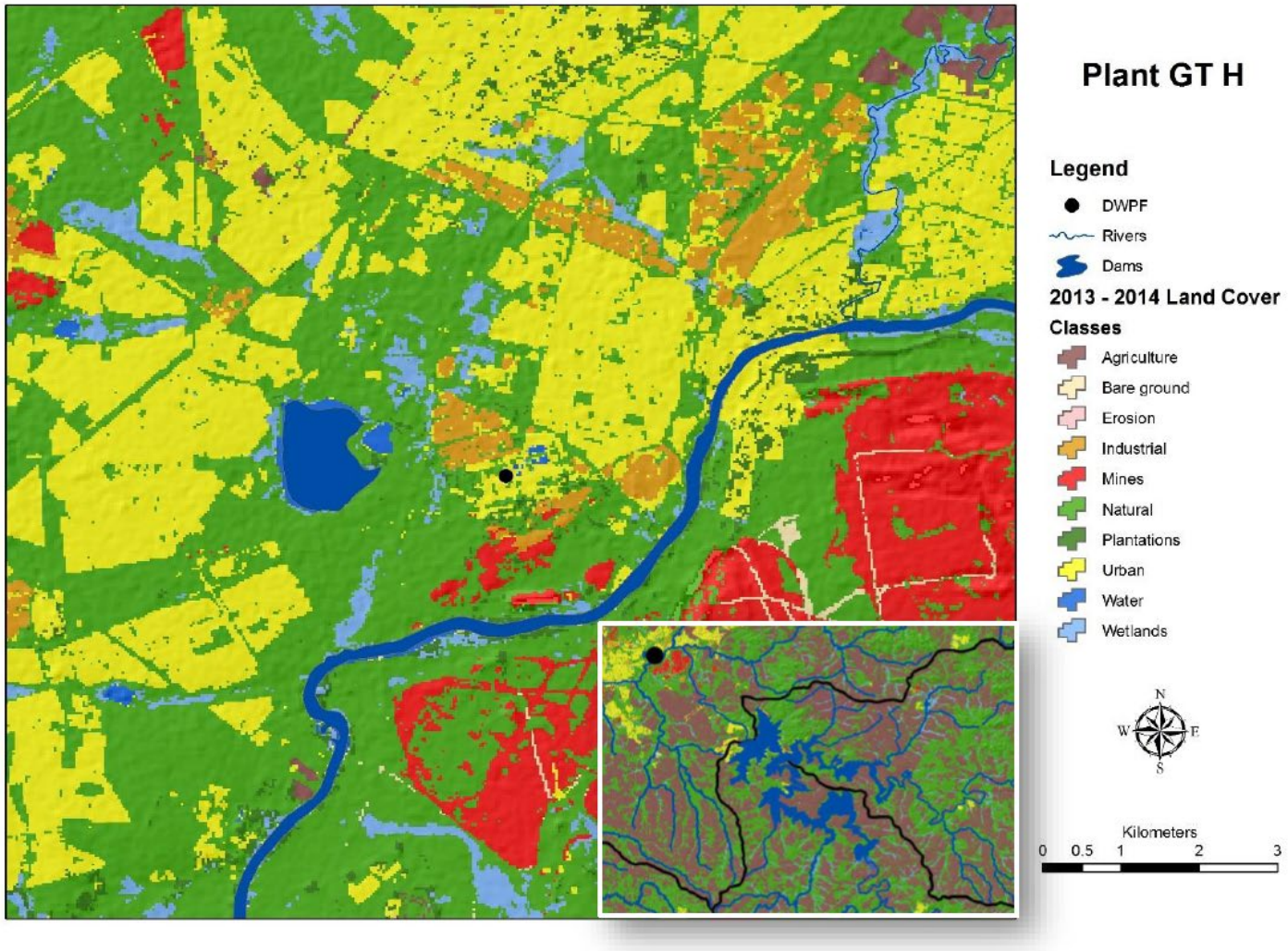


Figure 4.16: Land cover and activities upstream from GT-H

Table 4.11: Results of selected physical parameters of water before purification, after purification and during distribution at GT-H

		Turbidity (NTU)			pH level			Temperature (°C)			TDS (mg/ℓ)		
Sampling site		**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**
GT-H (June 2017)	**AV**	3.33	0.67	0.63	8.46	8.19	8.02	17.5	20.5	19.90	492	489	491
GT-H (November 2017)	**AV**	9.99	0.25	0.67	8.16	8.14	8.08	20.3	21.2	21.0	551	538	530

Raw – Raw water; AT – After treatment; Dis – Distribution system

Table 4.12: Results of selected chemical parameters of water before purification, after purification and during distribution at GT-H

	Phosphorus (mg/ℓ)			Nitrite (mg/ℓ)			Free chlorine (mg/ℓ)			COD (mg/ℓ)			Nitrate (mg/ℓ)		
Sampling site	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**	**Raw**	**AT**	**Dis**
GT-H (June 2017)	0.38	0.71	1.01	1.73	0.30	1.00	0.10	0.06	0.08	3.33	2.33	1.45	0.30	0.67	0.27
GT-H (November 2017)	2.00	1.50	1.30	0.17	0.21	0.03	1.02	1.56	1.66	4.24	3.42	3.19	6.09	5.03	4.12

Raw – Raw water; AT – After treatment; Dis – Distribution system

4.10 SUMMARY

The trends of the results are summarised in Table 4.13 and Table 4.14. In this study, DWPFs included in this study were classified as small (2.5 to 14 Mℓ/day; WC-A, NW-C, WC-F), medium (20 to 45 Mℓ/day; NW-B, NW-D, NW-E) and large (250 to 1 200 Mℓ/day; NW-G, GT-H). Population sizes that were dependent on the produced water ranged from 36 000 (WC-F) to a population of 12 million (GT-H; see Table 4.5). All systems used at least one filtration step before disinfection with chlorine. Some of the plants also incorporated coagulation/flocculation and sedimentation (NW-B, NW-E, WC-F, NW-G and GT-H). One of the larger plants also used dissolved air floatation and ozonation. There was a single direct potable reclamation plant (WC-A) that used NF, RO and advanced oxidation in addition to the other processes (Table 4.13). The source water was from surface sources (dams and rivers), as well as subsurface sources (boreholes and natural springs). Various land uses may impact on the water source or the quality of the water that reaches the DWPF. Agriculture (animal rearing and irrigation), upstream WWTPs, septic tanks, urbanisation (formal and informal), mining and industrial activities are the main impactors on the quality of the water (Table 4.5). These land uses could potentially also affect the presence of antibiotics and ARB in the source water.

Water temperatures in the distribution systems were in the range of 16.5 ±2.6 ˚C and 26.6 ±2.7 ˚C. This temperature range is suitable for regrowth and biofilm formation in distribution systems (Mulamattathil et al., 2015). Regrowth potential and biofilm formation could also be enhanced due to elevated TDS, nutrients (COD, nitrates and phosphates) and favourable pH levels. Increased regrowth could result in the depletion of free chlorine in the water. This could potentially explain the general low levels of this substance that was measured (Chowdhury, 2011).

In the case of at least one of the DWPFs (NW-G), the elevated TDS and very high turbidity (16.2 ±3.3 NTU) was linked to pollution of the source water. In this case, the source of the water was a major river that was prone to pollution from agriculture and non-functional WWTPs upstream of the DWPF (DWA, 2012). The quality of the source water, particularly surface water in South Africa, is further exacerbated by the low rainfall and drought patterns that the country has experienced over the past few years. This is expected to escalate (DWA, 2012; WWF, 2016), and solutions are urgently needed to deal with this trend of decreasing surface water quality. Despite such challenges of poor (deteriorating) quality source water, all plants produced water that is comparable to international and national drinking water standards (SANS 241) (SABS, 2015). The 2012 Blue Drop score for compliance to national standards for these plants was between 85.0% and 100% (DWA, 2013)

What was of concern was the fact that, in NW-C, the turbidity was lower (0.59 ±0.6 NTU) in the source water compared to the drinking water (1.6 ±1.1 NTU). This is probably due to the treatment process at this plant. After the raw water passes the rapid sand filtration, it is collected in a sump where it is not left long enough for the suspended particles to settle. The suspended material therefore increases the turbidity of the water. Normally treatment plants using filtration as part of its treatment process should be able to limit turbidity levels from rising above 0.5 NTU (WHO, 2003).

Physical and chemical parameters that mainly impacted on the water quality in the majority of plants were TDS, phosphates, nitrites and, in one case (WC-F), low pH (only in the source water; see Table 4.14). Figure 4.17 and Figure 4.18 provide principal component analysis (PCA) biplots to demonstrate these impacts.

Table 4.13: Summary of DWPFs' capacities, treatment processes and population supplied

	Water sources	Purification/treatment processes	Plant capacity (Mℓ/day)	Population served	Land-use issues
WC-A	Surface water: dam Ground water: boreholes WWTP effluent	Surface and ground water – sand filtration – chlorination WWTP effluent – sedimentation – sand filtration – ultrafiltration – RO – advanced oxidation – chlorination	4.92 2.5	51 080	Agriculture
NW-B	Surface water: dam WWTP immediate upstream	Coagulation-flocculation – sedimentation – sand filtration, chlorination	20	314 000	Agriculture, WWTP, urbanisation, informal settlements
NW-C	Natural spring	Sand filtration – chlorination	14	56 702	Agriculture, uncovered canal
NW-D	Ground water: natural spring, boreholes	Sand filtration – chlorination	45	314 000	Agriculture, septic tanks, Recreation – diving, uncovered canal
NW-E	Surface water: dam Ground water: borehole	Coagulation-flocculation – sedimentation – sand filtration, activated carbon filtration – chlorination	33.6	162 762	Agriculture, informal settlements urbanisation, mining, uncovered canal
WC-F	Surface water: dam Ground water: boreholes	Coagulation-flocculation – sedimentation – sand filtration, chlorination	8	36 000	None at the dam in the mountain, surface runoff to the mixing dam in town
NW-G	Surface water: river	Coagulation-flocculation – dissolved air floatation – ozonation – sedimentation – sand filtration, chlorination	250	417 282	Agriculture, informal settlements urbanisation, mining,
GT-H	Surface water: dam	Coagulation-flocculation – sedimentation – sand filtration, granular activated carbon – UV – chloramination	4 000	12 million	Agriculture, urbanisation, mining, energy generation, chemical and metal industries

Table 4.14: Results of selected physical parameters of water before purification, after purification and during distribution at all plants

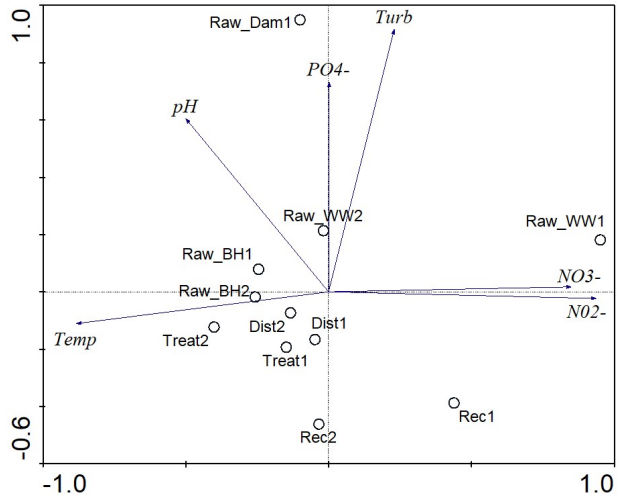
Sampling area	Turbidity (NTU)			pH (taken on site)			Temp. (°C)			TDS (mg/ℓ)		
	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis
WC-A	3.87±1.18	0.45±0.04	0.5±0.1	7.9±0.3	7.9±0.1	7.9±0.1	15.8±2.7	17.6±2.2	16.5±2.6	834.± 248.7	789.0±66.2	857.9±89.5
NW-B	Not done	Not done	Not done	8.9±1.2	8.5±0.9	8.6±0.9	24.1±4.1	21.7±2.6	24.3±3.4	483.8±2390	499.0±265.1	512.75±208.1
NW-C	0.59±0.6	1.21±0.8	1.6±1.1	8.7±0.1	8.6±0.08	8.6±0.1	17.8±2.2	16.9±1.5	20.2±3.3	362.9±23.2	364.8±23.8	362.4±23.2
NW-D	Not done	Not done	Not done	8.5±0.9	8.4±0.5	8.3±0.5	19.3±6.8	22.9±2.1	26.6±2.7	497.0±338.3	324.0±269.0	363.4±249.3
NW-E	2.6±1.2	1.2±0.7	0.4±0.2	8.3±0.3	8.1±0.3	8.3±0.4	17.6±3.7	18.6±3.7	17.7±3.4	493.4±38.7	502.9±38.2	514.4±23.6
WC-F	6.5±3.7	0.2±0.0	0.3±0.0	6.4±0.8	7.2±0.0	7.5±0.1	17.2±4.5	13.0±0.1	15.8±0.1	396.3±43.0	391±0.0	393.7±2.6
NW-G	16.2±3.3	1.2±1.4	1.3±1.9	9.3±0.4	8.4±0.2	8.5±0.4	16.8±4.8	18.0±5.4	19.5±4.3	493.6±52.7	508.1±58.2	525.3±61.8
GT-H	6.7±3.6	0.5±0.2	0.6±0.0	8.3±0.2	8.2±0.0	8.0±0.0	18.9±1.5	20.9±0.4	19.9±0.1	521.8±32.3	513.3±26.7	491.4±1.7

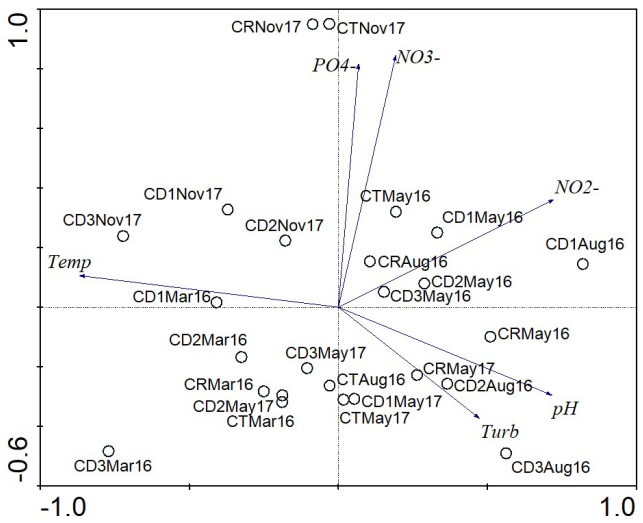
Raw – Raw water; AT – After treatment; Dis – Distribution system; Temp. – Temperature; TDS – Total dissolved solids; NTU - Nephelometric turbidity units; mg/ℓ – milligrams per liter

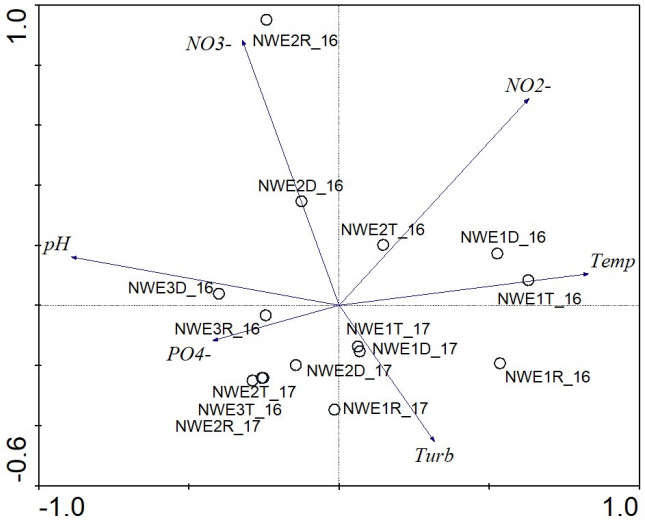
Table 4.15: Results of selected chemical parameters of water before purification, after purification and during distribution at all plants

Sampling site	Phosphorus (mg/ℓ)			Nitrate (mg/ℓ)			Nitrite (mg/ℓ)			COD (mg/ℓ)			Free chlorine (mg/ℓ)
	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis	Dis
WC-A	4.4±0.9	3.5±0.3	3.7±1.2	3.1±3.7	1.1±0.3	1.1±0.3	0.1±0.2	0.0±0.0	0.1±0.2	Not done	Not done	Not done	0.04±0.03
NW-B	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done
NW-C	0.8±1.2	1.2±1.5	0.5±0.6	2.3±1.1	2.1±0.8	2.1±0.7	3.6±1.4	2.8±2.0	3.0±2.1	0.0±0.0	18.0±25.4	7.4±13.6	0.4±0.2
NW-D	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done	Not done
NW-E	2.0±2.0	1.6±2.2	1.4±0.7	1.0±1.4	0.4±0.4	0.7±0.8	4.9±7.7	4.1±6.8	5.8±8.0	2.2±2.8	20.5±40.2	8.2±21.9	0.4±0.6
WC-F	3.0±1.2	3.8±1.2	2.3±0.9	0.2±0.2	0.3±0.1	0.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	Not done	Not done	Not done	0.04±0.0
NW-G	2.3±2.2	1.6±1.5	1.1±1.3	2.1±3.8	2.8±1.2	1.9±0.7	8.1±15.3	5.8±2.7	5.7±5.8	49.6±44.9	19.6±20.5	18.2±13.0	0.2±0.4
GT-H	1.2±0.9	1.1±0.4	1.0±0.0	3.2±3.2	1.9±2.4	0.3±0.0	1.0±0.9	0.3±0.0	1.0±0.1	3.8±0.5	2.9±0.6	1.5±0.0	0.1±0.0

Raw – Raw water; AT – After treatment; Dis – Distribution system; COD – Chemical oxygen demand; mg/ℓ – milligrams per liter

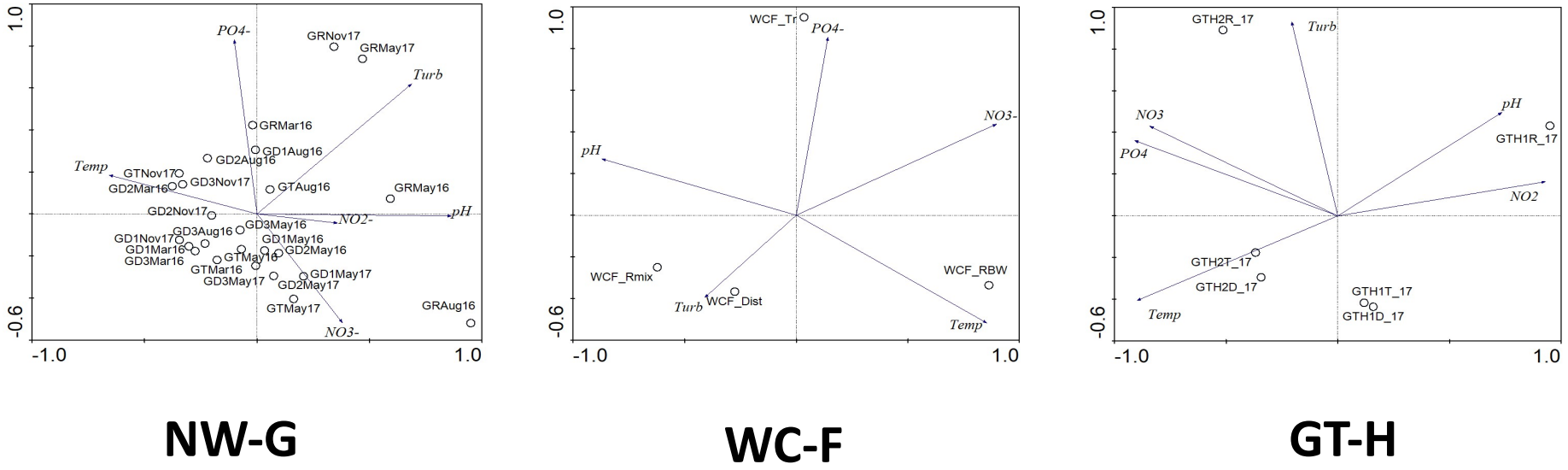






WC-A **NW-C** **NW-E**

Figure 4.17: PCA biplots of WC-A, NW-C and NW-E



NW-G **WC-F** **GT-H**

Figure 4.18: PCA biplots of WC-F, NW-G and GT-H

The following trends are evident in Figure 4.17 and Figure 4.18: There is a general separation of the clustering of raw water and the treated and drinking water. However, some of the raw and drinking water was clustered together. In the case of WC-A, pH level, phosphates and TDS impacted on the quality of the raw water, whereas temperature impacted on the quality of the treated and drinking water. At NW-C, pH level and nitrites impacted on the quality of the raw water. Temperature, turbidity and pH level impacted on the quality of the treated and drinking water. In some cases, nitrates, nitrites and phosphates also impacted on the quality of the water. The impacts on the raw water at NW-E varied and no trend could be observed. However, phosphates and, in some cases, turbidity, nitrates and nitrites impacted on the quality of the treated and drinking water. Turbidity impacted on the raw mixed (dam and borehole) and drinking water at WC-F. Nutrients (nitrates, nitrites, phosphates) and turbidity impacted on the raw water at NW-G. The treated and drinking water formed a cluster in the centre of the biplot and seemed to be affected by all the parameters to a different extent. The pH level and nitrites impacted on the raw water at GT-H, and temperature impacted on the treated and drinking water. One physical parameter that impacted on the raw water in four of the six plants was pH level. From these PCA analyses, it is evident that nutrients (phosphates and nitrates) impacted on the quality of both the raw and the drinking water.

Phosphorus in drinking water does not pose health risks. However, studies show that levels as low as 1 µg/L increased microbial growth in treated water, as well as distribution water (Glasser, 2000; Lehtola et al., 2002). The addition of polyphosphates to drinking water to limit and decrease corrosion could result in elevated levels of phosphorus in the distribution system (Cantor et al., 2000). Further attention should thus be given to this aspect.

Nitrates and nitrites are related nitrogen compounds that occur naturally in the environment. Other sources are from contamination, including commercial fertilizer, sewage, industrial wastes and livestock manure (Alemdar et al., 2009). The study of Alemdar et al. (2009) demonstrated that nitrate levels in drinking water from Turkey were not high (2.40 mg/ℓ to 2.80 mg/ℓ). However, the nitrite levels were a source of concern. Nitrates are converted to nitrites by microbial action and could explain why, in some cases, the nitrate levels were lower than the nitrite levels (Chapman and Kimstach, 1996; Dallas and Day, 2004). The presence of both nitrites and nitrates in drinking water might be an indication of biofilm formation. Thus, finding various HPC bacteria in the drinking water and investigating these for antibiotic susceptibility and virulence followed. In addition to determining the phenotypic antibiotic susceptibility, the presence of ARGs was also determined. This was done by end-point PCR and by the WGS of isolated bacilli. For the 16S rRNA profiling, eDNA from the plants WC-A, NW-C, NW-E, WC-F and NW-G were included. Raw metagenomic sequences from GT-H were obtained from the University of Pretoria.

CHAPTER 5: ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF BACTERIA IN DRINKING WATER

5.1 INTRODUCTION

The aim of this chapter is to screen raw and treated water for the presence of antibiotics, as well as ARB. Associated antibiotic patterns and the presence of ARGs were also determined. All the plants were considered for ARB and ARG analyses. The isolated bacteria were also identified and virulence profiles determined. However, water samples from NW-B, NW-D and GT-H were not included for the antibiotic screening.

