THE DETECTION OF ANTIBIOTIC RESISTANCE ORGANISMS AND THE DEVELOPMENT OF A QPCR PROTOCOL FOR THE ENUMERATION OF ARGS IN DOMESTIC GREYWATER SYSTEMS: A CASE STUDY CONDUCTED IN CAPE TOWN

Min-ghah Kariem, Raeesah Sonday, Busisiwe Kota, Marla Trindade, Bronwyn Kirby McCullough

WATER RESEARCH COMMISSION

TT 891/22

The Detection of Antibiotic Resistance Organisms and the Development of a QPCR Protocol for the Enumeration of ARGs in Domestic Greywater Systems: A Case Study Conducted in Cape Town

Report to the
Water Research Commission

by

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WRC Report No. TT 891/22
ISBN 978-0-6392-0475-8

July 2022

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EXECUTIVE SUMMARY

Over a three-year period (2015-2017) the Western Cape Province experienced a drought in which the six large reservoirs that serve as the raw water source for potable water to supply to the ~3.7 million residents were unable to meet the demand. Consequently, measures such as the use of alternative water sources, such as groundwater and greywater were put in place to prevent “Day Zero”. In particular, the use of greywater for flushing of toilets, gardening and outdoor cleaning was promoted as part of water conservation and demand management. Greywater is defined as wastewater which originates from showers, baths, hand basins, laundry tubs, and washing machines, and depending on the context may be extended to include wastewater from dishwashers and kitchen sinks. Importantly, all household wastewater which has faecal contamination, such as toilet wastewater and water from washing nappies, is excluded. Greywater is highly variable in composition and often contains significant microbial contamination, including pathogenic and resistant organisms. The risk of infection from pathogens and dissemination of resistant genes present in greywater is considered to be the most significant human health risk associated with greywater use.

This project was aimed at detecting and enumerating antibiotic resistant genes (ARGs) in domestic greywater systems, and to assess whether these systems can support the growth and proliferation of resistant organisms. The specific objectives were as follows:

1. Determine whether domestic greywater is a source of antibiotic resistance genes and pathogenic organisms
2. Enumeration of ARGs in various greywater sources using qPCR
3. Water analysis to measure the levels of antibiotics present in greywater
4. Development of novel FACS assays to screen for ARGs from environmental samples
5. Screen for the presence of integrons as markers of horizontal gene transfer

Microbial assays were performed to culture organisms from greywater and biofilm samples. The microbial abundance was found to be high, but the species diversity was low. This may point to the fact that the greywater within the systems had been stored for extended periods. The antibiotic resistance profile of these isolates was determined. Based on gradient plate assays, 64% of the strains isolated were resistant ampicillin and streptomycin, while only 13% were fully susceptible. Kirby-Bauer testing revealed that the greywater samples harboured organisms resistant to teicoplanin and cefpodoxime, while the biofilms had microbes resistant to teicoplanin, streptomycin and cephalosporins. Based on 16S rRNA gene sequence analysis the isolated strains were similar to clinical strains (such as *Klebsiella* and *Enterobacter*) as well as species which are typically found in the natural environment (soil/plants) or water (*Chryseobacterium, Sphingobacterium, Delftia, Rhizobium, Aquincola,* and *Bacillus* species).

Traditional culturing is only able to detect a fraction of the microorganisms present in any environment. While molecular methods can detect most microorganisms, these assays can be difficult, expensive and inhibitors present in samples can results in false negative results. In theory *in vivo* assay circumvents the limitations of culturing- and molecular assays. It was based on this assumption that we investigated the potential of developing an *in vivo* assay for the detection of metallo-β-lactamase producing microorganisms. The assay uses a fluorescent substrate (meropenem coupled to fluorescein) and initial testing looks promising. Future work will focus on adapting this assay from 96 well plate format to a FACS assay.

In order to determine whether the genes associated with antibiotic resistance were actively being expressed quantitative polymerase chain reaction (qPCR) assays were developed. In addition, a methodology was established which can be used to extract high quality RNA from greywater and biofilm samples. RNA was successfully extracted from all samples using this protocol and converted into cDNA

for the subsequent qPCR analysis. qPCR was used to screen for the presence of the *vanA* gene, which confers resistance to vancomycin. The optimised qPCR protocol was found to be highly sensitive, as it was able to detect very low levels of *vanA* gene, even with low input DNA concentrations. The vanA gene was found in three of the five samples, which confirms the findings from the culture-based screening which found that vancomycin resistant strains were present in the samples.

One concern about the improper use of greywater (particularly its long-term storage) is that this will allow the microorganisms which colonise these systems to exchange antibiotic resistance genes via horizontal gene transfer (HGT). As one cannot directly screen for HGT, the one solution is to rather screen for the genetic elements associated with gene transfer, such as integrons. As class 1 integrons are associated with the exchange of ARGs, a qPCR assay for the detection of the *intI* gene was developed. The *intI* was consistently detected at high levels and in fact was found in all the samples included in the molecular analysis (at all four sites, at all sampling points). This result would suggest that the microbes colonising these GW systems do harbour integrons, and HGT involving ARGs is likely occurring.

This study combined traditional microbiology tests with molecular biology to detect resistant microorganisms within domestic greywater systems, while qPCR was used to determine the levels of resistant genes. Our analysis supports the hypothesis that greywater systems are colonised by resistant organisms, these microbes are forming relatively stable communities (within the water and particularly the biofilms) and there are high levels of genetic exchange. We conclude that improper use of greywater does pose a risk to the environment and people's health.

ACKNOWLEDGEMENTS

Our sincere gratitude goes to the following:

- The Water Research Commission for funding the research

- The authors would like to thank the Reference Group of the WRC Project for their assistance and constructive discussions during the duration of the project:
  - Dr Nonhlanhla Kalebaila (Water Research Commission)
  - Dr Marilize Le Roes-Hill (Cape Peninsula University of Technology)
  - Dr Jo Barnes (Stellenbosch University)
  - Mrs Maronel Steyn (CSIR)
  - Prof Carlos Bezuidenhout (North-West University)
  - Dr Nosiphiwe Ngqwala (Rhodes University)
  - Prof E Chirwa (University of Pretoria)
  - Mr J Nathoo (NuWater)
  - Dr Hafizah Chenia (University of KwaZulu-Natal)
  - Dr W Edwards (Quorus Biotechnology)

- Dr Richard Greene for his invaluable assistance in designing the assay and synthesising the substrate

- Dr Lonnie Van Zyl (IMBM, UWC) for his technical assistance

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ACRONYMS & ABBREVIATIONS

AMP10	Ampicillin 10µg
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ARG	Antibiotic resistance gene
BF	Biofilms
BLAST	Basic Local Alignment Search Tool
CDC	Center for Disease Control
cDNA	Complementary DNA
CoCT	City of Cape Town
CPD10	Cefpodoxime 10µg
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethyl pyro carbonate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
eDNA	Environmental DNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
ESBL	Extended spectrum β-lactamase
GW	Greywater
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
HPLC	High Performance Liquid Chromatography
KF30	Cephalothin 30µg
LB	Luria-Bertani
$LiCl_2$	Lithium chloride
MDR	Multi-drug resistant
MH	Mueller-Hinton Agar
NA	Nutrient Agar
NaAc	Sodium acetate
NEB	New England Biolabs
NH_4Ac	Ammonium acetate
PCR	Polymerase chain reaction
pH	potential Hydrogen
PSC-B	Phosphate, Sodium dodecyl sulphate, Chloroform Bead-Beater
qPCR	Quantitative polymerase chain reaction

R2A	Reasoners 2A
RNA	Ribonucleic acid
RNase	Ribonucleases
RPM	Revolutions per minute
RT	Room temperature
S10	Streptomycin 10µg
SDS	Sodium Dodecyl Sulphate
spp.	species *pluralis*
SSF	Slow Sand Filters
TAE	Tris, Acetic acid and Ethylenediaminetetraacetic acid
TBE	Tris, Borate and Ethylenediaminetetraacetic acid
TE	Tris Ethylenediaminetetraacetic acid
TEC30	Teicoplanin 30µg
UV	Ultra Violet
VBNC	Viable but non-culturable
VRE	vancomycin resistance *Enterococcus*
WC	Western Cape
WHO	World Health Organisation
½LB	Half strength Luria-Bertani

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CHAPTER 1: BACKGROUND

1.1 INTRODUCTION

"Antibiotic Resistance is one of the biggest threats to global health, food security and development today"
− WHO

Globally, there is a growing concern about the availability of freshwater, and governments are increasingly looking at long term alternative solutions to meet the increasing water demand. South Africa is a water scarce country. Specifically, the Western and Eastern Cape Provinces has experienced severe droughts in recent years (Meissner & Jacobs-Mata, 2017). From the period 2015 to 2017 the winter rainfall region of South Africa (includes Cape Town) received below normal rainfall and these three years were considered to be drought years of increasing severity – with 2017 being the driest year recorded by several rain gauges (Otto *et al.*, 2018; Taing *et al.*, 2019). The introduction of stringent water restrictions and increased tariff rates likely motivated many Western Cape (WC residents to explore alternative approaches such as greywater. Treated greywater use is encouraged for flushing of toilets, gardening and outdoor cleaning. Domestic greywater collection can vary widely from simple hand-bucket-collection (bucketing) to automatic filtration treatment systems. For the manual bucket systems where the water is not treated the accepted usage guidelines are that water must be used within 24 hours, and if the greywater is to be stored it requires treatment. As per the City of Cape Town (CoCT) by-laws, the connection of alternative water systems to the City's water supply does require the City's approval and such systems must have approved backflow prevention installed to prevent contamination of drinking water (Guidelines for the installation of alternative water systems in Cape Town).

Greywater is defined as all wastewater generated in households or office buildings from streams without faecal contamination. This includes water from showers/baths, washing machines and kitchen sinks (Lin *et al.*, 2004). In order to reduce contaminants in greywater some countries (USA and Australia) exclude kitchen wastewaters as they may contain high levels of microorganisms, food particulate matter and fats/oils. Typically, 50-80% of indoor water used in the home can be reused as greywater (Rodda *et al.,* 2010). Greywater is a highly variable source of non-potable water, and its composition is governed by the household demographics and activities (Maimon *et al.,* 2010). Nationally, while the use of greywater has increased exponentially, there is limited regulatory information regarding its usage available. The WRC has published a strategic document (Carden *et al.,* 2018), while the CoCT has several by-laws (Summary guide to the City of Cape Town's Water By-law, May 2019), as well as published guidelines on how these alternative water systems should be designed, operated and maintained (Guidelines for the installation of alternative water systems in Cape Town). There have already been several WRC studies which investigated the use of greywater in various settings. It is estimated that toilet flushing consumes between 20-40% of domestic water demand (DWAF 2007), an application where greywater usage is a viable replacement option. Ilemobade *et al.* (2012; WRC Report No. 1821/1/11) investigated the use of greywater for toilet flushing in high density housing, in a study which focused on student accommodation at two South African Universities. Other WRC studies have focused on greywater use in agricultural settings (Carden *et al.*, 2007, Albertse, 2000, Ilemobade *et al.*, 2009a). Rhodda *et al.*, 2010 presented holistic guidelines for the sustainable use of greywater to irrigate gardens and small-scale agriculture in South Africa with a focus of protecting human health, the irrigated plants health, as well as the soil and environment.

Currently, there is no "global standard" to assess the safety and quality of greywater. Many countries only determine the COD and screen for the presence of faecal organisms. Many treatment methods (such as filtration) are ineffective at removing residual antibiotics. Unlike treated municipal water, domestic greywater is likely to contain high levels of organic substances which can support large bacterial numbers. These organisms may serve as a reservoir of ARGs2 which can be spread to humans directly or indirectly by seeping into groundwater or other surface water sources. Given the negative health and societal impacts arising from the spread of antibiotic resistance into natural habitats, improved environmental monitoring to investigate the occurrence, transport and fate of ARGs in different habitats is urgently required.

Due to climate change, it is expected that the use of alternative water sources in South Africa will increase, and more households are likely to install greywater systems. As such, additional research must be performed to assess the microbial safety of these systems, particularly DIY systems which may have little/no treatment. Thus, the aim and objectives of the present study are:

1.2 PROJECT AIMS

To detect and enumerate ARGs in domestic greywater systems, and to assess whether these systems can support the growth and proliferation of resistant organisms.

6. Determine whether domestic greywater is a source of antibiotic resistance genes and pathogenic organisms
7. Enumeration of ARGs in various greywater sources using qPCR
8. Water analysis to measure the levels of antibiotics present in greywater
9. Development of novel FACS assays to screen for ARGs from environmental samples
10. Screen for the presence of integrons as markers of horizontal gene transfer

1.3 SCOPE AND LIMITATIONS

The primary scope of this project was to investigate antimicrobial resistance in domestic greywater systems. Complementary approaches were employed to study resistance within these systems. Firstly, viable microorganisms were cultured from greywater and biofilm samples, and their resistance profiles to several antibiotics was determined via plate assays, Kirby-Bauer and gradient plates. Resistant organisms were identified by 16S rRNA gene sequencing. Secondly, a novel spectrophotometric assay was developed to screen for microorganisms which express β-lactamases (particularly metallo-β-lactamases). Ideally this assay will be further optimised so it can be used on a fluorescence activated cell sorter (FACS), which will have increased sensitivity. The third aspect of the research project focused on the detection and enumeration of three antimicrobial resistance genes using qPCR. The final study aim looked at screening for the presence of integrons in the greywater and biofilm samples. Integrons are mobile genetic elements which contain ARGs, which can be transferred between organisms by horizontal gene transfer. As such, the presence of integrons can be linked to occurrence of HGT of ARGs. qPCR was performed which targeted the *intI* gene, as the presence of this gene is associated with class 1 integrons (associated with the transfer of ARGs).

This project was undertaken from January 2019 until December 2021. The study site selected was an upper-middle class suburb in Cape Town's northern metropole. Four domestic greywater systems were sampled over the duration of the project. One of the limitations of this study was accounting for the variability of the greywater itself as well as variability in operational use. Factors such as household composition are also likely to influence the greywater composition generated at each site. In order to reduce variability due to operational use all four systems studied were the same brand and size, and three were installed by the same company. Although samples were only collected from four greywater

systems the fact that similar levels of resistance organisms and ARGs were detected at all four sites does provide us with some confidence that our findings are a reflection of what the microbial communities in similar systems would look like. However, as socioeconomic factors have been shown to influence greywater composition in households, a limitation of this research is that findings are probably only reflect of what would be found in middle or upper-middle class areas.

CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

Several studies have shown that domestic greywater harbours various contaminants, from chemicals to visible debris to microorganisms (Sorensen, 2017). In addition, given its high organic content greywater can support microbial growth and biofilm production. One often overlooked hazard is that greywater may contain residual antibiotics, derived directly from human consumption or indirectly from the preparation of foods contaminated with antibiotics. Ultimately greywater systems could act as an environmental reservoir of antibiotic resistance genes (ARG) with the biofilms serving as a platform for antibiotic resistance gene proliferation and exchange.

2.2 ANTIBIOTIC RESISTANCE – AN OVERVIEW

"Antibiotics are truly miracle drugs that have saved countless millions of lives.
But antibiotic resistance is a critical public health issue that is eroding the effectiveness of antibiotics and may affect the health of each and every one of us"
− Betsy Bauman

Since their widespread introduction in the 1950s antibiotics have revolutionized medicine, veterinary health and agriculture (Davies and Davies, 2010). While it is not possible to quantify the impact of antibiotics in human health, it has been estimated that penicillin alone has saved 200 million lives since its introduction. Unfortunately, for the last 60 years antibiotics have often been used with little or no regulations controlling their use. Globally, it is estimated that more than 70 billion doses of antibiotics are used in the clinical sector per annum (Woolhouse *et al.*, 2016), with a further 63 151 tons consumed by livestock as part of animal feed (van Boeckel *et al.*, 2014).

The global antibiotic resistance crisis can be attributed to the overuse and misuse of antibiotics in clinical therapy and animal husbandry (Tennstedt *et al.*, 2003; Ventola, 2015). Exposure to antibiotics (particularly sub-lethal doses) serves as one of the main drivers for the development and spread of ARGs which can be transmitted to humans by means of direct or indirect contact (van Boeckel *et al.*, 2014). The development of resistance to common antimicrobial therapies in important bacterial pathogens is increasing at an alarming rate. This coupled with the emergence of multi-drug resistant (MDR) bacteria is one of the most concerning problems faced by the healthcare industry in the twenty-first century (Lupo *et al.,* 2012; Frieri *et al.,* 2016). Globally, the number of antibiotic resistant infections is increasing. An estimated 23 000 deaths are attributed annually to resistant infections in the USA alone, at a direct cost of $20 billion with a further $35 billion in lost productivity. In developing countries, such as South Africa, resistant infections are increasing pressure on already overburden public health systems.

2.3 ANTIBIOTIC RESISTANCE GENES

Antibiotic resistance is the ability of a microorganism to resist the effects of an antibiotic it was once susceptible to. Resistant microorganisms are more difficult to treat, requiring alternative medications or higher doses of antimicrobials. These approaches are often more expensive, require more toxic drugs or both. Bacteria which are resistant to multiple antimicrobials are termed "multi-drug resistant" (MDR). Antibiotic resistance arises through one of three mechanisms: 1) natural resistance in certain types of

bacteria (particularly in antibiotic producing strains); 2) genetic mutation; 3) acquisition of resistance genes from other microorganisms (horizontal gene transfer) (Davies and Davies, 2010). While there are several mechanisms by which microbes resist antibiotics, resistance mechanisms can be broadly subdivided into four categories – 1) target bypass; 2) efflux pumps; 3) antibiotic inactivation; 4) target modification. Overcoming resistance is further compounded by the fact that resistance to an antibiotic may not be limited to a single resistance mechanism.

The three antibiotic resistance genes were targeted in this study based on their clinical relevance in targeting drug resistant nosocomial infections, in particular antibiotics used to treat ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species). The resistance genes, associated resistance mechanism and the prevalence of these genes in the environment, particularly aquatic environments, will be discussed briefly in the following sections.

2.3.1 Resistance to Aminoglycosides

Aminoglycosides are a group of therapeutic agents which contain an amino-modified glycoside sugar. Streptomycin, originally derived from *Streptomyces griseus*, was the first in-class aminoglycoside released in 1944 and was the earliest modern agent effective against *Mycobacterium tuberculosis*. Following on from the success of streptomycin, a series of other milestone compounds were released, namely kanamycin, gentamicin, tobramycin and neomycin. Aminoglycoside antibiotics display concentration-dependent bactericidal activity against Gram-negative aerobes and some anaerobic bacilli. Currently they are used for the treatment of *Acinetobacter baumannii, Enterobacteriaceae* species*, Escherichia coli, Klebsiella pneumonaie* and *Pseudomonas aeruginosa* (Garneau-Tsodikova and Labby, 2016). The motivation to include this class of compound in the present study was due to the fact that streptomycin is used in the treatment of drug-resistant tuberculosis. Aminoglycosides' primary mode of action is the inhibition of protein synthesis, and as such is most effective against rapidly multiplying susceptible bacteria. As aminoglycosides are poorly absorbed via the gut, they are typically administered intravenously or intramuscularly. Due to their limited antimicrobial spectrum and toxicity they were not widely used. However, with the emergence of resistance to other mainline drugs, there is renewed interest in this class of compounds.

Aminoglycoside resistance is conferred via direct deactivation of the antibiotic by enzymatic modification. There are over 50 enzymes involved in resistance which are classified, based on their biochemical action on the aminoglycoside substrate, into three groups; namely acetyltransferase (*aac* genes), phosphotransferases (*aph*) and nucleotidyltransferases (*ant*). Studies have shown that the *aac, aph* and *ant* genes are widely distributed in various genera isolated from polluted and natural aquatic environments including *Aeromonas, Escherichia, Vibrio, Salmonella* and *Listeria* species (Lee *et al.*, 1998; Heuer *et al.,* 2002; Titilawo and Okoh, 2015) (Table 2.1). The *aacC2* gene was targeted in this study and encodes an aminoglycoside-3-N-acetyltransferase. This gene is commonly used to detected aminoglycoside resistance in microbial communities in aquatic environments.

Table 2.1: Aminoglycoside resistance genes in aquatic environments

Gene	Biological source	Environmental source*	Function	Reference
*aac*A4	Plasmid pTB11	NW	Aminoglycoside-6'-*N*-acetyltransferase	Mukherjee and Chakraborty 2006
*aac*C1	Microbial communities	NW		Lee *et al.*, 1998
*aac*C2	Microbial communities	NW	Aminoglycoside-3-*N*-acetyltransferase	
*aac*C3	Microbial communities	NW		
*aac*C4	Microbial communities	NW		
*aad*A1	*Aeromonas, Citrobacter* and *Shigella*; Plasmid pTB11	NW		Henriques *et al.*, 2006a; Mukherjee and Chakraborty 2006
*aad*A2	*Aeromonas, Escherichia* and *Vibrio*	NW, SD	Aminoglycoside-3'-adenylyltransferase	Dalsgaard *et al.*, 200; Taviani *et al.*, 2008
*aad*A5	*Escherichia a*nd Vibrio	NW		Park *et al.,* 2003; Mohapatra *et al.*, 2008
*aph*A1	*Salmonella*	DW, NW	Aminoglycoside phosphoryltransferase	Cernat *et al.*, 2007; Poppe *et al.*, 2006
*aph*A2	*Escherichia*	DW		Cernat *et al.*, 2007
*aph*D	Microbial communities	NW		Heuer *et al.*, 2002
*npt*II	Microbial communities	NW	Neomycin phosphotransferase	Zhu 2007
*sat*1	*Aeromonas* and *Escherichia*	NW	Streptothricin acetyltransferase	Henriques *et al.*, 2006
*sat*2	*Aeromonas* and *Escherichia*	NW		Henriques *et al.*, 2006
*str*A	*Listeria, Salmonella* and *Vibrio;* Plasmid pB10	NW	Streptothricin phosphoryltransferase	Jacobs and Chenia, 2007; Mohapatra *et al.*, 2008
*str*B	*Salmonella* and *Vibrio*	NW		Poppe *et al.*, 2006; Mohapatra *et al.*, 2008

*The antibiotic resistance genes were detected in the following water environments: EW effluent water; NW natural water; SD sediments; and DW drinking water.

2.3.2 Resistance to Vancomycin

The second gene that will be targeted is associated with vancomycin resistance. The glycopeptide antibiotic vancomycin was initially isolated from the soil bacterium *Amycolatopsis orientalis* by a missionary in the jungles of Borneo. Due to its apparent potency it was called vancomycin derived from

the Latin word for “to vanquish”. Vancomycin is a so-called “last resort drug” and its use is limited to the treatment of serious, life-threatening infections by Gram positive bacteria which are unresponsive to other antibiotics, and vancomycin is on the WHO’s list of essential medicines. The recent emergence of vancomycin-resistant enterococci is of great concern and has resulted in the Centre for Disease Control (CDC) and WHO developing guidelines which restricts the use of vancomycin to a limited number of indications. Currently it is only recommended for the treatment of complicated skin infections, bloodstream infections, endocarditis, bone and joint infections, and meningitis caused by methicillin-resistant *Staphylococcus aureus*. It is also used for the treatment of severe *Clostridium difficile* colitis (Kang and Park, 2015).

It is believed that vancomycin resistance initially emerged in hospitals, firstly in enterococci before spreading to other species, including *S. aureus*. There are six types of vancomycin resistance genes with *vanA* and *vanB* most commonly detected in water environments (Messi *et al.*, 2006) (Table 2.2). The *vanA* gene has been detected in environmental water bodies such as wastewater and surface water biofilms (Schwartz *et al.*, 2003). Vancomycin resistance is manifested by the expression of the *van* gene clusters which encode proteins that alter and prevent the action of the antibiotic. The *vanA* gene encodes for an alteration in the peptidoglycan biosynthesis pathway and results in the production of modified peptidoglycan precursors to which glycopeptides exhibit low affinities (van Hoek *et al.*, 2011). The *vanA* gene is the most widespread of all the *van* gene clusters and has been detected in six Gram positive bacterial genera namely *Enterococcus, Erysipelothrix, Lactobacillus, Pediococus* and *Staphylococcus* (van Hoek *et al.*, 2011). Many environmental *Enterococcus* species harbouring the *vanA* gene are resistant to both vancomycin and teicoplanin.

Table 2.2: Vancomycin resistance genes in water environments

Gene	Biological source	Environmental source *	Function	Reference
vanA	*Enterococcus* *Staphylococci*	DW, EW, NW	Alter the peptidoglycan layer	Schwartz *et al.*, 2003; Messi *et al.*, 2006
vanB	*Enterococcus*	EW, NW, UW	Alter the peptidoglycan layer	Caplin *et al.*, 2008

*The antibiotic resistance genes were detected in the following water environments: EW effluent water; NW natural water; SD sediments; and DW drinking water.

2.3.3 Resistance to β-lactams

β-lactam antibiotics, particularly penicillin, have a long history. Penicillin (derived from *Penicillium* fungi) was discovered by Alexander Fleming in 1928. The process for its large-scale production was driven by World War II and it was first used to treat infections in 1942. β-lactam antibiotics are a class of broad-spectrum antibiotics which contain a beta-lactam ring in their molecular structure; this includes penicillin derivatives (penams), cephalosporins (cephems), monobactams and carbapenems. Due to their broad activity spectrum and low toxicity, β-lactams are the most widely used group of antibiotics worldwide, and until 2003 accounted for half of all commercially used antibiotics (by sales). While first generation β-lactams were only active against Gram positive bacteria, chemical modification has extended their range and broad spectrum β-lactams effective against both Gram positive and Gram negative organisms are now available. β-lactams are bacteriocidal and their mode of action is blocking of cell wall synthesis via the inhibition of peptidoglycan synthesis – the peptidoglycan layer is the outermost

and primary component of the bacterial cell wall, particularly of Gram positive organisms, and as such plays a critical role in maintaining cellular structural integrity.

Bacteria develop resistance to β-lactam antibiotics by producing β-lactamases which cleave the β-lactam ring. To overcome this resistance, β-lactam antibiotics are often given with a β-lactamase inhibitor such as clavulanic acid. Resistance to this class of compounds is particularly concerning as many developing countries with limited access to other antibiotics rely on β-lactams for the treatment of a host of bacterial infections. While there are several mechanisms of resistance the direct deactivation of antibiotics by β-lactamases is the most common, especially in Gram negative organisms. There are hundreds of different β-lactamases (*bla*) genes. The *bla* genes have been detected in water/sediment from aquaculture, dairy farms, sewage treatment plants, and surface water (Table 2.3). The *bla* genes have also been detected in environmental pathogens derived from animals including *Aeromonas, Enterobacter, Salmonella* and *Vibrio* species. Another resistance gene, *ampC*, has been detected in microbes colonizing wastewater, surface water and drinking water (Schwartz *et al.*, 2003). This finding is of clinical relevance as the *ampC* gene encodes for an extended-spectrum β-lactamase (ESBL) and the incidents of nosocomial infections caused by ESBL *Klebsiella pneumoniae* is a growing problem worldwide, especially in immune-compromised individuals. The *bla* genes often coexist with other antimicrobial resistance determinants associated with mobile genetic elements which increase the possibility of multidrug resistance and environmental dissemination. Plasmids containing *bla* genes detected in wastewater treatment plants are frequently associated with transposons (genetic element which can 'jump' to different locations within the genome) and integrons (genetic elements that can capture genes and integrate them into the genome) which may confer multi-drug resistance as they possess *aad* (aminoglycoside resistance), *cml* and *cat* (chloramphenicol resistance) genes.

Table 2.3: β-lactam and penicillin resistance genes in water environments

Gene	Biological source	Environmental source *	Function	Reference
*amp*C	*Enterobacter, Salmonella*	DW, NW	AmpC type β-lactamase	Schwartz *et al.*, 2003; Poppe *et al.*, 2006
bla$_{PSE-1}$	*Aeromonas, Salmonella* and *Vibrio*	EW, SD	PSE-1 β-lactamase	Dalsgaard *et al.*, 2000; Jacobs and Chenai 2007; Taviani *et al.*, 2008
bla$_{TEM-1}$	*Escherichia*	DW	TEM-type β-lactamase	Alpay-Karaoglu *et al.*, 2007; Cernat *et al.*, 2007
bla$_{OXA-2}$	*Aeromonas,* Plasmids pB8, pB10 and pTB11	AS, EW	OXA-2 β-lactamase	Schulter *et al.,* 2005; Tennstedt *et al.,* 2003
bla$_{OXA-}$	Plasmid pTB11	AS	OXA-10 β-lactamase	Tennstedt *et al.*, 2003
^{30}*mec*A	*Staphylococcus*	DW, NW	Penicillin-binding protein	Schwartz *et al.*, 2003
*pen*A	*Listeria*	DW,		Srinivasan *et al.,* 2005

*The antibiotic resistance genes were detected in the following water environments: EW effluent water; NW natural water; SD sediments; and DW drinking water.

2.4 HORIZONTAL GENE TRANSFER

Environmental bacteria are known to house antibiotic resistance genes and serve as a potential source of novel resistance genes in clinical pathogens (Li *et al.,* 2012). A study by Aminov (2011) reviewed the role of horizontal gene transfer (HGT) mechanisms (transformation, transduction and conjugation) in environmental microbiota. The study identified that HGT events are responsible for the acquisition of heterologous resistance mechanisms amongst bacterial species (Lupo *et al.,* 2012). HGT of genes between bacterial strains is facilitated by mobile genetic elements such as integrons, plasmids, transposons, bacteriophages, insertion elements and genomic islands (Li *et al.*, 2012). Integrons are non-mobile bacterial genetic elements that are able to promote the acquisition of genes embedded within a gene cassette. The gene transfer via the exchange of integrons (and their constituent gene cassettes) is a common pathway for the acquisition of ARGs (Domingues *et al.,* 2012). Integrons are composed of an *intI* gene encoding an integrase, a recombination site *attI* and a promoter, which constitute a system for gene capture and expression (Ploy *et al.,* 2000). Integrase facilitates the integration or excision of gene cassettes by a site-specific system of recombination (Domingues *et al.,* 2012). Integrons are characterised into different classes based on their integrase gene sequence. Antibiotic resistance genes cassettes are commonly associated with class 1 integrons and have been documented to be present in several natural environments (Ploy *et al.,* 2000; Domingues *et al.*, 2012). Integrons can incorporate one or more gene cassettes, with integrons containing up to five ARGs being identified in agricultural and urban wastewater in South Africa (Jacobs and Chenai, 2006). Integrons serve as a mode of rapid bacterial adaptation and evolution, therefore its close association and potential to spread ARGs in the environment is particularly concerning (Leverstein van-Hall *et al.,* 2002).

2.5 ANTIBIOTIC RESISTANCE AND THE ENVIRONMENT

2.5.1 Presence and occurrence of antibiotic resistant genes in the environment

Antibiotics are widely used globally, with a complex inter-relationship developing between antibiotic usage by humans, animals and the environment (the “one health” concept). Antibiotics are often excreted into the environment unchanged and have been known to contaminate water sources (Zhang *et al,* 2006). These off-spills into the environment serve as a driving force for the selection and proliferation of antibiotic resistant organisms. There is increasing evidence that environmental antibiotic contamination is widespread. In fact, antibiotics are so ubiquitous that researchers have even been able to detect antibiotics in groundwater at a depth of over 10 m (Batt *et al.*, 2006). Recent studies have shown that other than so called "pristine" mountainous sites, most surface water sources that come in contact with either urban or agricultural areas are contaminated with antibiotics (Yang and Carlson, 2003). Given the prevalence of antibiotics in nature, researchers have started to recognise the importance of screening for ARG in environmental microbial communities (including non-pathogenic organisms) and not limiting their studies to hospitals/clinics. When focusing specifically on water systems, most investigations focus on sewage treatment or wastewater emanating from commercial food production (meat processing, aquaculture, and agriculture), with sporadic studies investigating the natural environment. From published studies it is evident that ARGs associated with clinically relevant antibiotics can be detected in wastewater treatment plants, surface water, ground water, as well as rivers, estuaries (Auerbach *et al.*, 2007; Czekalski *et al.,* 2012; Fahrenfield *et al.,* 2013).