The quality of the raw water will impact on the quality of the produced water, as demonstrated in this study and a scoping study by Bezuidenhout et al., 2016. Physicochemical water properties determine the fitness and survival capability of organisms such as bacteria (Bezuidenhout et al., 2016). Associated with such survival abilities could be natural intrinsic characteristics, or these could be genetically acquired. Survival in water that contains antimicrobial substances could be associated with either intrinsic of genetic characteristics. When studies on ARB and ARGs are conducted, it is important that isolated bacteria are characterised for antibiotic resistance profiles and that these are identified. Screening for the presence of antibiotics provides further information about the potential of the water sources to act as reservoirs for ARB and ARGs (Biyela et al., 2004; Bezuidenhout et al., 2016). Additional information on characteristics regarding the pathogenic potential could be obtained by performing culture-based virulence tests (Prinsloo et al., 2013).

This approach is a practical one and demonstrates the existence of viable bacteria with the said properties. The occurrence of non-pathogenic or opportunistic pathogenic bacteria that are resistant to antibiotics due to genetic elements implies the potential of these elements to be transferred to susceptible pathogens or that the opportunistic pathogens may affect immunocompromised hosts. All these datasets could thus be used to demonstrate the potential impact of such water on health and the distribution of antibiotic resistance to vulnerable communities, and thus may have implications for management going forward.

5.2 SCREENING AND QUANTIFICATION OF ANTIBIOTICS IN DRINKING WATER

5.2.1 Screening for antibiotics in drinking water

Water samples were treated as described and subjected to liquid chromatography to screen for the presence of selected antibiotics. Antibiotics selected generally represented the antibiotics that were used to determine antibiotic resistance profiles. The results are presented in Table 5.1. Ciprofloxacin was present in all the water samples. Trimethoprim was not detected in the mixed water, but was present in the two drinking water samples. Most of the antibiotics were not detected by the crude liquid chromatography method used. Using ARB resistance phenotype data, more detailed studies should be conducted that could focus on the seasonal variability of antibiotics in drinking water. This data could then be analysed for land-use impacts.

5.2.2 Levels of antibiotics in drinking water

The antibiotics that were frequently detected by using both methods included beta-lactam antibiotics, Trimethoprim, Colistin and, in several cases, also Ciprofloxacin (Table 5.2). Trimethoprim levels in the raw water were much higher than in the drinking water. This was particularly the case in the raw water of NW-G, where levels were 38.983 ng/ℓ.

The levels of all three antibiotics were extremely low and below the predicted concentration where it could select for antibiotic resistance (10 to 1 000 times below the predicted no-effect concentration (PNEC) (Bengtsson-Palme and Larsson, 2016). What is important is that these antibiotics were present in detectable amounts in the raw and drinking water at these plants.

5.3 ANTIBIOTIC RESISTANCE PROFILES AND VIRULENCE ABILITY OF THE ISOLATED HPC BACTERIA

5.3.1 Overview

Heterotrophic plate count bacteria were successfully isolated from the various compartments of the selected DWTFs. Some HPC bacteria did not grow during the colony purification steps and could not be further analysed. After successive streak plating, Gram staining was performed to confirm the purity of isolates and classify them as Gram positive or Gram negative. This was important for the antibiotic susceptibility test using the Kirby-Bauer disk diffusion method. Only Gram-positive isolates were further tested for their susceptibility to Penicillin G and Vancomycin, in addition to 10 other antibiotic disks. Table 5.3 gives a list of the ARGs selected for this study. Figure 5.1 illustrates a 1.5% (w/v) agarose gel and demonstrates the positive controls for the various ARGs that were tested for.

Table 5.1: Antibiotics present (√) and absent (a) at different DWTPs

List of antibiotics	WC-A			WC-F		NW-C			NW-E			NW-G		
	mixed	Raw	RO	Raw	AT	Raw	AT	Dis	Raw	AT	Dis	Raw	AT	Dis
Ampicillin	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Cephalothin	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Chloramphenicol	a	√	a	a	a	a	a	a	a	a	a	√	a	a
Ciprofloxacin	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Erythromycin	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Kanamycin	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Neomycin	a	a	a	a	a	a	√	√	a	√	a	a	a	a
Oxytetracycline	a	√	a	a	a	a	a	a	a	a	a	a	a	a
Penicillin G	a	a	a	a	a	a	a	a	a	a	a	√	a	√
Penicillin	√	a	a	√	√	√	√	√	a	√	√	a	a	a
Streptomycin	a	√	a	a	a	a	a	a	√	a	a	√	√	a
Trimethoprim	a	√	√	a	a	a	a	a	a	a	a	a	a	a
Vancomycin	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Colistin	a	a	a	a	a	a	a	a	a	a	a	a	a	a
Sulfamethoxazole	a	a	a	a	a	a	a	a	a	a	a	a	a	a

Dis – Distribution system; RO – Reverse osmosis; AT – After treatment

Table 5.2: Concentrations of three selected antibiotics in raw and drinking water at selected DWPFs determined by ELISA

		PNEC	WC-A	NW-C	NW-E	WC-F	NW-G
Raw	**Trimethoprim (ng/ℓ)**	500	<LOD	1.100	0.849	0.413	38.983
	Colistin (ng/ℓ)	2000	142	123	75	128	56
	Beta-lactams (ng/ℓ)	250	3.284	3.673	2.442	3.968	4.498
Treated	**Trimethoprim (ng/ℓ)**	500	<LOD	0.495	0.481	0.417	0.562
	Colistin (ng/ℓ)	2000	30	118	46	104	77
	Beta-lactams (ng/ℓ)	250	<LOD	4.154	4.823	1.302	5.138
Distribution system	**Trimethoprim (ng/ℓ)**	500	1.384	0.292	0.253	N/D	0.218
	Colistin (ng/ℓ)	2000	87	115	152	N/D	41
	Beta-lactams (ng/ℓ)	250	3.661	3.469	4.563	N/D	4.653

PNEC – Predicted no-effect concentration; Dis – Distribution system; LOD – Limit of detection; N/D = Not done

Table 5.3: The ARGs selected in the present study and association with water sources

ARG	Antibiotics resistant to	Associated bacterial species	Reference
ampC	Cephalothin, Ceafazolin, Cefoxitin and most penicillins	*Citrobacter, Enterobacter, E. coli,* viable but non-cultivable bacteria	Schwartz et al., 2003; Volkmann et al., 2004; Zhang et al., 2009; Xu et al., 2016
TEM1	Cephalothin and penicillins	*E. coli*, HPC bacteria	Alpay-Karaoglu et al., 2007; Xi et al., 2009; Zhang et al., 2009; Xu et al., 2016
ermB, ermF	Macrolides, Lincosamide and Streptogramin	*Bacillus, Enterococcus*	Zhang et al., 2009; Xu et al., 2016
tetM	Tetracycline	*Aeromonas, Bacillus, Escherichia, Lactococcus, Pseudoalteromonas, Vibrio*	Zhang et al., 2009
IntI	Depends on genes present in cassettes	*E. coli, Vibrio*	Ozgumus et al., 2007; Taviani et al., 2008; Xu et al., 2016

5.4 ANTIBIOTIC RESISTANCE PROFILES AND VIRULENCE ABILITY OF THE ISOLATED HPC BACTERIA

5.4.1 Overview

Heterotrophic plate count bacteria were successfully isolated from the various compartments of the selected drinking water treatment plants. Some HPC bacteria did not grow during the colony purification steps and could not be further analysed. After successive streak plating, Gram staining was performed to confirm the purity of isolates and classify them as Gram positive or Gram negative. This was important for the antibiotic susceptibility test using the Kirby-Bauer disk diffusion method. Only Gram-positive isolates were further tested for susceptibility to Penicillin G and Vancomycin, in addition to 10 other antibiotic disks. Table 5.3 gives a list of the ARGs selected for this study. Figure 5.1 illustrates a 1.5% (w/v) agarose gel and demonstrates the positive controls for the various ARGs that were tested for.

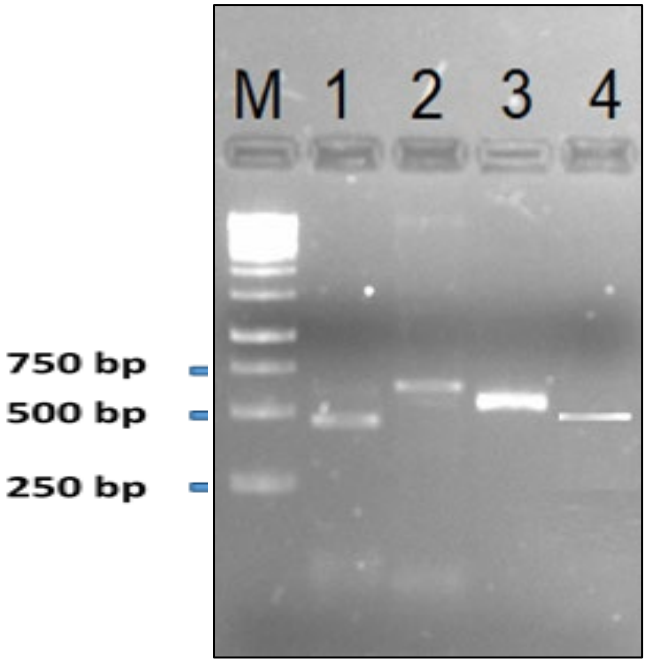


Figure 5.1: A 1.5% (w/v) agarose gel demonstrating the sizes of ARGs that were studied. Lane 1 represents *ermF.* Lane 2 represents *ermB.* Lane 3 represents *ampC.* Lane 4 represents *intI 1.* The lane marked M represents a 1 kb molecular weight marker (GeneRuler™ 1 kb DNA ladder, Fermentas, US).

5.4.2 A direct potable water re-use and reclamation plant – WC-A

5.4.2.1 Isolation and identification of HPC bacteria

Table 5.4 represents the identity of various isolates based on the 16S rRNA gene sequencing. *Pseudomonas* spp., *Rhodoferax* spp., *Rheinheimera* spp., *Undibacterium* spp., *Flavobacterium* spp., *Acidovorax* spp., *Arcicella* spp., *Paenibacillus* spp., *Hymenobacter* spp., *Rhizobium* spp., *Porphyrobacter* spp., *Cohnella* spp. and *Pantoea* spp. are the Gram-negative bacteria that were identified. Among the Gram-positive bacteria identified from WC-A were *Bacillus* spp. and *Novosphingobium* spp. *Bacillus* spp. were more prevalent throughout the different drinking water treatment stages (RO, after treatment and distribution stages) for sampling in June and November 2017. The elevated levels of *Bacillus* spp. are potentially caused by the ability of these species to form biofilm (Mulamattathil et al., 2015). *Pseudomonas* spp. were the most frequently detected bacteria in Raw 1 (borehole) for all sampling runs. The identity percentages were generally high at 99% to 100%. However, there were some cases where the identity percentage values were lower, indicating potentially novel strains.

Table 5.4: The identities of the HPC isolates from WC-A that were determined by 16S rDNA sequencing

Date	Site	GenBank ID	Percentage identity number	Associated accession number
June 2017	**Raw 1**	*Pseudomonas reinekei*	91.99	AM293565
		Pseudomonas reinekei	99.52	AM293565
		Pseudomonas reinekei	99.76	AM293565
		Pseudomonas reinekei	99.64	AM293565
		Pseudomonas reinekei	100.00	AM293565
		Pseudomonas reinekei	99.17	AM293565
		Pseudomonas reinekei	99.76	AM293565
		Pseudomonas reinekei	98.82	AM293565
		Pseudomonas reinekei	99.53	AM293565
	Raw 3	*Bacillus wiedmannii*	99.07	JH792383
		Rhodoferax saidenbachensis	98.11	AWQR01000064
		Rheinheimera texasensis	98.42	AY701891
		Undibacterium jejuense	97.56	KC735150
		Flavobacterium tructae	99.39	MUHH01000012
		Acidovorax delafieldii	99.88	jgi.1055345
		Acidovorax delafieldii	99.88	jgi.1055345
		Bacillus paramycoides	99.65	KJ812444
		Arcicella rigui	99.24	HM357635
		Undibacterium jejuense	98.41	KC735150
		Bacillus mobilis	99.65	KJ812449
	RO	*Bacillus tequilensis*	99.88	AYTO01000043
		Paenibacillus provencensis	98.84	EF212893
		Bacillus simplex	96.94	BCVO01000086
		Hymenobacter seoulensis	99.40	KU758880
	AT	*Bacillus zhangzhouensis*	99.30	JOTP01000061
November 2017	**R1**	*Novosphingobium subterraneum*	99.50	JRVC01000007
		Pseudomonas chengduensis	98.70	EU307111
		Pseudomonas chengduensis	98.57	EU307111
		Pseudomonas alcaligenes	98.45	BATI01000076
		Rhizobium rhizoryzae	98.85	EF649779
		Acidovorax temperans	99.17	AF078766
		Porphyrobacter colymbi	98.74	AB702992
		Bacillus wiedmannii	99.06	LOBC01000053
	Raw 2	*Bacillus wiedmannii*	99.77	LOBC01000053
	RO	*Bacillus mobilis*	93.41	KJ812449
	AT	*Bacillus glycinifermentans*	98.48	LECW01000063
		Bacillus wiedmannii	98.81	LOBC01000053
	Dis	*Cohnella xylanilytica*	97.04	FJ001841
		Pantoea eucrina	99.77	CP009880
		Bacillus wiedmannii	98.84	LOBC01000053
		Bacillus wiedmannii	96.26	LOBC01000053
		Bacillus wiedmannii	99.30	LOBC01000053
		Porphyrobacter mercurialis	98.61	JTDN01000001

Bacillus wiedmannii	99.77	LOBC01000053
Bacillus wiedmannii	99.30	LOBC01000053
Bacillus wiedmannii	99.88	LOBC01000053
Bacillus wiedmannii	98.81	LOBC01000053
Bacillus wiedmannii	99.43	LOBC01000053

Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 – Dam water; RO – Final treated reclaimed water; AT – After treatment, mixed water; Dis – Distribution system

5.4.2.2 Antibiotic susceptibility

Table 5.5 and Table 5.6 represent summaries of antibiotic susceptibility test results from WC-A. A trend was observed in antibiotic susceptibility tests in which isolates were generally resistant to Ampicillin, Cephalothin and Trimethoprim. Isolates from the reclamation plant were mostly susceptible to the antibiotics. However, isolates from the distribution network were resistant to the mentioned, as well as other antibiotics. Some 80% to 90% of isolates from Raw 1 (boreholes) were also resistant to Chloramphenicol and Erythromycin. This probably influenced the observed resistance patterns among isolates from the distribution network. Previous studies have also investigated the occurrence of ARB in DWPFs and distribution networks (Lu et al*.,* 2016; Xi et al. 2009) and found similar results. Data generated using the Kirby-Bauer disk diffusion method were used to determine the MAR indices shown in Table 5.6. The MAR Index was used to assess antibiotic use or the antibiotic exposure history of the isolates (Davis and Brown. 2016). An index of more than 0.2 indicates a high exposure rate of isolates to antibiotics (Davis and Brown. 2016). Raw 1 (borehole) had an MAR Index >0.2 for both sampling runs. The MAR Index after RO treatment in June was <0.2. However, in November, this value was higher (MAR index >0.2). Although the number of isolates was low, the higher MAR indices in the after treatment (0.46) and distribution stages (0.34) should be further considered as these index values indicate that the isolates were exposed to a higher number of antibiotics.

Table 5.5: Percentage of isolates that were resistant to the various antibiotics at WC-A

Site	Chl	CIP	Ery	Kan	O-T	Neo	TMP	Kf	Amp	Strep	PenG	Van
June 2017												
Raw 1	80	0	90	0	70	10	90	100	100	0	0	0
Raw 3	7.7	7.7	7.7	15.4	0	15.4	53.9	53.9	61.5	0	7.7	0
RO	0	20	0	20	0	0	20	0	60	0	20	20
AT	50	50	50	0	50	50	50	50	50	50	50	50
November 2017												
Raw 1	45.45	9.09	36.36	0	18.18	0	0	81.82	81.82	81.82	9.09	0
Raw 2	0	0	0	0	0	0	100	100	100	0	0	0
RO	0	0	50	0	0	0	0	0	50	0	100	100
AT	6.25	0	12.5	0	0	0	12.5	12.5	12.5	0	6.25	6.25
Dis	6.25	6.25	43.75	0	25	0	75	68.75	68.75	37.5	50	37.5

Amp – Ampicillin; Kf – Cephalothin; Chl – Chloramphenicol; CIP – Ciprofloxacin; Ery – Erythromycin; Kan – Kanamycin; Neo – Neomycin; O-T – Oxy-tetracycline; Pen-G – Penicillin G; Strep – Streptomycin; TMP – Trimethoprim; Van – Vancomycin; Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 – Dam water; RO – Final treated reclaimed water; AT – After treatment, mixed water

Table 5.6: Representation of MAR for WC-A

Sampling date	Raw 1	Raw 2	Raw 3	RO	AT	Dis
June 2017	0.45		0.19	0.13	0.46	
November 2017	0.30	0		0.25	0.46	0.34

Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 – Dam water; RO – Final treated reclaimed water; AT – After treatment, mixed water

5.4.2.3 Antibiotic-resistant profiles

Table 5.7 shows that ARGs were detected among isolates at WC-A. The *ermB* and *ermF* genes detected in raw and drinking water confer resistance to Erythromycin. These *erm* genes can also confer resistance to other classes of antibiotics, namely Chloramphenicol and Vancomycin (Zhang et al*.,* 2009). The resistance mechanisms that are associated with *erm* genes include rRNA methylation, efflux and inactivation (Zhang et al*.,* 2009). The *erm* genes are carried on the genetic elements, such as plasmids and transposons, thus making it easy for the genes to be shared between bacterial species (Zhang et al., 2009). Two of the isolates were positive for *ampC* genes. Class 1 integron genes (*intl 1*) were also detected among the isolates. The *intl 1* are associated with mobile elements such as transposons and plasmids that facilitate the transfer of resistance to antibiotics, heavy metals and disinfectants between bacteria (Gillings et al., 2015; Koczura et al., 2016).

Table 5.7: Antibiotic-resistant genes from WC-A in June and November 2017

Date	Site	Identity	ARG
June 2017	**Raw 2**	*Pseudomonas reinekei*	*erm B*
		Pseudomonas reinekei	*erm B*
		Pseudomonas reinekei	*erm B*
	Raw 3	*Rhodoferax saidenbachensis*	*erm F*
		Undibacterium jejuense	*erm B*
		Flavobacterium tructae	*erm B*
		Pseudomonas protegens	*erm B*
	RO	*Paenibacillus provencensis*	*erm F*
	AT	*Pseudomonas protegens*	*erm F, amp C*
November 2017	**Raw 2**	*Pseudomonas chengduensis*	*erm B*
		Bacillus wiedmannii	*erm B, int 1*
	Raw 1	*Bacillus wiedmannii*	*erm B*
	RO	*Bacillus mobilis*	*erm B*
	Dis	Unidentified	*Intl 1*
		Bacillus wiedmannii	*erm B, intl 1*
		Porphyrobacter mercurialis	*erm B*
		Bacillus wiedmannii	*erm B, intl 1*
		Porphyrobacter mercurialis	*intl 1*
		Bacillus wiedmannii	*erm B*
		Bacillus wiedmannii	*erm B*
		Unidentified	*erm B*
		Bacillus wiedmannii	*intl 1*
		Bacillus wiedmannii	*amp C*
	AT	*Bacillus wiedmannii*	*erm B*

Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 – Dam water; RO – Final treated reclaimed water; AT - After treatment, mixed water, RO – Reverse osmosis

5.4.2.4 Virulence ability of the isolated HPC bacteria

Table 5.8 and Table 5.9 represent potential pathogenicity patterns of HPC bacteria for WC-A in June and November. Most of the isolates for both sampling runs were beta-haemolytic (51.52% for the November 2017 and 57.58% for the June 2017 sampling runs). This denotes that many HPC bacteria have the ability to completely break down the host's red blood cells (Pavlov et al., 2004). Some isolates were alpha haemolytic (24.24% for the November 2017 and 6.06% for the June 2017 sampling runs). Alpha haemolytic isolates partially break down the host cell's red blood cells (Yeh et al., 2009). HPC bacterial isolates were then subjected to extracellular enzyme tests to determine whether isolates are potential pathogens. (An isolate is considered a potential pathogen if it produces two or more extracellular enzymes (Pavlov et al., 2004).) Many of the isolates tested were also positive for the production of a range of extracellular enzymes associated with pathogenic potential. Nine of the 32 isolates from June produced six enzymes and 15 produced five. The scenario was similar for the November sampling period. A general trend was that more than 50% of the haemolysin-producing HPC isolates also produced Dnase, lecithinase and proteinase. Among the June 2017 isolates, lipase was also produced by more than 50% of HPC bacteria.

Table 5.8: Extracellular enzyme tests for HPC isolates from WC-A in June 2017

Site	Identity	DNase	Gelatinase	Lipase	Lecithinase	Proteinase
Raw 1	*Pseudomonas reinekei*	+	+	+	+	+
	Pseudomonas reinekei	+	-	-	+	+
	Pseudomonas reinekei	+	+	+	+	+
	Pseudomonas reinekei	+	+	-	-	-
	Pseudomonas reinekei	+	+	-	+	+
	Pseudomonas reinekei	-	-	-	-	-
	Pseudomonas reinekei	-	-	-	+	+
	Pseudomonas reinekei					
	Pseudomonas reinekei	-	-	+	-	-
	Unidentified	+	-	-	+	+
Raw 3	*Bacillus wiedmannii*	+	+	+	+	-
	Unidentified	+	+	+	+	+
	Rhodoferax saidenbachensis	+	+	-	+	-
	Unidentified	+	-	+	+	+
	Undibacterium jejuense	+	-	+	+	-
	Flavobacterium tructae	+	-	+	+	+
	Acidovorax delafieldii	+	-	-	+	-
	Unidentified					
	Acidovorax delafieldii	-	-	-	+	+
	Unidentified	-	+	+	-	-
	Bacillus paramycoides	+	-	+	+	+
	Arcicella rigui					
	Undibacterium jejuense	-	+	-	-	-
	Bacillus mobilis	-	-	+	-	-
RO	*Unidentified*	+	-	+	+	+
	Unidentified	+	+	-	-	-
	Unidentified	+	-	+	+	+
	Paenibacillus provencensis					
	Bacillus simplex	-	+	+	-	-

Site	Identity	DNase	Gelatinase	Lipase	Lecithinase	Proteinase
	Hymenobacter seoulensis	+	+	+	+	+
	Unidentified	+	+	+	+	+
AT	*Bacillus zhangzhouensis*	-	-	-	+	+
	Unidentified	+	+	+	+	+
	Overall percentage	62.50	43.75	53.13	65.63	53.13

Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 – Dam water; RO – Final treated reclaimed water; AT - After treatment, mixed water; Dis – Distribution system

Table 5.9 Extracellular enzyme tests for HPC isolates from WC-A in November 2017

Site	Identity	DNase	Gelatinase	Lipase	Lecithinase	Proteinase
Raw 1	*Novosphingobium subterraneum*	+	-	-	-	-
	Pseudomonas chengduensis	-	-	-	+	-
	Unidentified	-	+	+	+	+
	Pseudomonas chengduensis	-	-	+	-	-
	Pseudomonas alcaligenes	+	-	+	+	-
	Unidentified	+	+	+	+	+
	Rhizobium rhizoryzae	+	-	-	+	+
	Acidovorax temperans	+	+	-	+	+
	Unidentified	+	+	-	+	+
	Porphyrobacter colymbi	+	+	+	-	+
	Bacillus wiedmannii	+	-	-	-	-
Raw 2	*Unidentified*	-	+	-	+	-
	Bacillus wiedmannii	-	-	-	-	-
RO	*Bacillus mobilis*	+	-	-	+	+
	Unidentified	-	-	-	-	-
	Cohnella xylanilytica	+	+	+	+	+
	unidentified	-	-	-	+	-
	Pantoea eucrina	+	-	-	-	-
	Unidentified	+	-	+	-	-
Dis	*Bacillus wiedmannii*	+	+	-	+	+
	Bacillus wiedmannii	+	-	+	+	+
	Bacillus wiedmannii	+	-	-	+	+
	Porphyrobacter mercurialis	+	+	-	+	+
	Bacillus wiedmannii	+	+	-	+	+
	Bacillus wiedmannii	+	+	-	+	+
	Unidentified	+	+	+	+	-
	Unidentified	+	-	-	+	+
	Unidentified	+	+	+	+	+
	Bacillus wiedmannii	-	-	-	-	-
	Bacillus wiedmannii	-	-	-	-	-
	Bacillus wiedmannii	-	-	-	-	-
AT	*Bacillus glycinifermentans*	+	+	-	+	+
	Unidentified	+	-	+	+	P
	Overall percentage	69.70	42.42	33.33	66.70	51.50

Raw 1 – Borehole; Raw 2 – WWTP effluent; Raw 3 - Dam water; RO – Final treated reclaimed water; AT – After treatment, mixed water; Dis – Distribution system.