Numerous studies have shown that ARGs can enter the environment either by direct discharge of untreated wastewater or as sludge/effluents emanating from sewage treatment plants (Auerbach *et al.*, 2007). ARGs can also enter soil from animal manure and/or biosludge, where they can leach into groundwater or contaminate shallow water. The resistance genes present in contaminated drinking

water can be further disseminated as the water passes through drinking water treatment facilities and re-enters the water distribution system (Schwartz *et al.*, 2003). Some researchers have gone so far as to consider ARGs to be “emerging” environmental contaminants, which like other pollutants are easily spread and difficult to clean up (even when selective pressure is removed) (Aminov and Mackie, 2007).

The *tet* genes appear to be the most frequently detected ARGs in environmental samples and have been detected in wastewaters worldwide (Agersø and Sandvang, 2005; Agersø and Peterson, 2007). Mackie and co-workers (2006) were able to show that the same *tetM, O, Q* and *W* genes present in swine lagoons could also be detected in groundwater downstream of these lagoons. However, ARGs in wastewater are not limited to tetracycline resistance. Chloramphenicol resistance genes (*catII, IV* and *B3*) (Dang *et al.*, 2007; Jacobs and Chenia, 2007) and sulphonamide resistance genes (*sulI, II, III* and *A*) (Agersø and Peterson, 2007) have been detected in aquaculture systems. Schwartz *et al.* (2003) reported the presence of methicillin resistant (*mecA*) *Staphylococcus* strains in hospital wastewater biofilms. Alarmingly ARGs are also present in slightly or non-polluted natural waters (Rahman *et al.,* 2008). The *vanA* gene has been detected in drinking water biofilms, even in the absence of enterococci, possibly indicating that gene transfer to autochthonous drinking water bacteria has occurred. Similarly, the enterobacterial *ampC* resistance gene which encodes a β-lactamase was detected via PCR in wastewater, surface water and drinking water biofilms (Schwartz *et al.*, 2003). β-lactamase genes were detected in nearly 80% of the ampicillin resistant *Enterobacteriaceae* and 10% of the resistant *Aeromonas* isolated from the Ria de Aveiro estuary (Henriques *et al.,* 2006). Multiple-antibiotic resistant *E. coli* carrying ARGs to aminoglycosides, β-lactams, tetracyclines, and trimethoprim-sulfamethoxazoles were detected from drinking water in the Rize region, Turkey (Alpay-Karaoglu *et al.,* 2007).

2.5.2 Antibiotic resistant genes in drinking water systems

Historically, concerns about the microbial quality of drinking water have focused on the occurrence of pathogens in water treatment and distribution systems. However, the presence of trace levels of antibiotics and ARGs in source water and finished drinking water may also affect public health, and is an emerging issue for the general public and government. Several studies have reported the presence of ARGs and heterotrophic resistant bacteria in finished water as well as tap water. Xi *et al.* (2009) examined the levels of eight ARGs [beta-lactam resistance genes (*blaTEM* and blaSHV), chloramphenicol resistance genes (*cat* and *cmr*), sulfonamide resistance genes (*sulI* and *sulII*), and tetracycline resistance genes (*tetO* and *tetW*)] in several small cities in USA (Michigan and Ohio). Except for *tetO* and *tetW* which were only detected in the source water, all the other genes were detected in every sample tested. The researchers also reported that the levels of most ARGs were higher in tap water than in finished and source water.

Metagenomic DNA analysis coupled to next generation sequencing is providing new insight into the diversity of ARGs in water systems. Shi *et al.* (2013) analysed drinking water (tap and filtered) plus water after chlorination using metagenomics. Genes associated with resistance to β-lactams accounted for 76% of the total sequence reads detected in tap water. In filtered water, the resistance genes were more diverse with 36% of the reads associating with β-lactam resistance, with 15% linked to tetracycline resistance and 15% to multi-drug resistance determinants. Surprisingly, chlorination treatment appeared to enrich for some resistance genes, particularly *ampC, aphA2, blaTEM-1, tetA, tetG, ermA* and *ermB.*

In several countries, including the European Union, the use of antibiotic agents as growth promoters in animal husbandry has been banned in order to prevent the selection and spread of antibiotic resistance.

However, in addition to antibiotic agents, heavy metals used in animal farming and aquaculture may promote the spread of antibiotic resistance via co-selection (Seiler and Berendonk, 2012). The heavy metals routinely used in agriculture and aquaculture include Hg, Cd, Cu, and Zn. Fish farmers frequently use antibiotics and metal containing products to prevent fouling, to feed and to treat fish in order to limit the spread of infections. This indirectly selects for resistance organisms due to a coupling of the resistance mechanisms against antibiotics and heavy metals. Those mechanisms can be coupled physiologically (cross-resistance) and/or genetically (co-resistance). Cross-resistance describes mechanisms that provide tolerance to more than one antimicrobial agent such as antibiotics and heavy metals, while co-resistance is defined as two or more genetically linked resistance genes, meaning that the genes responsible for resistance to different compounds are located next to each other on one mobile genetic element (Chapman, 2003). Due to this coupling, environmental heavy metals can be viewed as further drivers of antibiotic resistance.

2.5.3 Greywater systems as a source of ARGs

Greywater systems vary from hand-bucket-collection to automatic filtration treatment systems. The composition of the greywater is governed by the household or company from which it is produced and is mainly composed of effluents from washbasins, showers and laundry (Maimon *et al.*, 2010). Although the reuse of greywater for non-potable applications can substantially reduce potable water consumption, there are potential hazards often overlooked. Greywater is highly variable and often contains significant microbial contamination, including pathogenic and resistant organisms (Winward *et al.,* 2008). The environmental and health risks associated with this is great, as greywater could be a potential source of bacterial contamination of groundwater. In addition, antibiotic resistant organisms can proliferate and spread resistant genes to environmental strains (Birks and Hills, 2007; Maimon *et al.*, 2014; Busgang *et al.*, 2018).

The possibility of the greywater containing residual antibiotics is highly likely. Water sources such as greywater acquire bacteria and antibiotics from various sources, e.g. hand washing, cleaning of uncooked meats and food, and washing. These residual antibiotics can exert selective pressure on bacteria and contribute to the occurrence of resistant bacterial organisms (Li *et al.*, 2010; O'Toole *et al.*, 2012). Recent studies have suggested that water bodies such as reclaimed or greywater play a pivotal role in the transport and transfer of resistant organisms (Lupo *et al.*, 2012). Greywater systems also provide the perfect conditions for biofilms to form. Biofilms are defined as an aggregation of microorganisms embedded in a self-produced extracellular polymeric substance. Biofilms are a site of immense bacterial interactions due to its high bacterial density and nutritional richness (Madsen *et al.*, 2012). Recent studies have identified that horizontal gene transfer and biofilm formation in water bodies are interconnected (Madsen *et al.*, 2012). Biofilm formation and HGT interactions have been investigated in wastewater treatment plants (Schwartz *et al.*, 2003). Many of the conditions present in wastewater treatment plant biofilms are likely to be mimicked in domestic greywater tanks, such as periods of stagnation and high bacterial loads (Aminov, 2011). Thus, serving as a platform for biofilm formation. Ultimately greywater systems and its associate biofilms have the potential to act as an environmental reservoir for the proliferation and spread of ARGs.

As the number of antibiotic resistant infections increases globally, it should be acknowledged that greywater systems (biofilms) could be acting as potential environmental reservoir of antibiotic resistance bacteria (ARB) and ARGs.

2.6 MOLECULAR TECHNIQUES USED FOR THE DETECTION AND CHARACTERISATION OF ENVIRONMENTAL ANTIBIOTIC RESISTANCE

Annually microbiologists analyse thousands of environmental water samples to assess both the numbers and types of microorganisms present. Traditionally, culture-based assays were used, however, due to the widespread nature of ARGs in the environment, there is a need for the development of molecular methods to investigate the presence, occurrence and distribution of these environmental ARGs. Polymerase chain reaction (PCR) assays (either conventional, multiplex or quantitative) have been extensively used for the detection of specific ARGs in pure and mixed environmental samples. A limited number of studies have employed DNA hybridisation-based techniques such as microarray and FISH to study resistance, however due to the high cost, these methods are often limited to hospital settings. Most recently, metagenomics combined with next generation sequencing (NGS) has been used to detect ARGs in water samples.

2.6.1 Gene-specific PCR (Conventional PCR)

Environmental DNA (eDNA) is a DNA molecule that is released from organisms present in water or soil samples which is typically only present in low concentrations. Due to its sensitivity and reliability PCR is ideally suited for the analysis of eDNA (Tsuji *et al.*, 2018). Traditional two primer PCR has been extensively used for the analysis of environmental samples, as it allows for the amplification of a specific fragment of DNA present in a complex pool of DNA (Garibyan and Avashia, 2013; Fernando *et al.*, 2016). Gene-specific PCR has been used for the detection and characterization of antibiotic resistance genes from a range of environmental aquatic sources (Tavani *et al.*, 2007). Although the occurrence of false positives has been documented, the use of simple PCR assays for ARG detection remains one of the most sensitive approaches for environmental water sample analysis (Jansson and Leser, 1996). The main limitation of gene-specific PCR is that prior knowledge of the target DNA sequence is required in order to design primers, and as such, novel resistance genes will not be detected. An important consideration when employing any PCR-based technique is the intrinsic bias which may be introduced by PCR. This bias may be due to differences in primer binding to template DNA, as well as the quality and concentration of the template DNA itself. However, it can be costly to individually detect multiple genes using gene-specific PCR. Therefore, researchers are increasingly using multiplex PCR for the analysis of eDNA as it overcomes some of the limitations of conventional PCR. Additional problems of using PCR for the detection of genes in environmental genes is that eDNA may contain compounds which inhibit the polymerase and that low DNA concentrations obtained from samples may be below the detection limit of the assay.

2.6.2 Quantitative PCR

In quantitative PCR (qPCR) [also called real-time PCR (RT-PCR)], the accumulation of DNA after each amplification cycle can be visualised in real time (Arya *et al.*, 2015). Reactions are performed on a specialised thermocycler which detects the increased fluorescence of a reporter molecule as the amount of PCR product increases after each amplification cycle (Ponchel *et al.*, 2003). The inclusion of the fluorescent reporter in the reaction vessel allows for the simultaneous amplification and detection of nucleic acids to detect amplification products (Bustin *et al.*, 2005). qPCR can be used to quantify either the absolute copy number of a gene, or the concentration relative to a normalised gene. The three main types of reporter molecule systems used in qPCR are Taqman®, Molecular Beacons and SYBR-Green. These reporter molecules are either fluorescent dyes which intercalate with double stranded DNA (ds-DNA) or are modified DNA oligonucleotide probes which fluoresce when hybridized to complementary DNA. Unlike other PCR based methods which provide a simple presence/absence

result, RT-PCR allows for the direct quantification of the abundance of specific genes (Fierer *et al.*, 2005). Therefore, qPCR can be used to monitor water treatment and levels of contamination. RT-PCR has become an essential tool in molecular biology due to its specificity, reproducibility, high-sensitivity and simplicity (Gomes *et al.*, 2018).

qPCR is currently the most commonly used tool for the quantitation of resistance mediating genes (Volkmann *et al.*, 2004). The advantages of qPCR over conventional PCR for eDNA analysis include its speed, reproducibility and the ability to provide direct information regarding the abundance of the target ARG in an environmental sample (Luby *et al.*, 2016). However, the main advantage of RT-PCR is undoubtingly its exceptionally low detection level. Shannon *et al.* (2007) used RT-PCR to detect pathogens in wastewater and found that RT-PCR could detect DNA at concentrations as low as 100 fg (22 gene copies). qPCR using SYBR Green has been used to quantify ARGs such as *vanA, blaTEM-1, tetA* and *aacC1* in drinking water treatment plants and river water (Zhang *et al.*, 2016; Zhang *et al.*, 2012). Additionally, the methodology has been used to quantify sulphonamide resistance genes *sul(I), sul(II), tet(W)* and *tet(O)* in river sediment samples (Pei *et al.*, 2006). Genes such as *sul1* (Aminov *et al.*, 2001), *tet(O)* and *mecA* (McKinney and Pruden, 2012) have been quantified using qPCR in reclaimed water (Fahrenfield *et al.*, 2013). TaqMan assays have been developed for the quantifiable detection of *vanA* of enterococci, *ampC* of Enterobacteriaceae, and *mecA* of staphylococci in different municipal wastewater samples (Volkmann *et al.*, 2004). Using the assay *vanA* was detected in 21% of the samples, and *ampC* in 78%. The gene *mecA* was not found in municipal wastewater, but in two clinical wastewater samples. The main disadvantages highlighted by some authors is that a bias may be introduced due to difficulty in isolating pure genomic DNA from environmental samples (Smits *et al.*, 2004; Shannon *et al.*, 2007).

2.6.3 Flow-cytometry and cell sorting

Fluorescence-activated cell sorting (FACS) is a direct optical detection method which allows cells to be sorted based on a combination of fluorescence and light scatter (where light scatter is influenced by cell size). Some authors have even described FACS as "automated microscopy". One of the major advantages of flow cytometry over PCR-based assays is that the resulting data is not limited to presence/absence. As flow cytometry assays can test for the ability to reproduce, membrane integrity and metabolic activity, it allows researchers to study viability and physiological condition. Another major advantage is that single cell sorting allows for the recovery of a defined population (based on cellular properties) for further analyses.

Employing flow cytometry in ecology studies of mixed cell populations can be challenging. Firstly, as cytometry requires cells to be in suspension when studying soil communities, bacteria trapped in biofilms and closely associated with inert solid support will be missed. However, studies have shown that density gradient centrifugation using media such as Nycodenz can purify soil bacteria into liquid suspension, and the community analysis results for samples prepared in this manner are comparable to the original communities. A second challenge one must take into consideration is the variability of the source material and the potential of non-specific binding to other biological material such as proteins. Thirdly, there may be high background autofluorescence due to photosynthetic pigments produced by algae/cyanobacteria or minerals present in water samples. Endogenous DNA components such as flavin nucleotides and pyridine can also lead to autofluorescence.

While the most widely used assays in microbial ecology studies are viability assays, assays can test for specific a cellular function (see below). Most viability assays are based on membrane integrity. The LIVE/DEAD BacLight (Invitrogen) assay includes two nucleic acid dyes, green fluorescent SYTO9 and

red fluorescent propidium iodide (PI). Using this dye combination cells with intact membranes fluoresce bright green, whereas bacteria with damaged membranes fluoresce red. Unlike PCR-based methods which cannot differentiate between DNA derived from viable, dead and viable but non-culturable, the BacLight assay allows the researcher to differentiate between these populations.

2.7 THE CHALLENGES OF EXTRACTING NUCLEIC ACIDS FROM ENVIRONMENTAL SAMPLES

The extraction of high-quality biomolecules (such as RNA, DNA and proteins) from a variety of biological material has become the cornerstone of molecular ecology (El-Ashram *et al.*, 2016). In recent years there has been a need for simple and efficient novel methods of extracting both DNA and RNA, from both prokaryotic and eukaryotic organisms, especially with the rapid development of molecular techniques.

Isolated total RNA is widely used in several molecular assays, notably gene-expression analysis using real-time PCR (RT-PCR). Accurate and reliable gene expression data relies on the proper extraction of purified and high-quality RNA (Toni *et al.*, 2018). However, the 2' hydroxyl group attached to the pentose sugar ring of RNA makes the backbone intrinsically more sensitive to breakage than DNA. Hence, extracting intact total RNA for downstream applications is challenging and requires extensive optimisation (compared to DNA extraction which is relatively easy) (Nilsen, 2013).

Nucleic acid isolation can be divided into organic and inorganic extraction methods, as well as solid-phase extraction methods. However, there are four integral steps required for successful RNA isolation and purification which is common to all methods: (1) Effective cell lysis, (2) RNA separation and protein denaturation, (3) RNA precipitation and (4) an effective final RNA wash and solubilization step (John *et al.*, 2008). For microbial ecology studies each of these steps may need to be optimized.

Environmental samples contain a wide range of components that may interfere with molecular analysis techniques, especially when large volumes of water are concentrated into small volumes needed for effective molecular detection. In addition, microbes are typically present in water at low concentrations, which makes it difficult to optimize extraction methods to achieve both high nucleic acid recovery and purity (Hill *et al.*, 2015).

CHAPTER 3: PHENOTYPIC CHARACTERISATION OF MICROORGANISMS ISOLATED FROM GREYWATER SYSTEMS

3.1 INTRODUCTION

Traditional culturing is still widely used to assess water quality and for the detection of resistant microorganisms. Defined- and selective media allow for the rapid identification of specific microbial taxa, especially those which are indicators of faecal contamination. Culture-based testing is also low cost, requires minimal specialised equipment and generally results can be obtained quickly – within 16 to 48 hours depending on the target organisms. The main limitation of culture-based methods is that one is only able to detect organisms which are culturable under the test conditions, and viable but non-culturable (VBNC) organisms will be missed. Studies have shown that in most environments the vast majority of organisms are not culturable and as such, culture-based assays will only be able to detect a small fraction of the total bacterial population present in any environment. Despite this limitation, culturing is still an important tool for routine testing, and provides insight into the microbial population in an environment. The problem of low cell numbers in a water sample can relatively easily be overcome by performing a filtration or centrifugation step which concentrates the cells prior to plating onto solid agar plates.

There are several ways researchers can screen for antibiotic resistance phenotypes using culturing. One of the most widely used is to determine the minimal inhibition concentration (MIC) which is the lowest concentration of an antibiotic which prevents the visible growth of a microorganism. One can also use the disc diffusion methods (or Kirby-Bauer test) to test for antibiotic susceptibility. This chapter specially addresses Aim 1 as it describes the culture-based, phenotypic characterisation of strains isolated from greywater and biofilm samples. While the main focus of this study was the detection of ARGs used DNA-based methods (aka resistance genotyping), a limited complimentary culture-based experiments was performed to isolate microorganisms and determine their resistance phenotype. Antibiotic resistance profiles for the isolates were determined and resistant organisms were identified by 16S rRNA gene sequencing. Phylogenetic analysis was conducted to determine whether the isolated strains were similar to known clinical or environmental isolates.

3.2 METHODS AND MATERIALS

3.2.1 Site Identification

Samples were collected from Pinehurst, a suburb in Durbanville (Western Cape) (GPS -33.839, 18.665). The suburb is predominantly middle class and as a Garden Cities Development belongs to a Home-Owners Association (HOA). All four test systems were similar entry level/mid-range systems (minimal treatment) in order to ‘normalise’ the operational parameters such as treatment, retention time, etc. Two of the systems were operational for over six months at the time of first sampling while the remaining two systems were newly installed (less than three months old).

3.2.2 Sample Collection

Sampling equipment was prepared according to the *Handbook for Water-Resource Investigations* (Wilde, 2004). 10L Nalgene bottles and lids were washed using commercial dishwashing liquid, followed by two thorough rinses with tap water. The bottles were blotted dry and the interior and exterior was washed with 5% v/v hydrochloric acid (HCl) solution. The bottles were left to evaporate the remaining HCl solution in the fume hood. An additional rinse of the bottles was conducted using dH_2O. The bottles were then washed with HPLC Grade Methanol. Finally, the bottles were rinsed using UV-treated Millipore water and stored in clean plastic bags until sample collection.

At each sampling time point a 10L sample of greywater was collected from each respective sample site using the acid-washed 10L Nalgene Bottle. Biofilm samples were collected using a sterile buccal swab by scraping the inside of the tanks (~5cm^2 area) about 5cm below the waterline. As a control, 50 ml of the household main source of potable water (municipal tap water) was sampled at the start of the experiment to serve as a baseline and to ensure that no ARGs/microbes were being introduced by the input water itself. All samples were stored at 4°C at the Institute of Microbial Biotechnology and Metagenomics (IMBM), UWC until they were processed. After sample collection, 50 ml of greywater was aliquoted for microbial analysis and stored at 4°C until needed. A complete list of samples collected during the project is provided in Table 3.1.

Table 3.1: List of greywater and biofilm samples collected and analysed during the project

SEASON	TIME PERIOD
Summer	1 December-28/29 February
Autumn	1 March-31 May
Winter	1 June-9 September
Spring	20 September-30 November

SITE	SAMPLE	GREYWATER SAMPLE	BIOFILM SAMPLE	COLLECTION DATE
1	1	GWS1S1	BFS1S1	8/11/2018
	2	GWS1S2	BFS1S2	25/06/2019
	3	GWS1S3	BFS1S3	05/02/2021
	4	GWS1S4	BFS1S4	10/03/2021
2	1	GWS2S1	BFS2S1	11/11/2018
	2	GWS2S2		22/02/2019
	3	GWS2S3	BFS2S3	28/01/2021
	4	GWS2S4	BFS2S4	08/03/2021
3	1	GWS3S1	BFS3S1	11/11/2018
	2	GWS3S2	BFS3S2	28/02/2019
	3	GWS3S3	BFS3S3	8/11/2019
	4	GWS3S4		28/01/2020
	5	GWS3S5	BFS3S5	14/08/2020
	6	GWS3S6	BFS3S6	15/10/2020
	7	GWS3S7	BFS3S7	17/03/2021
4	1	GWS4S1	BFS4S1	29/01/2019
	2	GWS4S2	BFS4S2	24/05/2019
	3	GWS4S3	BFS4S3	30/05/2019

SEASON	TIME PERIOD			
	4	GWS4S4	BFS4S4	12/11/2019
	5	GWS4S5	BFS4S5	17/02/2020
	6	GWS4S6	BFS4S6	07/09/2020
	7	GWS4S7	BFS4S7	07/10/2020

3.2.3 Microbial Analysis

A ten-fold serial dilution was prepared using 1X Phosphate Buffered Saline (PBS) (pH 7.4) (137mM NaCl, 2.7mM KCl, 10MM Na_2HPO_4, 1.8mM KH_2PO_4). The dilutions were plated on Luria-Bertani (LB), half strength LB and Reasoners 2A agar (R2A) (Merck) plates, in duplicate, using the standard spread plate technique. Plates were incubated for 48 hours at 37°C. The plates were observed the following day to identify any difference in the colonies grown on the different media used and to perform colony counts. Colonies were selected based on unique colony morphologies and a Gram stain was conducted.

3.2.3.1 Phenotypic analysis of antibiotic resistance – Kirby Bauer Disk Diffusion test

Mueller-Hinton agar (Merck) and Nutrient agar (Biolab) were prepared according to the manufacturer's specifications. A sterile swab was placed into the greywater sample and streaked on the agar surface using aseptic techniques, in order to form a bacterial lawn. The entire plate was streaked in one direction followed by rotating the plate 90°C and then streaking the plate again in that direction. This rotation was repeated three times to obtain uniform growth over the entire plate. The plate was left to dry. A 90 mm antimicrobial susceptibility disc dispenser (Oxoid™, South Africa) was used to dispense 10µg Ampicillin, 10µg Cefpodoxime, 30µg Cephalothin, 30µg Teicoplanin or 10µg Streptomycin antibiotic discs on the various plates. The plates were incubated overnight at 37°C and observed the following day by measuring the zone sizes around each disk. The measurements obtained were compared to the CLSI guidelines (Cockerill *et al.*, 2012) to determine incidence of susceptibility to each antibiotic.

3.2.3.2 Microbial identification – Colony PCR, Amplified Ribosomal DNA Restriction Analysis and 16S rRNA gene analysis

A sterile toothpick was used to remove a small amount of cell mass and transfer it to an Eppendorf tube containing 10µl of 1M DTT-Proteinase K lysis buffer (pH 8.0) followed by heating it at 37°C for 20 minutes after which an additional incubation at 95°C for 10 minutes in the thermocycler was conducted. The tubes were centrifuged at maximum speed for 2 minutes. The supernatant was transferred to a sterile tube and 1µl was used in the PCR reaction as the DNA template. A PCR master mixture was set up. Each 25µl reaction contained 0.5U KAPA 2G Robust DNA Polymerase, 0.2mM dNTP mix, 1X KAPA Enhancer 1[3] and 1X KAPA 2G Buffer B, 0.5µM forward primer (F1), 0.5µM reverse primer (R5) (Table 3.2) and made to a final volume using PCR-grade water. A negative control containing all components in the experimental tube excluding DNA was included for all reactions. The PCR conditions were set up as 1 cycle of initial denaturation at 95°C or 3 minutes, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds and extension at 72°C for 15 seconds. The final extension was for 1 minute at 72°C for one cycle. Amplicons were analysed by gel electrophoresis (1% agarose gels supplemented with 0.5µg/ml ethidium bromide).

Table 3.2: 16S rRNA gene primers and amplification conditions used in this study

Primer	Sequence (5'-3')	Amplification conditions
Forward (F1)	AGAGTTTGATCITGGTCAG	95°C for 3 minutes 30 cycles at 95°C for 15 seconds 95°C for 15 seconds 72°C for 15 seconds 1 minute at 72°C
Reverse (R5)	GTATTACCGCGGCTGCTGGCAC	

As isolates may have different colony morphologies when grown on the various media, strains were dereplicated using amplified ribosomal DNA restriction analysis (ARDRA) prior to sequencing. After conducting colony PCR, 12µl PCR product, 5U *Rsa*I enzyme, and 2µl of 10X restriction enzyme buffer was added to a tube and the final volume was adjusted to 20 µl using PCR-grade water. The reactions were incubated overnight at 37b°C. Digestions were analysed on 1.7% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. To load the samples, 4µl of loading dye was added to each digestion and 14 µl was loaded. For the control, 10ul of undigested PCR product was mixed with loading dye. The samples were electrophoresed in TAE buffer for 60 minutes at 100 volts. The gel was visualized under long wavelength UV light at 312 nm. Banding patterns were analysed manually, to assign colonies to a ribotype. A representative from each ribotype was selected for sequencing. Amplicons generated from colony PCR were purified using the Nucleospin® Gel and PCR clean up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The concentrations of the samples were determined using the Qubit broad range DNA assay (Invitrogen) and samples were Sanger sequenced by the Central Analytical Facility at Stellenbosch University.

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation of Resistant Organisms

Microbial analysis of the greywater samples was conducted to determine the microbial community present by culture plate assays. Different media were used to allow for a wide range of organisms to grow – including Luria-Bertani (LB), half strength LB (½ LB) and Reasoners 2A agar (R2A). LB is a general-purpose culture medium which supports the growth of a wide range of facultative organisms and is also used for the analysis of bacterial colony morphology (MacWilliams and Liao, 2006). R2A agar is composed of reduced peptone, dextrose and yeast extract levels. The decreased level of nutrients increases the recovery of chlorine tolerant bacteria that may be present in treated wastewater (Reasoner and Geldreich, 1985). Phosphate Buffered Saline (PBS) is widely used to prepare serial dilutions as it is non-toxic and isotonic to cells. PBS maintains a constant pH of 7.4 which allows for optimal growth of bacteria following the spread plate procedure.

The average microbial numbers are reported in Table 3.2. Bacterial numbers were high with a lawn on the 10^{-1} and 10^{-2} plates, with the densest growth on LB (Figure 3.1). Cell counts on the three media were comparable and ranged from to 2.07 X 10^6 to 3.4 X 10^7 CFU/ml on LB, with lower numbers of R2A (3.05 X 10^5 to 1.56 X 10^7 CFU/ml). Given that greywater likely has high organic loads it is not unexpected that there was significant growth on the LB media – when analysing microbial growth in "natural" water systems one would typically see that there is minimal growth on the nutrient rich media (such as LB) as these organisms tend to be oligotrophic.

Table 3.3: Average bacterial counts for greywater samples.

Sample*	LB	½ LB	R2A
Site 1	2.4 X 10^7 CFU/ml	1.6 X 10^7 CFU/ml	2.7 X 10^6 CFU/ml
Site 2	7.9 X 10^6 CFU/ml	1.06 X 10^7 CFU/ml	5.2 X 10^6 CFU/ml
Site 3	2.07 X 10^6 CFU/ml	2.74 X 10^5 CFU/ml	3.05 X 10^5 CFU/ml
Site 4	3.4 X 10^7 CFU/ml	2.8 X 10^7 CFU/ml	1.56 X 10^7 CFU/ml

*Samples were collected in early winter 2020.

While the pour plating technique is often used for water analysis, it was decided to use a standard spread-plating procedure as it has many advantages including minimal interfering effects on microorganisms that are sensitive to temperature, uncomplicated selection of distinct colony types, and greater flexibility in handling during the procedure and prevents aerobic bacteria from getting trapped in the media (Thomas *et al.*, 2015). It also allows for easier recovery of the isolates which can be used in downstream applications such as genotypic identification. Bacterial numbers were high, based on colony morphology it appeared that the diversity was relatively low (Figure 3.1) with four common morphotypes present. Based on a preliminary visual inspection, it appeared that similar bacteria were present in the greywater and biofilm samples.

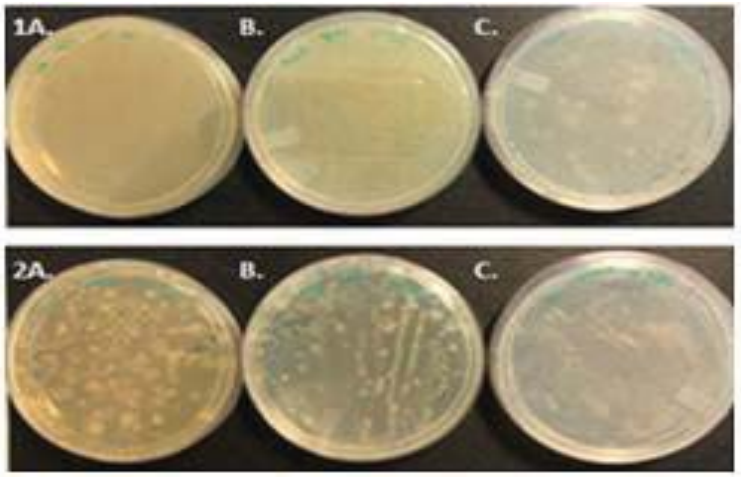


Figure 3.1: Microbial analysis of a greywater serial dilution on LB, ½ LB, and R2A agar. Plates were inoculated using the spread plate technique and incubated at 37° C for 24 hours. Plates 1A, B, and C are spread plates of a 10^{-1} dilution; Plates 2A, B, and C are spread plates of 10^{-4} dilution.

A Gram stain was conducted on a colony from each media to characterise the bacteria present in the sample and all the isolates were Gram-positive bacilli (Figure 3.2). Given the low bacterial diversity, it was also decided to test whether pre-treating the water samples with antibiotic would influence the diversity. Sand filtered and non-filtered greywater samples were treated with 100 µg/ml Ampicillin and spread-plated on the media again. Following incubation, the plates (Figure 3.3) were found to have similar growth as the plates shown in Figure 3.2, which would imply that many of the bacteria present in the greywater have intrinsic resistance to ampicillin. This result is not surprising as it has been reported that the abundance of ARGs which encode resistance to ampicillin were found to be greater in housing areas as compared clinical wastewater (Hong *et al.*, 2018).