5.4.3 Drinking water production using a mixture of raw sources (NW-B and NW-D)

5.4.3.1 Isolation of HPC bacteria

The isolates that were identified are listed in Table 5.10. Percentage similarities to sequences in GeneBank were generally 99%. However, there were cases where this percentage was below this and could be indicating novel strains from the specific genera. In this case, most of the isolated bacteria were Gram-negative bacteria and from the family Xanthomonads.

All of these are known producers of biofilm and some are known opportunistic pathogens. In previous studies, it was reported that *Aeromonas* sp. and *Pseudomonas* sp. were commonly isolated from a DWPF and distribution system that was part of such a semi-reuse scenario (Mulamatatthil et al., 2014a; Mulamatatthil et al., 2014b).

Table 5.10: The identities of the HPC isolates from NW-B and NW-D that were determined by 16S rDNA sequencing

Name	Top-hit taxon	Similarities (%)
1-2-_907-R_E08_14	*Escherichia coli*	99.33
4_907-R_A07_01	*Citrobacter pasteurii*	99.65
4-2-_907-R_F08_17	*Bacillus paramycoides*	92.75
6_907-R_B07_04	*Stenotrophomonas maltophilia*	99.41
7-2-_907-R_H08_23	*Klebsiella variicola*	99.76
8_907-R_C07_07	*Citrobacter amalonaticus*	99.33
10-2-_907-R_A09_03	*Delftia lacustris*	96.75
13-3-_907-R_B09_06	*Blastomonas natatoria*	98.24
16_907-R_D07_10	*Stenotrophomonas maltophilia*	99.53
19_907-R_E07_13	*Stenotrophomonas maltophilia*	99.42
19-2-_907-R_C09_09	*Pseudoxanthomonas mexicana*	97.02
19-3-_907-R_D09_12	*Bacillus wiedmannii*	84.74
22-2-_907-R_F09_18	*Stenotrophomonas maltophilia*	99.52
23-2-_907-R_G09_21	*Stenotrophomonas maltophilia*	99.65
23-3-_907-R_H09_24	*Stenotrophomonas pavanii*	99.49
24-4-_907-R_A10_01	*Pseudomonas indoloxydans*	95.03
25-3-_907-R_B10_04	*Klebsiella singaporensis*	80.51
27-3-_907-R_C10_07	*JHEE_s*	98.17
28_907-R_F07_16	*Stenotrophomonas maltophilia*	99.76
30_907-R_G07_19	*JQ084175_s*	64.32
32-3-_907-R_D10_10	*Enterobacter ludwigii*	99.63
34-3-_907-R_E10_13	*Serratia marcescens subsp. marcescens*	98.19
35_907-R_H07_22	*Klebsiella singaporensis*	96.96
38-2-_907-R_F10_16	*Paenibacillus typhae*	99.37
41-4-_907-R_G10_19	*Chryseobacterium gambrini*	84.77
43_907-R_A08_02	*Stenotrophomonas maltophilia*	100.00
44_907-R_B08_05	*Stenotrophomonas maltophilia*	99.53
44-4-_907-R_A11_02	*Klebsiella pneumoniae subsp. ozaenae*	94.11
46-4-_907-R_B11_05	*Serratia marcescens subsp. marcescens*	99.50
50-3-_907-R_C11_08	*Serratia marcescens subsp. marcescens*	98.66
51_907-R_C08_08	*Citrobacter europaeus*	99.07
52-4-_907-R_D11_11	*Serratia nematodiphila*	99.61
54-4-_907-R_B11_05	*Bacillus paramycoides*	99.88

5.4.3.2 Antibiotic susceptibility

In Table 5.11 and Table 5.12, resistance to beta-lactam antibiotics (Ampicillin), as well as Trimethoprim, was the most prominent and, in some cases, the percentage was higher in the samples collected after treatment. It is also evident that bacteria were, in some cases, resistant to aminoglycosides (Streptomycin and Kanamycin) and Oxy-tetracycline. Some of the isolates in the distribution system were also resistant to some antibiotics to which none of the isolates from the raw water were resistant;

for example, Kanamycin, Streptomycin, Oxy-tetracycline and Trimethoprim. This might be an artefact of the isolate selection process, but should be monitored in the other systems and metagenomic analyses.

Table 5.11: Percentage of isolates that were resistant to the various antibiotics at NW-B

Site	Antibiotic										
	Amp	**Pen-G**	**Ery**	**Chl**	**Strep**	**Kan**	**Neo**	**Van**	**O-T**	**CIP**	**TM**
						August 2016					
Raw	33.3	0	0	0	0	0	33.3	33.3	0	33.3	0
Inlet	28.6	0	0	0	42.9	28.6	0	0	28.6	0	57.1
AT	100	33.3	0	0	50	66.7	16.7	50	66.7	0	50
D1	16.7	0	16.7	0	0	0	0	16.7	16.7	0	50
D2	12.5	50	0	0	25	62.5	0	12.5	62.5	12.5	50
						November 2016					
Raw	50	50	16.7	33.3	33.3	0	0	33.3	16.7	0	100
Inlet	75	75	0	50	0	0	0	0	25.0	0	100
AT	100	100		50	0	0	0	0	0	0	100
D1	100	100	33.3	66.7	0	66.7	0	0	33.3	0	66.7
D2	0	0	16.7	66.7	88.3	33.3	16.7	0	66.7	0	66.7
						March 2017					
Raw	33.3	33.3	0	33.3	0	0	0	0	0	0	33.3
Inlet	0	0	0	20	0	0	0	0	0	0	40
AT	0	33.3	0	0	0	0	0	0	0	0	0
D1	16.7	16.7	0	33.3	0	0	0	0	0	0	83.3
D2	33.3	50	16.7	33.3	0	0	0	33.3	0	0	50
						May 2017					
Raw	40	40	0	0	0	0	0	0	0	0	60
Inlet	42.9	42.9	0	0	0	42.9	0	0	0	0	0
AT	40	40	0	0	0	60	0	0	20.0	0	0
D1	66.7	66.7	0	0	16.7	0	16.7	0	0	0	16.7
D2	100	80	0	0	0	60	0	0	0	0	20

Raw – Dam (NW-B); Inlet – Abstracted water prior to treatment; AT – After treatment; D1 – Random household tap water from treatment plant distribution system; D2 – Second random household tap water from treatment plant distribution system; Amp – Ampicillin; Pen-G – Penicillin G; Ery – Erythromycin; Chl – Chloramphenicol; Strep – Streptomycin; Kan – Kanamycin; Neo – Neomycin; Van – Vancomycin; O-T – Oxy-tetracycline; CIP – Ciprofloxacin; TMP – Trimethoprim

Table 5.12: Percentage of isolates that were resistant to the various antibiotics at NW-D

Site	Antibiotic										
	Amp	Pen-G	Ery	Chl	Strep	Kan	Neo	Van	O-T	CIP	TMP
						August 2016					
Raw	100	33.3	0	0	66.6	100	16.6	66.6	83.3	0	83.3
Inlet	85.7	71.4	0	0	0	57.1	0	57.1	85.7	14.3	71.4
AT	100	33.3	0	0	33.3	16.7	50	66.7	50	16.7	100
D1	50	0	0	0	66.7	50	50	66.7	66.7	0	100
D2	33.3	0	0	0	0	0	0	0	33.3	0	100
						November 2016					
Raw	0	0	0	100	100	0	66.7	0	66.7	0	100
Inlet	50	50	0	33.3	33.3	0	0	33.3	66.7	0	100
AT	0	0	33.3	100	100	100	33.3	0	100	0	100
D1	0	0	0	0	0	0	0	0	0	0	0
D2	0	0	0	0	0	0	0	0	0	0	25
						March 2017					
Raw	33.3	33.3	33.3	33.3	33.3	0	0	0	0	0	0
Inlet	0	0	0	100	0	0	0	0	0	0	33.3
AT	33.3	33.3	0	33.3	0	0	0	0	0	0	16.7
D1	30	30	0	30	10	0	10	10	0	0	40
D2	22.2	11.1	0	22.2	0	0	0	0	0	0	33.3
						May 2017					
Raw	100	100	0	0	0	0	0	0	0	0	100
Inlet	100	83.3	16.7	0	0	0	0	16.7	0	0	66.7
AT	60	80	0	0	0	60	0	0	0	0	0
D1	60	80	0	0	0	20	0	0	0	0	20
D2	50	50	0	0	16.7	0	0	0	0	0	33.3

Raw – Natural spring and boreholes (NW-D); Inlet – Abstracted water prior to treatment; AT – After treatment; D1 – Random household tap water from treatment plant distribution system; D2 – Second random household tap water from treatment plant distribution system; Amp – Ampicillin; Pen-G – Penicillin G; Ery – Erythromycin; Chl – Chloramphenicol; Strep – Streptomycin; Kan – Kanamycin; Neo – Neomycin; Van – Vancomycin;. O-T – Oxy-tetracycline; CIP – Ciprofloxacin; TMP – Trimethoprim

5.4.3.3 Antibiotic resistance genes

Table 5.13 provides a summary of the genes that were detected among the multiple-resistant bacterial isolates. The most common genes detected included those coding for resistance to Streptomycin (*strA* and *strB)* Trimethoprim (*dfrB1* and *dfrB2*) and Kanamycin (*aadA*). Various genes associated with conferring resistance to different classes of antibiotics were detected in the genomes of the selected bacteria isolated from these drinking water systems. These genes could be associated with antibiotic resistance phenotypes. Some of the genes (coding for streptomycin) were detected among isolates across all compartments. Resistance phenotypes to beta-lactam antibiotics was common among the isolates. However, only four of the isolates (three from raw water) had the *blaCTX-M* gene associated with the resistance phenotype. There are various other genes that also code for beta-lactam resistance that could have been responsible for the observed phenotype. Whole-genome sequencing and/or sequencing of the entire metagenome could reveal which of these genes are associated with the observed phenotypes.

Table 5.13: Summary of the genes that were detected among multiple ARB

		MAR HPC isolates positive for resistant genes tested					
Sample period	**Site**	***strA***	***strB***	***aadA***	**tetA**	***blaCTX-M***	***dfrB1, dfrB2***
August 2016	**Raw**		1	1		1	1
	Inlet	2	1				1
	AT	1	1	1			
	D2	2	1				1
November 2016	**Raw**	3			1	1	
	AT	1					
	D1	1	1				1
	D2	1	1				1
March 2017	**Raw**			1		1	
	Inlet	1		2		1	1
	AT	1					
	D1	2	2	1			1
	D2	2	1				
May 2017	**Raw**	1					
	Inlet	1					
	AT		1				
	D2		1	1			2
Total		**19**	**11**	**7**	**1**	**4**	**9**

Raw - NW-B and NW-D; Inlet **–** Abstracted water prior to treatment; AT **–** After treatment; D1 **–** Random household tap water from treatment plant distribution system; D2 **–** Second random household tap water from treatment plant distribution system

5.4.4 A system that uses groundwater source water with impacts from agriculture: NW-C

5.4.4.1 Antibiotic resistance data

Table 5.14 provides a summary of the antibiotic resistance data. A total of 112 HPC isolates (Raw 47; AT 23; Dis 42) were subjected to Kirby-Bauer disk diffusion analysis. More than 50% of HPC isolates were resistant to beta-lactam antibiotics (Ampicillin, Cephalothin, Penicillin G) and more than 70% to Trimethoprim. Among the raw water isolates, more than 40% were resistant to Erythomycin and Vancomycin. MAR indices (Table 5.15) in the raw water ranged from 0.29 to 0.47, with the highest value being recorded in August 2016. In the treated water, MAR values ranged from 0.12 to 0.38, with the highest value recorded in August 2016 and May 2017. The average MAR indices for the three compartments were generally above 0.2 (except for treated water in November 2017), indicating resistance to various classes of antibiotics and that these isolates had a history of exposure to various antibiotic classes.

5.4.4.2 Identification, virulence factors and antibiotic-resistant genes

Most of the isolates that were identified belonged to *Bacillus* spp. (Table 5.16). For this reason, isolates of this genus were selected for WGS. Percentage similarity to 16S rDNA from GeneBank were mostly >99%. In a few cases, these percentages were lower.

Table 5.14: Percentage of isolates that were resistant to the various antibiotics at NW-C

Site	n	Amp	Kf	Chl	CIP	Ery	Kan	Neo	O-T	Pen-G	Strep	TMP	Van
Raw	(47)	63	66	26	17	40	17	29	22	67	23	83	42
AT	(23)	60	61	10	20	13	7	11	0	100	0	73	25
Dis	(42)	64	56	8	14	24	22	18	31	76	10	75	30

Amp – Ampicillin; Kf – Cephalothin; Chl – Chloramphenicol; CIP – Ciprofloxacin; Ery – Erythromycin; Kan – Kanamycin; Neo – Neomycin; O-T – Oxy-tetracycline; Pen-G – Penicillin G; Strep – Streptomycin; TMP – Trimethoprim; Van – Vancomycin; Raw – raw water; AT – After treatment; Dis – Distribution system; n – Number of isolates tested

Table 5.15: MAR indices for the isolates sampling runs

	Raw	After treatment	Drinking water
March 2016	0.32	0.25	0.44
May 2016	0.29	0.31	0.24
August 2016	0.47	0.37	0.38
May 2017	0.39	0.38	0.28
November 2017	0.46	0.12	0.35

Table 5.16: The identities of the HPC isolates from NW-C that were determined by 16S rDNA sequencing

Sampling site	Organism identified	Similarity	bp
Raw	*Bacillus species*	99.70	338
	Bacillus species	99.38	327
	Novosphingobium acidiphilum	97.78	270
	Mitsuaria chitosanitabida	98.15	486
	Chitinivorax tropicus	97.34	338
	Bacillus species	99.71	347
	Bacillus species	100.00	329
	Bacillus safensis	100.00	231
	Novosphingobium acidiphilum	97.95	342
Treated	*Bacillus species*	99.67	306
	Flavobacterium aquidurense	99.06	318
	Bacillus species	100.00	404
	Bacillus species	99.70	334
	Rivibacter subsaxonicus	100.00	308
	Rivibacter subsaxonicus	98.66	371
	Bacillus safensis	99.07	323
Distribution system	*Bacillus species*	100.00	337
	Bacillus safensis	100.00	371
	Bacillus species	100.00	397
	Bacillus safensis	100.00	323
	Bacillus species	99.72	362
	Ornithinibacillus contaminans	100.00	361
	Bacillus species	99.70	339
	Bacillus species	99.69	326
	Bacillus species	100.00	442

A total of 63 isolates (23 from raw water and 40 from drinking water) were subjected to the haemolysin test. Of these, more than 80% tested positive and were mainly *β*-haemolytic (Table 5.17). More than 80% of the isolates from raw and drinking water produced at least three virulence factors (haemolysin, proteinase and lecithinase). This indicates that these multiple antibiotic-resistant HPC bacteria are also potential pathogens.

Table 5.17: A summary of the extracellular enzyme production patterns of the isolates from NW-C

Site	Haemolysis %	Proteinase %	Lecithinase %	Lipase %	DNase %
Raw	86.95 n = 23	85.00 n = 20	90.00 n = 20	45.00 n = 20	40.00 n = 20
Drinking	82.50 n = 40	93.94 n = 33	96.97 n = 33	39.39 n = 33	51.51 n = 33

5.4.5 A conventional system – upstream impacts from mining, agriculture and urbanisation: NW-E

5.4.5.1 Antibiotic resistance data

From Table 5.18, it is evident that large percentages of isolates were resistant to beta-lactam antibiotics (Ampicillin, Cephalothin, Penicillin G), as well as Trimethoprim. Some of the isolates were also resistant to several other antibiotics. The pattern varied over time. The MAR indices (Table 5.19) in the raw water ranged from 0.16 to 0.40, with the highest value recorded in March 2016. In the treated water, MAR values ranged from 0.16 to 39, with the highest value recorded in August 2016. The MAR indices were generally above 0.2 (except for raw and treated water in May 2017), indicating resistance to various classes of antibiotics.

Table 5.18: Percentage isolates that were resistant to various antibiotics at NW-E

Site	Amp	Kf	Chl	CIP	Ery	Kan	Neo	O-T	Pen-G	Strep	TMP	Van
March 2016												
Raw	83	67	25	10	10	33	16	42	42	16	83	50
AT	100	0	0	0	100	0	0	100	100	0	0	0
Dis	86	86	29	0	14	0	0	86	100	0	86	100
May 2016												
Raw	36	11	7	11	21	32	25	0	20	7	21	40
AT	50	13	0	13	13	13	0	0	17	0	13	33
Dis	40	13	29	7	0	36	20	10	13	20	40	33
August 2016												
Raw	83	83	0	20	0	0	0	33	0	20	83	0
AT	100	100	38	38	0	13	13	50	0	25	100	0
Dis	100	100	14	0	0	29	29	14	0	14	100	0
Date						**May 2017**						
Raw	100	90	0	44	11	22	0	10	100	0	100	11
AT	100	0	0	100	0	50	0	0	100	0	50	0
Dis	71	71	0	0	0	19	0	21	22	19	93	33
Date						**October 2017**						
Raw	36	11	7	11	21	21	32	25	100	18	100	83
AT	50	13	0	13	13	13	13	0	0	0	0	0
Dis	40	13	29	7	7	0	36	20	100	21	100	83

Amp – Ampicillin; Kf – Cephalothin; Ery – Erythromycin; Chl – Chloramphenicol; CIP – Ciprofloxacin; Kan – Kanamycin; Neo – Neomycin; O-T – Oxy-tetracycline; Pen-G – Penicillin G; Strep – Streptomycin; TMP – Trimethoprim; Van – Vancomycin; Dis – Distribution system. AT – After treatment

Table 5.19: Representation of MAR indices for NW-E

	Raw	AT	Dis
March 2016	0.40	0.33	0.37
May 2016	0.16	0.16	0.23
August 2016	0.30	0.39	0.33

Raw – Raw water; AT – After treatment; Dis – Distribution system

5.4.5.2 Identification, virulence factors and ARGs

The HPC bacteria that were identified included mainly *Bacilli* spp. (Table 5.20). In many cases, the percentage identity was below 99%, indicating potentially novel strains in the genera.

Table 5.20: The identities of the HPC isolates determined by 16S rDNA sequencing

Year (plant)	Sampling site	GenBank ID	Percentage identity number	Associated accession number
2016 NW-E	**Raw**	*Bacillus cereus*	87%	KR780449.1
		Bacillus thuringiensis	95%	KF818643.1
		Bacillus wiedmannii	99%	MG890254.1
		Bacillus cereus	94%	KX641888.1
		Bacillus cereus	95%	EU982473.1
	AT	*Bacillus cereus*	100%	CP026678.1
		Bacillus cereus	98%	KP813644.1
		Bacillus cereus	97%	KR780449.1
		Bacillus cereus	97%	KM596528.1
	Distribution system	*Bacillus thuringiensis*	97%	HF545006.1
		Bacillus thuringiensis	99%	KT714039.1
		Bacillus cereus	97%	HM179550.1
		Bacillus cereus	95%	KJ534420.1
		Bacillus cereus	98%	LC215052.1
		Bacillus toyonensis	98%	KX881447.1
		Bacillus cereus	96%	CPO15589.1
		Bacillus anthracis	91%	KF875584.1
		Bacillus cereus	98%	HQ238566.1
		Bacillus cereus	96%	CP020937.1
		Bacillus thuringiensis	90%	CPO15150.1
		Micrococcus luteus	99%	MG597316.1
2017 NW-E	**Raw**	*Bacillus thuringiensis*	98%	KF971833.1
		Bacillus cereus	99%	KC519400.1
		Chryseobacterium sp.	98%	JF899297.1
	AT	*Bacillus cereus*	100%	KJ812448.1
		Bacillus licheniformis	100%	MF321846.1
	Distribution system	*Shinella curvata*	98%	LT545981.1
		Bacillus cereus	98%	KP992166.1
		Bacillus cereus	97%	KU877653.1
		Bacillus safensis	98%	KR780976.1
		Bacillus pumilus	100%	JX680128.1
		Bacillus cereus	99%	KF295678.1
		Bacillus cereus	98%	MF953999.1

Year (plant)	Sampling site	GenBank ID	Percentage identity number	Associated accession number
		Bacillus thuringiensis	99%	KP997272.1
		Bacillus cereus	95%	KF731616.1
		Bacillus thuringiensis	100%	CP013274.1
		Bacillus toyonensis	99%	MG737481.1

Among all the isolates further tested for the potential to produce pathogenic features, a large proportion produced haemolysin (Table 5.21). Results presented in Table 5.22 indicate that several of the multiple ARB also produced haemolysin and several other extracellular enzymes. All these isolates were thus positive for two or more extracellular enzymes and are considered to be potential pathogens. Three of these isolates were also associated with ARGs. The *erm* gene codes for resistance to macrolides, but also for resistance to various other antibiotics.