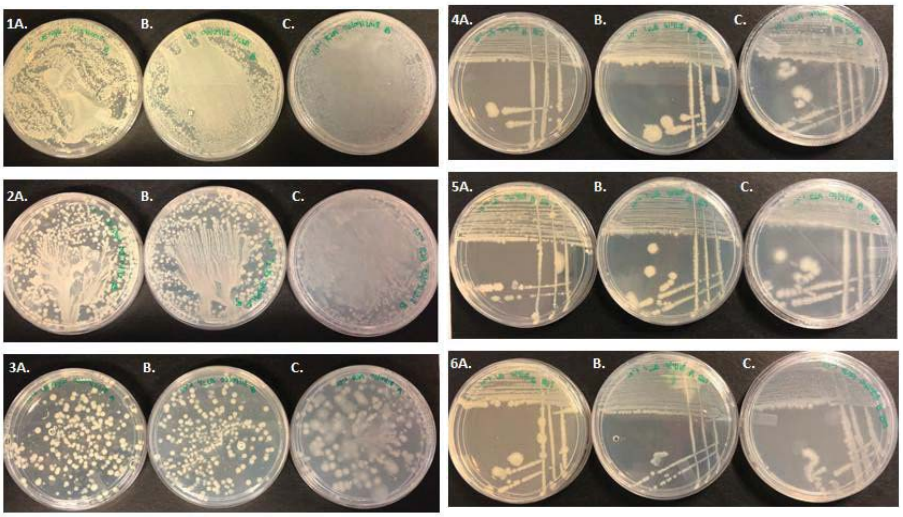


Figure 3.2: Microbial analysis of a greywater serial dilution on LB, ½ LB, and R2A agar. Plates 1 to 3 were prepared via the spread plate technique on LB, ½ strength LB and R2A agar, respectively and incubated at 37°C for 24 hours. Selected isolates were subsequently streaked onto fresh media in order to obtain single colonies.

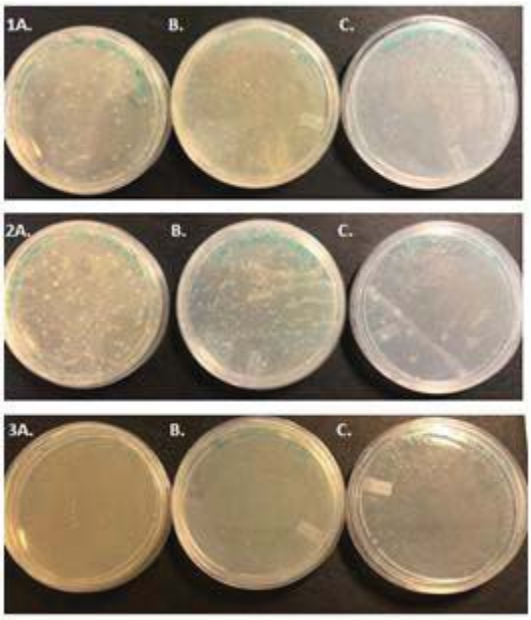


Figure 3.3: Microbial analysis of greywater samples treated with 100µg/ml ampicillin using the spread plate technique on LB, ½ strength LB and R2A agar after 24-hours incubation at 37°C. Plates 1A-C Sand filtered greywater; 2A-C Non-sand filtered greywater; and 3A-C Greywater sample.

The Kirby Bauer disc diffusion method is used for determining the resistance or sensitivity of microorganisms to a broad range of antimicrobial compounds particularly antibiotics (Vineetha *et al.*, 2015). According to literature, studies relating to wastewater treatment plants have indicated that there is an abundance of resistance organisms present (Davies and Davies, 2010). Many of these studies have utilised the Kirby Bauer disc diffusion method as it is easy and inexpensive compared to molecular

methods. The use of the Kirby Bauer method in the current study was to determine the phenotypic expression of antibiotic resistance genes in domestic greywater.

The antimicrobial susceptibility test was conducted on a trial basis using colonies obtained from the microbial analysis of the greywater and biofilm samples (Figure 3.4 and 3.5). For the greywater samples resistance was observed to teicoplanin and cefpodoxime (3rd generation cephalosporin, β-lactam) since no zone clearings were observed after incubation. While teicoplanin is not a routinely used antibiotic, it should be noted that it has a similar action and spectrum as vancomycin and is used to treat infections caused by multi-resistant Gram positive bacteria (Woodford and Johnson, 1994). Worryingly, various studies have shown that Enterococci with multi-drug resistance are resistant to both vancomycin and teicoplanin (Amyes and Gemmel, 1992), however, it was found that the form of resistance observed cannot be inferred from the resistant phenotype alone (Woodford and Johnson, 1994). Cefpodoxime is a third-generation cephalosporin, which displays activity against various Gram negative and Gram positive microorganisms (Chocas *et al.*, 1993). Clinically, the antibiotic is used to treat hospital-acquired infections and community acquired infections including blood, skin, soft tissue, intra-abdominal and urinary tract infections (Kester *et al.,* 2012). Cefpodoxime antibiotics are increasingly important for the treatment of infections resulting from Gram negative bacilli which are commonly resistant to β-lactam antibiotics (Craig and Andes, 2015).

Some of the isolates showed varying degrees of resistance to streptomycin (A*ppendix A, Table 13).* Streptomycin is an aminoglycoside antibiotic which is highly potent and used for the treatment of many life-threatening infections (Mingeot-Leclercq *et al.*, 1999). Streptomycin is not a widely used antibiotic (use predominately for the treatment of MDR infections), as such it was expected that there would be no/low levels of resistance to this drug. Based on the resistance patterns to streptomycin it may indicate the presence of *Enterobacteriaceae* and *Staphylococcus* species in the greywater samples.

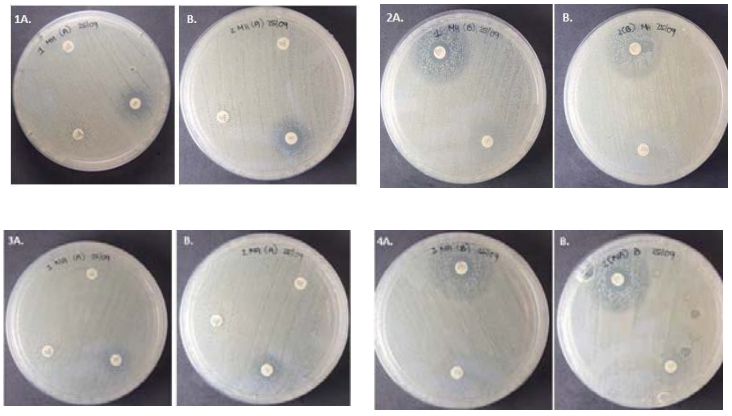


Figure 3.4: The Kirby Bauer test with Ampicillin (AMP10), Cefpodoxime (CPD10), Cephalothin (KF30), Streptomycin (S10) and Teicoplanin (TEC30) antibiotic discs. Greywater samples were plated on Mueller-Hinton (MH) and Nutrient agar (NA) 1A and B) Duplicate MH media plates with AMP10, S10, and TEC10,) 2A and B) Duplicate MH media with CPD10 and KF30, 3A and B) Duplicate NA media plates with AMP10, S10, and TEC10, 4A and B) Duplicate NA media plates with CPD10 and KF30.

Biofilms are proposed to provide a greater protection to both chemical and environmental stressors, with protection conferred from the extracellular polysaccharide matrix (Balcázar *et al.,* 2015). Biofilms contribute considerably to infections in humans and are difficult to destroy with antibiotic therapy (Mai-Prochnow *et al.*, 2016). The Kirby Bauer test was thus conducted on biofilm samples to determine if the phenotypic expression of genes were different as compared to the greywater samples (Figure 3.5).

Culturing directly from the swabs used to collect the biofilm revealed the presence of two distinct colony morphologies (referred to as the big and small organism) similar to what was seen from the water samples. These organisms were tested using the Kirby Bauer method and all the isolates were found to be resistant to teicoplanin *(Appendix A, Table 14).* The bacterium with the large colony morphology was resistant to teicoplanin, streptomycin and cephalothin, whereas the smaller colonies were resistant to cefpodoxime, ampicillin, as well as streptomycin and teicoplanin. Generally, the extracellular polymeric substances (EPS) of a biofilm increases antimicrobial resistance properties and low concentrations of antimicrobial compounds may result in various stress responses increasing HGT (Balcázar *et al.*, 2015; Donlan, 2002).

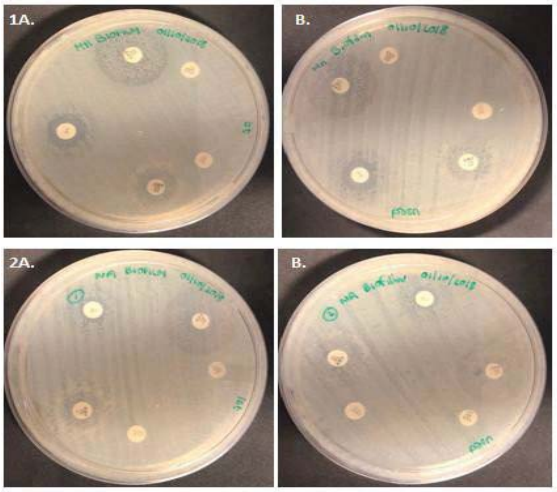


Figure 3.5: The Kirby Bauer test with Ampicillin (AMP10), Cefpodoxime (CPD10), Cephalothin (KF30), Streptomycin (S10) and Teicoplanin (TEC30) antibiotic discs. Biofilm samples were plated on Mueller-Hinton (MH) and Nutrient agar (NA) by streaking the collection swab directly on the agar surface. 1A and B) Duplicate samples on MH media plates with the 5 antibiotic discs, 2A and B) Duplicate samples on NA media with the 5 antibiotic discs.

Direct colony PCR is a technique commonly used for the identification of bacteria as it is fast and inexpensive. The 16S rRNA gene was amplified for all the isolates using this method (Figure 3.6). The resulting amplicons were then used for Amplified Ribosomal DNA Restriction Analysis (ARDRA) to dereplicate the strains. ARDRA is a straightforward technique that is used to classify microbes into phylotypes and was used in this study to dereplicate and select isolates for sequencing. For phylogenetic analysis the amplified bacterial 16S rDNA was digested with the restriction endonucleases *RsaI* (Gich *et al.*, 2000).

The results from the ARDRA are presented in Figure 3.7. Lanes 2 to 10 are digested products from colonies which had the larger colony morphology, while lanes 14 to 20 are the digested products of the smaller colonies. Overall, six different ribotypes were observed (lanes 4, 7, 8, 15, 16, and 20). Each of these unique profiles had corresponding profiles in different lanes from different samples alternating between greywater and biofilm samples. Thus, the same profile was observed in lanes 4, 5 and 10 indicating that the same bacterium is present in both greywater and biofilm samples. Lanes 6, 8 and 9 had the same profile. Lanes 14, 15, and 17 had the same profile, while lanes 16, 18 and 19 had the same profile. The band sizes in lanes 2 and 15 were greater than 1.5kb indicating that it was a mixture of two profiles, which may have resulted from using a mixed culture as template DNA for the colony PCR. Lane 3 had a smear indicating degradation of the sample.

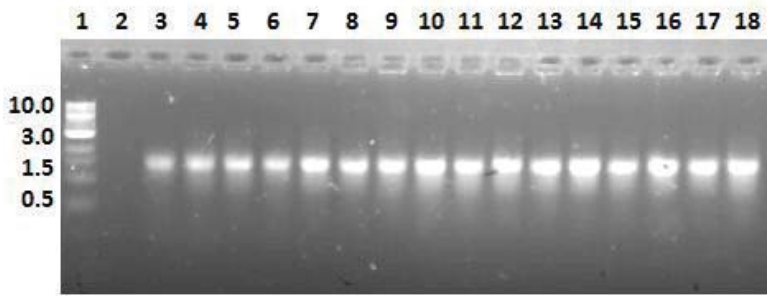


Figure 3.6: A 1% (w/v) agarose gel illustrating colony PCR using colonies obtained from the Kirby Bauer test of greywater and biofilm samples. Lane 1: 1KB NEB marker, Lane 2: Blank, Lane 3: 1, Lane 4: 2A, Lane 5: 2B, Lane 6: 3A, Lane 7: 3B, Lane 8: 4A, Lane 9: 4B, Lane 10: 5A, Lane 11: 5B, Lane 12: 6, Lane 13: 7A, Lane 14: 7B, Lane 15: 8A, Lane 16: 8B, Lane 17: 9A, and Lane 18: 9B.

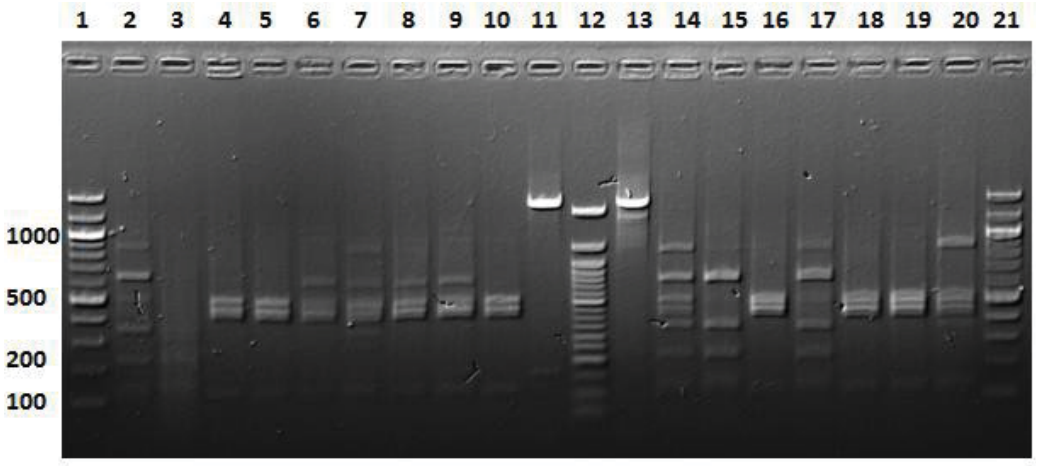


Figure 3.7: Amplified Ribosomal DNA Restriction Analysis of colony PCR products on a 1.7% v/v agarose gel. Lane 1: 100bp marker, Lane 2: 5B, Lane 3: 5A, Lane 4: 4B, Lane 5: 4A, Lane 6: 3B, Lane 7: 3A, Lane 8:2B, Lane 9: 2A, Lane 10: 1, Lane 11: PCR product of sample 1, Lane 12: 50bp marker, Lane 13: PCR product of sample 6, Lane 14: 6, Lane 15: 7A, Lane 16: 7B, Lane 17: 8A, Lane 18: 8B, Lane 19: 9A, Lane 20: 9B, and Lane 21: 100bp marker.

A representative clone from each of the six unique profiles was sequenced (Table 3.4). Isolates 3A and 4B (both greywater samples) were found to be *Klebsiella* species. The genus *Klebsiella* is a common cause of nonsocomial infections and is found in abundance in various environments (Podschun *et al.*, 2001). *Klebsiella oxytoca* is resistant to erythromycin, chloramphenicol, tetracycline, fluoroquinolones, cephalosporins and many others (Fenosa *et al.*, 2009); while *Klebsiella aerogenes* carries resistant determinants against streptomycin and chloramphenicol (Brenchley and Magasanik, 1972). Similarly, isolate 2B (a biofilm isolate) was found to be most similar to either a *Klebsiella* or a *Raoultella* species. *Raoultella* species inhabits natural environments, e.g. soil, plants and most importantly water; however, little is known regarding its antimicrobial resistance profile (Sekowska, 2017).

Enteric and human-associated pathogens are common in greywater. *Pseudomonas* species are widely distributed in aquatic and terrestrial environments having the ability to proliferate even in distilled water

(Gross *et al.*, 2006). Other studies have shown that environmental *Pseudomonas putida* strains were resistant to aminoglycosides, 3rd and 4th generation cephalosporins, carbapenems and extended-spectrum penicillins (Luczkiewicz *et al.*, 2015). *Pseudomonas* and *Aeromonas* species were previously isolated from raw water biofilms in Mafikeng, South Africa (Mulamattathil *et al.*, 2014). Multidrug-resistant *Enterobacteriaceae* were also identified in wastewater treatment plants (Texeira *et al.*, 2016).

In general, based on preliminary 16S rRNA gene analysis similar isolates are present in the greywater and biofilm samples, which is an interesting finding as the samples were collected from an established biofilm. This would imply that the microorganisms found in the water systems are not transient organisms, which has been reported elsewhere. However, this could also imply that the system sampled has a long residence time and it not routinely emptied (or not emptied to completion resulting in conditions which allow the microbes to form "established" communities in the water column). The culturing was repeated on samples collected at several time points and the same findings were observed – high microbial numbers, but limited diversity. The second interesting finding was that similar colony morphologies were found in the greywater and biofilm samples. Again, one would expect that there would be different organisms in the biofilms compared to the water column.

Table 3.4: 16S rRNA sequencing of colony PCR products for bacterial identification in greywater and biofilm samples.

Isolate name	Sample type	Colony morphology	Significant Alignments	Percentage similarity (%)
2B	Biofilm	Big	*Klebsiella aerogenes* *Raoultella* sp. *Enterobacteriaceae*	100% 100% 100%
3A	Greywater	Big	*Klebsiella oxytoca*	99%
4B	Greywater	Big	*Klebsiella oxytoca* *Enterobacter* sp. *Lactococcus lactis* subsp.	100% 100% 100%
7A	Biofilm	Small	*Pseudomonas*	
7B	Greywater	Small	*Enterobacter*	
9B	Greywater	Small	*Enterobacter*	100%

**all bacteria identified were resistant to the antibiotics used in this study*

3.3.2 Assessing antibiotic resistance with gradient plate assay

To successfully isolate bacteria from environmental samples, understanding and mimicking the environmental conditions in the laboratory is vital, especially the composition of the enrichment media used. Generally, growth media used for water samples include nutrient agar (basal media used for the cultivation of non-fastidious bacteria) and R2A agar (a low nutrient medium that supports growth of stressed bacteria used for heterotrophic plate counts from water samples). Varying strengths of nutrient agar were used to allow for the growth of bacteria that generally require reduced nutrient composition for optimal growth, i.e. ½ strength nutrient agar. R2A was expected to have the most bacterial growth,

however, ½ strength nutrient agar had the most bacterial growth (12) followed by R2A agar (6) and lastly full-strength nutrient agar (4) (Table 3.5). Initially bacteria were cultured on MH and R2A, however the MH plates were consistently covered with a slimy film which covered the entire agar surface (for all dilutions). As such, we had to substitute the MH with NA.

By definition, minimal inhibitory concentration is the lowest concentration of the assayed antibiotic that inhibits visible growth of the microorganism under investigation. In this study, the preliminary minimal inhibitory concentrations were tested by using standard antibiotic gradient plates (Table 3.5). Of the 22 isolates tested, three were susceptible to ampicillin and nineteen had visible ampicillin resistance. Of these, one isolate was susceptible to concentrations greater than 25µg/ml, three were susceptible to concentrations above 37.5µg/ml, and sixteen showed resistance levels greater than 50µg/ml.

Table 3.5: Resistance profiles against ampicillin and streptomycin for the strains isolated from biofilm and greywater samples.

Isolate	Sample type	Growth medium	Gram's stain	Ampicillin	Streptomycin
1(* ; **)	Biofilm	½ Nutrient agar	Positive	Resistant [50µg/ml]	Resistant [50µg/ml]
2(**)	GW	½ Nutrient agar	Positive	Resistant [50µg/ml]	Susceptible
3(**)	Biofilm	Nutrient agar	Negative	Resistant [50µg/ml]	Resistant [12.5µg/ml]
4(* ; **)	GW	½ Nutrient agar	Negative	Resistant [50µg/ml]	Resistant [50µg/ml]
5(* ; **)	GW	½ Nutrient agar	Positive	Resistant [50µg/ml]	Resistant [50µg/ml]
6	Biofilm	½ Nutrient agar	Positive	Resistant [50µg/ml]	Resistant [50µg/ml]
7	Biofilm	½ Nutrient agar	Negative	Resistant [50µg/ml]	Susceptible
8	Biofilm	R2A	Positive	Resistant [25µg/ml]	Susceptible
9	GW	½ Nutrient agar	Positive	Resistant [37.5µg/ml]	Susceptible
10	Biofilm	½ Nutrient agar	Positive	Resistant [50µg/ml]	Susceptible
11	Biofilm	R2A	Positive	Resistant [37.5µg/ml]	Resistant [50µg/ml]
12(**)	Biofilm	R2A	Positive	Resistant [50µg/ml]	Resistant [50µg/ml]
13(**)	GW	Nutrient agar	Negative	Resistant [50µg/ml]	Resistant [50µg/ml]
14	GW	Nutrient agar	Positive	Resistant [50µg/ml]	Resistant [50µg/ml]
15	Biofilm	Nutrient agar	Positive	Resistant [50µg/ml]	Resistant [50µg/ml]

(*) Streptomycin resistant colonies included in the phylogenetic characterisation; (**) Ampicillin resistant colonies included in the phylogenetic characterisation

Similarly, eight isolates were susceptible to streptomycin and fourteen had visible streptomycin resistance where one was susceptible to concentrations above 12.5µg/ml and thirteen had resistance greater than 50µg/ml. Of the 22 isolates, 63.64% were resistant to both ampicillin and streptomycin (multi-drug resistant) and 13.64% were susceptible to both ampicillin and streptomycin (Table 3.5).

Based on these findings, ampicillin resistance was the most prevalent in comparison to streptomycin resistance, which is likely due to the fact that bacteria in greywater systems are exposed to ampicillin more frequently. Ampicillin is quite a common antibiotic that it is highly likely that there was residual antibiotics in these systems at some time. On the other hand, streptomycin is only really used to treat selected infections (most notably TB) and the area we sampled is not a TB hotspot neighbourhood. However, Mulamattathil *et al.* (2014) saw a similar trend where all bacterial isolates from drinking water systems were resistant to ampicillin and isolates from one system displayed streptomycin resistance. Eight of the 14 MDR isolates were isolated from biofilm samples and six were isolated from GW samples (Table 3.5). The fact that a greater number of biofilm isolates were resistant is to be expected and can likely be attributed to the extracellular polymeric matrix, which provides a habitat for colonisation and transfer of antibiotic resistance genes (Mulamattathil *et al.,* 2014).

Figure 3.8: Gradient ampicillin plates. From right to left Plate 1 = isolate 9 and isolate 11; Plate 2 = isolate 6 and 7; Plate 3 = isolate 8

Of the 22 isolates the 16S rRNA gene was only successfully amplified from 14 strains for phylogenetic analysis. Of the fourteen isolates, nine belonged to the phylum Proteobacteria, including four from the class Alphaproteobacteria, three from class Gammaproteobacteria, and two belonged to the class Betaproteobacteria. In addition, four isolates belonged to the phylum Bacteroidetes and one isolate belonged to the phylum Firmicutes (Table 3.6). Isolate 1B (Biofilm isolate) appeared to have a slightly altered colony morphology in the presence of antibiotics – ampicillin supplementation made the colony appear matte, whereas streptomycin supplementation made it appear shiny (Table 3.6), which is not unexpected as β-lactams target cell wall synthesis and have been reported to modify cell wall morphology.

16S rRNA gene analysis confirmed isolate 1B was most similar to a *Chryseobacterium indologenes* strain. Typically, *C. indologenes* colonies are round, convex, and produce yellow to orange pigments (Whitman *et al.*, 2015) which are the colony features recorded for strain 1B. *C. indologenes* is a Gram-negative, aerobic bacilli ubiquitously found in plant, soil, food, and water environments (Mehta & Pathak, 2018; Whitman *et al.,* 2015). *Chryseobacterium indologenes* is a MDR bacterium with intrinsic resistance to multiple frontline antibiotics used for serious Gram-negative infections including aminoglycosides, β-lactams, and first generation cephalosporins, and aztreonam (Mehta & Pathak, 2018). In this study, strain 1B was isolated from a biofilm sample and had resistance to both ampicillin and streptomycin (Tables 3.5 and 3.6). Van Wyk (2008) found that *C. indologenes* strain isolated from

the human trachea were resistant to 10µg/ml ampicillin and streptomycin. Even though they are widely found in soil, plants, and food products, chryseobacteria are not considered to be a member of the typical human microflora (Mehta & Pathak, 2018). That being said they are frequently isolated from biofilms associated with hospital environments including patient catheters, feeding tubes, sinks and other equipment in contact with fluid substances (Mehta & Pathak, 2018).

Table 3.6: Species identification based on 16S rRNA gene analysis.

Isolate	Sample type	Colony morphology	% similarity	Significant alignment	Accession number
1B	Biofilm	Yellow, shiny, convex,round	100	*Chryseobacterium indologenes*	CP050961.1
2GW	GW	White, shiny, convex,round	92.23	*Sphingobacterium caeni*	KX664480.1
3B	Biofilm	Opaque, shiny, convex,round	84	*Klebsiella grimontii* strain	MW077302.1
3GW	GW	White, shiny, convex,round	98.34	*Sphingobacterium caeni*	KX664480.1
4GW	GW	Yellow, shiny, convex,round	98.46	*Sphingomonas zeae*	MK475024.1
5GW	GW	White, shiny, convex,round	100	*Sphingomonas zeae*	MK475024.1
6GW	GW	Yellow, shiny, convex,round	99	*Chryseobacterium indologenes*	CP050961.1
7GW	GW	White, shiny, convex,round	100	*Sphingobacterium multivorum*	MF348188.1
12B	Biofilm	Pink, shiny, convex,round	99.21	*Caulobacteraceae bacterium*	MT386225.1
13A	GW	Opaque, shiny, flat,round	100	*Bacillus thuringiensis*	MW242785.1
16A	GW	White, shiny, convex,round	99.39	*Klebsiella grimontii* strain 3830	MT538677.1
17GW	GW	Yellow, matte, flat,irregular	100	*Aquincola tertiaricarbonis*	MN733352.1
18B	Biofilm	Opaque, shiny, convex,round	100	*Delftia tsuruhatensis*	MT605296.1
19B	Biofilm	Opaque, matte, flat,irregular	100	*Rhizobium pusense*	MT573157.1

Phylogenetic analysis (Figure 3.9) showed that both *Chryseobacterium* isolates (1B and 2GW) were most similar to strains isolated from clinical samples. Isolates 2GW and 7GW was most similar to *Sphingobacterium* species. These microorganisms are ubiquitous in nature and can be found in soil, on plants, in foodstuffs and in water sources, but are rarely involved in human infections. A study which compared the microbial communities on different types of greywater filters (sand, charcoal and bark) found that Sphingobacteria accounted for between 7 to 18% of microbes growing on these filters (Dalahmeh *et al.,* 2014). Phylogenetic analysis revealed that isolates were predominantly related to strains isolated from water sources (activated sludge, wastewater, river sediments).

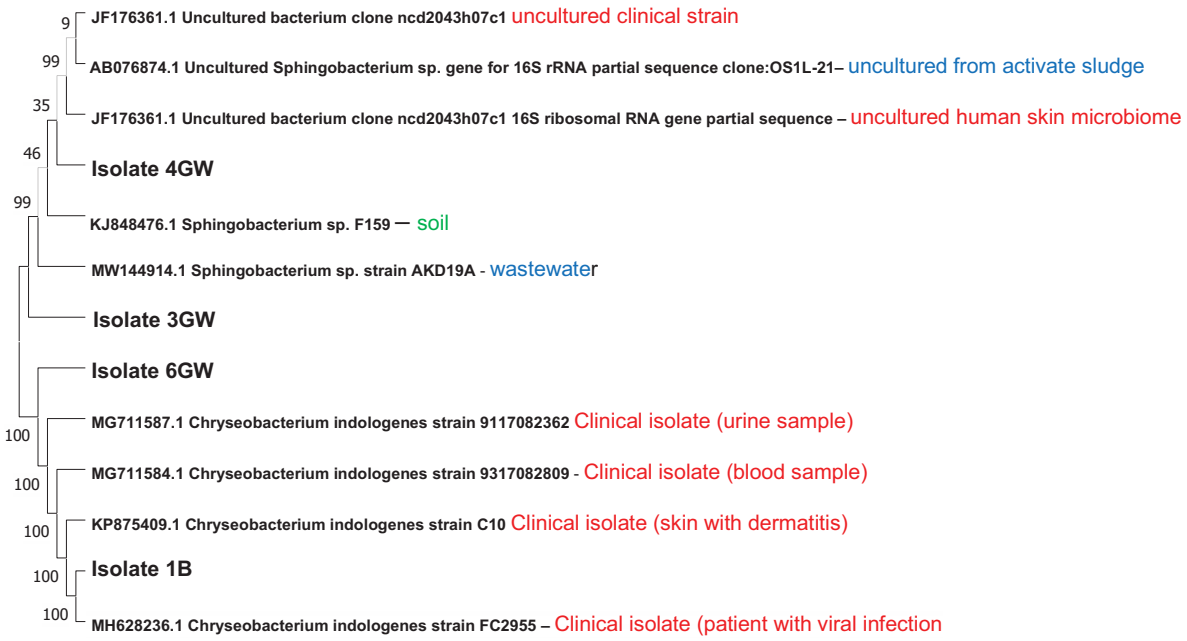


Figure 3.9: Phylogenetic analysis of isolates related to members of the Phylum Bacteroidetes. The tree is based on 540bp of conserved sequence and was constructed using the Maximum Parsimony model. Nearest phylogenetic neighbours are colour coded: red – strain isolated from a clinical sample; blue – associated with marine/wastewater; green – associated with soil/plants.

Figure 3.10 shows the phylogenetic relationships of isolates being to the phylum Alphaproteobacteria. Isolates 4GW and 5GW are greywater isolates, most similar to *Sphingomonas zeae* (98% and 100% similarity, respectively). Members of the genus *Sphingomonas* are Gram negative and widely distributed colonising plant surfaces, soils, groundwater, wastewater treatment plants, and clinical sources (Vaz-Moreira *et al.*, 2011). Phylogenetic analysis revealed that isolates 4GW and 5GW were related to strains associated with soil/plants and water environments (Figure 3.10). Studies have shown that streptomycin resistance is widespread in *Sphingomonas* species and four *Sphingomonas* strains investigated by Vanbroekhoven *et al.* (2004) showed streptomycin resistance to concentrations of up to 200µg/ml.

Isolate 18B (biofilm isolate) had a similarity index of 100% to *Delftia tsuruhatensis* (Table 3.6). *Delftia tsuruhatensis* is a Gram negative, β-Proteobacterium initially isolated from activated sludge from domestic wastewater treatment plant in Japan (Shigematsu *et al.,* 2003). *D. tsuruhatensis* have peptidoglycan-degrading and glucanase enzymes which aid in biofilm formation, hence these bacteria are predominantly found aggregated in biofilms. Bhattacharjee *et al.* (2015) found that *Delftia tsuruhatensis* isolated from a wastewater biofilm was resistant to ampicillin concentrations above 100µg/ml. In this study, isolate 18B showed resistance to both 50µg/ml ampicillin and streptomycin (Table 3.5). Isolate 18B appeared to be most similar to sequences derived from soil and agricultural settings, other than the *Delftia* strain CFB-28 which was isolated as part of a study which looked at used water from washrooms. Isolate 17GW was identified as *Aquincola tertiaricarbonis* (100% sequence similarity). *Aquincola tertiaricarbonis* is a floc-forming bacterium and is often found associated with activated sludge (Yu *et al.,* 2017). Isolate 17GW was similar to a Aquincola *tertiaricarbonis* RN12 isolated from well water (Figure 3.10). While there is limited information available about the strain's specific antibiotic resistance, strains found in wastewater treatment plants have been reported to carry antibiotic resistance cassettes. Lastly, isolate 19B (biofilm) was found to be 100% to *Rhizobium pusense.* While members of the genus *Agrobacterium* (or *Rhizobium*) are environmental bacteria mostly

associated with plants, there is evidence to show that *R. pusense* is an opportunistic pathogen and has been isolated from patients with a range of diseases, as well as the clinical environment (Aujoulat *et al.,* 2015). However, there is minimal data available on these strains' antibiotic susceptibility profiles. There is a study which showed that *R. pusense* strains isolated from the guts of plant feeding insects were resistant to a range of antibiotics including ampicillin (resistance t0 >64µg/ml), kanamycin (an aminoglycoside) and chloramphenicol (Ignasiak & Maxwell, 2017). Phylogenetic analysis showed that isolate 19B was related to strains isolated from agricultural settings, including root nodules and salt tolerant endophytes.

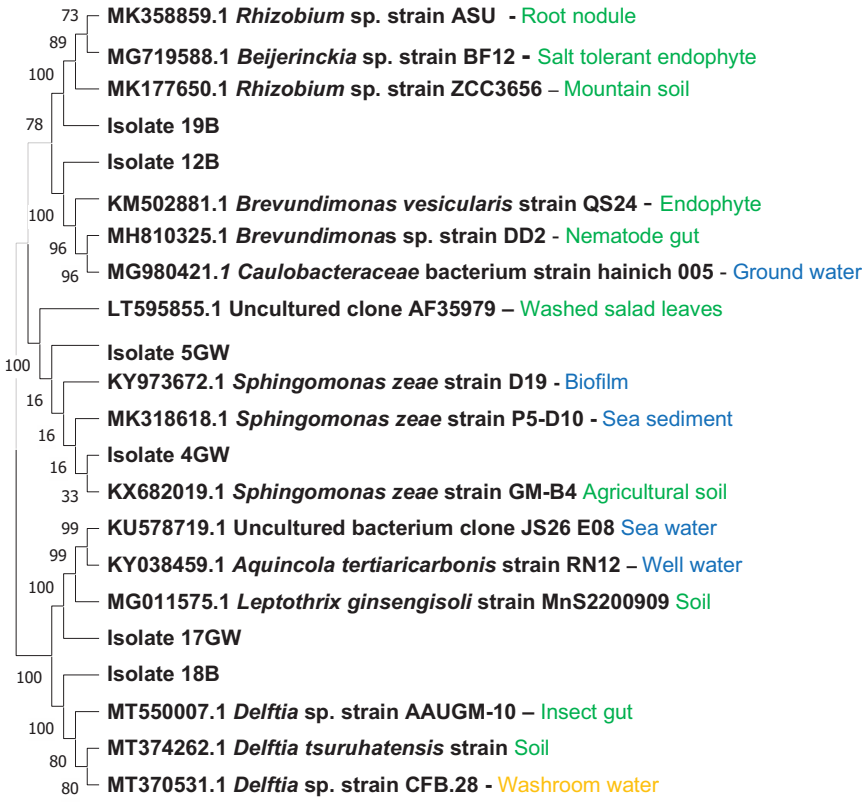


Figure 3.10: Phylogenetic analysis of isolates related to members of the Phylum Alpha Proteobacterium. The tree is based on 612bp of conserved sequence and was constructed using the Maximum Parsimony model. Nearest phylogenetic neighbours are colour coded: red – strain isolated from a clinical sample; blue – associated with marine/wastewater; green – associated with soil/plants; yellow – other

Isolates 3B (biofilm isolate) and 16GW (GW isolate) appeared to be most similar to *Klebsiella grimonti*, with similarity indices of 84% *Klebsiella grimontii* strain SB73 (isolate 3B) and 99.39% for *Klebsiella grimontii* strain 3830 (isolate 16GW) (Table 3.6). The low sequence similarity, may possibly be the result of the presumptive pure colonies used for DNA extraction were in fact a mixed bacterial culture with similar phenotypic appearances, making them appear as a single pure colony. Isolates 3B and 16GW were both Gram-negative and had a phenotypic resistance to both 50µg/ml ampicillin and streptomycin (Table 3.5). *Klebsiella* species are Gram-negative, rod-shaped coliforms (Passet & Brisse, 2018). Hubbard *et al.* (2019) found *Klebsiella grimontii* isolated from water bottle biofilm resistant to 16µg/ml ampicillin. Isolate 13 (GW isolate) was most similar to *Bacillus thuringiensis* (100% similarity). While *B. thuringiensis* is considered predominantly a soil-dwelling microbe, a Japanese study found that nearly 50% of fresh still and running water sources sampled harboured this organism (Ichimatsu *et al.*, 2000).

The bacterial community composition does appear to be affected by seasonal changes. The greywater systems sampled in spring appears to have more bacterial growth and diversity compared when sampled in winter and summer. Additionally, systems sampled in winter had more bacterial growth and diversity than when sampled in summer, and the GW systems sampled in summer had the least bacterial growth. While one should be cautious about inferring too much from relatively few samples, one possible explanation for this finding was that this change in population size is a reflection of changing utilisation patterns – i.e. systems are not used as frequently in winter and spring, so the water has a longer retention time. This may be coupled to the warmer temperatures in spring which would favour microbial growth.

3.4 SUMMARY, CHALLENGES AND FUTURE WORK

Culturing using standard serial dilutions revealed that microbial abundance (or total microbial numbers) in the biofilm and greywater samples were relatively high (3.05×10^5 to 3.4×10^7 CfU/ml), but the species diversity was low with 4-6 species identified in most samples. Similar organisms were consistently detected in all four systems, with *Enterobacter* sp. and *Sphingomonas zeae* detected in greywater, and *Chryseobacterium* found in the biofilms at several sampling points. Better growth was observed nutrient rich agar (nutrient and LB agar) compared to the oligotrophic medium R2A. This aligns with the hypothesis that these greywater systems have high organic loads and would be able support the growth of heterotrophic bacteria. High levels of antibiotic resistance was detected against a range of antibiotics using the Kirby-Bauer method. The greywater samples harboured organisms resistant to teicoplanin and cefpodoxime, while the biofilms had microbes resistant to teicoplanin, streptomycin and cephalosporins.

From the culture-based testing conducted in year 2 of the study, 22 strains were obtained in pure culture, of which 14 displayed phenotypic resistance to both ampicillin and streptomycin, of which 57.14% were biofilm isolates and 42.86% were GW isolates. Moreover, phenotypic resistance to ampicillin was the most prevalent in comparison to streptomycin resistance. Limited phylogenetic analysis identified that the isolates were most similar to clinically relevant strains or strains isolated from soil/water (and the associated environments). The latter likely entered the systems from washing foods such as fruit, vegetables and meat, as well as washing dishes; while the clinical strains are derived from hand washing and bathing. The identified isolates include species from the genera *Chryseobacterium, Sphingobacterium, Klebsiella, Delftia, Rhizobium, Aquincola,* and *Bacillus.* Lower bacterial numbers were recorded in summer, compared to the spring and winter samples. The species diversity likely reflects the “natural” organisms found in the surrounding environment – all four households had gardens (two had vegetable patches), access to clean water, had washing machines and dishwashes. It is likely that if one were to analyse greywater collected in buckets from a lower income household/informal dwelling that the diversity would be different.

The limitations of a culture-based study are that only organisms which were able to grow under the tested conditions would be detected and screened for resistance. As such, if one only performs a phenotypic study the resistance profiles generated may not be a true reflection of what is happening in these systems. Ideally, were possible both DNA-based and culture-based analysis should be performed. The major advantage of culture-based studies is that well established protocols are available, and one can obtain results relatively quickly, unlike DNA-based analysis which can take several months to optimise. As these methods are also relatively simple, it is easy to train personnel to perform tests and interpret the results. Another limitation of culturing is that in order to detect viable organisms and have plate counts which are a true representation in terms of diversity and abundance, the water samples cannot be stored. Ideally, the samples should be stored at cool conditions during transport and processed immediately.

CHAPTER 4: DEVELOPMENT OF NOVEL ASSAY FOR THE DETECTION OF B-LACTAMASE PRODUCING ORGANISMS

4.1 INTRODUCTION

One of the aims of this study was to develop a functional ARG-specific flow cytometry assay which utilises a fluorescent enzymatic substrate to label cells according to the expression of a specific enzyme activity. Due to the structural conservation of the lactam ring in β-lactam antibiotic which is essential for its mode of action it was decided that this would be a possible target to develop an assay for organisms with the ability to produce β-lactamases. Bacteria acquire resistance to these compounds by producing a β-lactamase which cleaves the β-lactam ring, rendering the cells resistant. CCF2-FA (Free Acid) is a fluorescence resonance energy transfer (FRET) substrate that consists of a cephalosporin core linking B7-hydroxycoumarin to fluorescein. While there are enzymatic assays using these substrates, currently there are no flow cytometry assays. A flow cytometry assay would have several advantages over other assays. The main advantage for environmental monitoring is that is does not require the cells to be obtained in pure culture prior to analysis and can be adapted to high throughput analysis. An ideal FACS assay would be one which required minimal sample processing prior to analysis. For the proposed assay the only processing which would be required is the concentration of cells via centrifugation, after which the cells would be incubated with the substrate in the dark (chromogenic substrate) for ~ 1 hour prior to flow cytometry analysis. Positive cells (cells which display the desired colour change) and negative (no colour change) 'events' will be collected separately for subsequent analysis. Cell sorting would allow the resistance phenotype (aka the cells that changed colour) to be confirmed by culturing in the presence of the target antibiotic.

The advantages of FACS assays over DNA- and culture-based assays are numerous. Firstly, the most important advantage is that the assay will only detect genes which are actively expressed under specific conditions and therefore reflects the changing dynamics in a community. As such it allows for real-time monitoring and will allow us to monitor how quickly a population responds to the addition of an antibiotic to the system. Secondly, FACS assays will detect enzyme activity in bacterial strains which are not culturable and therefore gives a true reflection of population numbers without the bias introduced by culturing which favours the fast-growing heterotrophs. Lastly, PCR methods rely on primers based on known sequences therefore may only detect specific bacterial taxa and “new” genes could also be missed. As FACS on gene expression not a DNA sequences it would not have the same limitations as PCR. Despite these advantages, there are disadvantages. Firstly, the initial development of new assays is time consuming and different sample types may require optimisation in terms of sample processing. Secondly, as FACS instruments are expensive there will always be concerns about accessibility, however with the development of newer benchtop models they are becoming more accessible (including instruments used for field stations). The last limitation is the stability/limited shelf-life of probes.

The work presented in this chapter specifically align with Aim 4 “Development of novel FACS assays to screen for ARGs from environmental samples”. We encountered several operational challenges in completing this work, including having to develop an entirely new assay as the original proposed substrate is no longer available. Chemicals required to synthesize the one substrate also took 8 months to arrive as it was on back order (global supply issues linked to Covid). Despite these problems, two different assays were tested and the assay using the fluorescein-meropenem substrate we synthesised in the lab gave promising preliminary results.

4.2 MATERIAL AND METHODS

4.2.1 CCF-AM Assay

Note: The β-lactamase substrate CCF2-AM is challenging to synthesize and therefore it was decided to rather purchase the substrate already labelled as it is part of a commercially kit (GeneBLAzer™ *In Vivo* Detection Kit cat no. 12578134). This substrate is used in gene reporter assays in eukaryotic cells, therefore we needed to optimise the assay in prokaryotic cells.

CCF2-AM stocks were prepared as per the manufacturer's instructions. A β-lactamase producing *P. aeruginosa* clinical isolate was obtained from a diagnostic laboratory at Tygerberg Hospital. The strain was cultured in 10 ml LB with and without 10μM ampicillin overnight at 37°C shaking. Cells were harvested by centrifugation at 6000 rpm for 2 minutes, and the cells were washed once in Hank's Balanced Salt Solution (HBSS) and centrifuged as before. Cells were resuspended in fresh HBSS and the optical density of the culture was determined at 600nm and the culture was diluted to 10^6 and 100μl aliquots were added to each well of a black walled microtitre plate. To perform the assay, 10μl CCF-AM prepared in 6X CCF-AM Enhanced Loading Solution was added to each well, to obtain a final substrate concentration of 1μM. To test the optimal loading temperature reactions were placed at room temperature (~22°C), 30°C and 37°C; and loading times of 15, 30 and 60 minutes were tested. The plates were protected from light at all times and incubated under the different test conditions. Readings were taken every 15 minutes using a fluorescence plate reader. Prior to reading the excess loading solution was removed and the cells were washed in HBSS, after which they were resuspended in fresh HBSS. The blue and green fluorescence signal was measured at 460nm and 530nm, respectively.

4.2.2 Synthesis of the substrate

The fluorescent substrate was synthesized according to the method of June *et al.*, 2014. Fluorescein-meropenem (FM) was prepared by mixing 44 mg of fluorescein-isothiocyanate with 45 mg meropenem/14 mg sodium bicarbonate in 1.0 mL N,N-dimethylformamide (DMF). The reaction proceeded at room temperature overnight, stirring in the dark. The product was isolated using preparative thin-layer chromatography on silica gel using ethyl acetate: acetic acid; 85:15 (v/v) as the mobile phase. The fluorescent product had a Rf of 0.6 and was scraped off the plate (Figure 4.1a). The product was eluted with 3 mL of DMF and precipitated by the addition of excess ethyl acetate. The purified fluorescein-meropenem was collected by centrifugation and stored at -80°C (protected from light). Fluorescence was confirmed by the viewing the TLC plates under long wave UV light (Figure 4.1b). Although the substrate was successfully synthesised the yield of labelled was relatively low (based on mass of labelled substrate) – it is likely that some substrate was lost during the preparative TLC step, and an alternative option would be to use HPLC in the future.

4.2.3 Optimisation of the assay

The substrate was tested on a pure culture of *Enterobacter cloacae* subsp. *cloacae* ATCC BAA-1143 (positive control) and *Escherichia coli* ATCC 25922 (negative control). The initial assay involved growing cultures overnight, harvesting the cells by centrifugation and resuspending the pellet in phosphate buffered saline. The substrate (50nM) was added to the cells which were incubated at room temperature (~22±2°C) for 2 hours in the dark. As the target β-lactamases are typically intracellular the cells were assayed for a change in fluorescence. Unfortunately, there was no difference in the detectible fluorescence between the test and control sample. The one possible reason for this is that the cultures do not constitutively express the β-lactamase gene. Therefore, trial experiments were set up in which the overnight cultures were incubated in the presence of different β-lactam antibiotics – penicillin,

ampicillin and cephalosporin at 12.5, 25 and 37.5µg/ml. The control strain was found to be able to grow in the presence of 6.25µg/ml of ampicillin. Following incubation, the cells were processed as before.

4.3 RESULTS AND DISCUSSION

4.3.1 Original Proposed Assays

In the initial funding application, it was proposed that two assays would be developed, one using CCF2 and the other using Fluorocillin Green as the substrates. However, as of 2019 Invitrogen (the sole supplier) has discontinued Fluorocillin Green. Initial attempts to source the labelled substrate elsewhere resulted in substantial delays (> 6 months) and the new supplier was ultimately unable to provide the substrate in small amounts – as the compound is synthesised to order the supplier would require us to purchase at least 100 mg and the quoted price for this substrate in March 2019 was R56 000 (excl. VAT). Therefore, it was decided to rather development a new assay using a different substrate. After an extensive literature search, it was decided to develop an assay using the carbapenem antibiotic meropenem as the substrate which would be conjugated to fluorescein. The first assay using the commercial substrate CCF2-AM (Thermo) was conducted as originally stipulated.

4.3.2 CCF2-AM Assay

CCF2 consists of a cephalosporin core linked to two fluorophores, 7-hydroxycoumarin and fluorescein (Figure 4.1). Based on a literature search it was decided that in order to optimise cellular uptake of the substrate the esterified form, CCF2-AM, and not the free acid form would be utilised. Unlike the free acid, esterified CCF2-AM is lipophilic and membrane permeable, therefore it readily enters the cell. Once inside the cell it is converted into CCF2 due to the action of endogenous cytoplasmic esterases. As the cleaved form is hydrophilic it is trapped inside the cell the principle of assay is that during the incubation period the cells become “loaded” with more substrate, increasing the intracellular concentration thereby improving the sensitivity of the assay.

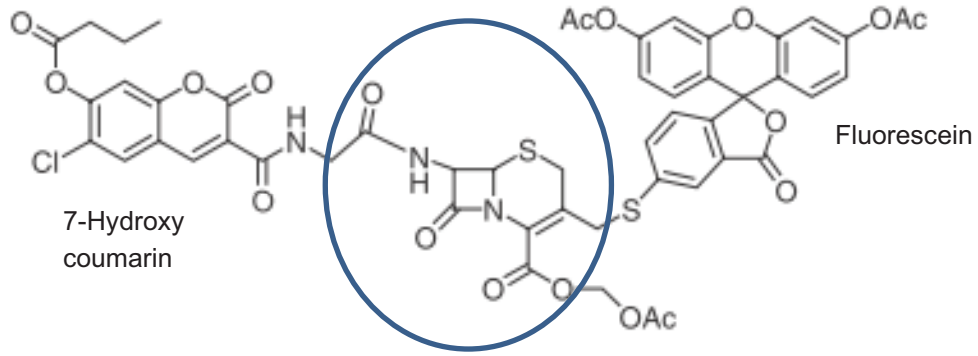
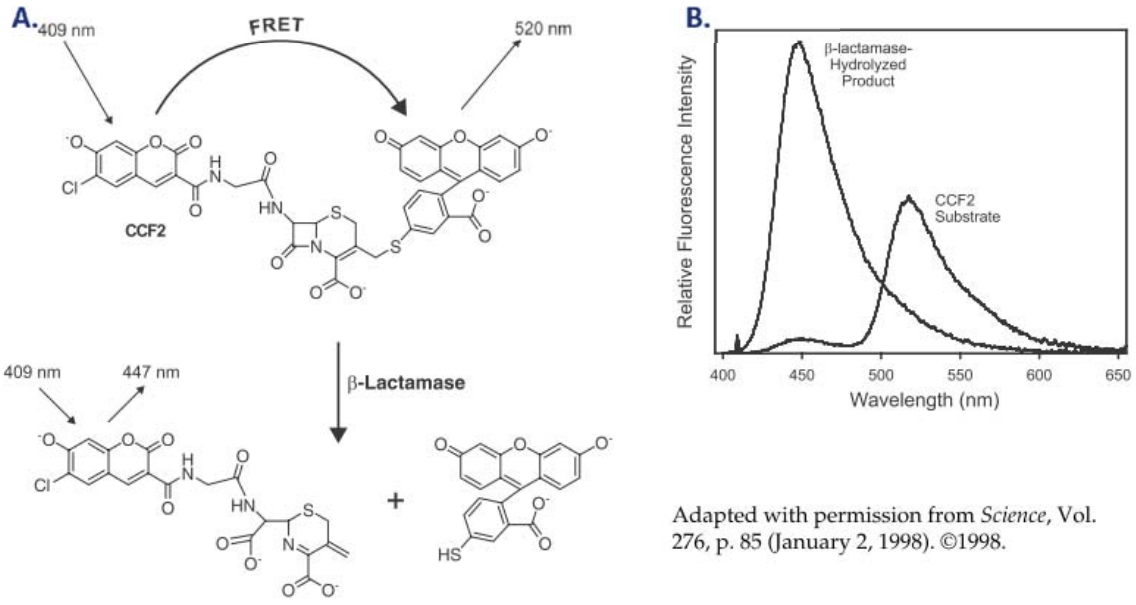


Figure 4.1: Chemical structure of CCF2-AM. The cephalosporin core structure is circled, while the two fluorophores have been identified.

In theory in the absence of β-lactamase the substrate molecule is not cleaved and remains intact. Excitation of the coumarin at 409 nm results in fluorescence resonance energy transfer (FRET) to the fluorescein moiety (Figure 4.2a). This energy transfer causes the fluorescein to emit a green fluorescence signal with an emission peak at 520nm. In the presence of β-lactamase activity the CCF2 substrate is cleaved, disrupting FRET. Excitation of the coumarin at 409 nm results in emission of a

blue fluorescence signal with an emission peak of 447 nm (Figure 4.2b). In a population of cells loaded with CCF2 substrate, those that fluoresce blue express cytoplasmic lactamases, while those that fluoresce green do not.



Adapted with permission from *Science*, Vol. 276, p. 85 (January 2, 1998). ©1998.

Figure 4.2: Overview of the β-Lactamase – catalysed hydrolysis of a CCF2 monitored by a change in fluorescence emission of a substrate.

The main limitation of this *in vivo* assay is that it would only be able to detect intercellular (cytoplasmic) enzyme activity. Research has shown that the β-lactamases produced by Gram negative bacteria are intracellular and located within the perisplasmic space, while in Gram positive bacteria they are mainly excreted from the cell and are thus extracellular. Therefore, it was decided to test the substrate using the Gram-negative pathogen *Pseudomonas aeruginosa,* which would produce an intracellular β-lactamase. In addition, as this organism had been detected in several of the sample sites, it was ideal to optimise the assay on an organism which would be present.

As the GeneBLAzer kit is designed for mammalian cells we needed to perform extensive optimisation testing different loading solutions, incubation time and temperatures, and different concentration of test cells (tested over range of 10^3 to 10^8). While we were able to detect fluorescent signal, absorption of the substrate was very unstable (this is stated in the manual supplier even when using mammalian cells). The detectible signal dropped within 15 minutes. Given the time it takes to prepare the loaded cells for analysis it was decided that it would not be ideal to have an assay which requires all the samples to be analysed within 10 minutes – especially if one is looking at developing a high throughput method for analysis of many samples. Also, optimal cell density was 10^6, with inconsistent results obtained when low densities were tested. Ideally, as the idea was to design an assay which could be used directly on environmental samples which would likely have low cell numbers, as such the assay would need to be sensitive to detect relatively few cells.

4.3.3 Development of fluorescein-meropenem Assay

Meropenem (sold as Merrem) is a broad spectrum carbapenem (Figure 4.3). It is listed on the World Health Organisation's *List of Essential Medicines* and is administered intravenously for the treatment of meningitis, intra-abdominal infection, pneumonia, sepsis and anthrax. Bacteria typically acquire resistance to meropenem by producing metallo-β-lactamases.

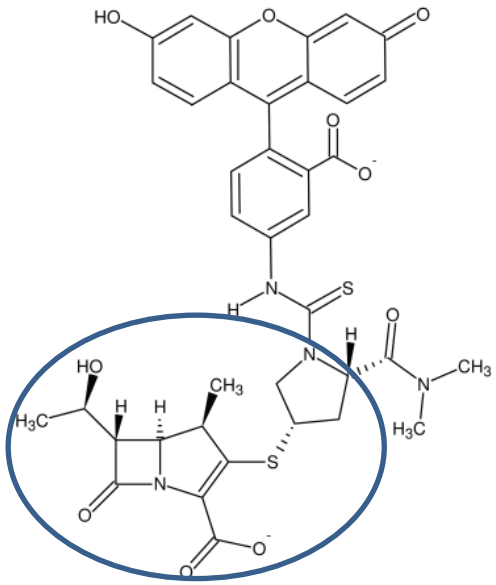


Figure 4.3: Chemical structure of meropenem coupled to the fluorophores. The meropenem core has been circled.

4.3.3.1 Synthesis of substrate

The fluorescent substrate was synthesized according to the method of June *et al.* (2014). Fluorescein-meropenem (FM) was prepared by mixing 44 mg of fluorescein-isothiocyanate with 45 mg meropenem/14 mg sodium bicarbonate in 1.0 mL N,N-dimethylformamide (DMF). The reaction proceeded at room temperature (~22°C) overnight, stirring in the dark. The product was isolated using preparative thin-layer chromatography on silica gel using ethyl acetate: acetic acid; 85:15 (v/v) as the mobile phase. The fluorescent product had a Rf of 0.6 and was scraped off the plate (Figure 4.4a). The product was eluted with 3 mL of DMF and precipitated by the addition of excess ethyl acetate. The purified fluorescein-meropenem was collected by centrifugation and stored at -80°C (protected from light). Fluorescence was confirmed by the viewing the TLC plates under long wave UV light (Figure 4.4b).

The appearance of a fluorescein containing spot at the expected Rf value confirmed that the substrate had successfully been synthesised. The one disadvantage of this method is that it uses preparative TLC so the substrate may not be of the required purity to be used in a FACS assay. If it is determined that the substrate is impure HPLC can be used to purify the substrate prior to analysis.

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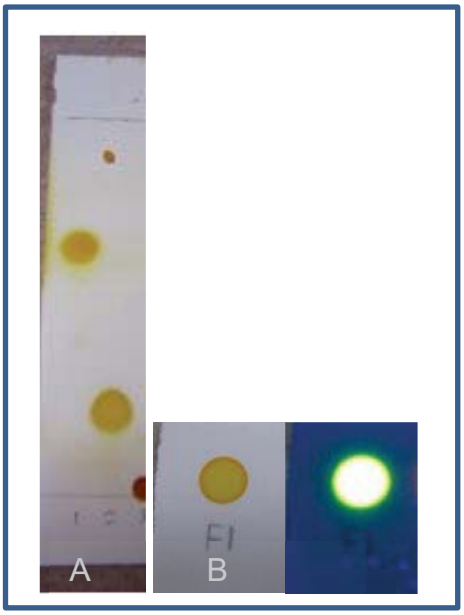

Figure 4.4: TLC used to monitor the synthesis of fluorescein-meropenem substrate. A) Putative substrate in lane 1 with an expected Rf of 0.6. B) Confirmation that fluorescein was detectible on via TLC.

4.3.3.2 Optimisation of the assay

The substrate was tested on a pure culture of *Enterobacter cloacae* subsp. *cloacae* ATCC BAA-1143 (positive control) and *Escherichia coli* ATCC 25922 (negative control). The initial assay involved growing cultures overnight, harvesting the cells by centrifugation and resuspending the pellet in phosphate buffered saline. The substrate (50 nM) was added to the cells which were incubated at room temperature 22°C for 2 hours in the dark. As the target β-lactamases are typically intracellular the cells were assayed for a change in fluorescence. Unfortunately, there was no difference in the detectible fluorescence between the test and control sample. The one possible reason for this is that the cultures do not constitutively express the β-lactamase gene. Therefore, trial experiments were set up in which the overnight cultures were incubated in the presence of different β-lactam antibiotics – penicillin, ampicillin and cephalosporin at 12.5, 25 and 37.5μg/ml. The control strain was found to be able to grow in the presence of 6.25μg/ml of ampicillin. Following incubation, the cells were processed as before.

An increase in fluorescence was detected for the *E. cloacae* culture incubated with the two lower concentrations of ampicillin (12.5 and 25μg/ml) compared to same strain incubated without antibiotic and the control *E. coli* culture. Interestingly, there was a negligible increase in fluorescence for the cultures pre-treated with the penicillin, while there was a high level of background signal for the cells incubated with cephalosporin. The findings in Table 4.1 strongly suggest that the substrate was taken up by the *E. cloacae* cells and is interacting with the β-lactamase produced by the strain. In the future anisotropy can be used to confirm the interaction between the fluorescent substrate and the putative β-lactamase if required – but this experiment would require a large amount of substrate. The labelled substrate was stable for 2 months when stored at -80°C for 2 months but dropped significantly after this. The article which described the synthesis (June *et al.* 2015) only reported stability to 2 months. Future work would include looking at methods to increase the stability of the substrate.

Table 4.1: Detecting an increase in fluorescence in β-lactamase producing strain due to the uptake of the substrate.

Culture	Antibiotic concentration (µg/ml)	Peak wavelength
E. cloacae (test sample)	12.5 amp	~522 nm
	25 amp	~530 nm
	37.5 amp	No peak
	No antibiotic	No peak
E. coli (negative control)	6.25 amp	No peak

Once the substrate synthesis and assay are optimised it would be necessary to first perform DEAD/LIVE Assay to ensure that the experimental samples are viable, and to test for the presence of background fluorescence due to fluorescent compounds or autofluorescence from photosynthetic organisms. Additional optimisation will also be required to determine how soon after the samples are collected, they need to be assayed without any loss of viability.

4.4 SUMMARY, CHALLENGES AND FUTURE WORK

Two different substrate/assays were tested. The first assay used a commercial cephalosporin substrate, CCF2-AM and could be used to detect isolates which are producing intracellular β-lactamases. As such the assay is likely limited to detecting Gram negative strains. However, although the initial results looked promising there were several problems with using this substrate. Firstly, CCF2-AM is currently only available as part of a kit and all components of the kit are optimised for eukaryotic cells. Therefore, although the cells did take up the substrate it was unstable, which means that the samples would need to be analysed as soon as the reaction was completed – limiting its use as a high throughput assay. In addition, the cost of the substrate is also likely to be prohibitive and as such it was decided to rather focus on the second assay.

This assay required us to synthesize our own substrate by coupling the carbapenem antibiotic meropenem to fluorescein. Initial assay development was performed with substrate we obtained from a collaborator. The synthesis procedure was optimised and the preliminary data looks promising. Unfortunately, this occurred just before lockdown and when we later tested the substrate we found that it had lost significant activity (likely only stable for 6 weeks) and would therefore need to synthesis more substrate. Future work will include testing the substrate on known β-lactamase producing clinical isolates, as well as greywater strains isolated in this study. We will then proceed to test the assay using a FACS, initially with pure cultures and then with greywater samples. From our preliminary results we are optimistic that the assay will be adaptable to a FACS, however, if this is not possible it can also be performed using a fluorescence plate reader. Additionally, the substrate is significantly cheaper than other commercially available β-lactamase substrates. The main disadvantage of this assay is that it would only detect metallo-β-lactamases. Within a clinical setting strain which produce metallo-β-lactamases are of particular concern, as due to the enzymes activity these strains are effectively resistant to all β-lactam antibiotics. Despite the challenges in developing this assay, the initial findings are promising, and it is still worthwhile pursuing this substrate.

The main advantage of this assay is that traditional culture-based antibiotic resistance assays are limited to organisms that can be grown on media in the laboratory, and as such, do not provide a full assessment of the resistance present in an environmental sample. While DNA-based methods can circumvent some of the limitations of culture-based testing, there is still a need for phenotyptic assays which complement molecular testing. Hopefully, once this assay is fully optimised, it would allow for the *in vivo* detection of enzyme activity, without having to culture organisms first. By coupling to assay to a FACS, cells with activity can be collected for additionally testing (both molecular and culture-based).

CHAPTER 5: OPTMISATION OF NUCLEIC ACID (DNA AND RNA) EXTRACTION PROTOCOL

5.1 INTRODUCTION

The extraction of high-quality biomolecules (such as RNA, DNA and proteins) from a variety of biological material has become the cornerstone of molecular ecology (El-Ashram *et al*., 2016). In recent years there has been a need for simple and efficient novel methods of extracting both DNA and RNA, from prokaryotic and eukaryotic organisms, especially with the rapid development of molecular techniques.