Table 5.21: Number of isolates that were haemolytic

Year	Sampling site	Number of isolates inoculated	Number of α- haemolysis	Number of β- haemolysis
NW-E 2016	**Raw**	20	7	8
	AT	16	5	6
	Dis	15	1	9
	Total	**51**	**13**	**23**
NW-E 2017	**Raw**	23	2	2
	AT	9	1	2
	Dis	12	3	3
	Total	**44**	**6**	**7**

Raw – Raw water; AT – After treatment; Dis – Distribution system

Table 5.22: Summary of haemolytic reaction, production of extracellular enzymes and ARGs detected of specific HPC from NW-E

DWPF	Sampling site	identity	Haemolysis	Extracellular enzymes	Resistance genes
NW-E 2016	Raw	*Bacillus cereus*	β	H, D, P	-
		Bacillus cereus	β	G, Li	-
		Bacillus cereus	β	G, D, P	*ampC*
		Bacillus cereus	β	D, P	-
	Distribution system	*Bacillus cereus*	β	G, D, Le, P	-
		Bacillus cereus	β	H, D, P, Li	*ermB*, *ermF*
		Micrococcus sp.	α	G, H, D, Le, P	-
NW-E 2017	Distribution system	*Shinella sp*	β	C, Le	*ermF*
		Bacillus cereus	α	D, P	_
		Bacillus pumilus	β	G, Le	_
		Bacillus cereus	α	H, D, P	_
		Bacillus thuringiensis	α	D, P, Li	_
		Bacillus thuringiensis	α	D, P, Li	_
		Staphylococcus aureus	β	G, P	_
		Veillonella tobetsuensis	α	D, P	_

β – Beta-haemolytic; α – haemolytic; D – DNase; P – Proteinase; Le – Lecithinase; Li – Lipase; H – Hyaluronidase

5.4.6 A conventional system with minimal upstream impacts: WC-F

5.4.6.1 Antibiotic resistance data

A large percentage of isolates were resistant to a range of antibiotics (Table 5.23). Overall, more than 50% of the isolates were resistant to beta-lactam (Ampicillin, Cephalothin) antibiotics and Trimethoprim. More than 40% of raw water isolates were resistant to Streptomycin. The MAR indices (Table 5.24) in the raw water ranged from 0.26 to 0.48. In the treated water, MAR values ranged from 0.39 to 0.47 with the highest value recorded in August 2016. The MAR indices were generally above 0.2 (except for raw and treated water in May 2017), indicating resistance to various classes of antibiotics.

Table 5.23: Percentage of isolates for WC-F that were resistant to antibiotics

Site	Chl	CIP	Ery	Kan	O-T	Neo	TMP	Kf	Amp	Strep	Pen-G	Van
Raw	28.571	14.29	28.57	0	28.57	28.57	57.14	57.14	57.14	42.86	0	0
Final	33.33	33.33	33.33	33.33	11.11	22.22	55.56	66.67	77.78	22.22	33.33	44.44

Amp – Ampicillin; Kf – Cephalothin; Chl – Chloramphenicol; CIP – Ciprofloxacin; Ery – Erythromycin; Kan – Kanamycin; Neo – Neomycin; O-T – Oxy-tetracycline; Pen-G – Penicillin G; Strep – Streptomycin; TMP – Trimethoprim; Van – Vancomycin

Table 5.24: MAR indices for plant WC-F 2017

Sampling date	Raw 1	Raw 2	Raw 3	AT	Dis
June 2016	0.48	0.38	0.29	0.45	0.47
June 2-17	N/A	0.26	N/A	0.39	N/A

Raw 1 – Borehole water; Raw 2 – Mixed raw water; Raw 3 – Dam water; AT – After treatment; Dis – Distribution

5.4.6.2 Identification, virulence factors and ARGs

A variety of bacterial species were identified (Table 5.25). Among these were several *Bacillus* spp. Some of these bacilli were subjected to WGS. The percentage identifications were generally above 99%. There were at least five where the percentage was below 99%, indicating potentially novel strains of the various genera.

From Table 5.26, it is evident that all the isolates that produced haemolysin also produced more than one other extracellular enzyme. These are thus all potential opportunistic pathogens. Two of the species produced all six virulence factors and six of them produced five of the virulence factors. More than 60% of the species produced all the virulence factors (extracellular enzymes). The only ARG that was successfully amplified was *ermB* (Table 5.27).

Table 5.25: The identities of the HPC isolates from WC-F that were determined by 16S rDNA sequencing

Site	Organism	Percentage similarity	Accession number association
Raw 1 (borehole)	*Acinetobacter johnsonii* cip 64.6 (T)	97.94	APON01000005
	Bacillus toyonensis BCT-7112(T)	100	CP006869
	Chryseobacterium piscium LMG 23089 (T)	98.09	AM040439
	Streptomyces albogriseolus NRRL B-1305 T	100	AJ494865
Raw 3 (dam)	*Acinetobacter johnsonii* cip 64.6 (T)	99.03	APON01000005
	Rheinheimera aquatic GR5 (T)	100	GQ168584
	Chryseobacterium scophthaimum LMG 13028 T	98.37	AJ271009
	Chromobacterium aquaticum CC-SEYA-1 (T)	96.47	EU109734
	Chromobacterium aquaticum CC-SEYA-1 (T)	96.05	EU109734
	Streptomyces flavovirens NBRC 3716 (T)	99.76	AB184834
	Kocuria arsenatis CM1E1 (T)	100	KM874399
Raw 2 Mixed (borehole and dam)	*Bacillus cereus* ATCC 14579 (T)	99.13	AE016877
	Chitinimonas taiwanensis cf (T)	96.29	AY323827
	Bacillus vietnamensis B-23890 (T)	97.47	CLG48530
	Bacillus cereus ATCC 14579 (T)	99.77	AE016877
	Arthrobacter humicola KV-653 (T)	99.88	AB279890
	Bacillus safensis FO-36b (T)	100	ASJD01000027
	Massilia suwonensis 5414S-25 (T)	99.39	FJ969487
	Chryseobacterium sediminis IMT-174 (T)	98.84	KR349467
	Bacillus cereus ATCC 14579 (T)	99.87	AE016877
	Bacillus cereus ATCC 14579 (T)	99.88	AE016877
	Chryseobacterium lactis NCTC 11390 (T)	98.46	JX100821
	Bacillus cereus ATCC 14579 (T)	99.65	AE016877
	Chryseobacterium hispanicum VP48 (T)	99.52	AM159183
D1	*Bacillus cereus* ATCC 14579 (T)	99.65	AE016877
	Bacillus cereus ATCC 14579 (T)	99.77	AE016877
	Bacillus cereus ATCC 14579 (T)	99.88	AE016877
	Bacillus cereus ATCC 14579 (T)	99.77	AE016877
	Bacillus cereus ATCC 14579 (T)	99.88	AE016877
D2	*Bacillus cereus* ATCC 14579 (T)	100	AE016877
	Bacillus cereus ATCC 14579 (T)	99.88	AE016877
	Staphylococcus epidermidis ATCC 14990 (T)	93.13	L37605
	Bacillus bingmayongensis FJAT-13831 (T)	86	AKCS01000011
	Bacillus cereus ATCC 14579 (T)	99.88	AE016877
	Bacillus cereus ATCC 14579 (T)	100	AE016877
	Bacillus cereus ATCC 14579 (T)	99.46	AE016877
	Bacillus cereus ATCC 14579 (T)	99.87	AE016877
AT	*Bacillus cereus* ATCC 14579 (T)	100	AE016877
	Bacillus cereus ATCC 14579 (T)	99.14	AE016877
	Chryseobacterium hispanicum VP48 (T)	99.40	AM159183
	Bacillus cereus ATCC 14579 (T)	99.88	AE016877

Table 5.26: Extracellular enzyme tests for HPC from WC-F

Site	Identity	Haemolys	DNase	Gelatinase	Lipase	Lecithinase	Proteinase
Raw 2	*Massilia timonae*	α	-	+	-	+	+
	Novosphingobium panipatense	β	+	-	+	+	+
		β	-	+	+	-	-
		α	+	+	+	+	+
	Massilia timonae	β	+	-	+	+	+
		β	+	-	+	+	+
	Pseudomonas protegens	β	+	+	+	-	-
AT	*Massilia brevitalea*	γ	+	-	-	+	+
	Massilia pinisoli	β	+	-	+	+	+
	Bacillus wiedmannii	β	+	-	-	+	+
	Bacillus wiedmannii	β	+	+	-	+	+
	Bacillus wiedmannii	γ	+	+	-	+	+
	Bacillus wiedmannii	γ	-	-	-	+	-
	Bacillus wiedmannii	β	+	+	+	-	-
	Bacillus wiedmannii	β	+	+	+	+	+
		α	-	-	-	-	-
	Percentage	100	80.0	66.7	75.0	80.0	73.0

Raw 2 – Mixed water; AT – After treatment; Haemolys – Haemolysis reaction

Table 5.27: ARGs detected among isolates from WC-F

Site	Identity	ARG
Raw	*Bacillus wiedmannii*	*erm B*
	Massilia timonae	*erm B*
AT	*Massilia brevitalea*	*erm B*
	Massilia pinisoli	*erm B*
	Bacillus wiedmannii	*erm B*
	Bacillus wiedmannii	*erm B*
	Bacillus wiedmannii	*erm B*

AT – After treatment

5.4.7 A system that uses ozone in the drinking water production process: NW-G

5.4.7.1 Antibiotic resistance data

In NW-G, a large percentage of isolates were resistant to a range of antibiotics (Table 5.28). Overall, more than 50% of the isolates were resistant to beta-lactam (Ampicillin, Cephalothin, Penicillin G) antibiotics and Trimethoprim. More than 40% of raw water isolates were resistant to Streptomycin.

Table 5.28: Percentage of isolates that were resistant to the various antibiotics at NW-G

Site	n	Amp	Kf	Chl	CIP	Ery	Kan	Neo	O-T	Pen-G	Strep	TMP	Van
Raw	(66)	63	56	12	4	38	9	8	7	59	9	62	10
AT	(8)	47	47	11	0	11	22	11	0	58	11	56	0
Dis	(50)	72	67	18	6	50	23	12	7	61	22	72	23

Amp – Ampicillin; Kf – Cephalothin; Chl – Chloramphenicol; CIP – Ciprofloxacin; Ery – Erythromycin; Kan – Kanamycin; Neo – Neomycin; O-T – Oxy-tetracycline; Pen-G – Penicillin G; Strep – Streptomycin; TMP – Trimethoprim; Van – Vancomycin; Raw – Raw water; AT – After treatment; Dis – Distribution system; n – Number of isolates tested

The MAR indices (Table 5.29) in the raw water ranged from 0.15 to 0.35. In the treated water, the MAR values ranged from 0.08 to 0.56 with the highest value recorded in May 2017. The MAR indices were generally above 0.2 (except in May 2016), indicating resistance to various classes of antibiotics.

Table 5.29: Presentation of the MAR indices for NW-G

	NW-G sampling sites		
Sampling date	**Raw**	**after treatment**	**Distribution system**
March 2016	0.28	-	0.51
May 2016	0.15	-	0.13
August 2016	0.35	0.27	0.28
May 2017	0.28	0.35	0.56
November 2017	0.35	0.08	0.35
Average	**0.28**	**0.23**	**0.37**

5.4.7.2 Identification, virulence factors and ARGs

A variety of bacterial species were identified (Table 5.30). Among these were several *Bacillus* spp. Some of these bacilli were subjected to WGS. The percentage identifications were generally above 99%. There were at least eight where the percentage was below 99%, indicating potentially novel strains of the various genera.

Table 5.30: Alphabetical list of the identified HPC isolates from NW-G that were determined by 16S rDNA sequencing

Identification	No isolates	Site	Similarity (%)
Aeromonas media	1	Raw	100
Aeromonas salmonicida	1	Dis	100
Aeromonas veronii	2	Raw	100
Bacillus licheniformis	1	Raw	100
Bacillus megaterium	1	AT	100
Bacillus mycoides	1	Raw	100
Bacillus paramycoides	2	Dis	100
Bacillus species	2	Dis	100
Curvibacter delicatus	1	Dis	98
Deefgea rivuli	1	Raw	98
Dongia rigui	1	Dis	99
Flavobacterium buctense	1	Raw	98
Flavobacterium tructae	2	Raw	99
Massilia aurea	1	AT	99
Nevskia ramosa	1	Dis	97
Novosphingobium subterraneum	1	Dis	100
Pedobacter quisquiliarum	1	AT	99
Pseudomonas coleopterorum	1	Dis	99
Pseudomonas guineae	1	Dis	97
Pseudomonas koreensis	1	Raw	100
Pseudomonas moorei	2	Raw	99
Rheinheimera chironomi	1	Raw	99

Rheinheimera mesophila	1	Dis	98
Rheinheimera tangshanensis	1	Dis	98
Roseomonas stagni	1	Dis	98
Shewanella profunda	1	Raw	99
Sphingobium yanoikuyae	1	Dis	99
Sphingorhabdus contaminans	1	Dis	99
Staphylococcus argenteus	1	Dis	100
Williamsia spongiae	1	AT	99

Raw – Raw water; AT – After treatment; Dis – Distribution system

From Table 5.31, it is evident that several of the isolates that produced haemolysin also produced more than one other extracellular enzyme. These are thus all potential opportunistic pathogens. More than 70% of the species produced three virulence factors (extracellular enzymes).

Table 5.31: Percentage of HPC isolates that produced extracellular enzymes at NW-G

Plant	Site	Haemolysis	Proteinase	Lecithinase	Lipase	DNase
NW-G	**Raw**	76.47 n = 34	96.15 n = 26	96.15 n = 26	38.46 n = 26	42.31 n = 26
	Drinking	86.67 n = 30	96.15 n = 26	96.15 n = 26	19.23 n = 26	50.00 n = 26

5.4.8 A system that uses advanced purification and a combination of chlorination and monochloramine as disinfection: GT-H

5.4.8.1 Antibiotic resistance data

A large proportion (80 to 100%) of the isolates were resistant to beta-lactam antibiotics (Ampicillin, Cephalothin) and Trimethoprim (Table 5.32). Some were also resistant to Kanamycin, Chloramphenicol, Oxy-tetracycline and, to a certain extent, Streptomycin.

MAR indices (Table 5.33) in the raw water were 0.30 and 0.35. In the treated water, the MAR values ranged from 0.33 to 0.52, with the highest value recorded in November 2017. The MAR indices were generally above 0.2, indicating resistance to various classes of antibiotics.

5.4.8.2 Identification, virulence factors and ARGs

Almost 70% of the isolates that were tested for haemolysin production tested positive (Table 5.34). Of these, 41% were β-haemolytic, indicating that they could completely lyse red blood cells (Table 5.35). Among the haemolysin-producing isolates, several were bacilli and produced more than two other extracellular enzymes as well (Table 5.36). This demonstrates the pathogenic potential of the isolates. Six of isolates also carried ARGs. Two of the genomes were positive for *ampC* and one was positive for *bla*$_{TEM}$. Most of the other ARGs detected were *erm* genes (Table 5.37).

Table 5.32: Percentage of HPC isolates that were resistant to various antibiotics at GT-H

Site	Amp	Kf	Chl	CIP	Ery	Kan	Neo	O-T	Pen-G	Strep	TMP	Van
						June 2017						
Raw	80	80	17	17	0	33	0	80	80	67	100	60
AT	100	83	0	0	0	0	0	50	50	0	100	0
Dis	75	75	0	0	25	0	0	75	100	50	100	67
						November 2017						
Raw	100	100	18.1	0	0	63	0	81	100	18	100	83
AT	100	100	0	0	0	100	0	100	0	0	0	0
Dis	100	100	100	0	7.1	29	93	0	100	21	100	83

Amp – Ampicillin; Kf – Cephalothin; Chl – Chloramphenicol; CIP – Ciprofloxacin; Ery – Erythromycin; Kan – Kanamycin; Neo – Neomycin; O-T – Oxy-tetracycline; Pen-G – Penicillin G; Strep – Streptomycin; TMP – Trimethoprim; Van – Vancomycin; AT – After treatment, Dis – Distribution system

Table 5.33: Representation of the MAR indices for GT-H

Sampling date	Raw	AT	Dis
June 2017	0.30	0.39	0.33
November 2017	0.34	0	0.52

MAR – Multiple antibiotic resistance; AT – After treatment; Dis – Distribution system

Table 5.34: The identities of the HPC isolates determined by 16S rDNA sequencing

Sampling site	GenBank ID	Percentage identity number	Associated accession number
Raw	*Bacillus cereus*	96%	KX495491.1
	Paenibacillus chitinolyticus	99%	NR_113797.1
	Bacillus cereus	98%	EF535591.1
	Bacillus thuringiensis	100%	CP016589.1
	Bacillus cereus	100%	CP017060.1
AT	*Bacillus thuringiensis*	100%	CP016588.1
	Bacillus cereus	99%	KU551240.1
	Bacillus thuringiensis	100%	CP016588.1
	Bacillus thuringiensis	99%	KC414686.1
	Bacillus cereus	96%	MG407612.1
	Bacillus thuringiensis	99%	KT714050.1

Table 5.35: Summary of isolates that were positive for haemolysin production

Sampling site	Number of isolates streaked	Number of α- haemolysis	Number of β-haemolysis
Raw	14	7	2
AT	10	2	5
D	13	1	8
Total	**37**	**10 (27%)**	**15 (41%)**

Table 5.36: Percentage haemolysin-producing HPC isolates that also produced various extracellular enzymes

Site	DNase	Lipase	Proteinase	Hyaluronidase	Chondoitinase	Gelatinase	Lecithinase
Raw	30	None	80	None	None	None	None
AT	45	30	70	30	100	20	10
Dis	70	50	None	None	70	None	None

Table 5.37: Summary of characteristics of potentially pathogenic bacteria

Sampling site	Identity	Haemology	Extracellular enzymes	Resistant genes
Raw	*Brevibacillus laterosporus*	α	P, Li	
	Acinetobacter sp.	β	P, D	
	Bacillus cereus	α	P, D	*ampC*
	Paenibacillus chitinolyticus	β	D, Le	bla_{TEM}
AT	*Bacillus thuringiensis*	β	G, D,	
Distribution	*Bacillus cereus*	α	G, Le, Li,	*ermB*
system	*Bacillus cereus*	β	H, P	*ermB*
	Bacillus cereus	α	P, D	*ermF*
	Bacillus cereus	α	P, Li	*ampC*

5.5 SUMMARY

Several ARGs, in particular *ermb* and *ermF,* were detected in the source and drinking water of all plants. The *ampC*, *blaTEM, blaCTX-M* and several others were also detected. Several of these were detected in the source and drinking waters (Table 5.38).

Table 5.38: Summary of antibiotics and antibiotic resistance data

	Water sources	Antibiotics most isolates were resistant to	MAR in source water	MAR in drinking water	Antibiotics detected in source water	Antibiotics detected in drinking water	ARGs in source water	ARGs in drinking water
WC-A	Surface water (dam) Ground water (boreholes) WWTP effluent	Ampicillin, Cephalothin, Trimethoprim; also Erythromycin and Chloramphenicol	Borehole >0.3 Dam <0.19	0.13-0.25 (RO) 0.34-0.46	Chloramphenicol, Ciprofloxacin, Oxy-tetracycline, Streptomycin, Trimethoprim, Colistin	Ciprofloxacin, Penicillin, Trimethoprim, Colistin	*ermB, ermF, int1*	*ermB, ermF, int1*
NW-B	Surface water (dam) WWTP immediate upstream	Ampicillin, Trimethoprim, Streptomycin, Oxy-tetracycline	Not done	Not done	Not done	Not done	*strA, strB, aadA, blaCTX-M, dfrB1*	*strA, strB, aadA, blaCTX-M, dfrB1*
NW-C	Natural spring	Ampicillin, Cephalothin, Penicillin G, Trimethoprim; also Erythromycin and Vancomycin	0.29-0.47	0.12-0.38	Ciprofloxacin, Beta-lactams, Trimethoprim, Colistin	Ciprofloxacin, Neomycin, beta-lactams, Trimethoprim, Colistin	PCRs unsuccessful	PCRs unsuccessful
NW-D	Ground water (natural spring, boreholes)	Ampicillin, Trimethoprim, Streptomycin, Oxy-tetracycline	Not done	Not done	Not done	Not done	*strA, strB, aadA, blaCTX-M, dfrB1*	*strA, strB, aadA, blaCTX-M, dfrB1*
NW-E	Surface water(dam) Ground water (borehole)	Ampicillin, Cephalothin, Penicillin G, Trimethoprim	0.16-0.40	0.16-39	Ciprofloxacin, beta-lactams, Streptomycin, Trimethoprim, Colistin	Ciprofloxacin, beta-lactams, Neomycin, Trimethoprim, Colistin	*ampC*	*ermB, ermF*

	Water sources	Antibiotics most isolates were resistant to	MAR in source water	MAR in drinking water	Antibiotics detected in source water	Antibiotics detected in drinking water	ARGs in source water	ARGs in drinking water
WC-F	Surface water (dam) Ground water (boreholes)	Ampicillin, Cephalothin, Trimethoprim	0.26-0.48	0.39-0.47	Ciprofloxacin, beta-lactams, Trimethoprim, Colistin	Ciprofloxacin, beta-lactams, Trimethoprim, Colistin	*ermB*	*ermB*
NW-G	Surface water (river)	Ampicillin, Cephalothin, Penicillin G, Trimethoprim	0.15-0.35	0.08-0.56	Chloramphenicol, Ciprofloxacin, beta-lactams, Streptomycin, Trimethoprim, Colistin	Ciprofloxacin, Beta-lactams, Streptomycin, Trimethoprim, Colistin	PCRs unsuccessful	PCRs unsuccessful
GT-H	Surface water (dam)	Ampicillin, Cephalothin, Trimethoprim, Kanamycin, Chloramphenicol, Oxy-tetracycline	0.30 and 0.35	0.33-0.52	Not done	Not done	*ampC, bla*$_{TEM}$	*ermB, ermF, ampC*

Table 5.39: Summary of virulence data

	Water sources	Dominant virulence phenotype – source water	Dominant virulence phenotype – drinking water
WC-A	Surface water (dam) Ground water (boreholes) WWTP effluent	Haemolysin, DNase, Lecithinase, Proteinase	Haemolysin, DNase, Lecithinase, Proteinase
NW-B	Surface water (dam) WWTP immediate upstream	Not done	Not done
NW-C	Natural spring	Haemolysin, DNase, Lecithinase, Proteinase	Haemolysin, DNase, Lecithinase, Proteinase

NW-D	Ground water (natural spring, boreholes)	Not done	Not done
NW-E	Surface water (dam) Ground water (borehole)	Haemolysin, DNase, Proteinase	Haemolysin, DNase, Lipase, Proteinase
WC-F	Surface water (dam) Ground water (boreholes)	Haemolysin, DNase, Lipase, Lecithinase, Gelatinase, Proteinase	Haemolysin, DNase, Lipase, Lecithinase, Gelatinase, Proteinase
NW-G	Surface water (river)	Haemolysin, DNase, Lecithinase, Proteinase	Haemolysin, DNase, Lecithinase, Proteinase
GT-H	Surface water (dam)	Haemolysin, Proteinase	Haemolysin, DNase, Lipase, Proteinase, Chondroitinase

In Table 5.3, nine virulence factors (extracellular enzymes produced) are summarised. Besides haemolysin proteinase, DNase and lecithinase were also commonly produced among the isolated bacteria. In some cases, lipase was also produced. This is an indication that these isolated HPC bacteria are potentially pathogenic; that is in addition to the antibiotic resistance features.

The *erm* genes encode for resistance to macrolides, such as Erythromycin (Choi et al., 2018) and were detected in DNA from bacteria that were isolated from four of the six DWPFs (WC-A, NW-E, NW-G and GT-H), where PCRs for ARGs were positive. These genes were detected in source and drinking water isolates.