Isolated total RNA is widely used in several molecular assays, notably gene-expression analysis using qPCR. Accurate and reliable gene expression data relies on the proper extraction of purified and high-quality RNA (Toni *et al*., 2018). However, the 2' hydroxyl group attached to the pentose sugar ring of RNA makes the backbone intrinsically more sensitive to breakage than DNA. Hence, extracting intact total RNA for downstream applications is challenging and requires extensive optimisation (compared to DNA extraction which is relatively easy) (Nilsen, 2013). Environmental samples contain a wide range of components that may interfere with molecular analysis techniques, especially when large volumes of water are concentrated into the smaller input volumes required for nucleic acid extraction. In addition, microbes are typically present in water at low concentrations, which makes it difficult to optimize extraction methods to achieve both high nucleic acid recovery and purity (Hill *et al.,* 2015).

Nucleic acid isolation can be divided into organic and inorganic extraction methods, as well as solid-phase extraction methods. However, there are four integral steps required for successful RNA isolation and purification which is common to all methods: (1) Effective cell lysis, (2) RNA separation and protein denaturation, (3) RNA precipitation and (4) an effective final RNA wash and solubilization step (John *et al.,* 2008). For microbial ecology studies each of these steps may need to be optimized.

Numerous studies have shown that due to inherent biases and differences in efficiencies, the DNA extraction method employed can ultimately affect which microbes are detected in environmental sample, specifically in water distribution systems (Hwang *et al*., 2012). The co-extraction of PCR-inhibitors, incomplete cell lysis, cell damage or degradation of DNA often occurs resulting in unsuccessful DNA isolation (Miller *et al*., 1999), which subsequently will influence all downstream analyses. For this reason, in the present study different DNA extraction protocols were initially tested to compare the quality and quantity of DNA yields from greywater and biofilm samples.

The work presented in Chapter 5 describes the foundational work and optimisation process which was required for the successful completion of Aim 2 (Enumeration of ARGs in various greywater sources using qPCR) and Aim 5 (Screen for the presence of integrons as markers of horizontal gene transfer). The various steps involved in developing the extraction protocols needed to reproducibly extract high quality DNA and RNA from greywater and biofilm samples is described. Several protocols were initially tested, and the outcomes of the different methods is compared. Lastly, the essential quality tests which ensures that the extracted nucleic acids are pure and free from inhibitors is outlined.

5.2 METHODS AND MATERIALS

5.2.1 Pre-processing of greywater and biofilm samples for DNA/RNA extraction

For molecular analysis, 8L of greywater was sequentially centrifuged, 500 ml at a time in metal-screw capped bottles (Nalgene) at 5000 rpm for 20 minutes using the Sorvall Lynx 6000 centrifuge (Thermo Fischer Scientific, MA, USA). The resulting pellet was resuspended in 100 ml of sterile diethyl pyrocarbonate (DEPC)-water. Sample processing of biofilm samples was conducted by submerging the buccal swab in 250µl of 1X Phosphate Buffer Saline (PBS) and vortexing for 5 minutes at maximum speed to dislodge bacteria. Cells were harvested by centrifugation at 13 500 rpm for 10 minutes and the pellet was resuspended in 500µl of 1X TE (10mM Tris, 1mM EDTA, pH 8.0), of which 250µl of the resulting suspension was then used for DNA extraction.

5.2.2 DNA extractions of greywater samples

5.2.2.1 Phosphate, SDS, and Chloroform Bead-Beater method

DNA was extracted from greywater samples using a modified version of Miller *et al.,* 1999. Bead-beater vials were made using 15 ml Greiner tubes filled with 0.5 g of 0.5 mm and 0.3 mm silica-zirconium beads. A greywater sample of 500 µl was centrifuged in 300 µl of phosphate buffer. After centrifugation, 800µl of the resuspension and 300 µl of SDS lysis buffer was added to the bead-beater vials and vortexed to thoroughly mix the sample. 300µl of chloroform:isoamyl alcohol (24:1; v/v) was added to the sample and vortexed at maximum speed for 2 minutes. To pellet the cellular debris the samples were centrifuged in a microfuge at 15000 x*g* for 5 minutes. The supernatant (~650µl) was transferred to a new tube and 7M NH_4OAc was added to a final concentration of 2.5M. The samples were shaken by hand, and then centrifuged at full speed for 5 minutes. Clear supernatant (~580µl) was transferred to a clean tube followed by the addition of 315µl of cold isopropanol. Samples were incubated at room temperature (~22±2°C) for 5 minutes. The supernatant was carefully removed, and the pellet was washed with 1 ml of 70% ethanol. The samples were centrifuged at 12000 x*g* for 5 minutes. The supernatant was removed, and the pellet was allowed to dry for 15 to 45 minutes. DNA was resuspended in 100µl of water or 1M Tris buffer (pH 7.0) and stored at -20°C for short term storage.

5.2.2.2 PowerSoil® DNA Isolation Kit

DNA was also extracted using the PowerSoil® DNA Isolation kit (MoBio Laboratories, Carlsbad) according to the manufacturer's instructions. DNA was extracted by adding 250µl of the centrifuged greywater samples to the PowerBead Tubes. DNA concentration was determined as outlined earlier.

5.2.2.3 16S rRNA gene Polymerase Chain Reaction

PCR was carried out in 25 µl reaction volumes. Each reaction contained 0.5U KAPA 2G Robust DNA Polymerase, 0.2 mM dNTP mix, 1X KAPA Enhance and 1x KAPA 2G Buffer B, 0.5µM forward primer (F1), 0.5µM reverse primer (R5) and made to a final volume using PCR-grade water and approximately 1 ng of DNA. The PCR primers and amplification conditions are given in Table 3.1. PCR products amplified were electrophoresed on 1% w/v agarose gels. All gels contained 10µg/ml ethidium bromide and were electrophoresed in 1X TAE buffer at 90V for 1 hour.

5.2.3 RNA extraction from greywater samples

5.2.3.1 Sample Processing for RNA Extractions

Figure 5.1 provides a schematic presentation of the protocol used to extract RNA from the greywater samples. For RNA extractions using the Hot-Phenol SDS Method, a modified method of Jahn *et al.* (2008) was used. Following sequential centrifugation, the resulting pellet was resuspended in sterile DEPC-water. Prior to extraction, the resuspended pellet was re-centrifuged at 5000 rpm for 15 minutes.

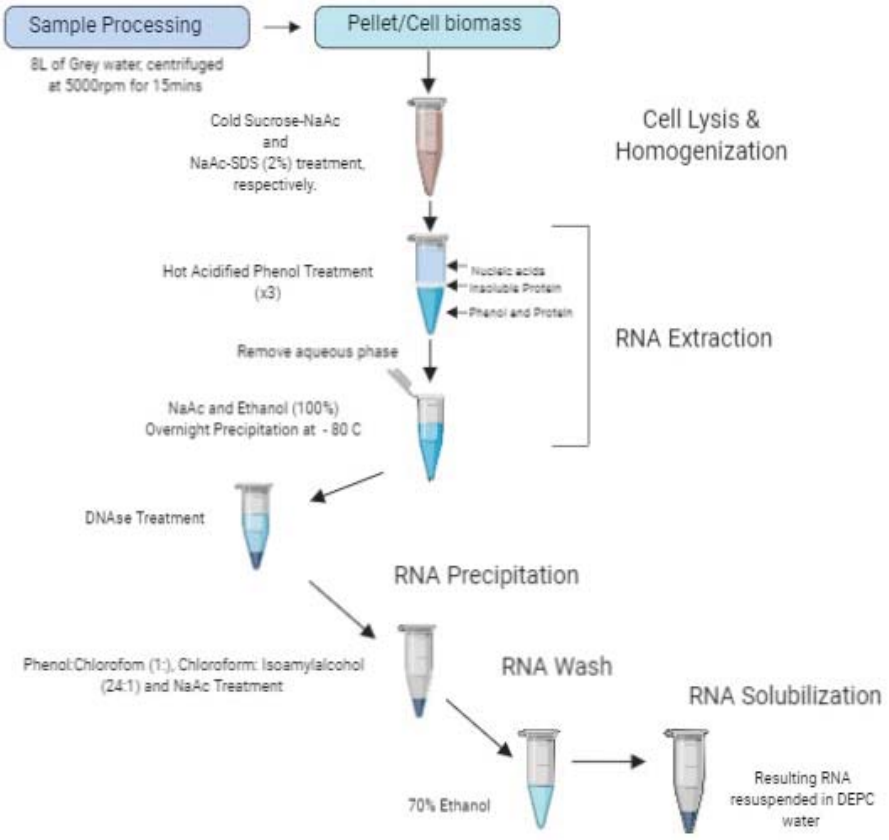

Figure 5.1: Schematic overview of Hot Acid Phenol RNA Isolation Method

5.2.3.2 *Hot Phenol RNA extraction*

RNA was extracted using a modified hot phenol method (Figure 5.1) as described by Ares *et al.,* (2018). Following centrifugation, the resulting pellet was resuspended in ice cold sucrose (0.3M) and sodium acetate (0.01M) (pH 4.5). The sample was split into two Eppendorf tubes and 500µl of sodium acetate (0.01M)-SDS (2% v/v) solution was added to each. The reaction was heated at 65°C for 90 seconds. An equal volume of heated acidified phenol:chloroform:IAA (125:24:1; v/v/v) pH 4.5 (Ambion) at 65°C was added. The samples were vortexed and incubated for three minutes at 65°C. Samples were then frozen rapidly on ice for 30 seconds, followed by centrifugation at room temperature for 10 minutes at maximum speed. The aqueous phase was transferred to a clean tube and re-extracted twice, as outlined above. RNA was precipitated using 1/10 volume sodium acetate (3M) (pH 5.2) and three volumes of 96% (v/v) Ethanol (AR Grade), overnight at -80°C.

Post-precipitation, the samples were centrifuged at 4°C for 30 minutes at maximum speed. The samples were then treated with DNAse 1 (NEB), according to the manufacturer's instruction, however omitting the heat inactivation step. Equal volumes of phenol:chloroform (1:1; v/v) was added to the sample followed by centrifugation at room temperature (22±2°C) for two minutes at maximum speed. The top layer was transferred to a clean tube, and an equal volume of chloroform: *iso*-amyl alcohol (24:1; v/v) was added. The sample was vortexed and centrifuged at room temperature for two minutes at maximum speed. The aqueous layer was transferred, and the RNA re-precipitated by the addition of 1/10 volume sodium acetate (3M) (pH 5.2) and three volumes of 96% Ethanol (AR Grade) at -80°C for one hour. The samples were centrifuged at 4°C for 15 minutes at maximum speed and washed with 70% (v/v) ethanol, after which the sample was centrifuged again at 4°C for 15 minutes at 10 000 x*g* speed. The resulting RNA pellet was resuspended in 40µl of sterile DEPC-water.

5.2.3.3 RNA extraction

A modified CTAB method (Simister *et al.*, 2011) was followed. The pellet obtained following centrifugation of a 250 ml greywater sample was resuspended in DEPC-water. Two hundred and fifty micro-litres (250µl) of sample was transferred to a clean Eppendorf tube. Extraction buffer (1.5 ml) was immediately added and the samples were vortexed for 30 seconds followed by incubation at 65°C for 30 minutes. Samples were centrifuged for 10 minutes at 16000 x*g* at RT and 950µl of the supernatant was transferred to a new tube. One volume (950µl) of chloroform:*iso*-amyl alcohol (24:1; v/v) solution was added and vortexed for 30 seconds. The samples were centrifuged for 10 minutes at 16000 x*g* at 4°C and the supernatant was transferred to a new tube. One volume (~950µl) of chloroform:*iso*-amyl alcohol (24:1; v/v) solution was added and the samples were vortexed for 30 seconds. Samples were centrifuged for 10 minutes at 16000 x*g* at 4°C and the supernatant was transferred to a new tube. LiCl was added to a final concentration of 2M and the samples were incubated overnight at 4°C to precipitate the RNA. The precipitated samples were centrifuged (pre-chilled) for 1 hour at 4°C at 16000 x*g*. The supernatant was discarded, and the pellet was washed with 70% (v/v) ethanol. The ethanol was removed with a pipette and the tubes were centrifuged for 20 minutes at 16000 x*g* at 4°C to remove any residual ethanol. The tubes were left to dry on ice for 10 minutes followed by resuspension of the pellet in 60µl of DEPC water.

5.2.3.4 RNeasy PowerWater Kit

RNA was extracted using the RNeasy PowerWater Kit (Qiagen). RNA was extracted by adding 250µl of the centrifuged samples to the PowerBead Tubes after which the instructions of the manufacturer was followed for RNA isolation. In addition, greywater samples were filtered to remove cellular debris prior to RNA extraction. The vacuum pump was set up with the filter funnel and a 500 ml glass bottle. Filter sizes of 20µm Nylon Net Filters (Merck Millipore Ltd, Tullagreen), 10µm Nylon Net Filters, and 0.45µm MCE Membrane filters were used for sequential filtering of the greywater sample. The filters were inserted directly into the PowerBead Tubes and the RNA was extracted according to the manufacturer's instructions.

5.2.3.5 RNA quality and integrity

The concentration of the RNA (at 230 nm) was determined using a Nanodrop blanked with 2µl of resuspension solution (dependant on the RNA extraction protocol used). After quantification, the samples were immediately analysed by agarose gel electrophoresis by mixing 4µl RNA, 1 µl loading dye, 2 µl formamide and 3µl DEPC-treated H_2O. The samples were incubated at 65°C for 5 minutes in the heating block followed by incubation on ice for 2 minutes. A 1.2% (w/v) gel was prepared by

dissolving 1.2 g of agarose in 100 ml of TBE buffer. The 1kb NEB ladder was included in all gels. The samples were electrophoresed in TBE buffer for 45 minutes at 90 volts. The agarose gels were viewed using long wavelength UV light at 312 nm.

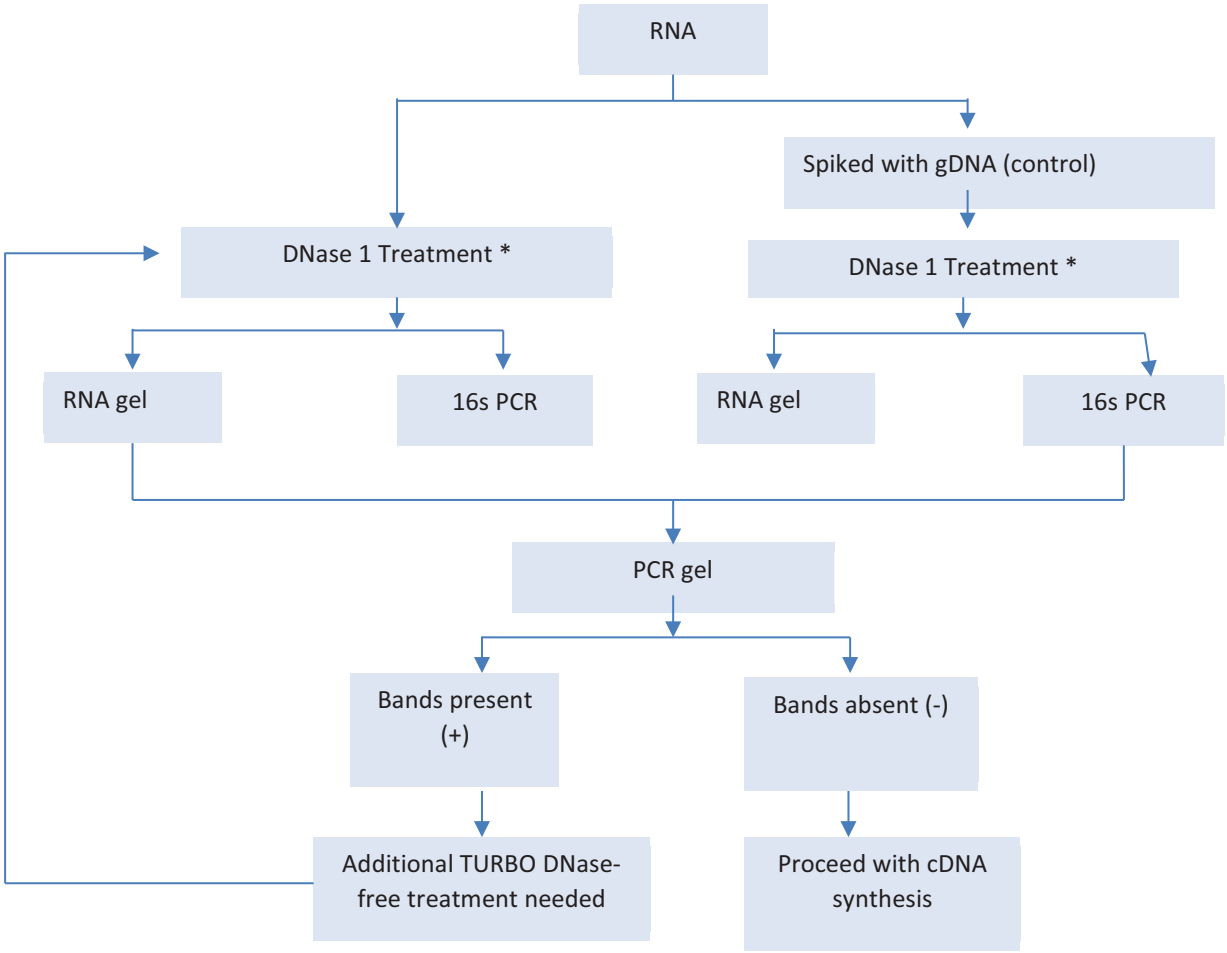

Figure 5.2: Schematic diagram of optimization process of contaminating DNA removal

5.2.3.6 cDNA synthesis

Prior to cDNA synthesis, each RNA sample was evaluated using the Qubit BR RNA Assay kit (Invitrogen) as per the manufacturer's instructions. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) was used to convert RNA extracted from a greywater sample using the RNeasy PowerWater Kit. The Random Hexamer primer supplied in the kit was used, and cDNA was synthesized according to the manufacturer's instructions. PCR was conducted on the cDNA sample using the KAPA2G Robust PCR Kit (KAPA Biosystems). The integrity of the cDNA was determined by agarose gel electrophoresis on a 1% (w/v) agarose gel.

5.2.3.7 16S rRNA gene PCR test for contamination

To ensure no contaminating DNA was present in RNA samples prior to cDNA synthesis, the necessary checks, depicted in the flow diagram in Figure 5.2, were conducted. A 16S rRNA gene PCR was carried out in 25µl reaction volumes. Each reaction contained 0.5U KAPA 2G Robust DNA Polymerase, 0.2mM

dNTP mix, 1X KAPA Enhancer, 1X KAPA 2G Buffer B, 0.5µM forward and reverse primer and made to a final volume using PCR-grade water and 10 ng of RNA. The primers and amplification conditions are provided in table 2. PCR amplicons were electrophoresed on 1% (w/v) agarose gels. All gels contained 10µg/ml ethidium bromide and were electrophoresed in 1X TAE buffer at 90V for 1 hour.

5.3 RESULTS AND DISCUSSION

5.3.1 Comparison of DNA extraction protocols

"Lab" (aka non-kit) and commercial kits were tested during the optimisation phase. Initially a Phosphate, SDS, Chloroform Bead-beater (PSC-B) method was tested on two greywater and two biofilm samples. DNA was isolated from one of the greywater samples as seen by the faint 10.0kb band in Figure 5.3 lane 10. However, the PSC-B method was unsuccessful in extracting DNA from the two biofilm samples examined. Biofilms are predominantly composed of extracellular polymeric substances (EPS). As this method includes an initial physical lysis step it was hoped that this would be sufficient to break up the EPS and release any bacterial cells within. Environmental biofilms have not been studied to a great extent and as such there is limited information available comparing the efficacy of DNA extraction procedures (Hwang *et al.*, 2012). Compared to many other environmental samples, biofilm samples often have limited biomass which is additional problem for DNA extraction (Hwang *et al.,* 2012). The EPS also contains an abundance of negatively charged complex polysaccharides, which have been shown to affect the isolation of DNA (Lear *et al.,* 2010). In addition, the presence organic matter found in greywater systems may interfere with the extraction of DNA from biofilms resulting in the inhibition of downstream molecular applications, such as PCR and RT-PCR (Hill *et al.,* 2015; Hwang *et al.,* 2012).

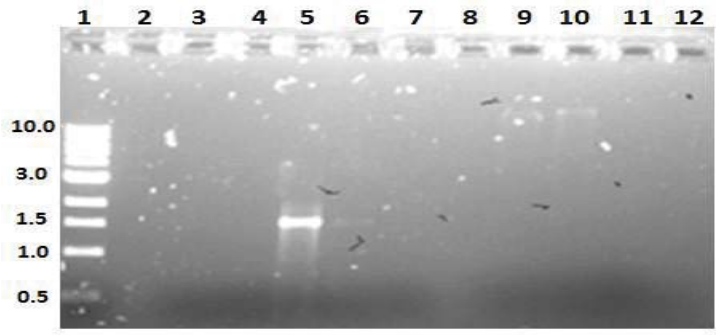

Figure 5.3: 16S rRNA gene amplification using the PSC-B (Phosphate, SDS, Chloroform Bead-beater) method. Lane 1: 1kb NEB marker, Lane 2: Blank, Lane 3: Tap water (-) control, Lane 4: 16S PCR of GW1, Lane 5: 16S PCR of GW2, Lane 6: 16S PCR of BF1, and Lane 7: 16S PCR of BF 2, Lane 8: Blank, Lane 9: Genomic DNA of GW1, Lane 10: Genomic DNA of GW2, Lane 11: Genomic DNA of BF1, Lane 12: Genomic DNA of BF2.

The A260/A280 ratio for the greywater and biofilm samples ranged from 0.5-1.51 indicating the presence of contaminants. The DNA concentrations were also very low indicating that the PSC-B method was unsuccessful. While it is likely that the samples did have very low biomass, it does also appear that the physical lysis step may have been ineffective. For the biofilm samples, compounds present in the EPS may have bound to the nucleic acid during the extraction which could have resulted in further DNA loss (Corcoll *et al.*, 2017). In addition, the presence of fine materials on silica-zirconium beads often interferes with isolation procedures and hence need to be removed using an acid wash. Other studies have shown that bead beating techniques are more effective on soil

samples as compared to water samples depending on the speed and duration of agitation (Fatima *et al.*, 2011).

All DNA extraction protocols were further tested by performing a 16S rRNA gene PCR. This type of PCR is commonly used as a tool for bacterial identification due to the presence of the 16S rRNA gene in all bacteria (Woese, 1987). The method is routinely used for environmental samples where a broad range of pathogens are expected to be present or organism-specific PCRs are not suited, including when analysing metagenomic DNA (Jenkins *et al.*, 2012). As PCR can be used to analyse metagenomic samples (which does not require culturing) it allows for the identification of bacteria which are suppressed by antibiotic treatments, have not survived sampling, viable but non culturable organism, as well as microbes with complex growth requirements (Edwards *et al.*, 2012). As expected, when the DNA extracted using the PSC-B method was analysed via PCR (Figure 5.3), only one greywater sample (GW2, lane 5) and one biofilm (BF1, lane 6) amplified using the universal primers as indicated by the 1.5kb band in lane 5. The rest of the samples did not amplify, including both biofilm samples. Since the DNA extracted using the PSC-B method was of poor quality it was decided to rather try other methods, such as commercial kits.

Many conventional DNA extraction methods are time consuming; the reagents may be toxic (e.g. Phenol-Chloroform-Isoamyl alcohol (PCI) reagents) and often do not isolate good quality DNA, which is required for downstream processing of water samples. The development of commercial kits has allowed for the processing of various sample types and relatively high yields can be achieved in a less time-consuming manner since standardised reagents are provided (Felczykowska *et al.*, 2015; Hinlo *et al.*, 2017). The PowerSoil DNA Isolation Kit was designed for samples with high humic acid contents, such as compost, manure and sediment soil types. However, as the kit has been shown to extract good quality DNA from a diverse range of sample types (Santos *et al.*, 2012), it is widely used in metagenomic studies and is in fact the recommended kit for the Human Microbiome Project. The kit makes use of a patented Inhibitor Removal Technology (IRT) and it is thus effective at removing a wide range of PCR inhibitors.

In the present study, the Powersoil kit was tested on greywater samples (both filtered and non-filtered), as well as biofilm samples. Many greywater systems include a filtration step prior to treatment, and Slow Sand Filters (SSF) are commonly used for the removal of microorganisms (Khalaphallah, 2012). The removal of particles with SSFs is dependent on sand pore size. Generally, contaminants larger than the pore size get trapped in between sand particles preventing their accumulation in the water sample. However, small pore sizes have the ability to trap bacteria within the filter cake (Khalaphallah, 2012) which would then not be present in the filtered water. For this reason, it was decided to assess whether microbes could still be detected in sand filtered greywater – as if the filters reduced the bacterial numbers significantly to below detection levels than these types of systems would need to be excluded from the study.

Based on Figure 5.4a, DNA was successfully isolated from non-sand filtered, sand filtered GW and the BF2 sample using the Powersoil kit. Based on the A260/A280 ratios many of the samples had pure DNA extracts (Appendix A, Table S2) however, the DNA concentration of the biofilm samples were lower compared to that of the greywater samples. The A260/A230 ratio was below 2.0 indicating the presence of protein contaminants absorbing at 230 nm. Generally, for pure DNA samples the A260/A230 ratios should be higher than their respective A260/A280 ratios. The 16S rRNA gene was successfully amplified for all samples (Figure 5.4b). The smears present on the gel are indicative of PCR artefacts which is common for environmental DNA (eDNA).

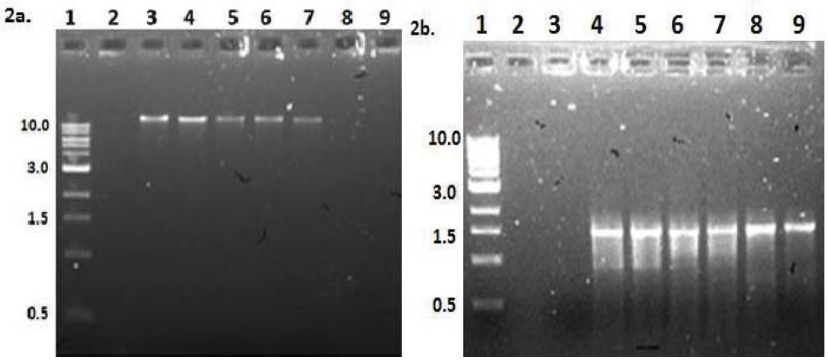

Figure 5.4: Agarose gels confirming the integrity of nucleic acid extracted using the PowerSoil DNA Isolation kit. Gel A: Genomic DNA extracted using the kit. 2a) Lane 1: 1kb NEB marker, Lane 2: Blank, Lane 3: DNA of non-filtered GW 1A, Lane 4: DNA of non-filtered GW 1B, Lane 5: DNA of sand filtered GW 2A, and Lane 6: DNA of sand filtered GW 2B, Lane 7: DNA of BF1, Lane 8: DNA of BF2, and Lane 9: Tap water (-) control. Gel B: 16S rRNA gene amplification of extracted samples. 2b) Lane 1: 1kb NEB marker, Lane 2: Blank, Lane 3: Tap water (-) control, Lane 4: 16S PCR of non-filtered GW 1A, Lane 5: 16S PCR of non-filtered GW 1B, Lane 6: 16S PCR of sand filtered GW 2A, and Lane 7: 16S PCR of sand filtered GW 2B, Lane 8: 16S PCR of BF1, and Lane 9: 16S PCR of BF2.

The quantity and quality of DNA recovered from a sample is highly affected by sample collection and preservation prior to the extraction of nucleic acids (Tatangelo *et al.*, 2014). Sample preservation methods are commonly used for protecting the viability of bacteria thus preventing the degradation of nucleic acids present in environmental samples. However, it has been shown that some solutions may impede the extraction of nucleic acids from specific microbes and thus skew downstream community analysis (Tatangelo *et al.*, 2014). The Forensic DNA Laboratory at the University of the Western Cape has developed an in-house storage buffer (subsequently referred to as FDL solution) which has been shown to preserve DNA from various samples. In addition to the FDL solution, LifeGuard Soil Preservation Solution (LFG) was also tested. LFG soil preservation solution is reported to preserve nucleic acids in environmental samples for long periods at lower temperatures and can even maintain RNA integrity in samples stored at room temperature (22°C) for short periods.

In this study it was found that DNA could only be extracted from the GW FDL 1 preservation solution, based on the faint 10.0kb band in Figure 5.5a, lane 5. Greywater samples in the LFG had intense DNA bands of 10kb in size indicating that the preservation solution was able to preserve the DNA integrity of the samples after collection. According to the A260/A280 ratios the quality of the DNA extracted was pure for all samples except for one GW sample in FDL preservation solution (Appendix A, Table S3). It is also evident that more DNA was extracted from the LFG preserved samples compared to the FDL preservation solution. The FDL preservation solution was developed and optimised on buccal swabs in the forensic DNA laboratory and had not yet been tested on environmental samples. During the extraction, the GW samples preserved in the FDL solution precipitated and the resulting white coagulates did not pellet following centrifugation and thus it may have reduced DNA yield. These extractions were then used for 16S rRNA gene PCR. The intense bands in lane 5 and 6 of Figure 5.5b indicate successful amplification of the LFG samples (GW 2A and 2B) confirming that these samples were good quality, inhibitor free DNA extractions. None of the GW samples in the FDL solution amplified. This may be due to the solution introducing PCR inhibitors, which were not removed during the extraction process or the DNA itself was degraded prior to the PCR. Given these results it was decided to use LFG for all subsequent experiments.

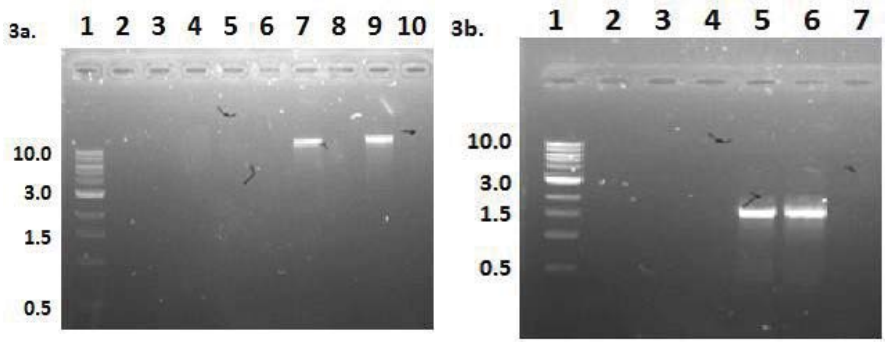

Figure 5.5: A comparison of the quality of the genomic DNA extracted from greywater and biofilm samples using two different preservation solutions. DNA was extracted from the preserved samples using the PowerSoil DNA isolation kit. Gel A: Genomic DNA from preserved greywater and biofilm samples. Gel B: 16S rRNA gene amplification of preserved greywater and biofilm samples. 3a) Lane 1: 1KB NEB marker, Lane 2: Blank, Lane 3: GW in FDL 1A, Lane 4: Blank, Lane 5: GW in FDL 1B, Lane 6: Blank, Lane 7: GW in LFG 2A, Lane 8: Blank, and Lane 9: GW in LFG 2B. 3b) Lane 1: 1kb NEB marker, Lane 2: Blank, Lane 3: 16S PCR of FDL GW 1A, Lane 4: 16S PCR of FDL GW 1B, Lane 5: 16S PCR of LFG GW 2A, and Lane 6: 16S PCR of LFG GW 2B.

5.3.2 Optimisation of RNA Extraction Protocol

Intact RNA is an important requirement for the success of many molecular methods such as qPCR, micro-array analysis, and *in situ* hybridization. The accurate analysis of gene expression is strongly influenced by the quality and quantity of the RNA template (Fleige & Pfaffl, 2006). Therefore, obtaining high quality RNA in sufficient quantities is the most important preliminary step for any investigation in molecular biology (Ma *et al.,* 2015). However, the extraction of total RNA from greywater, which is rich in inhibitors such as detergents, debris and various dissolved inorganic and organic compounds, is a time-consuming and tedious task. Large amounts of these substances can interfere with the RNA isolation procedures. This problem is further compounded by the variability of water samples. Therefore, it was necessary to develop an efficient method for the extraction of RNA from greywater. As such, a considerable amount of time was spent at the start of this project optimising the extraction process as this was critical for the success of the downstream analyses.

A conventional hot phenol RNA extraction method was first tested on preserved, non-preserved greywater samples, as well as an *E. coli* culture (positive control). Unfortunately, based on the presence of a 3.0kb and 1.5kb band in Figure 5.6, lanes 7 and 8 it was only successful on the positive control. These bands correspond to the 23S and 16S ribosomal RNA, respectively, which are an indication that the RNA was intact. No RNA was detected in the experimental samples (both the preserved and non-preserved greywater). Both FDL and LFG preservation solutions appeared to have no impact on the amount of RNA extracted and did not prevent the RNA from degrading. It is likely that there was insufficient starting material in order to obtain good RNA yields. When the extractions were repeated using an increased sample volume the concentration of the extracted RNA was the lowest for the preserved greywater samples in comparison to the remaining samples (Appendix A, Table 7) which would imply that the preservation solution in fact contains chemicals which are impeding RNA extraction – it should be noted that LifeGuard is designed to preserve nucleic acid in soil, however the manufacturers do state that it can be used for a wide range of sample types, including water. Another widely used preservation solution, RNAlater was not used as in a previous project we found that the resulting RNA was contaminated with a lot of salts which inhibited all downstream applications.

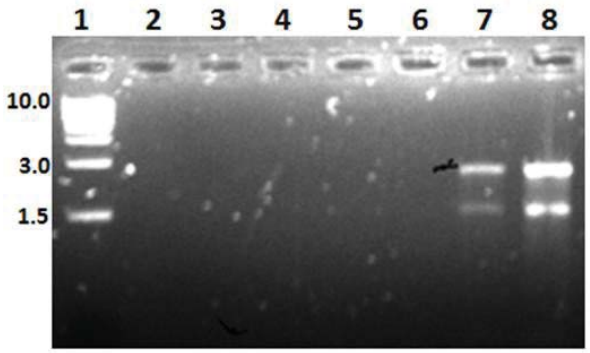

Figure 5.6: RNA extracted from greywater samples using the Hot-Acid Phenol RNA extraction method. Lane 1: 1kb NEB marker, Lane 2: Blank, Lane 3: FDL GW1, Lane 4: LG GW2, Lane 5: GW 2A, Lane 6: GW 2B, Lane 7: *E. coli* 1 (+) control, and Lane 8: *E. coli* 2 (+) control.

Despite the low yields, the extracted RNA appeared to be relatively pure, based on A260/A280 ratios for most samples being ~2.1. However, A260/A230 ratios indicated high protein contamination for the greywater samples. RNA of this quality could not be used for qPCR analysis. It is likely that these samples contain contaminating residual solvents (e.g. phenol) or salts. Therefore, it was decided to test other extraction protocols, including commercial kits.

The cetyltrimethylammonium bromide (CTAB) protocol is rapid and inexpensive as compared to other expensive and time-consuming methods (Gambino *et al.*, 2008). This method utilizes β-mercaptoethanol and PVP as reducing agents in the extraction buffer to increase the overall yield and quality of RNA extracted. CTAB is used as a non-ionic detergent, which has the ability to precipitate acidic polysaccharides and nucleic acids from various low ionic strength solutions (Tan and Yiap, 2009). For this reason, it may be better for biofilm samples. An added benefit of this method is that it avoids toxic chemicals such as guanidium isothiocyanate, phenol or guanidium hydrochloride (Chang *et al.*, 1993).

The CTAB extraction method only worked for one water sample (GW 2B) and the two *E. coli* cultures based on the faint bands present (Figure 5.7a). A faint band was also detectible for the filtered tap water which is supposed to serve as a negative control. The gel also shows the presence of genomic DNA for lanes 4, 5, 6, and 9, which indicates that a DNase treatment would need to be included for future experiments. As the RNA yields were relatively low, the CTAB extraction was repeated. For this experiment several RNA extractions were performed from one greywater sample and the RNA was pooled in the final resuspension step. The presence of the 23S and 16S ribosomal bands in lane 3 (Figure 5.7b) indicated that the RNA extraction was successful and the concentration of the pooled sample was 123.2 ng/µl as compared to the other samples which had concentrations in the range of 4.0 to 31 ng/µl using the standard protocol. However, genomic DNA was also present in the sample which would indicate that a DNase treatment should be included.

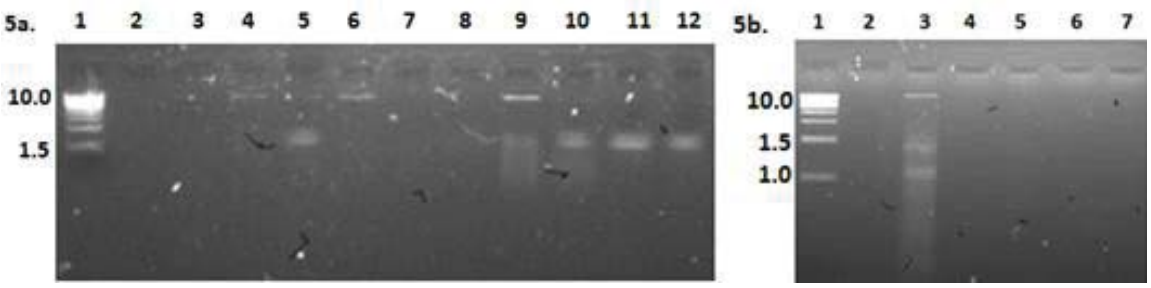

Figure 5.7: RNA extracted using the CTAB RNA extraction method. Gel a) Lane 1: 1kb NEB marker, Lane 2: GW 1A, Lane 3: GW 1B, Lane 4: GW 2A, Lane 5: GW 2B, Lane 6: BF 1, Lane 7: BF 2, Lane 8: Blank, Lane 9: Tap water 1 (-) control, Lane 10: Tap water 2 (-) control, Lane 11: *E. coli* 1 (+) control, and Lane 12: *E. coli* 2 (+) control. Gel b) Lane 1: 1KB NEB marker, Lane 2: Blank, Lane 3: Pooled samples (3x into one tube)

While numerous kits have been developed to improve the yield and quality of the extracted nucleic acid, these vary in the amount and quality of RNA extracted, which may affect the results of various downstream applications in biotechnology (Kaluźna *et al.*, 2016). The yield and quality of RNA obtained using kits are often dependent on the sample type. Water samples always require a concentration step of the sample prior to any extraction procedure, which may be achieved through filtration, centrifugation or a combination of methods (Felczykowska *et al.*, 2015). Figure 5.8 and 5.9b is a comparison of the RNA extracted using the RNeasy PowerWater Kit where the samples were treated with either centrifugation or filtration prior to extraction. A pellet obtained from a 250 ml centrifuged greywater sample was used for the extraction of RNA (Figure 5.8). Lanes 3 and 4 indicate faint 23S and 16S bands, however, the samples look degraded. The presence of smearing on the gel may be due to secondary structure or other RNA transcripts, i.e. mRNA. The quality of the RNA extracted was in the range of 1.9 to 2.2 ng/µl based on the A260/A280 ratio (Table 9, Appendix A). Greywater was also sequentially filtered (20µm, 10µm, and 0.45µm filters) which allowed an increased volume to be filtered (430 ml greywater). Based on the RNA integrity analysis in Figure 5.9b, faint intact 23S and 16S bands are present in lane 5.

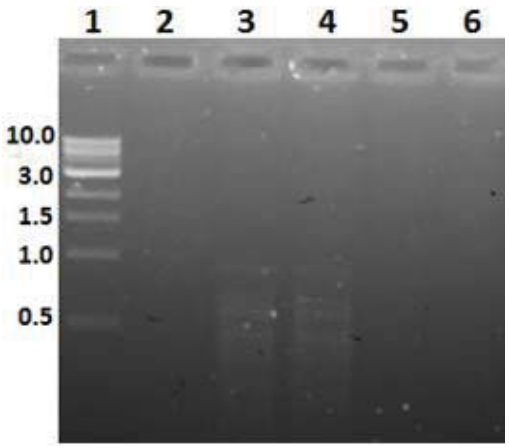

Figure 5.8: RNA extracted from filtered greywater samples using the RNeasy PowerWater Kit. Lane 1: 1kb NEB marker, Lane 2: Blank, Lane 3: GW A, Lane 4: GW B and Lane 5: Tap water (-) control.

The reason for including the 20µm and 10µm filters was to remove larger debris from the greywater which would clog the filters with a smaller pore size. As expected, RNA is only present for the 0.45µm filters since smaller pore sizes were able to capture bacteria. The quality of the RNA extracts was measured using the Nanodrop and an alternative spec, the DeNovix which is reported to be superior for determining very low nucleic acid concentrations. Overall, the DeNovix gave better results for the samples (Appendix A, Table 10). For all the samples analysed the RNA concentrations were low and appeared to contain impurities. The same filters were also used for the extraction of DNA using the PowerSoil DNA Isolation Kit. As with the RNA extraction, DNA was successfully extracted for the 0.45µm filters. In addition, it was found that the DeNovix was more accurate than the Nanodrop.

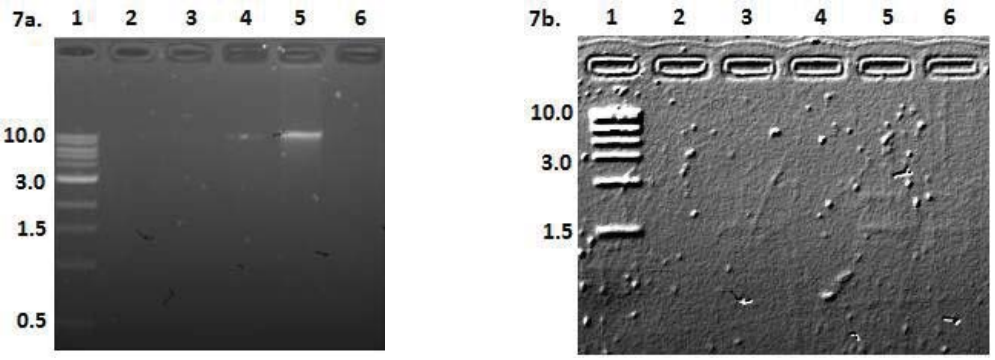

Figure 5.9: A comparison of the DNA and RNA extracted from filtered greywater samples using the commercial kits. a) DNA extracted using the PowerSoil DNA Isolation Kit Lane 1: 1kb Neb marker, Lane 2: Blank, Lane 3: 20µm filter, Lane 4: 10µm filter, Lane 5: 0.45µm filter, and Lane 6: 0.45µm filter. b) RNA extracted using the RNeasy PowerWater Kit. Lane 1: 1kb Neb marker, Lane 2: Blank, Lane 3: 20µm filter, Lane 4: 10µm filter, Lane 5: 0.45µm filter, and Lane 6: 0.45µm filter.

During the testing phase all protocols were first tested on bacterial cultures before being tested on greywater samples. Two in-house lab protocols were tested, and it was found that the conventional organic method using hot phenol was highly successful on bacterial trial samples. Therefore, it was decided to optimize this method to adapt to greywater samples. Initially, low-to-no RNA was extracted when tested on small sample volumes (<1L). Therefore, it was decided to increase the sample volume to allow for more cell mass to be harvested. A larger cell pellet should result in a higher yield of total RNA. Therefore, it was decided to scale up the extraction to 8L of greywater where the cell mass was harvested by sequential centrifugation leading to an increase in cell mass for RNA extractions. High quality and quantity RNA were obtained when using an increased sample size, especially in comparison to the commercial RNEAsy PowerWater Kit, as shown in Table 5.2. The Hot-Phenol SDS isolation technique resulted in an over 100-fold increase in yield and a significant increase in RNA quality. These findings were supported in a study done by Jahn *et al.*, in 2008, which found that a modified version of a hot-SDS phenol method proposed by Linchao and Bremer in 1986, had significantly higher yields than that of the RNEasy Kit (Qiagen) and even TRIzol reagent (Invitrogen), when extracting RNA from *D. dadantii*, a phytopathogenic member of the *Enterobacteriacea* family.

Table 5.1: Total RNA Extractions from Greywater samples

SAMPLE NAME	EXTRACTION METHOD	CONCENTRATION (ng/µl)	A260/A280	A260/A230
GWS1S1	RNEasy kit	0,7	3,05	0.22
GWS1S1	RNEasy kit	5	1,53	0.67
GWS1S2A	Hot Phenol	274,9	2,1	2.16
GWS1S2B	Hot Phenol	330,9	2,04	2.16
GWS2S1	RNEasy kit	1,6	0,9	0.3
GWS2S1	RNEasy kit	1,4	2,34	0.24
GWS2S2A	Hot Phenol	614,9	2,18	2.27
GWS2S2B	Hot Phenol	427,4	2,01	2.35
GWS3S1	RNEasy kit	3,7	1,26	0.45
GWS3S1	Hot Phenol	493,8	1,9	2.0
GWS3S2	Hot Phenol	399	1,82	1.01
GWS3S2	Hot Phenol	144	2,05	2.29
GWS4S1	Hot Phenol	366,9	1,97	1.68
GWS4S1	Hot Phenol	532,5	2,08	2.14
GWS4S2	Hot Phenol	446,1	1,96	1.15
GWS4S2	Hot Phenol	279	1,87	1.01
GWS4S3	Hot Phenol	638,6	2,18	1.88

Yield variability per sample can be seen in Table 5.1. In relation to the success of the Hot-Phenol SDS method, phenol-chloroform based methods rely on the use of phenol-chloroform to promote phase separation and ultimately the selective isolation of the molecules of interest (Toni *et al.*, 2018). Additionally, phenol-chloroform based methods, as the one described above, are advantageous when extracting RNA from small and complex cells, because it yields 2.4 to 9.3 times more RNA than silica-column based protocols (Toni *et al.*, 2018). A vital step in any purification techniques is solubilization of the material from which RNA is to be extracted. The basic goal is to minimize nuclease activity while simultaneously optimizing recovery. It is advisable to inactivate nucleases as efficiently and effectively as possible. This can be achieved by solubilizing biological material in a chaotropic salt, such as guanidine isothiocyanate or a denaturing detergent such as SDS, which is used as a solubilizing agent in the method proposed above (Nilsen, 2013). Careful consideration should be taken when choosing a precipitating salt, as SDS and potassium salts for instance are not compatible. This combination forms an insoluble potassium dodecyl sulfate precipitate (Rio *et al.*, 2010a).

RNA is inherently hydrophilic and therefore dissolves readily in water. However, its hydrophobicity can be reduced by the presence of salt at acidic pH and furthermore by the addition of ethanol (Rio *et al.*, 2010b). Therefore, the precipitation method used in the modified hot-phenol SDS method is highly effective, especially when recovering RNA from aqueous solutions. However, it should be noted that ethanol precipitation is concentration dependent and careful consideration should be taken when precipitating samples which are likely to have either very high or very low amount of RNA present. The purity and integrity of RNA are critical elements for the overall success of RNA-based analysis, as low-quality RNA may compromise the results of downstream applications. The assessment of RNA integrity is especially crucial when aiming to obtain meaningful gene expression data (Fleige and Pfaffl, 2006). The most popular RNA quantification methods use a spectrophotometer/ nano-spectrophotometer to measure the absorbance at 260 nm (A260). This is because the nucleotides present in RNA absorb ultraviolet (UV) light in the 250 to 265 nm range, thus this property can be used to quantitatively measure the concentration of an RNA solution by using the average absorbance for the four nucleotide bases

(Rio *et al.*, 2010c). Spectrophotometric methods can also be used to determine the purity of the sample, by looking at ratios of the absorbances at 230 260, and 280 nm. For purified RNA, the A260/280 ratio should be greater than 1.8, as the unpaired bases in RNA absorb more UV light than the base-paired bases in DNA (Rio *et al.*, 2010c; Fleige & Pfaffl, 2006). Additionally, the A 260/230 ratio is also considered an indicator for nucleic acid purity, with ratios between 2.0 and 2.2 considered to indicate that the sample is pure. The A260/280 ratio for many samples extracted using the hot-phenol SDS Method were within the range of 1.8-2.0, which is considered relatively pure. As well as their A260/230 ratios for many samples were between 2.0 and 2.2. However, for the RNEasy PowerWater kit, the A260/280 ratio was often variable ranging from 0.14 to 3.05 (Table 5.1). Although analysing quality based on the A260/280 ratio is generally reliable, this method can be hindered if the samples are contaminated with DNA, protein or phenol, all of which absorb UV light at 260 nm. An indicator of protein contamination is absorbance at 280nm and phenol is absorbed at 270 nm (Rio *et al.*, 2010c). If the ratio exceeds 2.0 as seen for greywater samples, protein contamination is probable and re-extraction with phenol is recommended. DNA contamination is harder to detect using a spectrophotometer, as RNA and DNA essentially have identical absorbance spectra. Therefore, if abnormal A260 and A260/280 ratios are present and phenol and protein contamination has been ruled out, the RNA sample is most likely contaminated with DNA (Rio *et al.*, 2010c). The presence of genomic DNA can compromise absorbance leading to an over-estimation of the actual RNA present (Fleige and Pfaffl, 2006).

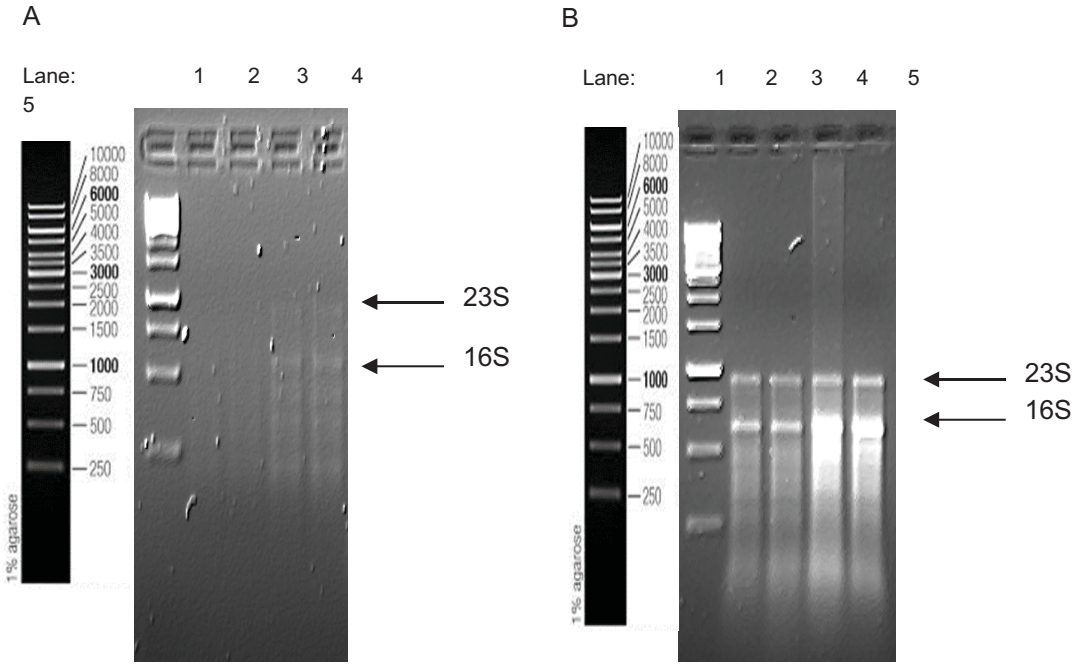

Figure 5.10: Comparison of RNA integrity on 1,2% TBE Agarose gel containing RNA extracted using the RNEasy PowerWater Kit (Qiagen) and the Hot Acid Phenol Method, respectively. Gel (A): Lane 1: 1kb O'Gene Ladder, Lane 2: GWS1S2; Lane 3: GWS2S2; Lane 4: GWS3S2 and Lane 5: GWS4S2. Gel (B): Lane 1: 1kb O'Gene Ladder, Lane 2: GWS1S2; Lane 3: GWS2S2; Lane 4: GWS3S2 and Lane 5: GWS4S2.

In addition to spectrophotometric methods, RNA integrity was also assessed by gel electrophoresis. While there are several methods of quantifying RNA, agarose gels remain very popular as it is cost-effective, scalable and requires limited chemical/reagents. The major disadvantage of this technique is that large amounts of RNA is required when running an RNA agarose gel. Gel analysis of the RNA extracted using the different methods confirmed the Nanodrop results. The concentration of RNA extracted using the RNEasy PowerWater Kit was low as the bands at 3.0kb and 1.5kb are relatively

faint. These bands correspond to the 23S and 16S ribosomal RNA, respectively, which are an indication that the RNA is in intact. However, in figure 5.10B, RNA extracted using the Hot-Phenol SDS method produced bright, intense bands at the same positions, indicating that large amounts of intact RNA were extracted. Most samples extracted using this method also appeared to be relatively pure as they generally did not have the characteristic smear which typically indicates the presence of DNA.

DNA contamination remains a problem with many RNA extraction methods, especially from prokaryotic organisms. Its removal often requires rigorous DNAse treatments, which may affect the amount and purity of extracted RNA. The RNEasy PowerWater kit has a patented DNAse digestion step which appeared to be effective at removing DNA, however, given the low yields and purity of RNA extracted with this kit, it was not feasible to include these samples in subsequent analysis.

To confirm the complete digestion of gDNA in RNA samples, the presence of the 16S rRNA gene was assessed (Lim *et al.*, 2016) using RNA as a template. Figure 5.11 illustrates the presence of a 1.5kb band in RNA samples extracted using the hot-phenol SDS method which would indicate that contaminating DNA was present. Based on the flow diagram depicted in Figure 3.2, these samples required an additional DNAse I treatment. Additionally, the presence of residual gDNA alongside samples with no amplifiable gDNA highlights the importance of checking all samples for the presence of contaminating gDNA (Lim *et al.*, 2016). Lim *et al.* stated that a large proportion of publications fail to indicate or demonstrate that their RNA extracts are DNA-free. Hence, it is strongly recommended including methods that can detect even trace amounts of gDNA in your workflow to avoid the overestimation of active microbial communities in greywater due to the presence of contaminating genomic DNA (Lim *et al.*, 2016).

After conducting extensive optimisation if was found that when using the hot-phenol SDS method, two rigorous DNAse treatments were required to render the samples genomic DNA free, as shown in Figure 5.12. It should be noted that a study by Jahn *et al.*, in 2008, identified that RNA isolated using a phenol-SDS approach, required fewer DNAse treatments compared to commercial proprietary reagents.

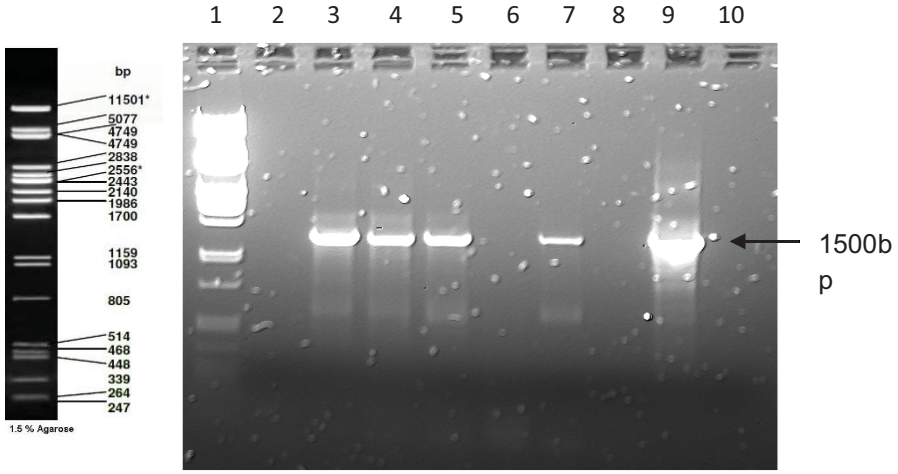

Figure 5.11: 1% Agarose gel depicting the 16S rRNA PCR for DNA contamination after 1X DNAse I treated RNA samples. Lane: 1: Lambda PST1; 2: GWS1S2A; 3: GWS1S2B; 4: GWS2S2A; 5: GWS2S2B; 6: GWS4S3B; 7: GWS3S2; 8: gDNA treated with DNAse; 9: gDNA (+) control; 10: NTC.

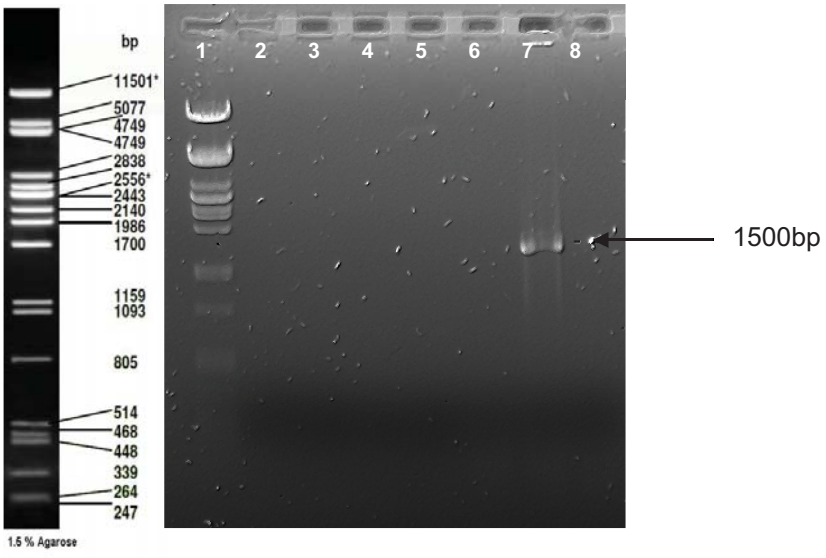

Figure 5.12: 1% TAE Agarose gel of 16S rRNA PCR for DNA contamination after 3X DNAse 1 treated RNA samples. Lane 1: Lambda PST, Lane 2: GWS2S2A; Lane 3: GWS2S2B; Lane 4: GWS2S2C; Lane 5: GWS2S2D; Lane 6: *E. coli* RNA; Lane 7: *E. coli* gDNA, Lane 8: NTC.

5.3.3 cDNA synthesis

cDNA, also known as complementary DNA, is generated from a single strand RNA template by reverse transcription using the enzyme Reverse Transcriptase. The generation of cDNA is often required for gene expression analysis using qPCR, as it indicates which genes are being actively transcribed under a particular set of conditions. In the proposed workflow, RNA samples are only converted to cDNA once all the necessary checks have been conducted. This takes into consideration the presence of contaminating genomic DNA as well as the presence of any PCR inhibitors introduced during the workflow. Figure 5.13 represents RNA samples which were converted to cDNA after two additional DNAse digestion treatments. The cDNA was then used as template DNA in a 16S rRNA PCR to check for the presence of any contaminating DNA. For this experiment the absence of bands is a positive result. This optimised protocol consistently produced cDNA without contaminating gDNA, and as such, the cDNA was deemed suitable for gene expression analysis.

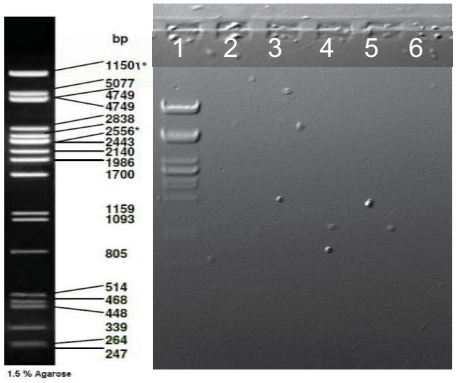

Figure 5.13: 1% TAE Agarose gel illustrating the 16S rRNA PCR of cDNA synthesized using the QuantiNova Reverse Transcription Kit. Lane 1: Lambda PST, Lane 2: GWS2S2A; Lane 3: GWS2S2B; Lane 4: GWS2S2C; Lane 5: GWS2S2D; Lane 6: NTC.

As shown in Table 5.2, total RNA was extracted from 19 samples of greywater over the period of the study. However, due to the rigorous steps taken to eliminate contaminating genomic DNA, as well as variations in yield, the extraction of RNA did not always directly result in the synthesis of cDNA. In total, cDNA was successfully synthesized from 16 greywater samples for gene expression studies.

Table 5.2: Total RNA Extractions from greywater samples

	Greywater Samples	RNA	Extraction Method	16S rRNA PCR for gDNA contamination	cDNA conversion
Site 1	GWS1S1				
	GWS1S2	✓	HPM	✓	✓
	GWS1S3	✓	HPM	✓	✓
	GWS1S4	✓	HPM	✓	✓
Site 2	GWS2S1				
	GWS2S2	✓	HPM	✓	✓
	GWS2S3	✓	HPM	✓	
	GWS2S4	✓	HPM	✓	✓
Site 3	GWS3S1				
	GWS3S2	✓	HPM	✓	✓
	GWS3S3	✓	HPM	✓	
	GWS3S4	✓	HPM	✓	✓
	GWS3S5	✓	HPM	✓	✓
	GWS3S6	✓	HPM	✓	✓
	GWS3S7	✓	HPM		✓
Site 4	GWS4S1	✓	HPM	✓	✓
	GWS4S2	✓	HPM	✓	✓
	GWS4S3	✓	HPM	✓	✓
	GWS4S4	✓	HPM	✓	
	GWS4S5	✓	HPM	✓	✓
	GWS4S6	✓	HPM	✓	✓
	GWS4S7	✓	HPM	✓	✓

5.4 SUMMARY AND CHALLENGES AND FUTURE WORK

Methods for isolating intact total RNA from bacteria are often cumbersome and difficult (Jahn *et al.*, 2008). These tedious methods often make use of toxic and expensive chemicals in order to inhibit RNAses. In addition, while commercial kits that are supposed to decrease time and cost, they often introduce bias and do not have reproducible results. Numerous studies have shown that the extraction method used may affect RNA quality and yield, and therefore careful consideration must be taken when choosing isolation procedures. Ideally, a method should be reproducible and able to effectively lyse the source of the RNA (Bustin and Noal, 2004), which in this case is microbes present in greywater. A reliable isolation technique must yield intact, high-quality RNA that is free of RNAses, proteins and genomic DNA. The extraction and purification procedures must also generate RNA which is free of PCR- and real-time PCR inhibitors (Jahn *et al.*, 2008). In addition to these requirements, as this study was not focused on specific organisms, but was rather looking at the total microbial diversity it was essential that the extraction technique used was able to isolate RNA from both Gram negative and Gram positive bacteria.