Finding these genes in these water sources is cause for concern since the *erm* genes can also confer resistance to other classes of antibiotics, namely Chloramphenicol and Vancomycin (Zhang et al., 2009). Mechanisms that are associated with *erm* genes include rRNA methylation and efflux pumps (Zhang et al., 2009). These *erm* genes are carried in the genetic elements, such as plasmids and transposons, thus making it easy for the genes to be shared between bacterial species (Dzyubak and Yap; 2016; Zhang et al., 2009).

On the other hand, Erythromycin is effective against Gram-positive cocci and bacilli, as well as some Gram-negative bacteria (Choi et al., 2018). It can be used for infections in the respiratory, gastrointestinal and genital tract, as well as skin and soft tissue (Jelić and Antolović, 2016). It is thus a widely applicable broad-spectrum antibiotic. This antibiotic Erythromycin can also cause broad specific and non-specific resistance to different classes of antibiotics.

In the context of the present study, genes coding for β-lactamases, conferring resistance to penicillins, were found in the raw and drinking water of three of the six DWPFs. Furthermore, beta-lactam antibiotics were detected in the raw and drinking of all six the DWPFs that were tested. There is thus a potential of selection pressure to the main antibiotics in the water environments. Resistance to Ampicillin was common among the isolates from all the plants

Penicillin was one of the first antibiotics introduced more than 60 years ago, and finding genes coding for resistance in water environments should be a surprise. What is of concern is that these genes could be disseminated by water systems, and particularly in drinking water into general communities.

Resistance to beta-lactam antibiotics is mainly due to the production of beta-lactamases that render the cell walls of bacteria unstable or unable to further produce cell walls. Ampicillin, one of the beta-lactam antibiotics, is effective against both Gram-positive and Gram-negative bacteria (Kaushik et al., 2014) and are commonly used for treatment against enteric fever, respiratory infections, urinary tract infections, skin and soft tissue infections (Kaushik et al., 2014). The *bla*$_{TEM}$ gene is one the most studied genes from the extended spectrum beta-lactamases (ESBLs) and is one of the Class A antibiotics (Shahid et al., 2011; Lachmayr et al., 2009). There are more than 220 different distinct alleles, and these are associated with mobile genetic elements (Zhang et al., 2009). The spread of the *bla*$_{TEM}$ gene in the water environment is facilitated by transposons and integrons (Lachmayr et al., 2009). The ESBLs render the treatment of beta-lactam antibiotics less effective, leading to therapeutic failure, thus requiring the application of broader spectrum and more costly therapeutic agents (Sageerabanoo et al., 2015). This calls for processes to reduce the occurrence of these antibiotics and genes in drinking water systems.

Class 1 integrons genes are associated with the capture and dissemination of ARGs in the environment. Class 1 integrons are involved in the acquisition of gene cassettes that are associated with antibiotic resistance (Koczura et al., 2016). According to Lin et al. (2015), these gene cassettes confer resistance to a wide range of antibiotics, such as aminoglycosides, beta-lactams and Chloramphenicol.

Class 1 integrons and the associated ARGs pose a threat in the water environment and are not removed by conventional water treatment processes. This increases their chances of being present in and being disseminated by drinking water (Gillings et al., 2015; Chen et al., 2015).

Finding these genes and antibiotics widely distributed in source (ground and surface water) and drinking water in geographically separated areas (±600 to ±1 200 km apart), in which the impacts of land use also varies, implies that the genes are naturally common in the environment. The antibiotics may be originating from land-use activities. Antibiotics (various classes) could thus potentially select for the accumulation of the observed ARGs in the water environment. What was also evident is that, in some cases (Table 5.2), there was a reduction of some of the antibiotics, and, in other cases, this was not observed. The current analyses could not link the antibiotic removal capacity to the drinking water production processes or to the physicochemical quality of the raw water.

CHAPTER 6: ANALYSIS OF THE DRINKING WATER MICROBIOME

6.1 INTRODUCTION

The whole genomes of *Bacillus* spp. were sequenced using the Illumina MiSeq procedures. Various genes associated with conferring resistance to different classes of antibiotics were detected in the genomes of the selected bacilli isolated from the various drinking water production systems. The identified genes could be analysed and associated with 22 different antibiotic classes.

6.2 WHOLE-GENOME SEQUENCING STUDIES

6.2.1 A direct potable water re-use/reclamation plant: WC-A

Whole-genome sequencing results are summarised in Table 6.1. It shows amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes (VIRs). Prior to assembly, the obtained sequences were trimmed with a trimmomatic program. Low-quality value fragments from each end (lower than 15) were trimmed off, as well as ambiguous coding extending for more than two nucleotides. Short reads, less than 50 nucleotides, were removed from further analysis. SPAdes Assembler was used to assemble *de novo* whole genomes of each specimen using the trimmed reads. Each genome was assembled independently.

Table 6.1: Summary of WGS data for bacilli from WC-A

	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
						ID	Predicted	ID	Predicted
WC-A – Raw	3789	115	10504	10383	5	146	239	577	220
WC-A – Raw	207	58	5865	5803	3	127	144	632	212
WC-A – Raw	2419	88	6066	5971	6	116	158	444	151
WC-A – Raw	2537	79	6830	6742	7	113	155	436	126
WC-A – Raw	480	127	10695	10560	6	157	266	680	244
WC-A – AT	207	62	5243	5174	6	83	161	370	140
WC-A – AT	3869	101	5326	5216	7	66	97	304	124
WC-A – AT	2652	81	5211	5126	3	85	132	341	141
WC-A – Dis	1474	118	8483	8355	8	152	228	559	166

Various genes associated with conferring resistance to different classes of antibiotics were detected in the genes of the selected bacilli isolated from a single drinking water system. These genes could be associated with antibiotic resistance phenotypes. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species. The identified genes were further analysed and could be associated with 22 different antibiotic classes as indicated in Figure 6.1. The most predominant genes were associated with multidrug, glycopeptide and macrolide, lincosamide and streptogramin (MLS) resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented.

The virulence genes identified belonged to nine different classes (Figure 6.2). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production.

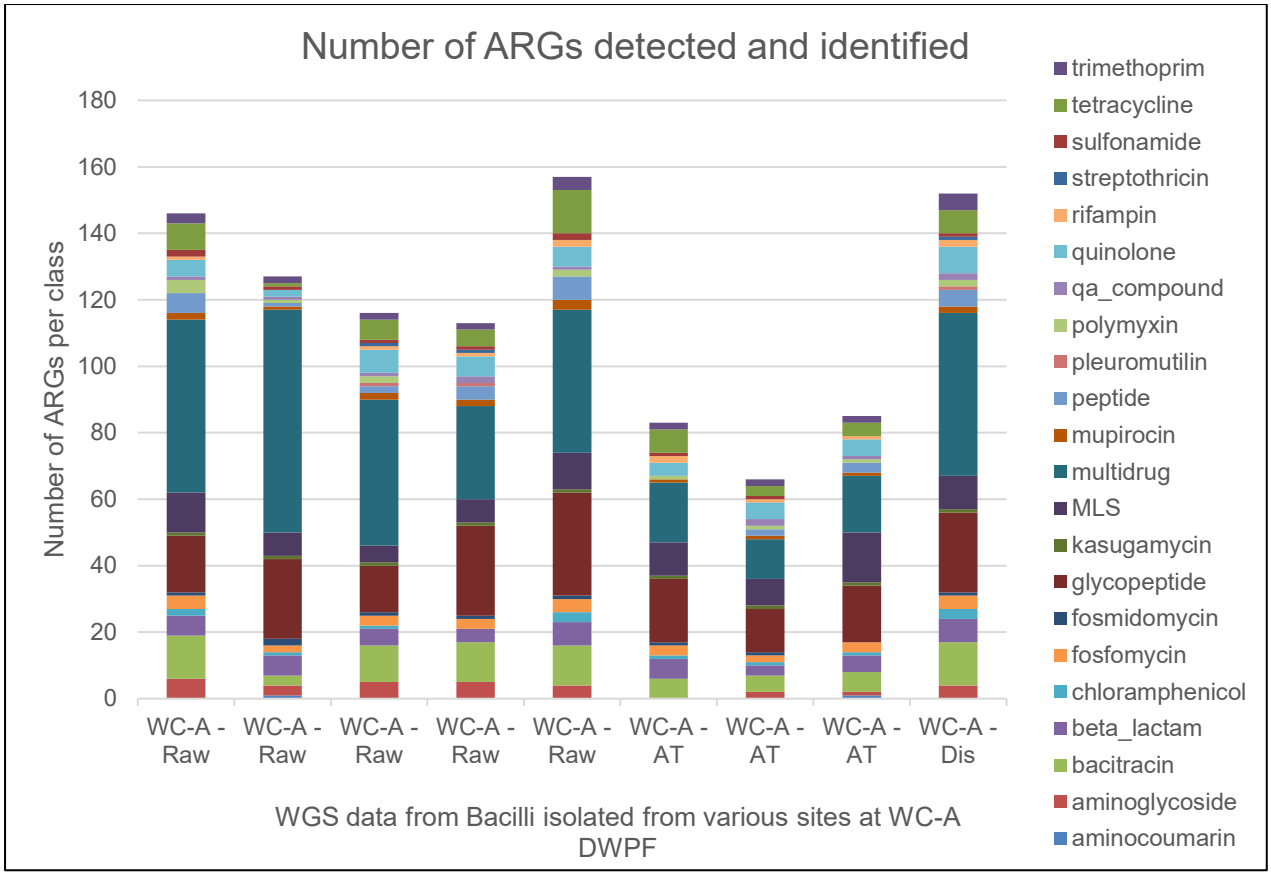


Figure 6.1: A breakdown of the various ARG classes from WGS data of bacilli that were isolated from various sites at WC-A

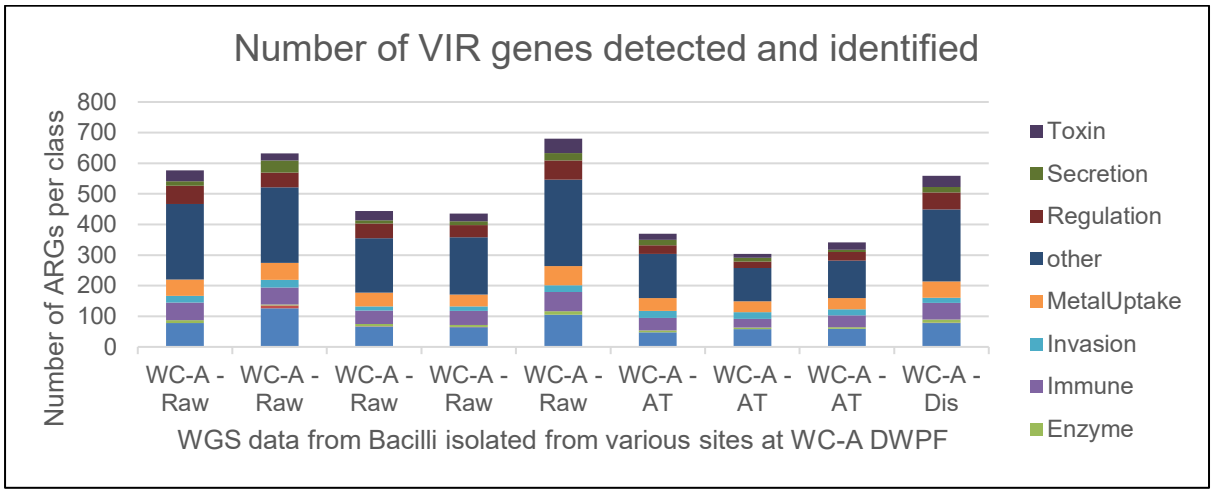


Figure 6.2: A breakdown of the various virulence gene classes from WGS data of bacilli that were isolated from various sites at WC-A

6.2.2 A conventional system with minimal upstream impacts: WC-F

Whole genome sequencing results are summarised in Table 6.2. It shows amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes. Prior to assembly, obtained sequences were trimmed with a trimmomatic program. Low-quality value fragments from each end (lower than 15) were trimmed off, as well as ambiguous coding extending for more than two nucleotides. Short reads, less than 50 nucleotides, were removed from further analysis. SPAdes Assembler was used to assemble *de novo* whole genomes of each specimen using the trimmed reads.

Each genome was assembled independently. Various genes associated with conferring resistance to different classes of antibiotics were detected in the genes of the selected bacilli isolated from a single drinking water system. These genes could be associated with antibiotic resistance phenotypes. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species.

Table 6.2: Summary of WGS data of bacilli from WC-F

Source	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
						ID	Predicted	ID	Predicted
WC-F – Raw	1362	81	5993	5902	9	129	158	429	129
WC-F – AT	2640	82	7151	7063	5	141	182	482	142
WC-F – AT	316	75	5817	5737	4	133	165	448	134

The identified genes were further analysed and could be associated with 22 different antibiotic classes as indicated in Figure 6.3. The most predominant genes were associated with multidrug, glycopeptide and MLS resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented. The virulence genes identified belonged to nine different classes (Figure 6.3). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production.

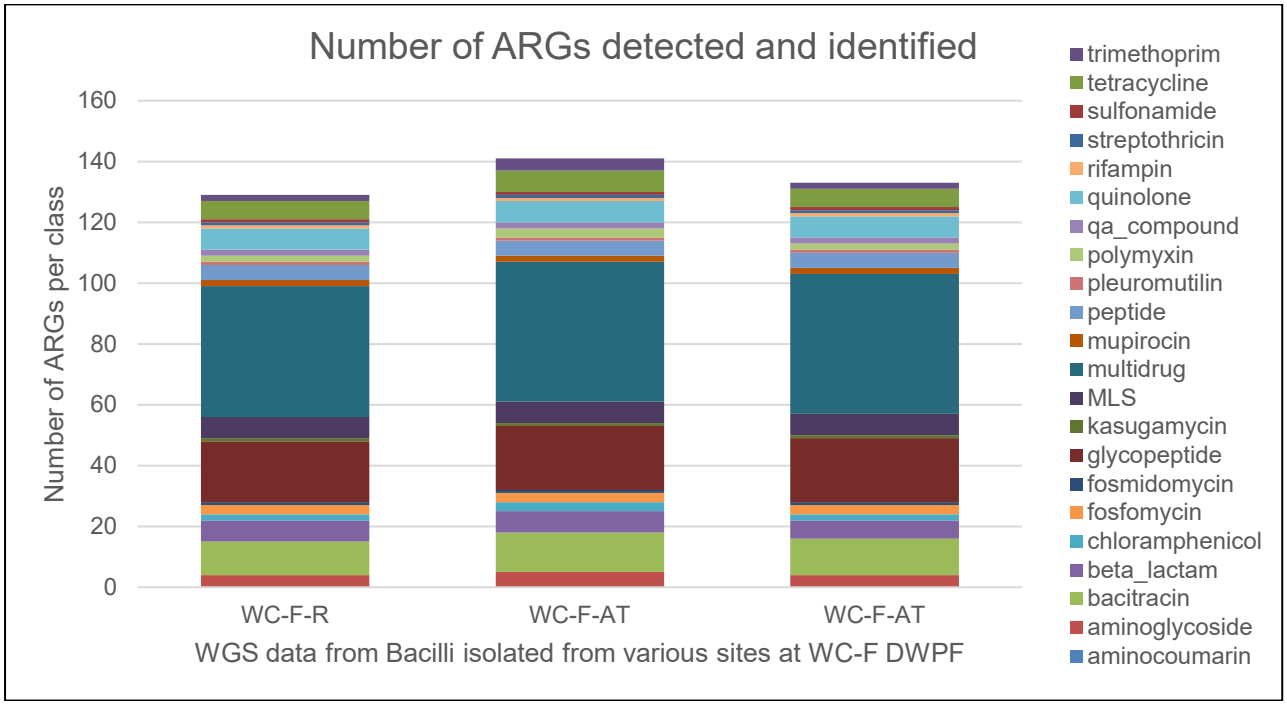


Figure 6.3: A breakdown of the various ARG classes from WGS data of bacilli that were isolated from various sites at WC-F

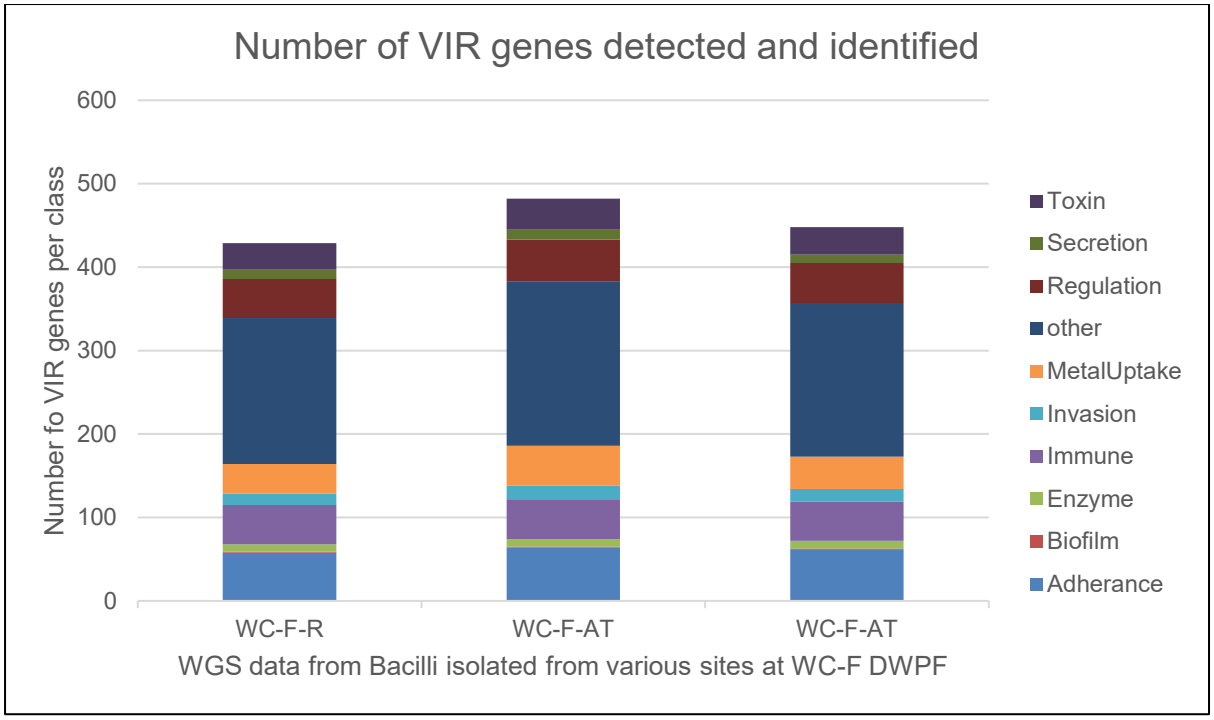


Figure 6.4: A breakdown of the various virulence gene classes from WGS data of bacilli that were isolated from various sites at WC-F

6.2.3 A system that uses groundwater source water with impacts on agriculture: NW-C

Next-generation sequencing resulted in two files for each of six genomes (2D25, 2D33, 2O2, 2O3, 2R9 and 2R10). Whole genome sequencing results are summarised in Table 6.3. It shows the amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes. Prior to assembly, obtained sequences were trimmed with a trimmomatic program. Low-quality value fragments from each end (lower than 15) were trimmed off, as well as ambiguous coding extending for more than two nucleotides. Short reads, less than 50 nucleotides, were removed from further analysis. SPAdes Assembler was used to assemble *de novo* whole genomes of each specimen using the trimmed reads. Each genome was assembled independently. Basic statistics of the generated assembly is shown in Table 6.3.

Table 6.3: Summary of WGS data for the bacilli from NW-C

Source	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
						ID	Predicted	ID	Predicted
NW-C – Raw	134	64	3885	3816	4	50	36	233	97
NW-C – Raw	324	85	3898	3800	12	52	103	307	120
NW-C – AT	385	79	3929	3837	12	67	109	294	140
NW-C – AT	793	99	6298	6180	18	120	162	455	157
NW-C – Dis	206	101	6089	5972	15	120	161	454	156
NW-C – Dis	181	99	6087	5970	17	119	162	453	156

In Table 6.4, the results of this step of the analysis and the percentage of similarities for 16S rRNA gene and each genome to its closest reference species is shown. The results for each housekeeping gene (HKS) were very similar with the bias towards *gyrB* and *recA* genes as best markers to distinguish between very closely related species. Various genes associated with conferring resistance to different classes of antibiotics were detected in the genes of the selected bacilli isolated from a single drinking

water system. These genes could be associated with antibiotic resistance phenotypes. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species. Beta-lactam resistance, for example, was one of the prominent resistance phenotypes. Two genes associated with beta-lactam resistance was detected in isolates from the distribution system. Furthermore, genes associated with Vancomycin resistance were also detected in isolates from the distribution system. Various genes or mutations that cause non-specific resistance were also detected. The identified genes were further analysed and could be associated with 22 different antibiotic classes as indicated in Figure 6.5. The most predominant genes were associated with multidrug, glycopeptide and MLS resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented. The virulence genes identified belonged to nine different classes (Figure 6.6). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production.