For this reason, commercially available kits were initially chosen for extracting RNA from greywater samples, as they should theoretically allow for the extraction of high-quality RNA and have reliable (and highly reproducible) performance. The RNEasy PowerWater kit (Qiagen) was ideal as it had an initial concentration step by filtration, which is required for water samples. However, we found that the RNEasy PowerWater Kit gave low yields and extraction efficiency/quality was highly variable. Reproducibility was a problem, as the amount of sample being processed per filter often differed between samples. Due to the nature of greywater, the filters would often reach capacity quickly (become clogged with cellular debris) thus affecting the yield and quality of the isolated RNA. Low yield may also be due to poor lysis of bacterial cells prior to extraction. Ultimately, it was decided that despite the fact that other extraction methods may be more time-consuming, and they may be better than commercial kits.

After an extensive optimisation process, the optimal methodology was developed to ensure the reproducible extraction of high-quality RNA for gene expression analysis. Although laborious, the Hot-phenol SDS RNA isolation methodology is cost-effective, and efficient when working with our biological sample. DNA extraction using the DNA PowerSoil kit was found to be optimal compared to the beating and CTAB methods tested. Having completed the optimisation process, RNA and DNA was extracted from all environmental samples collected and cDNA was successfully synthesised for 16 of the 19 samples. This cDNA was used in the subsequent qPCR experiments (described in Chapter 6).

In the original workplan only 3 months was allocated to the optimisation process. However, this was a major underestimation, and it took significantly longer to test and optimise the extraction process. Given that extraction of nucleic acid is critical in obtaining meaningful results, the initial 6-8 months spent developing the methodologies was worthwhile, as we ultimately were able to extract good quality RNA and DNA from the majority of the samples.

CHAPTER 6: QUANTIFICATION OF ARGS IN GREYWATER SYSTEMS VIA QPCR

6.1 INTRODUCTION

Quantitative PCR (qPCR) is a highly sensitive technique able to detect and quantify minute amounts of nucleic acids and is widely used in gene expression studies. qPCR employs fluorescent reporter molecules to monitor the synthesis of amplicons during each cycle of the PCR reaction. In one homogenous assay, nucleic acid amplification and detection is combined to detect amplification products (Bustin *et al*., 2005). qPCR has become the method of choice for gene expression quantification due to its specificity, reproducibility, high-sensitivity and simplicity (Gomes *et al*., 2018).

When conducting quantitative real-time PCR (RT-qPCR) the choice of reference genes is essential for obtaining accurate normalization of gene expression data (Rocha *et al.*, 2015). For accurate quantification of gene expression there are a series of experimental parameters which must be considered: (1) determining the appropriate number of biological replicates; (2) strict quality control during RNA extraction and reverse transcription, and (3) the selection and validation of suitable reference genes. According to MIQE standards, the most accurate experimental design would require between three and five good reference genes (Rocha *et al.*, 2015). Based on a literature search of other microbial ecology studies (including those which investigated greywater systems) it was decided to initially test the "standard" set of house-keeping genes namely: *rpoB, gapA, mdh* and 16S rRNA genes.

In this study the target antibiotic resistance genes (*aacC2*, *vanA* and *ampC*) were selected based on their clinical relevance, particularly, their occurrence in the ESKAPE pathogens (acronym for ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumonaie,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter* species) which are the leading cause of nosocomial infections (Santajit and Indrawattana, 2016). Many of these pathogens have acquired multiple-drug resistance and currently serve as one of the greatest challenges in clinical practice. The presence of these antimicrobial resistant pathogens places a significant burden on the health care systems and has adverse global economic costs (Santajit and Indrawattana, 2016).

The first gene, *vanA,* encodes resistance to glycopeptides. Glycopeptides are a class of antibiotics, often used to treat severe infections caused by Gram-positive pathogens such as Enterococci, methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile* (Kang and Park, 2015). The glycopeptide vancomycin is considered to be a 'last resort antibiotic' and is reserved for the treatment of infections that have failed to respond to all other antibiotics (Kang and Park, 2015). Vancomycin resistance is manifested by the expression of the *van* gene cluster which encode proteins that alter and prevent the action of the antibiotic. Expression of the *vanA* gene results in an alteration in the peptidoglycan biosynthesis pathway leading to the production of modified peptidoglycan precursors to which glycopeptides exhibit low affinities (van Hoek *et al*., 2011). The *vanA* gene is the most widespread of all the *van* gene clusters and has been detected in six Gram-positive bacterial genera: *Enterococcus, Erysipelothrix, Lactobacillus, Pediococus* and *Staphylococcus* (van Hoek *et al.,* 2011). The *vanA* gene has been detected in environmental water bodies such as wastewater and surface water biofilms (Schwartz *et al*., 2003).

The second gene, *ampC,* encodes resistance to β-lactam antibiotics. β-lactam antibiotics are amongst the most commonly prescribed and thus widely used drugs. They are characterized by the presence of a β-lactam ring and include antibiotics such as penicillin, cephalosporins, cephamycins and carbapenems (van Hoek *et al*., 2011). The *ampC* gene encodes for a range of β-lactamases, including extended-spectrum β-lactamases (ESBLs). The *ampC* gene has been detected in microbes isolated

from surface water, drinking water and wastewater along with their associated biofilms (Schwartz *et al.*, 2003).

The third gene, *aadA*, mediates bacterial resistance to streptomycin and spectomycin, thus inferring resistance to aminoglycoside antibiotics. Clinically, the most prevalent bacterial resistance mechanism is their chemical modification by aminoglycoside-modifying enzymes, such as aminoglycoside nucleotidyl-transferases (ANTs). The gene *aadA* encodes an aminoglycoside adenylyl-transferase that O-adenylates positions 3'' of streptomycin and 9'' of spectomycin (Stern *et al.,* 2018). Streptomycin serves as a first-line antibiotic used for the treatment of tuberculosis, as well as an agricultural bactericide in animal husbandry and plant disease control (Gu *et al*., 2018).

The experiments described in this Chapter specifically aligns with Aim 2, namely the "Enumeration of ARGs in various greywater sources using qPCR" and follows on from the work described in Chapter 5 as the synthesised cDNA was used as the template for qPCR reactions. In this Chapter the optimisation of the qPCR for the six primer sets is described, after which the generation of the standard curves for each primer set is explained. Lastly, preliminary qPCR analysis and absolute quantification of test greywater samples is described.

6.2 METHODS AND MATERIALS

6.2.1 Bacterial Strains for qPCR analysis

The following bacterial strains were used for the optimisation of the reference genes and antibiotic resistance genes (ARGs) (Tables 6.1). *Escherichia coli* ATCC 25922 was grown at 37°C shaking at 180 rpm for 16 hours in Luria-Broth (LB), while *E. coli* NCTC 13846 and *Enterobacter cloacae* ATCC BAA-1143 were grown at 37°C shaking at 180 rpm for 16 hours in Tryptic Soy Broth. *Enterococcus faecium* ATCC 700221 was grown in Brain Heart Infusion broth, supplemented with 50µg/ml vancomycin hydrochloride, at 37°C shaking at 180rpm for 16 hours.

Table 6.1: Bacterial Control Strains used for Optimization of qPCR

Bacterial Control Strain	Target gene	Target gene type
Escherichia coli ATCC 25922	*mdh*	House keeping
Escherichia coli NCTC 13846	*gapA*	House keeping
Enterobacter cloacae subsp. *cloacae* ATCC BAA-1143	16S rRNA	House keeping
Enterococcus faecium ATCC 700221	*vanA*	ARG
Escherichia coli NCTC 13846	*aadA*	ARG
Enterobacter cloacae subsp. *cloacae* ATCC BAA-1143	*ampC*	ARG

6.2.2 Genomic DNA Extraction using the Ammonium Acetate Method

Genomic DNA was extracted according to Crouse *et al.* (1987). Bacterial cells were harvested from 5 ml of overnight culture by centrifugation at 5000 rpm for 5 minutes and washed with sterile 1X TE (10mM Tris, 1mM EDTA, pH 8.0). Cells were then resuspended in 1 ml SET buffer (25% sucrose, 2mM EDTA, 50mM Tris, pH 8.0) and lysozyme (1 mg/ml) was added. Suspensions were incubated for 30 minutes at 37°C, after which Proteinase K was added to a concentration of 1 mg/ml and further incubated for 30 minutes at 37°C. Post-incubation 500µl of 1X TE (10mM Tris, 1mM EDTA pH 8.0) was added in addition to 50µl of 10% SDS to lyse the cells. The bacterial suspensions were incubated overnight at 50°C to

allow the Proteinase K to degrade protein debris. 500µl of 7.5M Ammonium acetate was addedand the sample was incubated at room temperature (22+/-2°C) for 1 hour. Samples were centrifuged at 13 000 rpm for 15 minutes at room temperature after which 500µl of supernatant was transferred to a new tube. DNA was precipitated by the addition of 2 volumes of 99.9% Absolute Ethanol and the samples were placed on ice for 5 minutes. Samples were centrifuged at room temperature for 30 minutes at 13 000 rpm and the resulting pellet was washed with 70% (v/v) ethanol. Samples were re-centrifuged for 5 minutes at 13 000 rpm, the DNA pellet was resuspended in 1X TE (10mM Tris, 1mM EDTA, pH 8.0) and RNAse A (Thermo Fisher Scientific) was added to a final concentration of 100 ng/ml. The reaction was incubated at 37°C for 30 minutes. Extracted DNA was stored at 4°C. Prior to quantification using the Nanodrop ND-1000 spectrophotometer (Thermo Fischer Scientific, MA, USA) genomic DNA samples were centrifuged at 13 000 rpm for 15 seconds.

6.2.3 Plasmid Miniprep used for the generation of the standard curve

Plasmid DNA was extracted from *E. faecium* ATCC 700221 and *E. coli* NCTC 13846 using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions from a 5 ml overnight bacterial culture. Plasmid Minipreps were quantified using the Qubit dsDNA High Sensitivity (HS) assay (Invitrogen). For the purpose of this study, previously published reference genes with universal primer sequences were evaluated for use in the antibiotic resistance genes expression analysis (Tables 3.2).

6.2.4 Selection of primers and bioinformatic analysis

A comprehensive list of potential target genes and the relevant primers was compiled. Selection criteria for primers were as follows: a) primer set must amplify amplicons within the optimal size range (50-200 bp), b) primers should target a wide range of bacterial genera, c) be specific for the target gene. Selected primers were analysed via primer-BLAST to confirm their specificity and identify their species range. Selected primers are listed in Table 6.2.

Table 6.2: List of primers used in this study

Gene	Encoded protein and function	Size (bp)	Reference
16S rRNA	*Ribosomal RNA* Translation of mRNA	194	Parnanen *et al*., 2019
gapA	*Glyceraldehyde-3-phosphate dehydrogenase A* Energy production – involved in glycolysis	185	Parnanen *et al*., 2019
mdh	*Malate dehydrogenase* Catalyzes the interconversion between malate and oxaloacetate	197	Parnanen *et al*., 2019
rpoB	*β-subunit of RNA polymerase* Transcription of RNA	148	
vanA	*Dehydrogenase (VanH)* Reduces pyruvate to d-Lac, thereby inhibiting cell wall synthesis	65	Volkmann *et al*., 2004
ampC	*Beta-lactamase* Hydrolysis of the β-lactam ring	67	Volkmann *et al*., 2004
aadA	*Aminoglycoside-3-N acetyltransferase* Chemical modification of target	295	Wang *et al*., 2018

6.2.5 PCR optimisation of Reference Genes

Prior to conducting qPCR all primers were first optimised by conventional PCR. Each 50µl reaction contained 1.25U Dream*Taq* Polymerase (Thermo Fischer Scientific), 0.2mM dNTP mix, 1X Dream*Taq* Reaction buffer, 0.2µM forward and reverse primer, 500ng of genomic DNA and was made to the final volume using PCR grade water. The reaction components were the same for all reference genes tested, except for the *gapA* gene which included adding $MgCl_2$ to a final concentration of 2.5 mM. The primers and amplification conditions are listed in Table 6.3. Amplicons were electrophoresed on 2% (w/v) agarose gels containing 10µg/ml Ethidium bromide and were electrophoresed in 1X TAE at 90V for 1 hour.

Table 6.3: Reference genes Primer sets Optimised Amplification conditions

Gene	Primers	Sequence	Amplification Conditions
16S rRNA	*16S rRNA* FP	CCTACGGGAGGCAGCAG	95°C for 3 minutes 40 cycles at 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minute 5 minutes at 72°C
	16S rRNA RP:	ATTACCGCGGCTGCTGGC	
gapA	*gapA* FP	CCGTTGAAGTGAAAGACGGTC	95°C for 3 minutes 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute 5 minutes at 72°C.
	gapA RP	AACCACTTTCTTCGCACCAGC	
mdh	*Mdh* FP	AAGAAACGGGCGTACTGACC	95°C for 3 minutes 40 cycles at 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute 5 minutes at 72°C.
	Mdh RP	GTGGCTGATCTGACCAAACG	
rpoB	rpoB-F	TGGCAACATCGTTCAAGGTG	95°C for 3 minutes, 98°C for 20 seconds, 55-57°C for 15 seconds, 72°C for 15 seconds 72°C for 1 minute.
	rpoB-R	ATCGATGGACCATCTGCAAGG	

6.2.6 PCR Optimisation of Antibiotic Resistance Genes

6.2.6.1 *Optimisation of the vanA PCR*

PCR optimisation was carried out in 50µl reaction volumes. Each reaction contained 1.25U Dream*Taq* Polymerase, 0.4 mM dNTP mix, 1X Dream Reaction buffer, 3 mM Magnesium Chloride, 1 µM forward and reverse primer, 1 ng of *E. faecium* ATCC 700221 plasmid DNA, and made to final volume using PCR grade water. The primers and amplification conditions are summarised in Table 6.4. The resulting amplicons were electrophoresed on 2% agarose gels. All gels contained 10µg/ml Ethidium bromide and were electrophoresed in 1X TAE at 90V for 1 hour.

6.2.6.2 Optimisation of the ampC PCR

PCR optimisation was carried out in 50 µl reaction volumes. Each reaction contained 1.25U Dream*Taq* Polymerase, 0.4 mM dNTP mix, 1X Dream *Taq* Reaction buffer, 2.5 mM Magnesium Chloride, 0.4 µM forward and reverse primer, 1 µg of *E. cloacae*subsp. *cloacae* ATCC BAA-1143 genomic DNA and made to final volume using PCR grade water. The primers and amplification conditions are provided in Table 6.4. Amplicons were analysed as stated above.

6.2.6.3 PCR Optimisation of aadA PCR

PCR optimisation was carried out in 50µl reaction volumes. Each reaction contained 1.25U Dream*Taq* Polymerase, 0.2mM dNTP mix, 1X Dream*Taq* Reaction buffer, 0.2µM forward and reverse primer, 500ng of *E. coli* NCTC 13846 genomic DNA, and made to final volume using PCR grade water. The primers and amplification conditions listed in Table 6.4, was used. Amplicons were analysed as stated above.

Table 6.4: Antibiotic Resistance Genes for RT-PCR Optimised Amplification Conditions

Gene	Primers	Sequence	Amplification Conditions
vanA	vanA-FP	CTGTGAGGTCGGTTGTGCG	95°C for 3 minutes 40 cycles at 95°C for 1 minute,
	vanA-RP	TTTGGTCCACCTCGCCA	60°C for 45 seconds, 72°C for 1 minute 5 minutes at 72°C.
ampC	ampC-FP	GGGAATGCTGGATGCACAA	95°C for 3 minutes 40 cycles at 95°C for 1 minute,
	ampC-RP	CATGACCCAGTTCGCCATATC	60°C for 45 seconds, 72°C for 1 minute 5 minutes at 72°C.
aadA	aadA-FP	CAGCGCAATGACATTCTTGC	95°C for 3 minutes 40 cycles at 95°C for 30 seconds,
	aadA-RP	GTCGGCAGCGACAYCCTTCG	60°C for 30 seconds, 72°C for 1 minute 5 minutes at 72°C.

6.2.7 Quantitative PCR (qPCR)

Real-Time PCR was performed on a LightCycler 480 II (Roche Diagnostics, IN, USA). qPCR reactions were set up in white 96-well PCR plates, with optically clear sealing films supplied with the plates (Roche).

6.2.7.1 Construction of the Standard Curves

Amplicons for each of the respective genes were generated using PCR (four 50 µl reactions per set). Amplification was conducted using the optimised conditions provided in Tables 6.3 and 6.4. The amplicons for each primer set were pooled and purified using the NucleoSpin Gel and PCR Clean Up Kit (Macherey-Nagel) according to the manufacturer's instructions, eluting in 30µl of elution buffer. The purified amplicons for each primer set were quantified using the Qubit dsDNA HS assay (Invitrogen)

and the number of molecules/µl was determined using Equation 1. A serial dilution was prepared over the range of 10^8 to 10^2 copies.

Equation 1. Formula used to calculate number of gene copies in a specific amount of DNA of a particular size.

$$Number\ of\ copies = \frac{Amount\ (ng) \times 6.022\ \times 10^{23}}{Length\ (bp)\ \times 1 \times 10^{9}\ \times Mass\ of\ DNA\ bp}$$

ng – nanograms, bp – base pairs

For the reference genes, 16S rRNA and *gapA*, qPCR was performed using the Quantinova SYBR Green I Kit (Qiagen). Each 10µl reaction contained 9µl of Quantinova SYBR GreenI master mix and 1 µl of template DNA. For the generation of a standard curve, serially diluted purified PCR amplicons served as the input DNA. qPCR cycling conditions were as follows: initial activation step at 95**°**C for 2 minutes, followed by 40 cycles of denaturation at 95**°**C for 10 s, followed by a combined primer annealing and extension step at 60°C for 30 s. Melt curve analysis was performed from 60°C with a gradual increase in temperature to 97**°**C, during which time changes in fluorescence were monitored. Quantification was performed using the LC480 software (version 1.5) and Microsoft Excel.

For the *mdh* reference gene, qPCR was performed using the LightCycler 480 SYBR Green I Master mix (Roche) in 10µl reaction volumes, containing 9µl of the master mix and 1µl template DNA. For the generation of a standard curve, serially diluted purified PCR amplicons served as the input DNA. qPCR cycling conditions were as follows: initial activation step at 95°C for 5 minutes, followed by 45 cycles of 95°C for 10 s followed by a combined primer annealing and extension at 57°C for 15 s. Melt curve analysis was performed from 56°C with a gradual increase in temperature to 97°C during which time changes in fluorescence were monitored. Quantification was performed using the LC480 software and Microsoft Excel.

For the ARGs, qPCR was performed in 10µl reaction volumes, using the Quantinova SYBR Green I Kit (Qiagen,) in 10µl reaction volumes, containing 9µl of master mix and 1 µl of template DNA. For the generation of a standard curve, serially diluted purified PCR amplicons served as the input DNA. qPCR cycling conditions were as follows: initial activation step at 95°C for 2 minutes, followed by 40 cycles of denaturation 95°C for 10s, followed by a combined primer annealing and extension at 60°C for 30s. Melt curve analysis was performed from 60°C with a gradual increase in temperature to 97°C, during which time changes and fluorescence were monitored. Quantification was performed using the LC480 software and Microsoft excel.

6.2.7.2 Absolute Quantification of the gapA gene PCR

qPCR of the *gapA* reference gene was achieved using LightCycler 480 SYBR Green I Mastermix and the primer set as indicated in Table 3.3. The following PCR parameters were used: denaturation (1 cycle) at 95°C for 5 minutes; amplification (45 cycles) at 95°C for 10 s, 60°C for 15 seconds and 72°C for 15 s; melting curve analysis at 59 with a gradual increase in temperature to 97.

6.2.7.3 Absolute Quantification of 16S rRNA using qPCR

qPCR of the 16S rRNA reference gene was achieved using the LightCycler 480 SYBR Green I Mastermix and the primer set as indicated in Table 6.4. The following PCR parameters were used: denaturation (1 cycle) at 95°C for 5 minutes; amplification (45 cycles) at 95°C for 10s, 60°C for 15 seconds and 72°C for 15 s; melting curve analysis at 95°C for 5s, 59°C for 1 minute and 97°C continuous for 5°C and cooling for 1 cycle at 40°C.

6.2.7.4 Absolute Quantification of vanA using qPCR

qPCR of the *vanA* reference gene was achieved using the LightCycler 480 SYBR Green I Mastermix and the primer set as indicated in Table 6.4. The following PCR parameters were used: denaturation (1 cycle) at 95°C for 5 minutes; amplification (45 cycles) at 95°C for 10s, 60°C for 15 s and 72°C for 15 s; melting curve analysis at 95°C for 5 s, 59°C for 1 minute and 97°C continuous for 5°C and cooling for 1 cycle at 40°C for 30 s.

6.3 RESULTS AND DISCUSSION

6.3.1 *In silico* analysis of primer sets

As there are numerous published qPCR primer sets available (especially for the housekeeping genes) it is critical that one should first conduct proper *in silico* analysis to decide which primers to employ. In this study the primers were selected as they were specific, would target the relevant taxa and the product size was within the optimal range for accurate qPCR analysis (50-200bp).

For primer-BLAST testing of the house keeping gene *rpoB*, the parameters were all set to default. BLAST analysis confirmed that the primers targeted the correct gene and that the predicted product was the expected size of ~150bp (Figure 6.1).

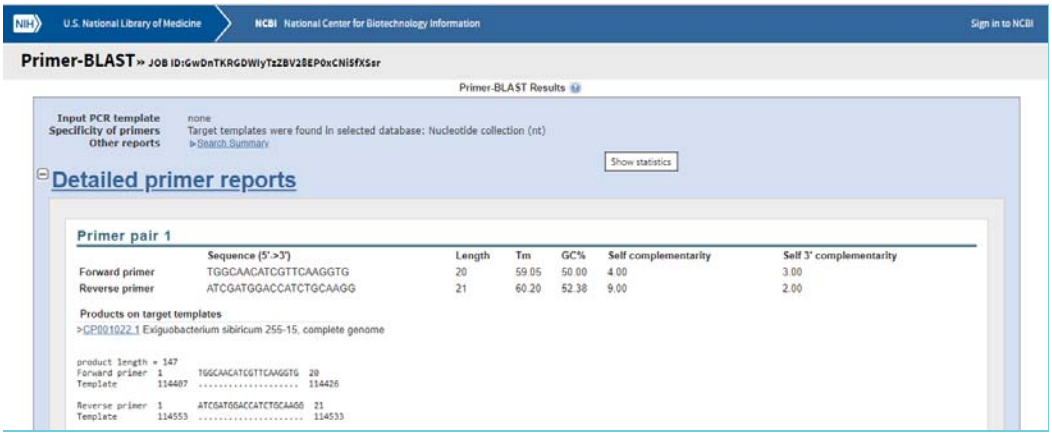

Figure 6.1: Primer-BLAST results confirming that the *rpoB* primers target the correct housekeeping gene.

Primer-BLAST analysis was also performed on the three primer pairs which target the antibiotic resistance genes. Figure 6.2 shows that the *vanA* primers would amplify a ~65bp product from various *Enterococcus* species.

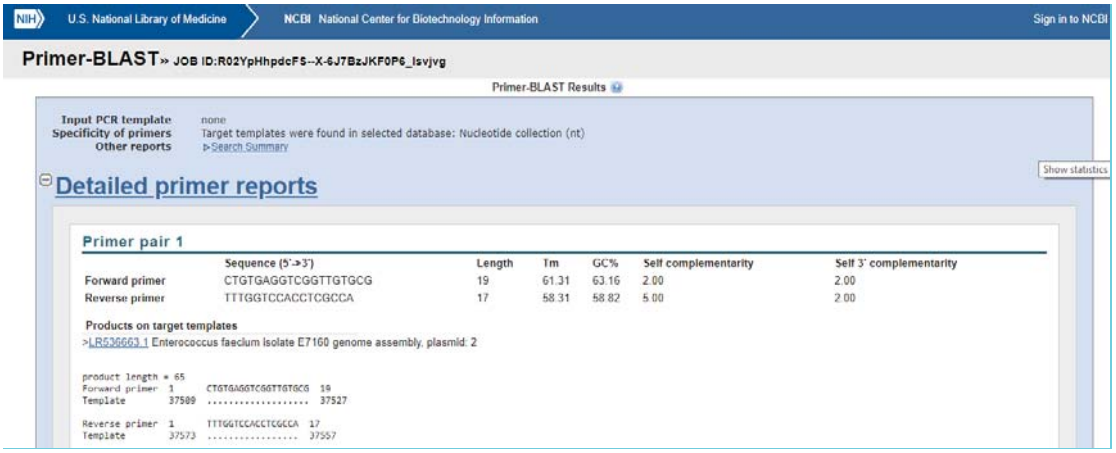

Figure 6.2: Primer-BLAST results confirming that the primers would amplify the *vanA* gene.

Similarly, Figure 6.3 is the BLAST analysis for the *ampC* gene primers which are associated with ampicillin resistance, would target chromosomal *ampC* gene, blaATC, and amplifying a 66bp fragment in β-lactamase producing *Enterobacter* species.

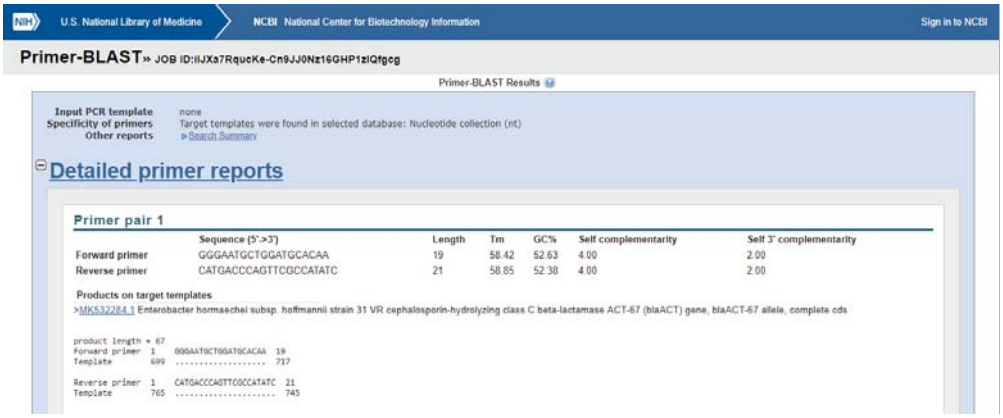

Figure 6.3: Primer-BLAST results confirming that the primers would amplify the *ampC* gene.

BLAST analysis on the final primer set, *aacC2*, also confirmed that it should specifically target the gene (Figure 6.4).

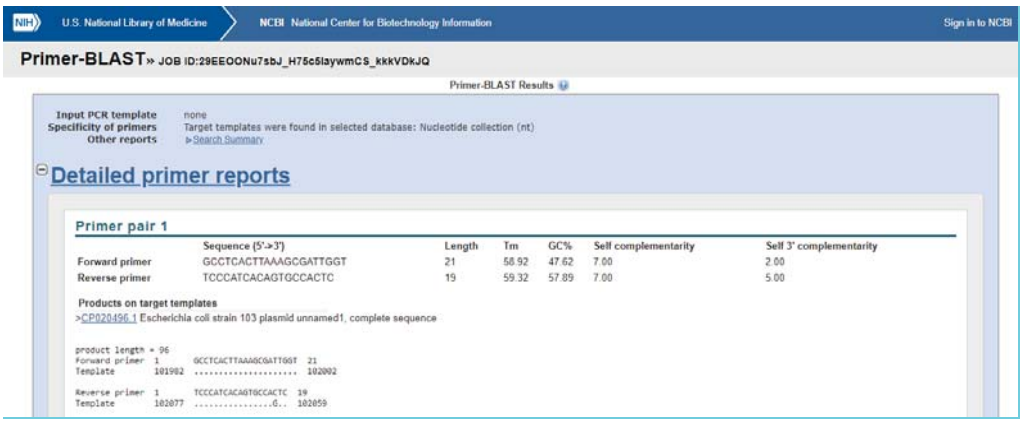

Figure 6.4: Illustrates a Primer-BLAST of the *aacC2*-gene.

6.3.2 Optimisation of reference gene primer sets using conventional PCR

Prior to qPCR analysis conventional PCR was employed to optimise the PCR cycling conditions. Optimisation of the respective PCRs was based on identifying the optimal cycling conditions, the ideal bacterial strain, which gave consistent amplification, as well as the concentration of vital PCR components such as primers and dNTPs. Figure 6.5 illustrates positive amplification for the 16S rRNA gene, with an amplicon size of 194bp, *gapA* as seen in Figure 6.6 has a positive amplicon size of 185bp, which is represented by bright bands on the agarose gel. Figure 6.7 represents the positive amplification of the *mdh* gene of which is 197bp in size. All PCR reactions were optimised using Dream*Taq* Polymerase. As a single fragment of the correct size had been generated for the three primer sets, the resulting amplicons could be used as a template to generate the standard curve required for qPCR analysis. The optimised PCR conditions were used as the initial conditions for qPCR experiments.

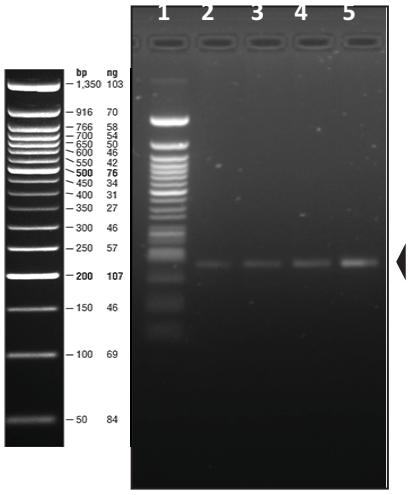

Figure 6.5: Optimised amplification conditions for the 16S rRNA primer set. The amplicons represented above were purified and used to generate a standard curve for each target gene. Lane 1: 50bp NEB Quick Load Ladder Lane 2-5: 16S rRNA gene amplicons generated using *E. cloacae* subsp. *cloacae* ATCC BAA-1143 genomic DNA.

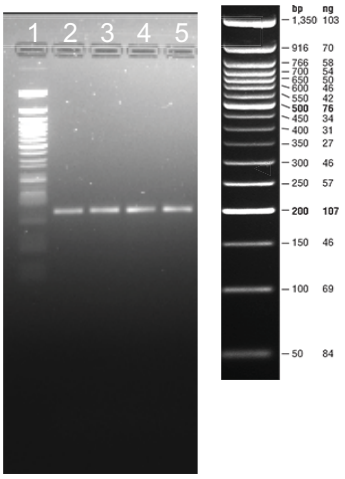

Figure 6.6: Optimised amplification conditions for the *gapA* gene primer set. The amplicons represented above were purified ad used to generate a standard curve for each target gene. Lane 1: 50bp NEB Quick Load Ladder Lane 2-5: *gapA* gene amplicon generated from *E. coli* NCTC 13846 genomic DNA.

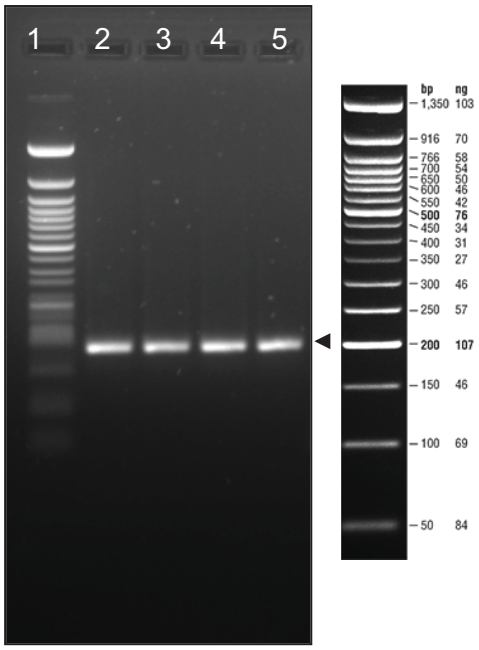

Figure 6.7: Optimised amplification conditions for the *mdh* gene primer set. The amplicons represented above were purified ad used to generate a standard curve for each target gene. Lane 1: 50bp NEB Quick Load Ladder Lane 2-5: *mdh* gene amplicons generated from *E. coli* ATCC25922 genomic DNA.