Table 6.4: Identities of the isolates from NW-C used for WGS analysis

	16S rRNA percentage of identity	Species
2D25	99.8	*B. thuringiensis*
2D33	99.7	*B. thuringiensis*
2O2	100	*B. pumilus*
2O3	99.7	*B. thuringiensis*
2R9	99.9	*B. pumilus*
2R10	93.4	*B. subtilis*

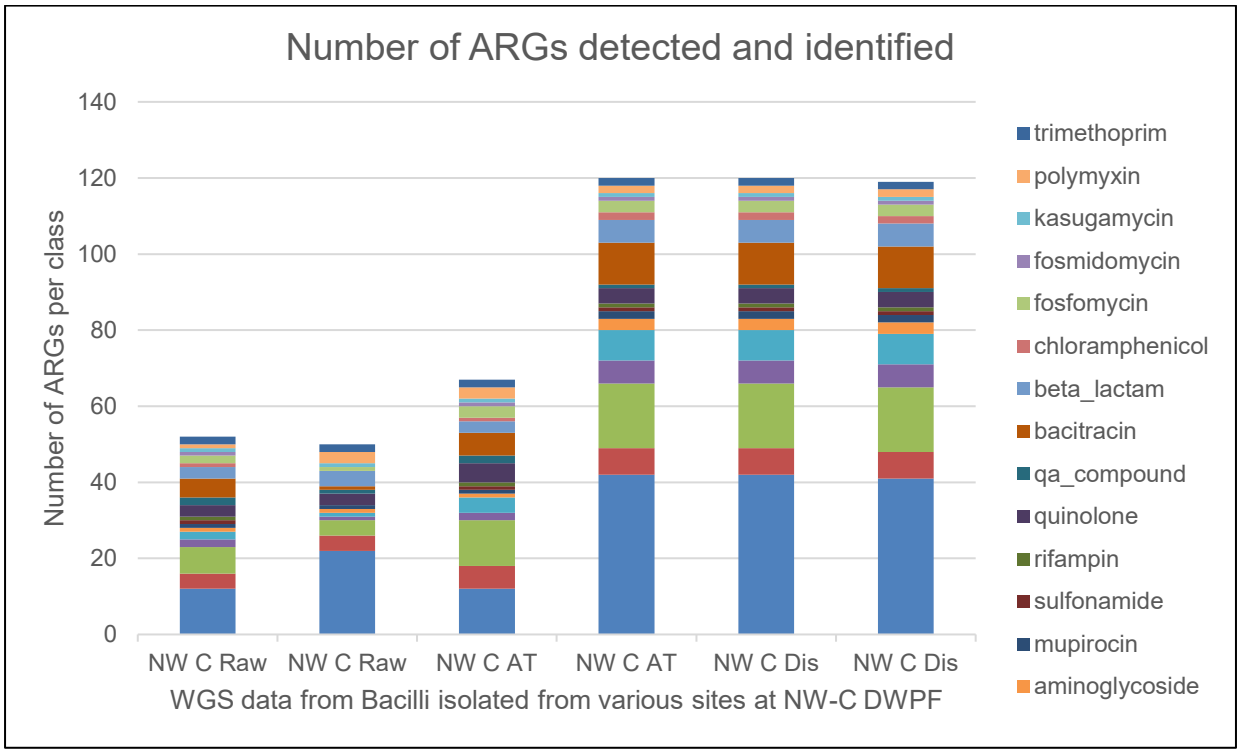


Figure 6.5: A breakdown of the various ARG classes from WGS data of bacilli that were isolated from various sites at NW-C

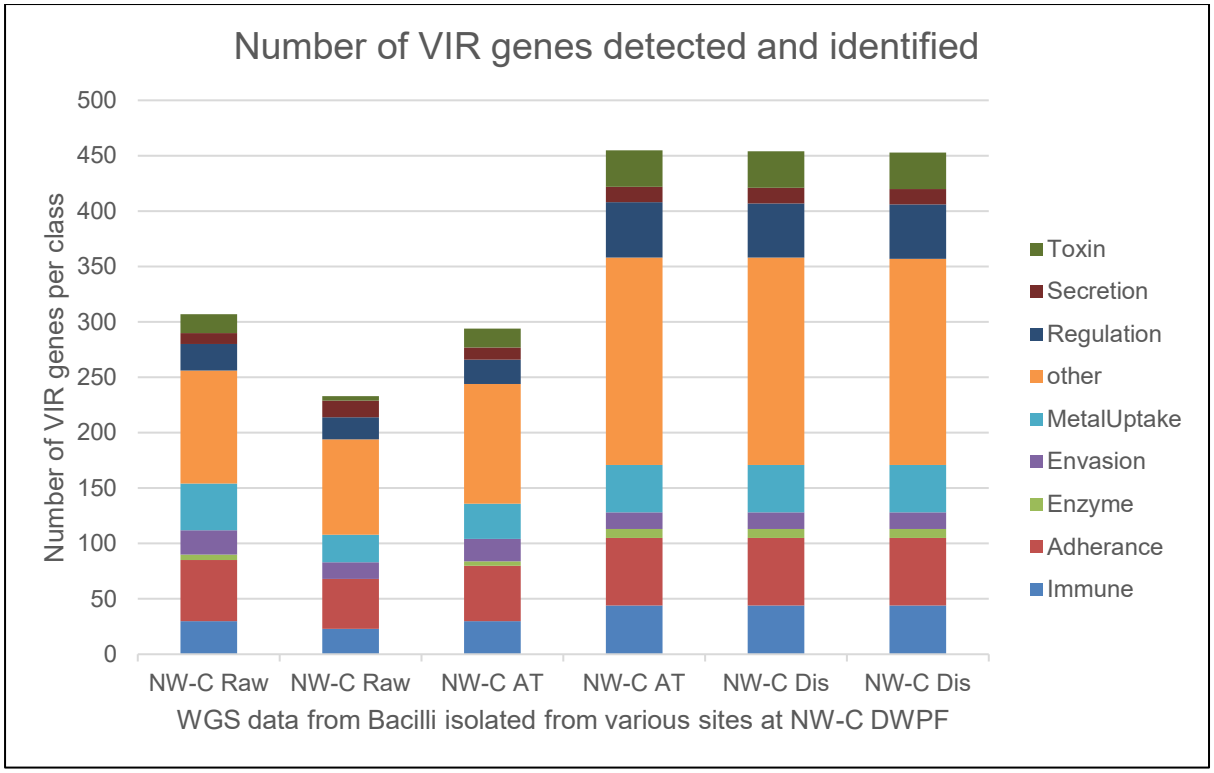


Figure 6.6: A breakdown of the various virulence gene classes from WGS data of bacilli that were isolated from various sites at NW-C

6.2.4 A conventional system with upstream impacts from mining, agriculture and urbanisation: NW-E

Whole genome sequencing results are summarised in Table 6.5. It shows amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes. Prior to assembly, obtained sequences were trimmed with a trimmomatic program. Low-quality value fragments from each end (lower than 15) were trimmed off, as well as ambiguous coding extending for more than two nucleotides. Short reads, less than 50 nucleotides, were removed from further analysis. SPAdes Assembler was used to assemble *de novo* whole genomes of each specimen using the trimmed reads. Each genome was assembled independently. Basic statistics of the generated assemblies are shown in Table 6.5. Various genes associated with conferring resistance to different classes of antibiotics were detected in the genes of the selected bacilli isolated from a single drinking water system. These genes could be associated with antibiotic resistance phenotypes. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species.

Table 6.5: Summary of WGS data for the bacilli from NW-E

Source	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
						ID	Predicted	ID	Predicted
NW-E – Raw	7317	110	9408	9291	6	128	179	484	159
NW-E – Raw	2878	142	10057	9902	11	143	249	629	198
NW-E – AT	105	75	4232	4153	3	70	106	302	121
NW-E – Dis	4382	78	7290	7205	6	91	297	492	190
NW-E – Dis	1387	69	8444	8369	5	155	229	577	187

The identified genes were further analysed and could be associated with 22 different antibiotic classes as indicated in Figure 6.7. The most predominant genes were associated with multidrug, glycopeptide and MLS resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented. The virulence genes identified belonged to nine different classes (Figure 6.8). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production.

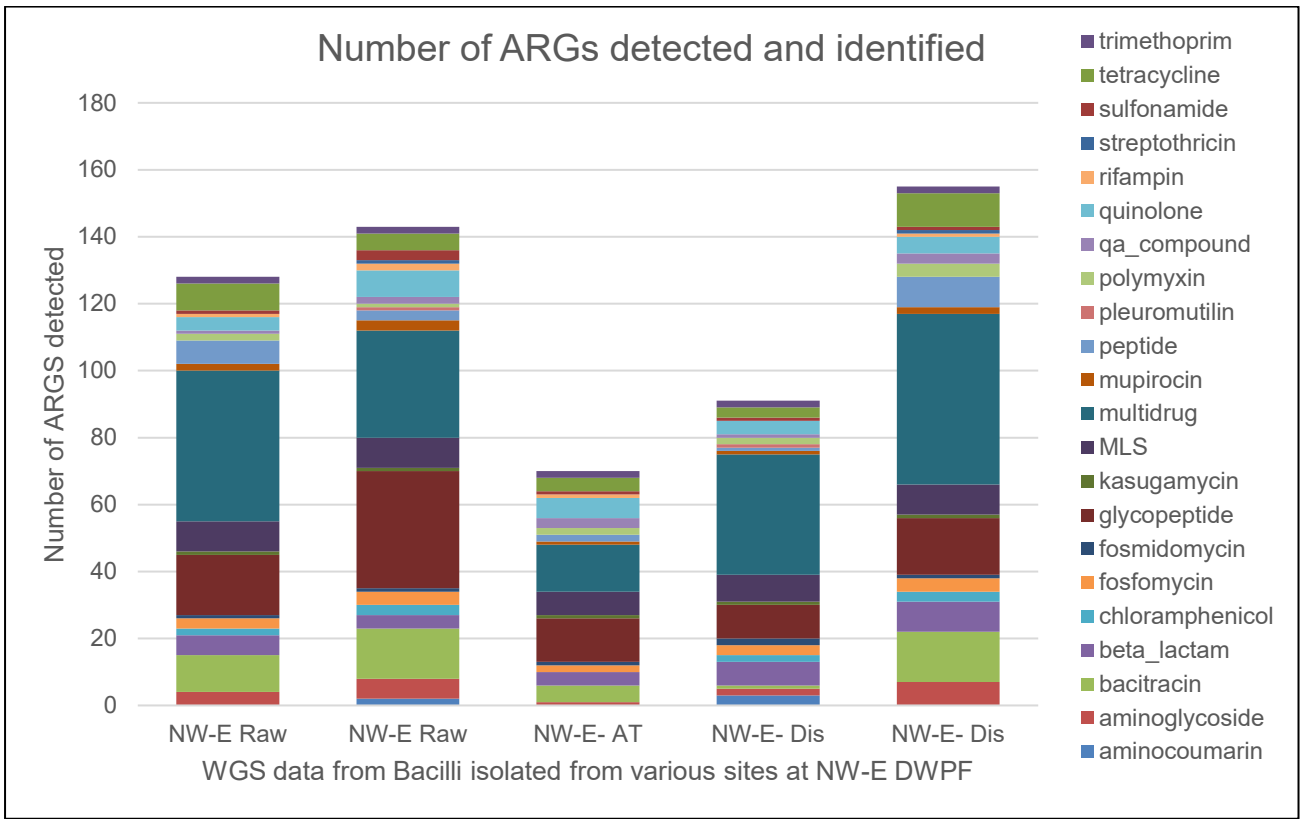


Figure 6.7: A breakdown of the various ARG classes from WGS data of bacilli that were isolated from various sites at NW-E

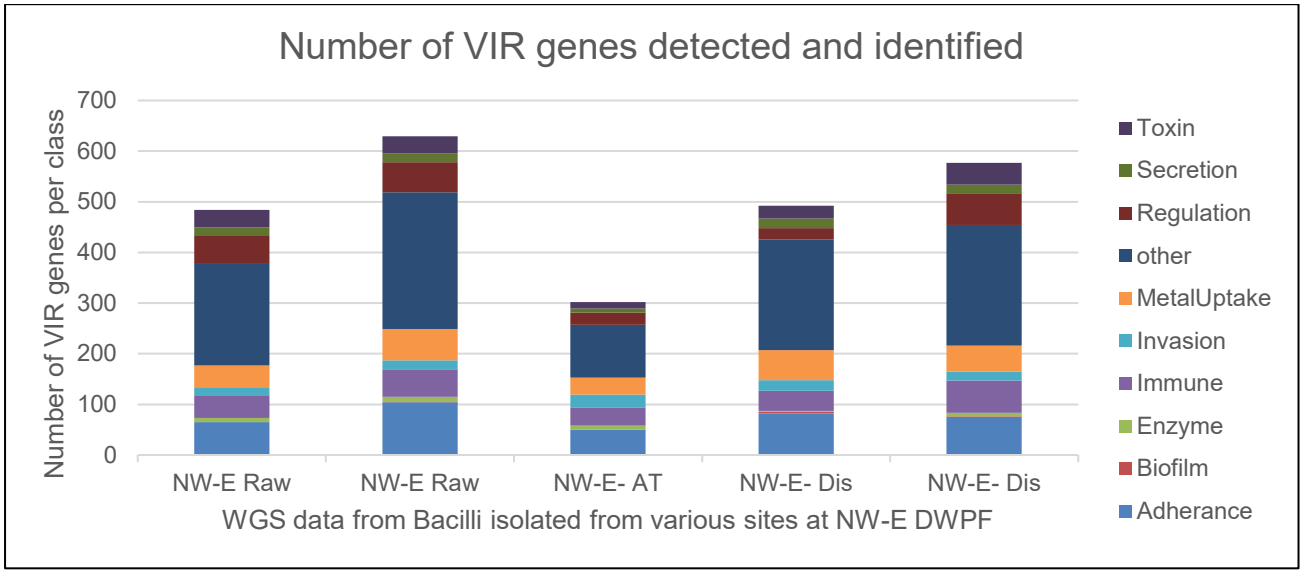


Figure 6.8: A breakdown of the various virulence gene classes from WGS data of bacilli that were isolated from various sites at NW-E

6.2.5 A system that uses ozone in the drinking water production process: NW-G

Whole genome sequencing results are summarised in Table 6.6. It shows amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes. Prior to assembly, obtained sequences were trimmed with a trimmomatic program. Low-quality value fragments from each end (lower than 15) were trimmed off, as well as ambiguous coding extending for more than two nucleotides. Short reads, less than 50 nucleotides, were removed from further analysis. SPAdes Assembler was used to assemble *de novo* whole genomes of each specimen using the trimmed reads. Each genome was assembled independently. Basic statistics of the generated assembly is shown in Table 6,6.

Table 6.6: Summary of WGS data for the bacilli from NW-G

Source	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
						ID	Predicted	ID	Predicted
NW-G – Raw	1892	152	11069	10907	8	212	291	989	301
NW-G – Raw	51	74	4512	4434	3	76	132	318	129
NW-G – AT	304	72	6596	6518	5	102	148	376	151
NW-G – Dis	141	66	5989	5919	3	119	165	450	142
NW-G – Dis	735	47	5571	5518	5	108	143	381	120
NW-G – Dis	153	58	5625	5561	5	115	149	423	113

Various genes associated with conferring resistance to different classes of antibiotics were detected in the genes of the selected bacilli isolated from a single drinking water system (Table 6.6). These genes could be associated with antibiotic resistance phenotypes. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species. The identified genes were further analysed and could be associated with 22 different antibiotic classes as indicated in Figure 6.10. The most predominant genes were associated with multidrug, glycopeptide and MLS resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented. The virulence genes identified belonged to nine different classes (Figure 6.11). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production

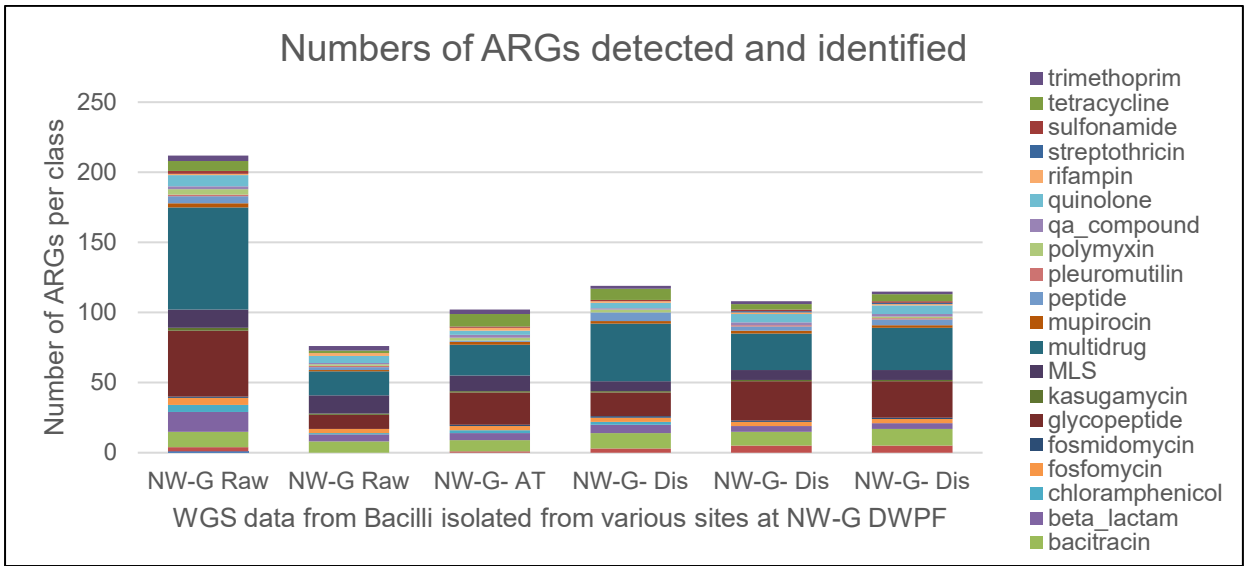


Figure 6.9: A breakdown of the various ARG classes from WGS data of bacilli that were isolated from various sites at NW-G

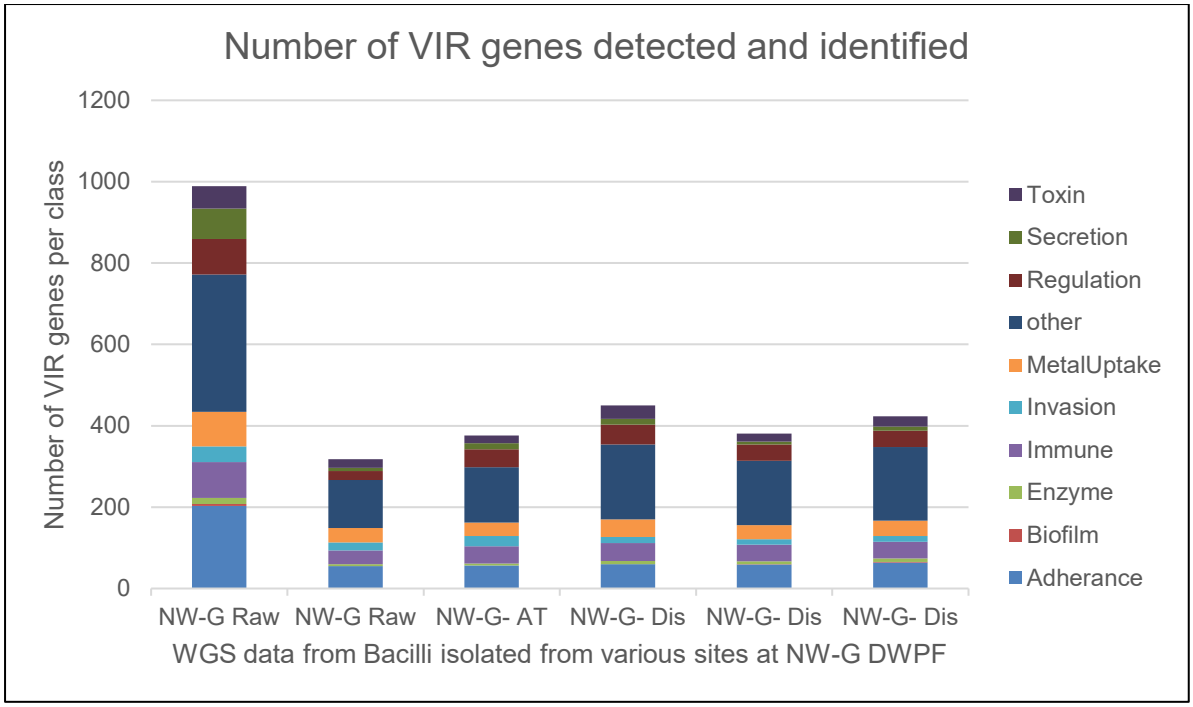


Figure 6.10: A breakdown of the various virulence gene classes from WGS data of bacilli that were isolated from various sites at NW-G

6.2.6 A system that uses advanced purification and a combination of chlorination and monochloramine as disinfection: GT-H

Whole genome sequencing results are summarised in Table 6.7. It shows amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes. Prior to assembly, obtained sequences were trimmed with a trimmomatic program. Low-quality value fragments from each end (lower than 15) were trimmed off, as well as ambiguous coding extending for more than two nucleotides. Short reads, less than 50 nucleotides, were removed from further analysis. SPAdes Assembler was used to assemble *de novo* whole genomes of each specimen using the trimmed reads. Each genome was assembled independently.

Table 6.7: Summary of WGS data for the bacilli from GT-H

Source	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
						ID	Predicted	ID	Predicted
GT-H – Raw	1802	79	8329	8241	7	136	165	468	167
GT-H – AT	166	68	5762	5686	7	119	172	455	137
GT-H – AT	7670	109	9673	9554	8	137	189	481	152
GT-H – Dis	190	66	6199	6128	4	125	167	466	149
GT-H – Dis	6229	127	16423	16288	6	212	316	773	283

Various genes associated with conferring resistance to different classes of antibiotics were detected in the genes of the selected bacilli isolated from a single drinking water system (Table 6.7). These genes could be associated with antibiotic resistance phenotypes. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species. The identified genes were further analysed and could be associated with 22 different antibiotic classes, as indicated in Figure 6.11. The most predominant genes were associated with multidrug, glycopeptide and MLS resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented.

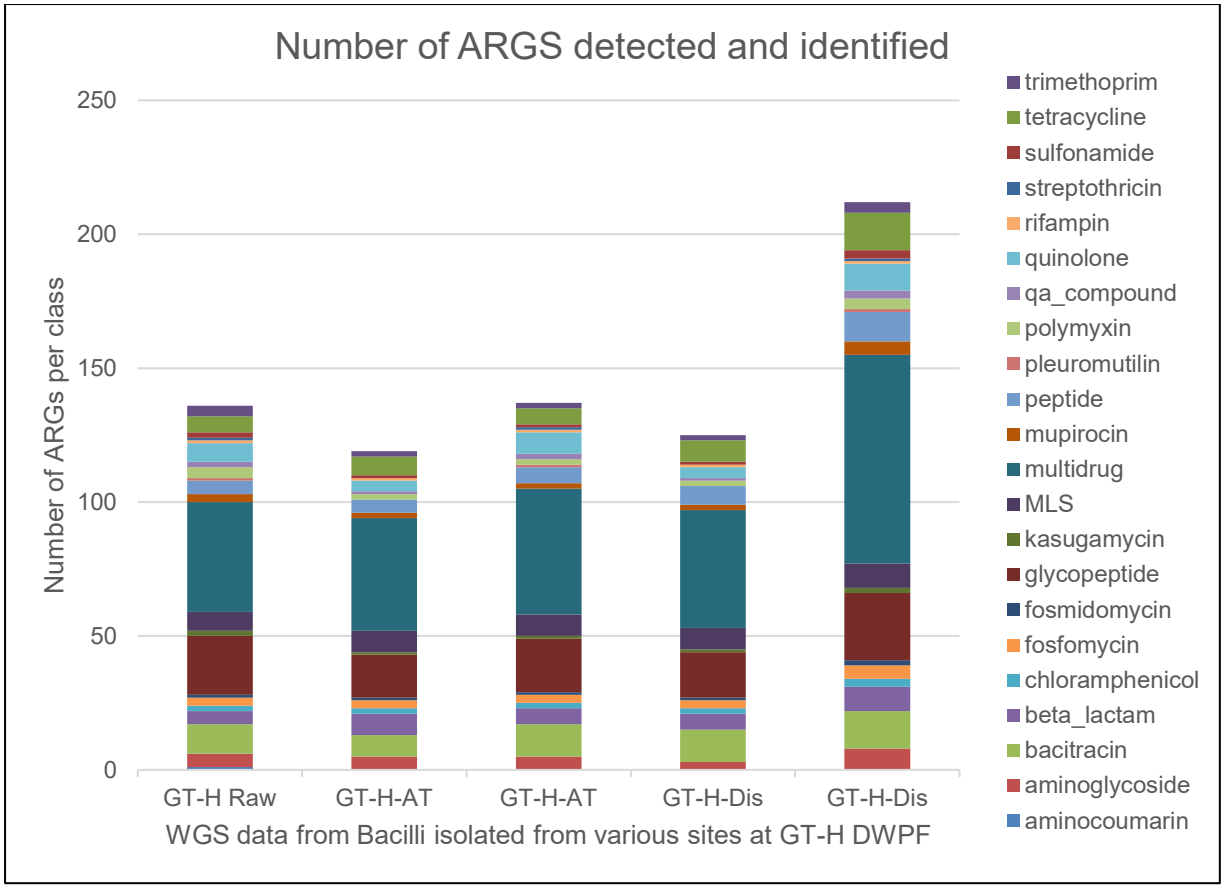


Figure 6.11: A breakdown of the various ARG classes from WGS data of bacilli that were isolated from various sites at GT-H

The virulence genes identified belonged to nine different classes (Figure 6.12). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production.

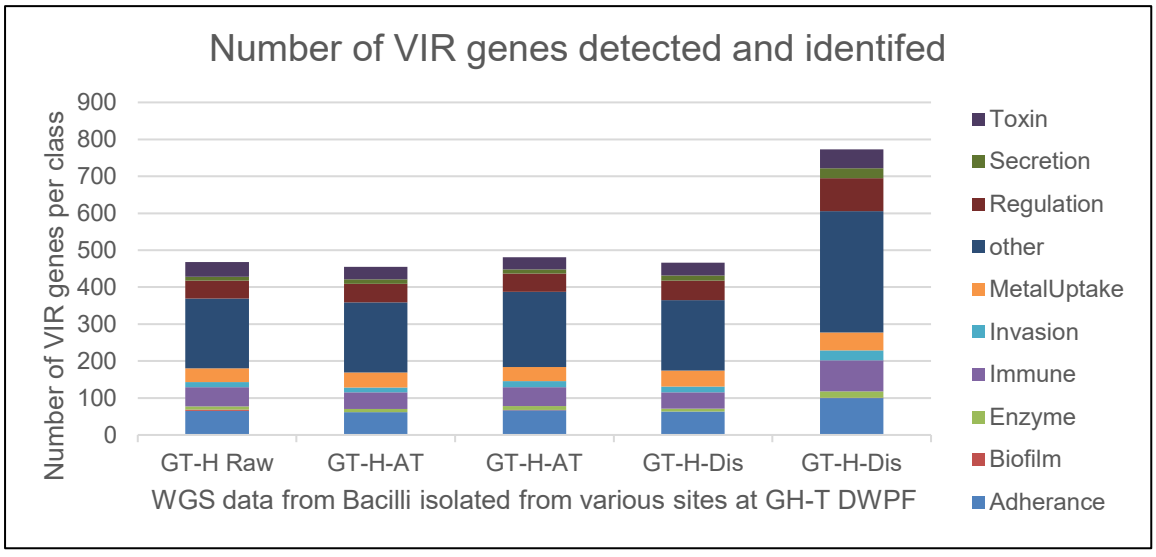


Figure 6.12: A breakdown of the various virulence gene classes from WGS data of bacilli that were isolated from various sites at GT-H

Metagenomics data (raw reads) for filter beds and reservoirs of this system was provided by Prof Fanus Venter and Sarah Potgieter of the University of Pretoria. The sequencing results are summarised in Table 6.8. It shows amount of contigs, tRNA, mRNA (genes), rRNA, identified and predicted antibiotic resistance and virulence genes (some basic statistics). The identified genes were further analysed and could be associated with 22 different antibiotic classes as indicated in Figure 6.13. The most predominant genes were associated with multidrug, glycopeptide and MLS resistance. Beta-lactam, bacitracin, quinolone and tetracycline-associated ARGs were also well represented. These genes could be associated with antibiotic resistance phenotypes, as well as WGS data of bacilli isolates from this system. The genes were not all initially isolated from the genus *Bacillus,* but originated from various Gram-positive and Gram-negative species. The virulence genes identified belonged to nine different classes (Figure 6.14). A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production.

6.2.7 Summary

Similar patterns were observed in the genomes of bacilli from source water, water after treatment, as well as drinking water. It was also similar across the various DWPFs, as well as the metagenomics analysis of the filter beds and reservoir samples. The WGS also demonstrated that virulence genes were common in the genomes of the bacilli (Figure 6.1 and Figure 6.2). The various classes of genes could be associated with the phenotypic extracellular enzyme production and belonged to nine different classes. A large number was not classified and was grouped as “other”. Most of the virulence genes were associated with adherence, immunity, metal uptake, regulation and toxin production. Similar patterns were observed in the genomes of bacilli from source water, water after treatment, as well as drinking water. It was also similar across the various DWPFs, as well as the metagenomics analysis of the filter beds and reservoir samples. Major genes detected in the genomes of the bacilli included the following order of abundance (Tables 6.9 to 6.11):

Multidrug resistance > Glycopetides > MLS~Bacitracin>beta-lactams~Quinolone~Tetracycline

Table 6.8: Summary of eDNA sequencing data for the bacilli from GT-H

Name		Reads: Raw (F or R)	Number of bins percentage similar = 0.9 len ≥ 300 bp	Contigs	tRNA	Genes/ mRNA	CDS	rRNA	ARGs		VIRs	
									ID	Predicted	ID	Predicted
S41_17	Res 1	7 584 113	10	124554	929	83753	82780	27	490	695	2674	1183
S41_18	Res 1	9 577 829	11	109606	854	66891	65993	29	416	617	2401	1049
S41_19	Res 1	9 033 763	15	124223	920	91515	90554	26	510	929	3106	1408
S41_20	Res 1	5 370 295	13	165272	962	83034	82033	28	417	641	2204	1014
S41_21	Res 1	8 483 857	6	147674	650	59320	58641	21	163	308	998	412
S41_4	Res 1	7 362 731	12	109025	857	85966	85069	24	489	727	2526	1177
S41_5	Res 1	6 841 582	15	127967	904	89907	88956	31	514	751	2827	1282
S41_6	Res 1	6 800 216	15	103779	957	82793	81798	23	584	857	3060	1402
S73_10	Res 2	7 004 401	13	748368	2833	300407	297468	75	832	1303	5505	2549
S73_17	Res 2	7 319 913	12	444210	1561	158719	157103	37	396	639	2350	1141
S73_18	Res 2	7 401 362	11	225251	1166	104772	103559	33	462	727	2502	1103
S73_19	Res 2	12 101 710	12	103648	765	62259	61456	25	391	596	2106	935
S73_1	Res 2	6 343 357	30	96120	770	69112	68302	27	429	635	2362	1059
S73_20	Res 2	6 507 475	9	86785	642	58340	57668	21	347	547	1860	827
S73_5	Res 2	6 201 514	27	272871	1779	205564	203687	69	926	1810	5200	2530
S73_6	Res 2	3 622 649	22	214541	1494	156485	154907	61	797	1456	4447	2153
S73_7	Res 2	7 164 623	23	174144	1483	150467	148899	55	1006	1780	5764	2811
S73_8	Res 2	6 858 566	17	231255	1418	139705	138227	41	756	1185	4131	2037
SS_110	Filter bed A	5 858 457	50	1324567	7534	596388	588637	113	975	2537	6993	3508
SS_112	Filter bed A	5 432 346	33	980316	7443	452185	444558	79	782	2047	5372	2714
SS_114	Filter bed A	11 001 007	69	1868398	10461	867373	856639	126	1493	3349	10622	5455
SS_44	Filter bed B	7 267 077	24	526517	3275	238829	235465	52	1066	1337	5544	2208
SS_45	Filter bed B	15 113 320	27	744062	5164	345990	340690	64	1164	1768	6555	2715
SS_93	Filter bed B	9 061 654	40	1135939	8377	511895	503332	65	684	1368	4639	2111

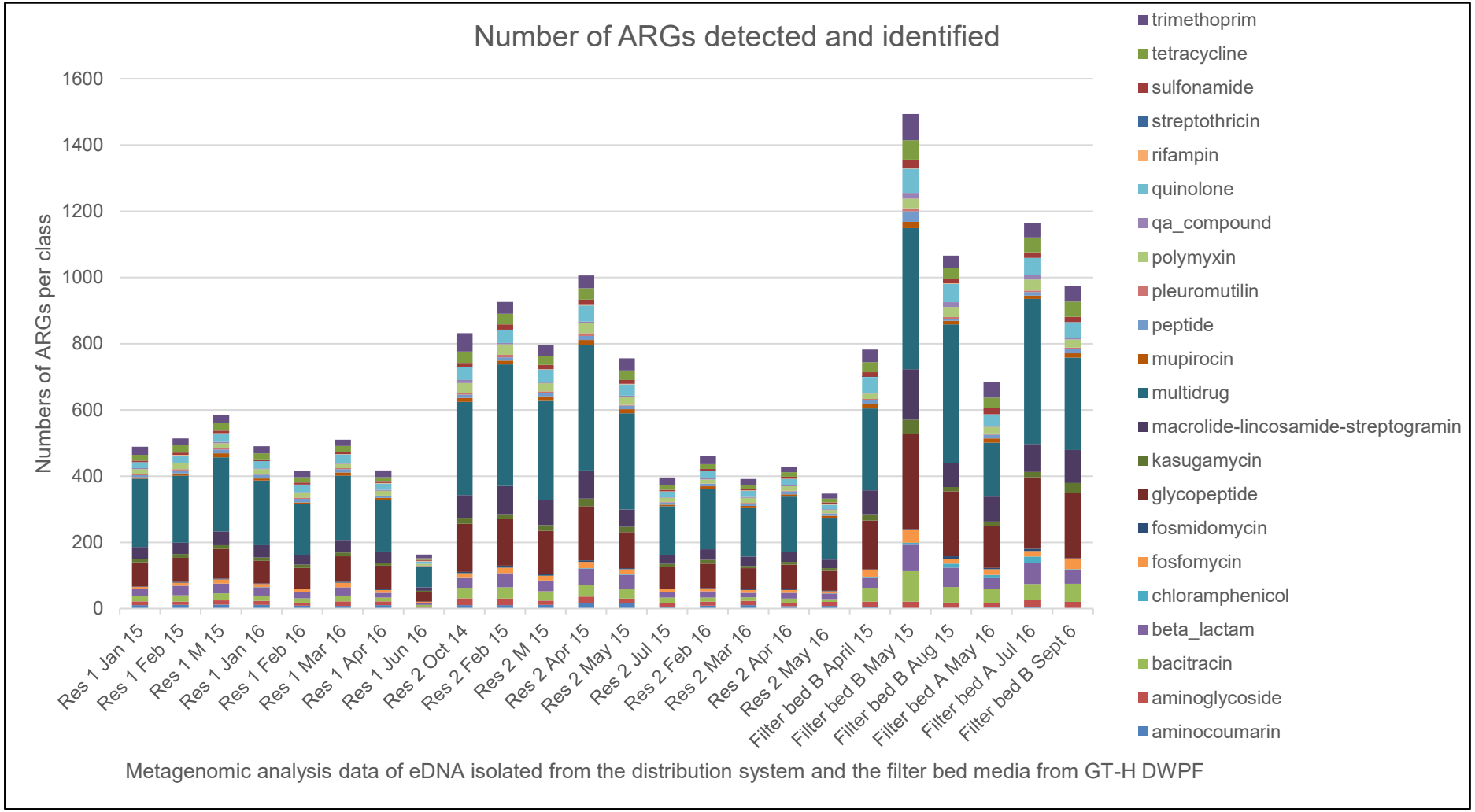


Figure 6.13: Metagenomics eDNA analysis: a breakdown of the various ARG classes from the reservoirs and filter bed media from GT-H

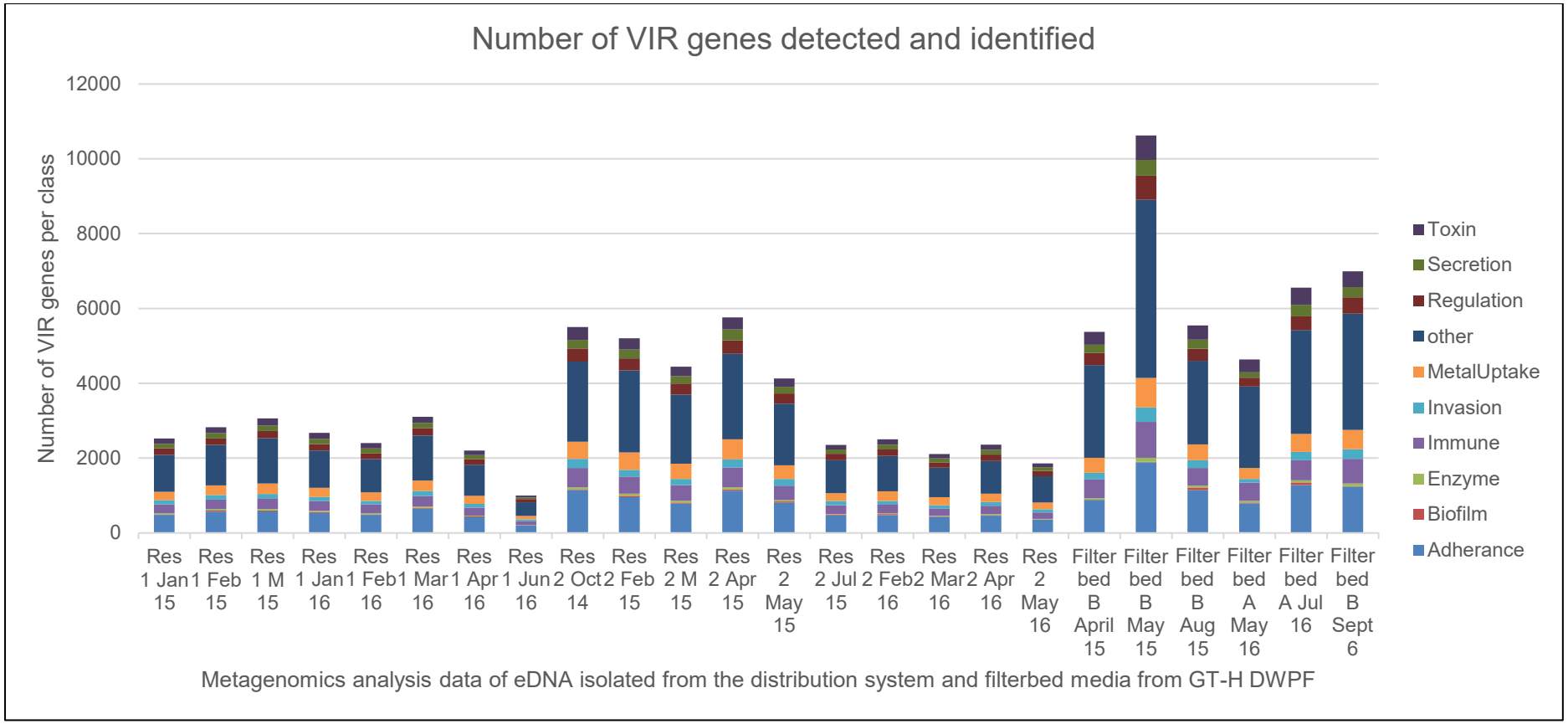


Figure 6.14: Metagenomics eDNA analysis: a breakdown of the various virulence gene classes from the reservoirs and filter bed media from GT-H

Table 6.9: Summary of WGS data from bacilli isolated from source waters

	WC-A	WC-A	WC-A	WC-A	WC-A	NW-C	NW-C	NW-E	NW-E	WC-F	NW-G	NW-G	GT-H	Total	Average
Bacitracin	13	3	11	12	12	5	1	11	15	11	11	8	11	124	9,54
Beta-lactam	6	6	5	4	7	3	4	6	4	7	14	5	5	76	5,85
Glycopeptide	17	24	14	27	31	7	4	18	35	20	47	10	22	276	21,2
MLS	12	7	5	7	11	4	4	9	9	7	13	13	7	108	8,31
Multidrug	52	67	44	28	43	12	22	45	32	43	73	17	41	519	39,9
Quinolone	5	2	7	6	6	3	3	4	8	7	8	5	7	71	5,46
Sulfonamide	2	1	1	1	2			1	3	1	2	0	2	16	1,45
Tetracycline	8	1	6	5	13	2	1	8	5	6	7	2	6	70	5,38
Trimethoprim	3	2	2	2	4	2	2	2	2	2	4	3	4	34	2,62

Table 6.10: Summary of WGS data from bacilli isolated immediately after treatment

	WC-A	WC-A	WC-A	NW-C	NW-C	NW-E	WC-F	NW-G	GT-H	GT-H	Total	Average
Bacitracin	6	5	6	6	11	5	13	8	8	12	80	8
Beta-lactam	6	3	5	3	6	4	7	5	8	6	53	5,3
Glycopeptide	19	13	17	12	17	13	21	23	16	20	171	17,1
MLS	10	8	15	6	7	7	7	11	8	8	87	8,7
Multidrug	18	12	17	12	42	14	46	22	42	47	272	27,2
Quinolone	4	5	5	5	4	6	7	3	4	8	51	5,1
Sulfonamide	1	1	0			1	1	1	1	1	7	0,88
Tetracycline	7	3	4	4	8	4	7	9	7	6	59	5,9
Trimethoprim	2	2	2	2	2	2	4	3	2	2	23	2,3

Table 6.11: Summary of WGS data from bacilli isolated from distribution systems

	WC-A	NW-C	NW-C	NW-E-	NW-E	WC-F	NW-G	NW-G	NW-G	GT-H	GT-H	Total	Average
Bacitracin	13	11	11	1	15	12	11	10	12	12	14	109	11,09
Beta-lactam	7	6	6	7	9	6	6	4	4	6	9	63	6,364
Glycopeptide	24	17	17	10	17	21	17	28	26	17	25	195	19,91
MLS	10	7	7	8	9	7	7	7	7	8	9	76	7,818
Multidrug	49	42	42	36	51	46	41	26	30	44	78	436	44,09
Quinolone	8	4	4	4	5	7	4	6	6	4	10	54	5,636
Sulfonamide	1			1	1	1	1	1	1	1	3	10	1,222
Tetracycline	7	8	8	3	10	6	8	4	5	8	14	74	7,364
Trimethoprim	5	2	2	2	2	2	2	2	2	2	4	22	2,455

6.3 ANALYSIS OF THE DRINKING WATER MICROBIOME

All steps were performed in Qiime2, embedded within the program modules and BLAST+. It entailed importing data to the pipeline, OTU picking, phylogenetic tree generation, taxonomic analysis and alpha and beta diversity analysis. Figure 6.15 is a heat map of the results. It is a classification method that is used to determine which OTUs discriminate between groups, and a heatmap is used to visualise the over- or under-representation of these OTUs in the groups. The abundances of the 26 samples (columns) are coloured from low abundance (blue) to high abundance (red) in the 13 349 discriminatory OTUs (rows). These OTUs represent a large number of species.

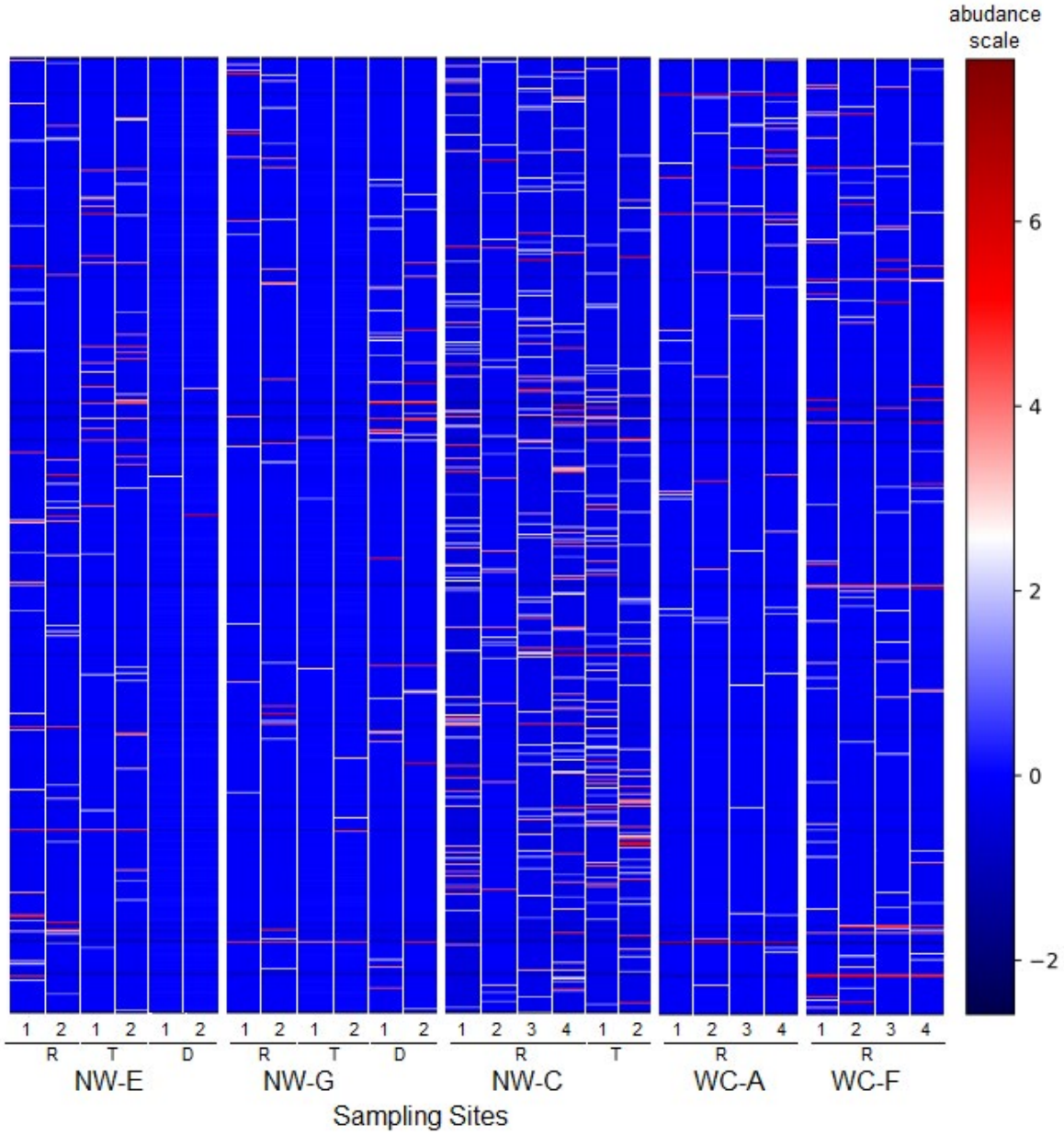


Figure 6.15: Abundance heat maps of the various genera of the five DWPFs

The diversity of sequences of bacteria detected were greater than the HPC isolation processes. Table 6.12 shows the list of genera obtained. The sequences of the less dominant strains are not shown, but could be present in the database. What these two datasets are demonstrating is that the microbiomes between source, after treatment and in the distribution system, are represented by different dominant resident taxa (Pinto et al., 2014; Bruno et al., 2018). Treatment processes have impacts on the downstream dominant populations. Various ecological diversity indices may or may not show major shifts associated with these diversity changes. Even with the latest NGS technologies and software pipelines, a large group of sequences either classified as others or unknown make up a considerable part of the population and present a real challenge (Bruno et al., 2018). Solden et al. (2016) refer to this as “microbiological dark matter”, a term borrowed from astronomy.

Table 6.12: Genera that were identified by direct 16S rRNA gene sequencing using the Illumina MiSeq protocols and QIMME 2 pipelines; eDNA from five of the participating DWPFs were analysed

	Genus	WC-A	NW-C	NW-E	WC-F	NW-G
Raw water	*Aciditerrimonas* sp.	√	√	√	√	
	Actinobacterium spp.	√		√	√	
	Bacillus sp.		√			
	Bacteria sp.	√		√	√	√
	Clostridium sp.	√			√	
	Cyanobium sp.				√	
	Flavobacterium sp.	√				
	Gemmata sp.		√			
	Isosphaera sp.			√		√
	Iamia sp.				√	
	Luteolibacter sp.			√	√	
	Mycobacterium sp.	√				
	Pirellula sp.		√			
	Planctomycetaceae sp.			√	√	
	Planctomycete sp.		√			√
	Proteobacterium sp.		√			√
	Planktophila sp.				√	
	Sphingomonas sp.					√
	Synechococcus sp.				√	
	Verrucomicrobium sp.		√			√
	Unidentified	√	√	√	√	
	Others	√	√	√	√	√
After treatment	*Bacillus* sp.		√			
	Bacteria sp.			√		
	Bythopirellula sp.		√			
	Clostridium sp.		√			
	Cyanobacterium sp.			√		√
	Gemmata sp.		√	√		
	Isosphaera sp..			√		√
	Pasteuria sp.		√			
	Phreatobacter sp.			√		
	Pirellula sp.		√			
	Planctomycetaceae sp.		√	√		√
	Planctomycete sp.		√	√		

	Genus	WC-A	NW-C	NW-E	WC-F	NW-G
	Proteobacterium sp..			√		
	Sphingomonas sp.			√		
	Unidentified			√		
	Others		√	√		√
Distribution system	*Bacillus* sp.			√		
	Bacteria sp.		√			√
	Bythopirellula sp.		√			
	Clostridium sp.		√			
	Gemmata sp.		√			
	Isosphaera sp.					√
	Phreatobacter sp.					√
	Planctomycetaceae		√			
	Planctomycete sp.		√			√
	Proteobacterium sp.					√
	Sphingomonas sp.					√
	Others		√	√		

Beta diversity metrics provide a measure of the degree to which samples differ from one another and can reveal aspects of microbial ecology that are not apparent from looking at the composition of individual samples. Generally, beta diversity metrics are remarkably robust to issues such as low sequence counts and noise. In the present dataset, there is no significant correlation (the sample types group together in one point on the plot) between the sample types. It means there are no significant differences in abundance between the sample types. Unweighted UniFrac distance is a qualitative measure of community dissimilarity that incorporates phylogenetic relationships between the features. The group significance plots for distances between raw water, treated water and distribution water show similar distances between raw water, distribution water and treated water (Figure 6.16). The amount of taxon diversity stays the same during the processes in all systems. Faith's phylogenetic diversity is a qualitative measure of community richness that incorporates phylogenetic relationships between the features (Figure 6.17). Phylogenic diversity according to sample type also shows that there is no significant diversity between them.

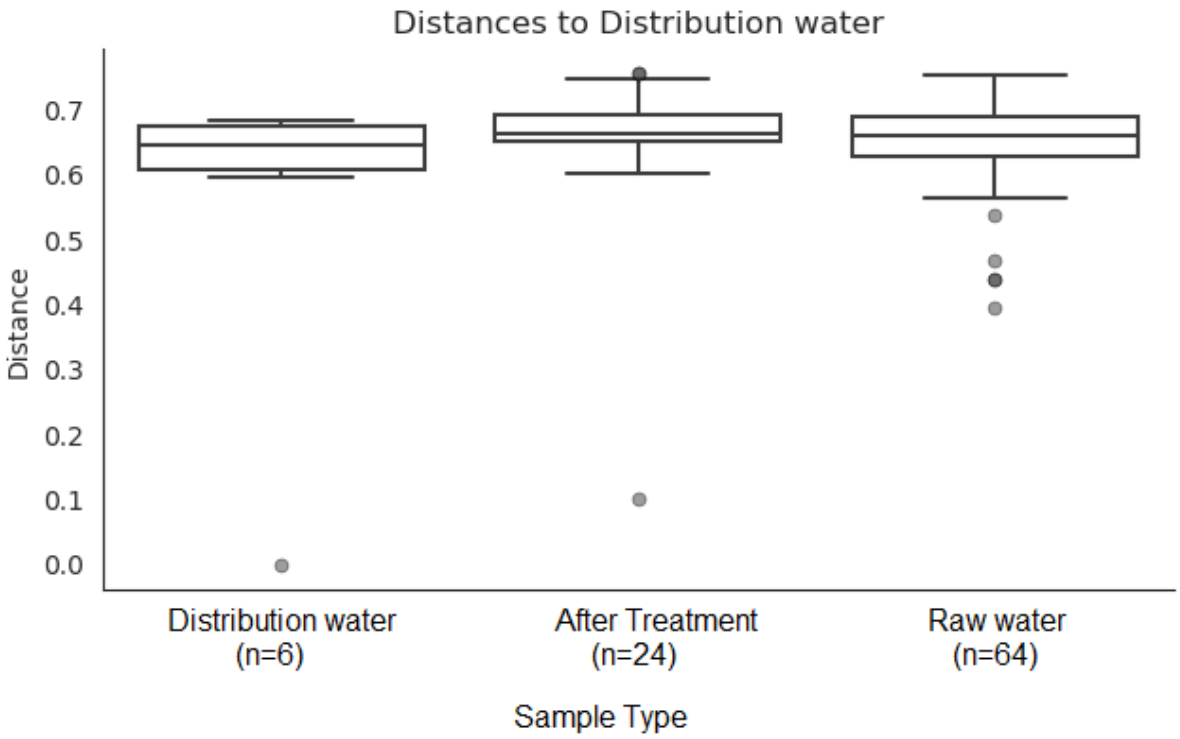


Figure 6.16: Beta diversity data based on unweighted UniFrac distance for sample types

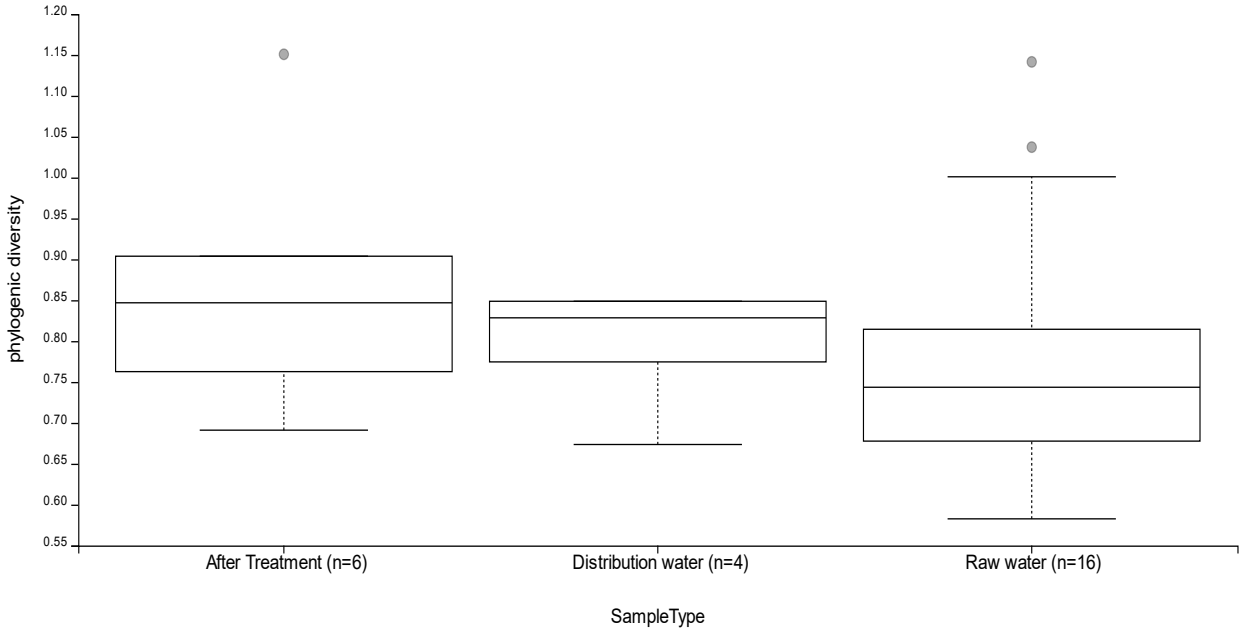


Figure 6.17: Alpha diversity based on Faith's phylogenetic diversity for the various sample types

According to the phylogenic diversity computations, the most taxon divergent sampling site is WC-A (Figure 6.18). This could be the case as this DWPF is represented by only raw water. The rest of the sampling sites are less divergent. This lack in demonstrating major differences in diversity indices data is thus supported by work that was conducted by observations and concerns raised by Solden et al. (2016) and Bruno et al. (2018).

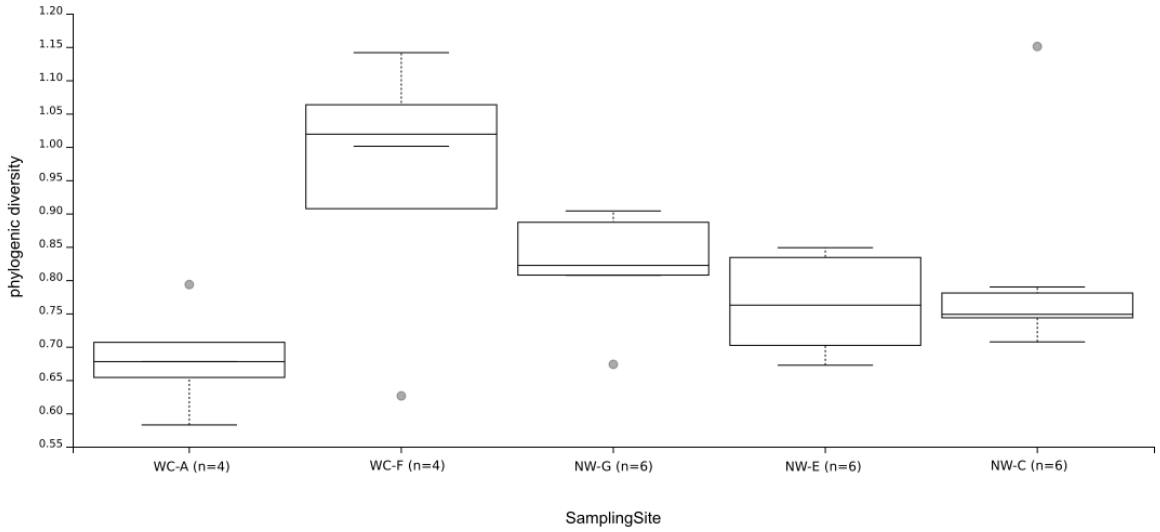


Figure 6.18: Alpha diversity based on Faith's phylogenetic diversity for the various sampling sites

Curated databases linked to antibiotic resistance are available for clinically relevant bacteria, but do not exist for the majority of bacteria. These databases for clinical species could be usefully explored. What is a real advantage is the approach described by Bowman and Ducklow (2015) to extrapolate and predict metabolic and ecosystem functioning to the 16S rRNA gene sequence data. In the present study, microbial metagenomes were predicted from 16S rRNA gene sequences using the online PICRUSt pipeline (Langille et al., 2013) as previously described (Zaura et al., 2015). Prevalence of ARDs was evaluated by blasting OTUs against ARGs downloaded from the ARDB (Liu and Pop, 2009).

In Table 6.13, selected predicted genes and pathways are summarised. Predicted ARGs for five antibiotic classes are listed and these efflux pumps' coding for multiple antibiotic resistance (*emrA, B* and *F*). There was also a prediction for genes coding for resistance to Chloramphenicol, Ciprofloxacine and Methicillin. These could potentially be present in multiple copies (<10 to >300) in raw and treated water samples. Such prediction results confirm the WGS and the end-point PCR results from previous sections.

Table 6.13: Predicted KEGG Orthology groups (KOs) associated with multidrug or antibiotic resistance that were observed in the different water treatment plants and the treatment stages

Antibiotic	KO ID	Description	WC-F		WC-A			NW-G			NW-E			NW-C		
			Raw 1	Raw 2	Raw 1	Raw 2	Treated	Raw	Dis	Treated	Raw	Dis	PT	Dis	Raw	Treated
Clindamycin	K00012	• *ugd, M00671 Polycationic antibiotics resistance, arn lipopolysaccharide (LPS) modification operon*	344	352	268	337	114	316	215	88	482	24	293	709	847	655
	K07552	• *bcr, MFS transporter, DHA1 family, bicyclomycin/chloramphenicol resistance protein*	202	205	161	204	78	213	146	50	276	20	162	424	515	341
Ciprofloxacin	K03543	• *emrA, multidrug resistance protein A*	182	178	140	178	69	204	141	41	247	8	156	394	481	328
	K00897	• *E2.7.1.95 kanamycin kinase, M00640 Aminoglycoside antibiotics resistance*	3	5	7	9	6	4	3	4	9	2	5	14	20	12
	K03446	• *emrB, MFS transporter, DHA2 family, multidrug resistance protein B*	182	175	129	165	63	193	137	40	226	7	152	391	473	321
	K05515	• *mrdA, penicillin-binding protein 2, ko00312 beta-Lactam resistance, M00626 beta-Lactam resistance, penicillin-binding protein variants*	286	317	261	336	114	279	180	71	452	13	244	563	666	492
	K13888	• *macA, macrolide-specific efflux protein MacA*	57	55	62	86	24	59	52	23	87	10	73	146	171	155
Minocycline	K03543	• *emrA, multidrug resistance protein A*	182	178	140	178	69	204	141	41	247	8	156	394	481	328
	K08223	• *fsr, MFS transporter, FSR family, fosmidomycin resistance protein*	107	110	81	95	24	104	66	38	137	13	98	256	303	266
	K12340	• *tolC, outer membrane channel protein, ko00312 beta-Lactam resistance, M00646 Multidrug resistance, efflux pump AcrAD-TolC, M00647 Multidrug resistance, efflux pump MexAB-OprM/SmeDEF/AcrAB-TolC*	173	184	154	192	84	194	143	31	250	6	149	331	408	235
	K07576	• *metallo-beta--lactamase family protein*	141	164	144	174	51	181	112	47	259	5	165	348	418	357
Amoxicillin	K00012	• *ugd, M00671 Polycationic antibiotics resistance, arn lipopolysaccharide (LPS) modification operon*	344	352	268	337	114	316	215	88	482	24	293	709	847	655

	K03327	• *TC.MATE, SLC47A, norM, mdtK, dinF, multidrug resistance protein, MATE family*	232	227	189	236	84	259	198	70	358	22	249	544	651	503
	K03543	• *emrA, multidrug resistance protein A*	182	178	140	178	69	204	141	41	247	8	156	394	481	328
	K03585	• *acrA, mexA, adeI, smeD, membrane fusion protein, ko00312 beta-Lactam resistance, M00646 Multidrug resistance, efflux pump AcrAD-TolC, M00647 Multidrug resistance, efflux pump MexAB-OprM/SmeDEF/AcrAB-TolC*	210	208	175	222	84	233	151	50	318	11	187	481	574	415
	K05595	• *marC, multiple antibiotic resistance protein*	240	240	161	204	81	217	137	49	296	11	166	471	565	386
	K08169	• *yebQ, MFS transporter, DHA2 family, multidrug resistance protein*	31	32	31	36	15	27	21	5	38	2	13	31	43	20
	K08223	• *fsr, MFS transporter, FSR family, fosmidomycin resistance protein*	107	110	81	95	24	104	66	38	137	13	98	256	303	266
	K12340	• *tolC, outer membrane channel protein, ko00312 beta-Lactam resistance, M00646 Multidrug resistance, efflux pump AcrAD-TolC, M00647 Multidrug resistance, efflux pump MexAB-OprM/SmeDEF/AcrAB-TolC*	518	552	461	576	255	583	429	92	482	18	447	992	1223	704
	K02547	• *mecR1, methicillin resistance protein, ko00312 beta-Lactam resistance, M00625 Methicillin resistance*	1	4	23	36	0	3	0	6	276	7	7	7	10	16
	K03712	• *marR, MarR family transcriptional regulator, multiple antibiotic resistance protein MarR*	515	558	426	534	159	453	231	154	174	45	323	855	1016	846
Vancomycin	K07260	• *vanY, D-alanyl-D-alanine carboxypeptidase [EC:3.4.16.4], ko00550 Peptidoglycan biosynthesis, M00651 Vancomycin resistance, VanB type, ko02020 Two-component system, M00652 Vancomycin resistance, VanE type*	140	179	198	299	87	171	60	117	48	39	179	261	316	317

	K10012	• *arnC, pmrF, undecaprenyl-phosphate 4-deoxy--4-formamido-L-arabinose transferase [EC:2.4.2.53], ko00520 Amino sugar and nucleotide sugar metabolism, M00671 Polycationic antibiotics resistance, arn lipopolysaccharide (LPS) modification operon*	76	65	92	147	45	67	15	22	15	5	54	82	103	65
	K08641	• *vanX, M00651 Vancomycin resistance, VanB type*	122	129	81	84	36	132	76	38	174	4	99	237	288	218

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 CONCLUSIONS

7.1.1 Physicochemical and general microbiological parameters of the different water sources

The results presented here indicated that upstream land-use could impact on the quality of raw water to such an extent that general drinking water production processes may be inefficient to restore the drinking water fully to the highest quality. It is thus of cardinal importance that physical and chemical parameters are continually monitored in order to take immediate corrective steps (Nel and Haarhof, 2011). The results also demonstrated that the participating DWPFs all had drinking water production processes in place to ensure that the water they produced complied with national standards. However, this was not the focus of the study, but was a constant factor that could be used to indicate that, in terms of South African standards, all the drinking water was similar.

7.1.2 Isolating and determining the antibiotic resistance profiles of isolated bacteria for comparison to the next-generation molecular evaluation methodologies

Upstream land use and human activities may be a source of antibiotics, antibiotic-resistant heterotrophic bacteria and associated genes. In the present study, it was demonstrated that these could be reduced, but are not completely removed by the current drinking water production systems. Even though the levels of individually measured antibiotics were very low, combinatorial effects of the suite of antibiotics could be of concern. These antibiotic substances, bacteria and genetic material eventually land in the drinking water distribution system and would ultimately be taken in by consumers. Effects of these are currently unknown.

Genes potentially responsible for antibiotic resistance phenotypes could be detected in isolated and purified bacteria that were resistant to multiple antibiotics. This is an indication that the genes are functional, and the dissemination of such genes could potentially have detrimental consequences should they be transferred to infective pathogenic species. Such multiple antibiotic resistance pathogens will be difficult and very costly to treat.

In all DWPFs, *Bacillus* spp. were the genus that was constantly isolated from source and drinking water. Known opportunistic species strains were also detected. A large percentage of the various isolated bacteria produced the virulence features (extracellular enzymes) that could allow for the invasion of the host tissue.

The whole genomes of *Bacillus* spp. from six of the eight DWPFs had similar ARGs and virulence genetic determinants. These were isolated from raw and produced drinking water. This indicates that these species survive the drinking water production barriers.

A large proportion of the various multiple antibiotic-resistant HPCs were haemolytic and could produce more than one extracellular enzyme, an indication of the potential pathogenicity of these bacteria. The species that were isolated were, in some examples, also isolated and characterised in clinical settings and some foodborne infection scenarios.

7.1.3 Perform qPCR and environmental metagenomic analysis of DNA isolated directly from water and evaluate the analysis processes

Hollow fibre membranes are suitable for the isolation of sufficient eDNA for metagenomics studies. A system was developed that would be suitable to harvest eDNA from between 1 000 and 10 000 litres of water in such a manner as to prevent water wastage. The current sampling system is, however, costly (R1 500 for the filtration system per sample) and should be further optimised using more cost-effective materials.

The 16S rRNA microbiome data could be generated for bacterial species. There were some overlaps with regard to the species that were isolated. Ecological indices analyses did not show major differences between the various water compartments. This could be explained by taking into account the fact that a large proportion of the sequence data belonged to the uncultivated majority. Future analysis of the current data may cast some light on this proportion of the population and how they are contributing towards the microbiome.

7.1.4 Evaluate the next-generation molecular method data and determine their implications

The microbiome data could be used to predict metagenomes. The latter was used to evaluate the occurrence of ARGs. In this case, it was demonstrated that predicted ARGs for five antibiotic classes included similar efflux pumps' coding for multiple antibiotic resistance (*emrA*, *B* and *F*). There was also a prediction for genes coding for resistance to Chloramphenicol, Ciprofloxacin and Methicillin. These could potentially be present in multiple copies (<10 to >300) in raw and treated water samples. Such prediction results confirm the WGS and end-point PCR results from previous sections.

Metagenomics analysis data for one plant is available that provided the raw data – courtesy of Prof Fanus Venter and Sarah Potgieter of the University of Pretoria. These metagenomics ARG and virulence genes showed similar trends when compared to the WGS analysis of the bacilli of the various plants.

7.1.5 Potential mitigation strategies

Taking all the data into account, there is evidence that the quality of the raw water impacts on the drinking water production processes, particularly with respect to physicochemical properties and the microbiome of the final produced water. This study has shown that ARGs and virulence genes are present in bacterial isolates, as well as in eDNA in distribution systems. There is consensus that attention should be paid to making contributions for reducing antibiotic resistance. It is a multidisciplinary issue that involves microbiologists, engineers, environmental scientists and city planners and needs to adapt existing technologies to capacitate the major contributors (wastewater treatment plants, animal rearing facilities, etc.), as well as drinking water production facilities so that the effective and simultaneous removal of antibiotics and ARGs can occur. There are efforts internationally that investigate various options, including combining, adapted sedimentation, filtration and oxidation technologies. These studies should be linked to the dynamics of the microbiome of the drinking water distribution systems, as well as the microbiomes within treatment plants. The ideal approach would be to use a metagenomics approach and/or HT-qPCR.

7.2 RECOMMENDATIONS

Internationally, a considerable body of knowledge is being generated to establish the occurrence of antibiotics, ARB and ARGs in aquatic systems, particularly in drinking water distribution systems. The South African context is different with respect to the quality and quantity of water sources, and the

impacts of land use. The present study provided some data for examples of drinking water production systems typically in operation in South Africa. Further investigation using the methods (chemical and molecular techniques) optimised in the present study should be conducted to reflect whether seasonal (particularly rain) meteorological conditions affect the water quality and how this may impact on the final produced water.

Connecting contaminants of emerging concern in aquatic ecosystems to waste and impacts on human health is a theme that is poorly understood and needs to be explored. This is particularly the case for antibiotics, ARB and ARGs that are disposed of in water sources that are used for drinking water production. A review of all the work that has been funded by the WRC and their implications should be considered.

Due to the data gathered, and as parameters have shown, a further investigative study is necessary to look into the health-related impacts of the bacterial species identified and their associated virulence factors.

Rapid ELISAs are sensitive and can detect very low antibiotic residues. This could be conducted at DWPFs as part of WSP, particularly where upstream land use would involve antibiotic use in human or animal medicine. The cost for setting up the equipment and analysis is not prohibitively high. It would allow for the quantification of antibiotic residues in water samples and provide trends over time.

Furthermore, with such substantial data that was gathered in the current study, there is a need for linking WGS data to inhibition zone analysis data. This will not only give insight into the world of these identified bacterial species, but will also be able to trace their lineage and possibly find innovative remediation solutions. The WGS will provide an overview of ARGs associated with target genera.

It is also very important that findings from studies such as this one should be circulated to the relevant stakeholders. Attempts should be made to get this to those who were not part of this initial study. Such data must also be made available to communities in such a manner that would make it easily understandable to all members.

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