Despite numerous attempts to optimise the PCR, the primer set for the fourth potential housekeeping gene, *rpoB*, consistently produced non-specific bands and failed to amplify a band of the expected size (148bp) (Figure 6.8). There are several reasons for non-specific amplification including incorrect cycling protocols, the target GC content, DNA concentration, annealing temperature, and $MgCl_2$ concentration and poor primer design. As the same non-specific bands were amplified under all the test conditions it is possible that the primers bound to the wrong targets (possibly a gene coding for another type of polymerase). As we already had three housekeeping gene primer sets optimised it was decided to exclude the *rpoB* primers from the study.

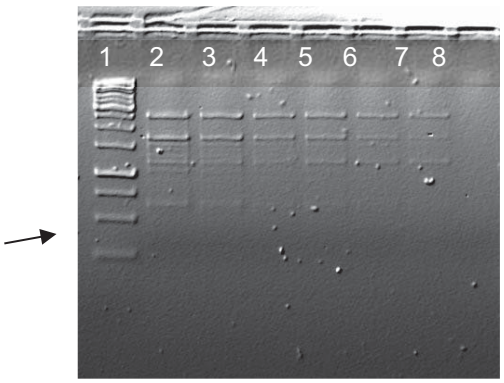

Figure 6.8: *rpoB* gene-specific gradient PCR using KAPA Hifi HotStart. Non-specific banding can be seen across the full range of annealing temperatures tested. There doesn't appear to be a band at the expected size (148bp) – indicated by the arrow. Lane 1: 100bp Quickload Ladder, lane 2-lane 7: Gradient annealing temperature using *E. coli* gDNA, lane 8: NTC.

6.4.3 Optimisation of ARG gene primer sets using conventional PCR

The optimisation of target ARGs were of vital importance to move forward to the qPCR analysis. As seen in Figure 6.9, amplification of the *vanA* amplicon was achieved (presence of the 65bp target band) using Dream*Taq* polymerase. The *vanA* gene which encodes vancomycin resistance is plasmid-borne and therefore plasmid DNA extracted from *E. faecium* ATCC 700221 was used as the template for this PCR (McKenney *et al*., 2016).

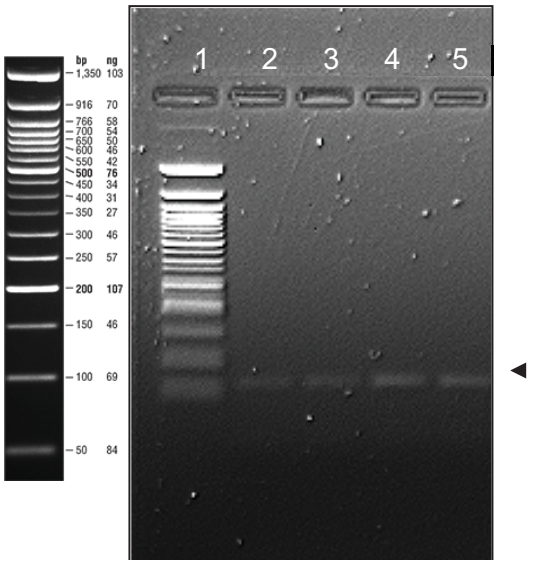

Figure 6.9: Optimised amplification conditions for the *vanA* gene primer set. The amplicons represented above were purified and used to generate a standard curve. Lane 1: 50bp NEB Quick Load Ladder Lane 2-5: *vanA* gene amplicons generated using *Enterococcus faecium* ATCC 700221 plasmid DNA.

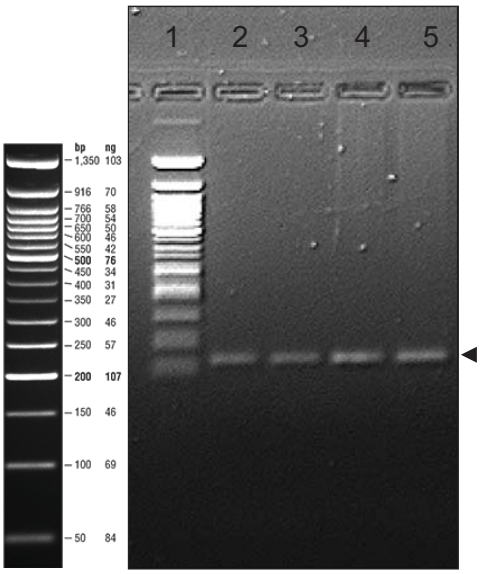

Figure 6.10: Optimised amplification conditions for the ampC gene primer set. The amplicons represented above were purified ad used to generate a standard curve. Lane 1: 50bp EB Quick Load Ladder Lane 2-5: ampC gene amplicons generated using Enterobacter cloacae subsp. cloacae ATCC BAA-1143 genomic DNA.

As seen in Figures 6.10 and 6.11, amplification for the *ampC* and *aadA* genes were achieved, with bright bands at 67bp and 295bp, respectively. All amplicons represented in these two figures were used as template for standard curve generation.

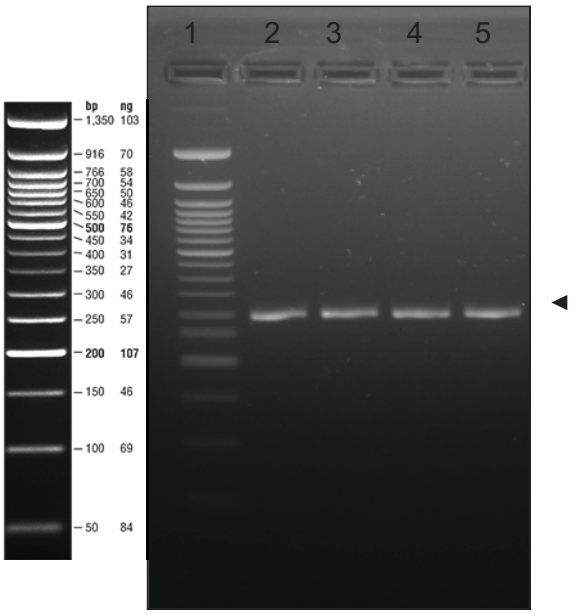

Figure 6.11: Optimised amplification conditions for the *aadA* gene primer set. The amplicons represented above were purified ad used to generate a standard curve. Lane 1: 50bp NEB Quick Load Ladder Lane 2-5: *aadA* gene amplicons generated using *Escherichia coli* derived from NCTC 13846 genomic DNA.

6.3.3 Generation of the Standard Curve for Reference Genes

A standard curve has been widely used to assess the performance of qPCR assays by estimating its efficiency, in parallel with identifying the assays dynamic range, limit of quantification and limit of detection. The standard curve remains the most effective and robust approach to estimate PCR efficiency (Svec *et al*., 2015). To estimate PCR efficiency by means of a standard curve one generates a series of samples with a controlled amount of target template. A dilution series of known template concentrations is used to establish a standard curve for determining the amount of target template in experimental samples, as well as assessing reaction efficiency. A qPCR standard curve is a plot of the Threshold cycle (C_t) versus the logarithm of the amount of RNA/DNA target template. In a typical standard curve, the C_t is shown on the y-axis and the template concentrations on the x-axis. The slope, y-intercept and correlation coefficient values are used to extrapolate information about the performance of a reaction. The correlation coefficient (R^2) reflects the linearity of the standard curve and is a measure of how effectively the data fits the standard curve produced. The slope of the log-linear phase of the amplification reaction is a measure of the reaction efficiency. For accurate and reproducible results reactions should have an efficiency as close to 100% as possible,which is equivalent to a slope of -3.32.

Efficiency is calculate using the formula: Efficiency = $10^{(-1/\text{slope})} - 1$.

Therefore, standard curves for each of the target reference and antibiotic resistance genes were produced in addition to conducting melting curve analysis for each primer set. A melting curve, also known as a dissociation curve, charts the change in fluorescence observed when double-stranded DNA (dsDNA) coupled with dye molecules 'melts' or dissociates into single-stranded DNA as the temperature of the reaction increases.

Standard curves were generated for each primer set within MIQE guidelines. The Melt curve analysis revealed that the 16S rRNA gene, seen in Figure 6.12, was specific as only a single size amplicon was generated. The efficiency of the PCR was within the accepted range of 90-100%, as the slope of the standard curve in Figure 6.13 was -3.406 and the R^2 value was 0.9934.

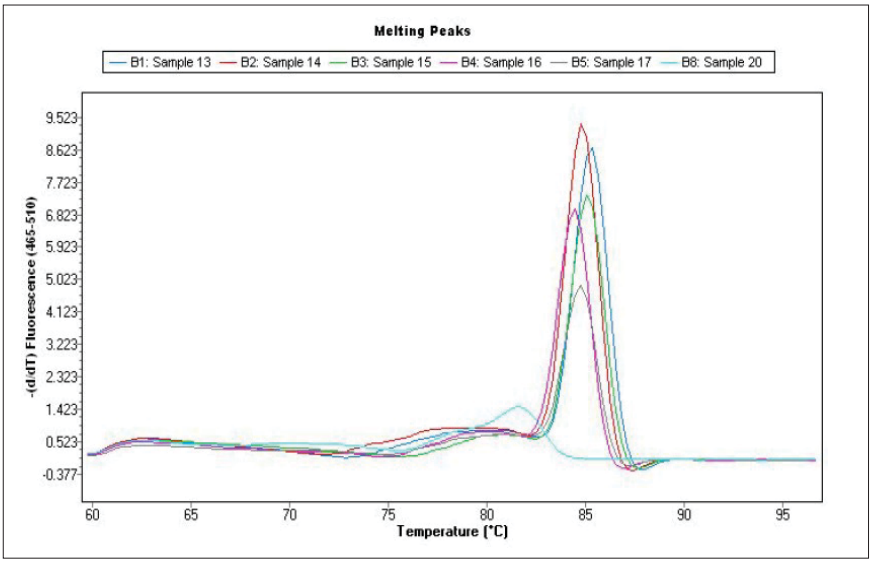

Figure 6.12: Melt Curve Analysis of 16S rRNA reference gene.

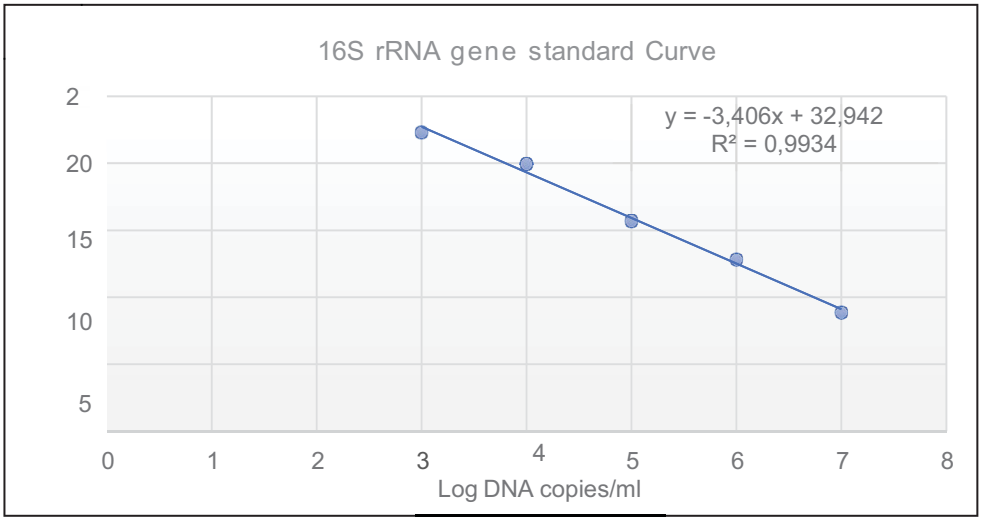

Figure 6.13: Standard curve generated for the 16S rRNA reference gene primers.

Similarly, the *gapA* melt curve analysis also had a single size amplification. It should be noted that the A8 sample present in Figure 6.14 had a peak prior to the desired temperature, this was due to the omission of water in the non-template control (NTC). The standard curve generated for *gapA* yielded positive results as seen in Figure 6.15, with a slope of -3.95 and a R^2 value of 0.98, falling within the 90-100% range.

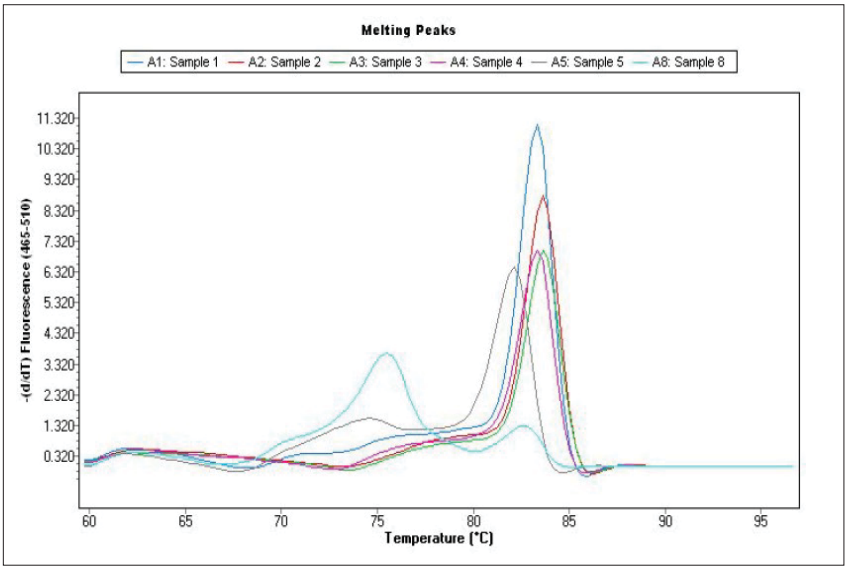

Figure 6.14: Melt Curve analysis of gapA gene

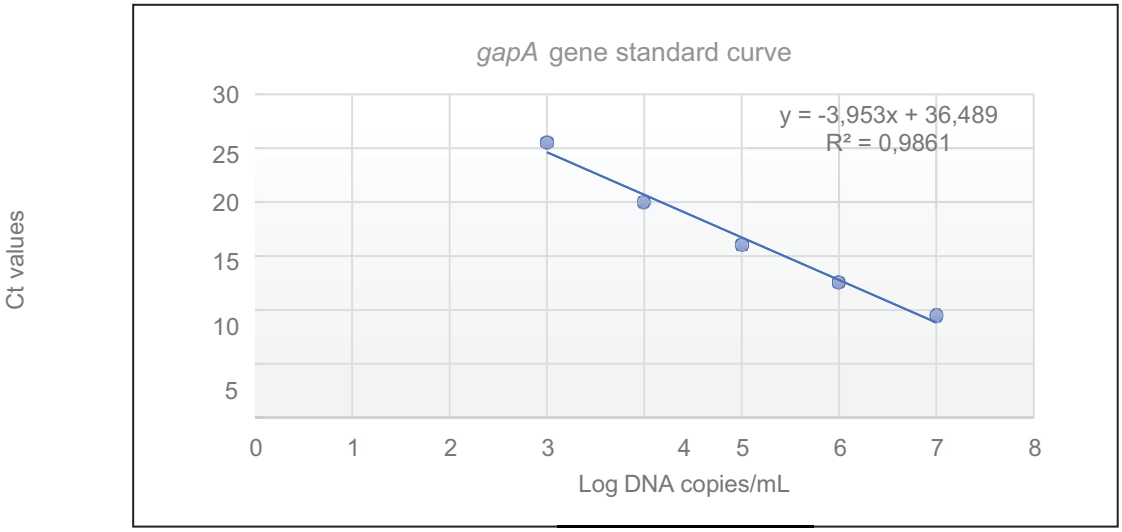

Figure 6.15: Standard curve generated for the *gapA* reference gene primers.

The melt curve produced for the *mdh* gene primer set was specific and generated a single size amplification as seen in Figure 6.16. The standard curve produced for the *mdh* primer set (Figure 6.17) had a slope of -3.4208, which correlated to a R^2 value of 0.9964. Based on the results obtained, the standard curve for *mdh* has been improved to an efficiency of 99%.

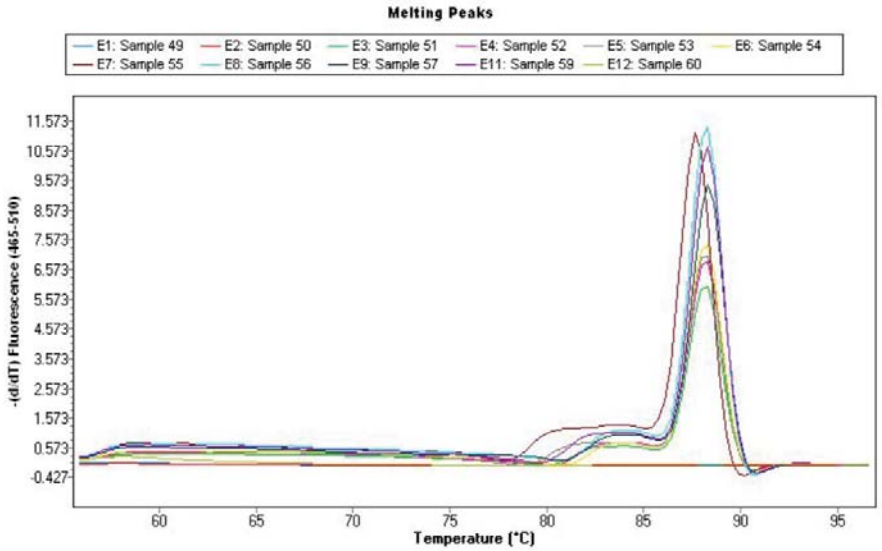

Figure 6.16: Melt Curve Analysis of the mdh reference gene.

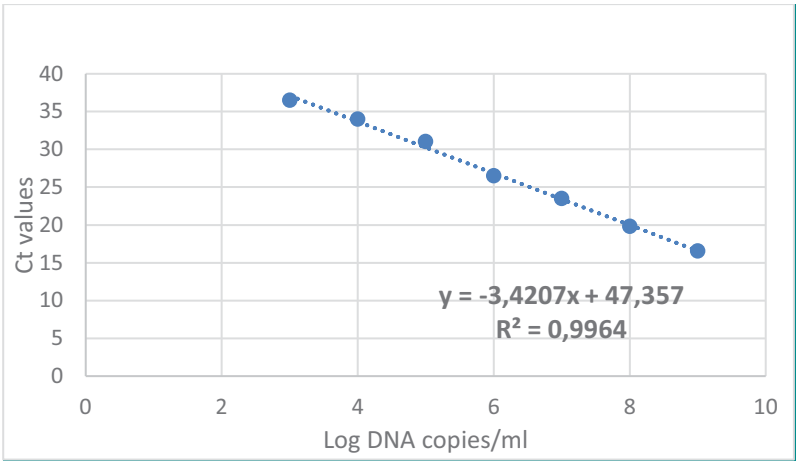

Figure 6.17: Standard curve generated for the *mdh* reference gene primers.

The efficiency of the PCR assays for the reference genes described above was of great importance for the progression of the study. As mentioned before reference genes need to be stably expressed and accurate quantification of the reference genes is essential to eliminate sample to sample variation especially when conducting relative quantification.

6.3.4 Generation of the Standard Curves for ARG primer sets

In this study, we focused on three antibiotic resistance genes (ARGs) of three different classes. For the progression of the study, it was important that the previously published primers used for these genes were accurately quantified in the bacterial control strains used, as this will serve as the external control when conducting relative quantification. In order to determine the efficiency of the PCR assays, a melt curve analysis and standard curve was produced for each gene primer set. As seen in Figure 6.18, the melt curve for the *vanA* gene was highly specific and produced a single sized amplicon. The standard curve had a slope of -3.325, with a R^2 coefficient of 0.9929 (Figure 6.19).

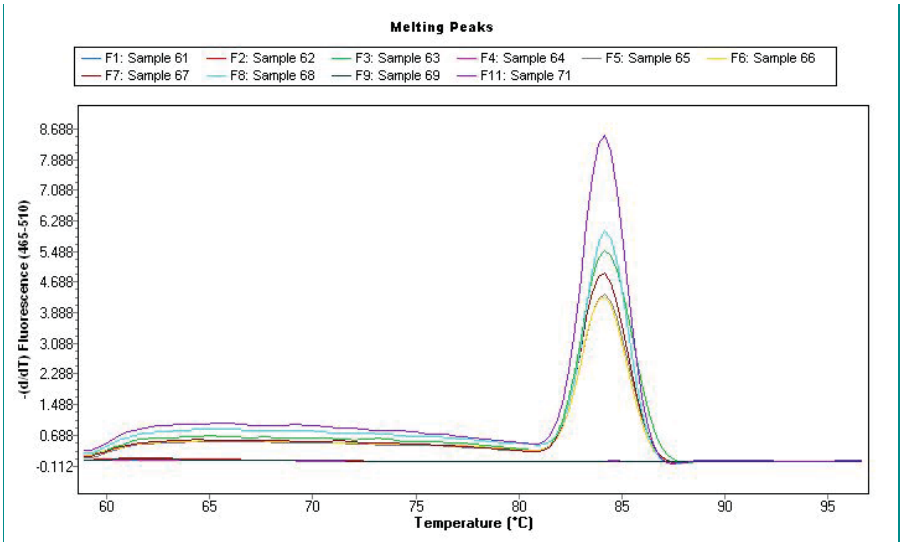

Figure 6.18: Melt Curve Analysis of the *vanA* gene

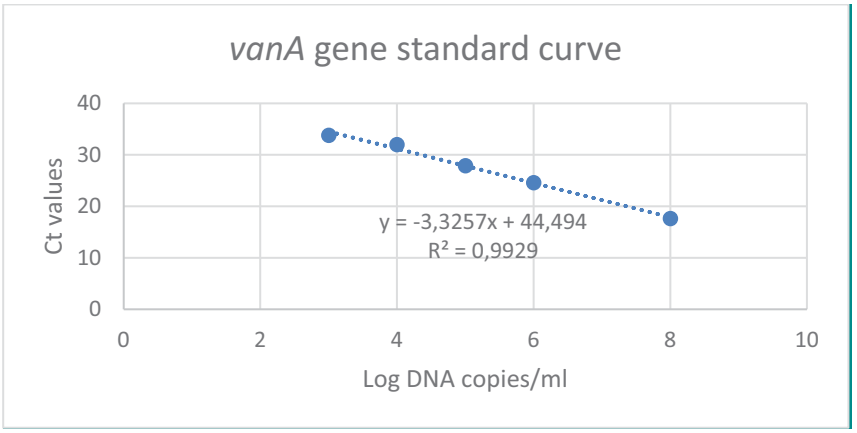

Figure 6.19: Standard curve generated for the vanA gene primers.

Figure 6.20, illustrates the melt curve analysis of the *ampC* gene primer set. The optimised conditions were specific (single size amplicon is produced) and the standard curve generated for the *ampC* primer set had a slopeof -3.4283 and a R^2 value of 0.9926 (Figure 6.21).

The melt curve produced for the *aadA* primer set also had a specific and single sized amplicon present, which was indicative of the efficiency of the primers and PCR assay. Additionally, the standard curve provided valuable information regarding the efficiency of the reaction. The *aadA* primer set produceda standard curve with a slope of -3.3049 and a R^2 value of 0.9982, falling within the 90-100% range of assay efficiency.

A considerable amount of time was spent constructing these standard curves as they are essential for accurate quantification. In qPCR gene copy number can be quantified in two ways; either *absolute quantification* – where the actual number of template molecules is determined by comparing to known standards (hence the need for a standard curve); or *relative quantification* – which can be used to determine the levels of expression of a target gene compared to a reference gene. For our analysis we were interested in quantifying and comparing the number of genes present in the different samples, hence absolute quantification was performed.

Absolute quantification is performed by constructing a standard curve for each gene of interest and plotting the quantification cycle (Cq) values against the log {quantity} of a dilution series of known gene of interest amount. These standards, comprising of purified PCR products, plasmid DNA constructs or synthetic oligonucleotides spanning the PCR amplicon, are amplified, as are any experimental errors. This is important as the standard curve provides both the efficiency of the amplification primers and the number of gene copies in the unknown sample (Boutler *et al.,* 2016).

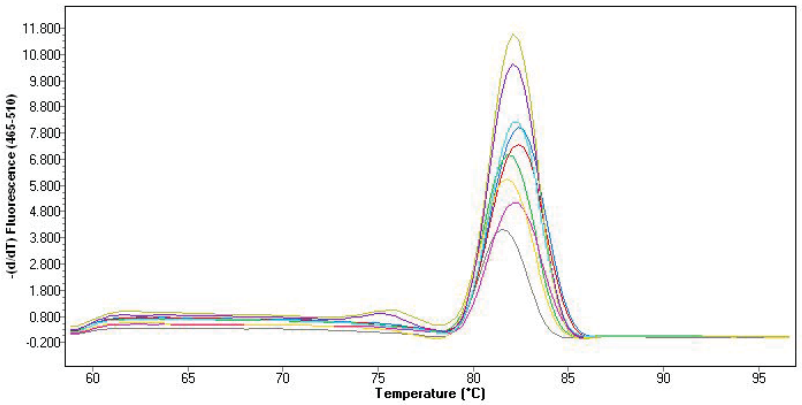

Figure 6.20: Melt Curve Analysis of *ampC* gene

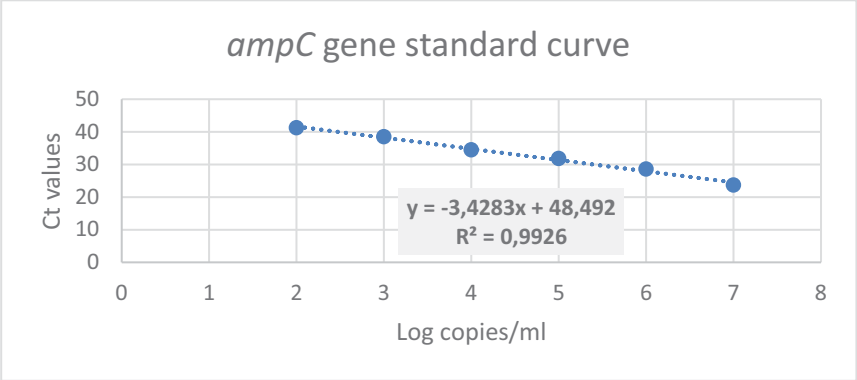

Figure 6.21: Standard curve generated for the *ampC* gene primers.

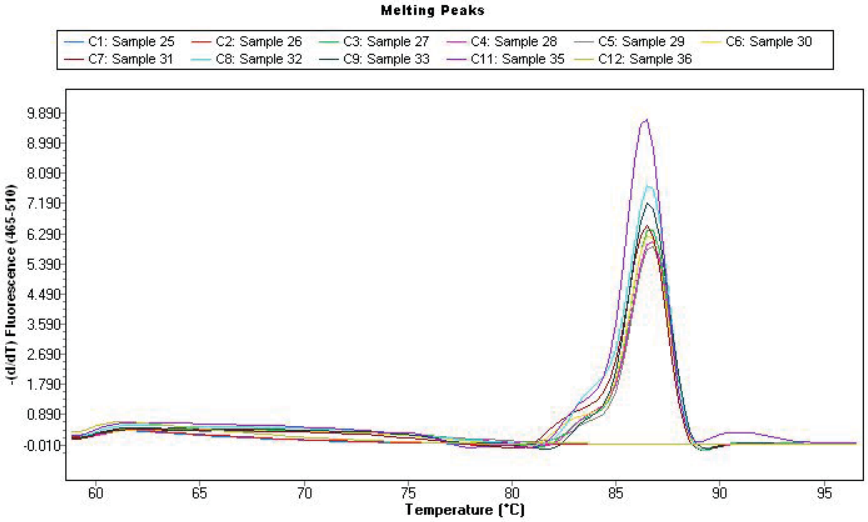

Figure 6.22: Melt Curve Analysis of *aadA* gene

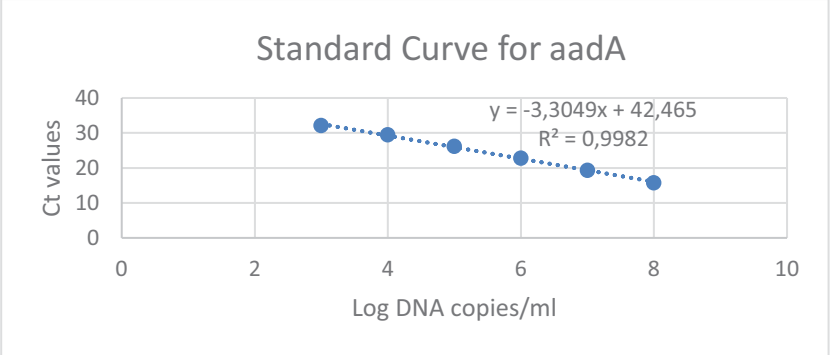

Figure 6.23: Standard curve generated for the *aadA* gene

6.3.5 Preliminary quantification experiments of the *vanA* gene using eDNA Samples

Once the cycling conditions had been optimised for all the primer sets using conventional PCR the next step was to test the efficiency of the primers using eDNA. Initially we tested the protocol on six eDNA samples, representing both greywater and biofilm samples, from summer and winter respectively. Figure 6.24 illustrates the melt curve analysis of the *vanA* ARG gene, for the standards (samples B1 to B8) and eDNA samples (F1 to F9), and it can be seen that only the specific band of interestis being amplified in the eDNA samples.

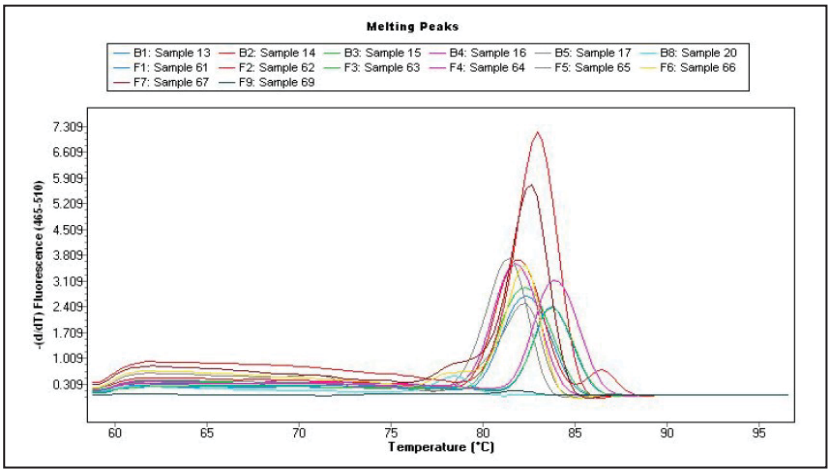

Figure 6.24: Melt curve of the vanA gene using ampC standards and eDNA samples. Samples F1 to F7 are eDNA samples extracted from greywater and biofilms, while samples B1 to H5 are standards made from purified amplicons generated with ampC primers with Enterobacter cloacae subsp. cloacae genomic DNA. F1 – GWS3S4, F2 – GWS3S5, F3 – BFS3S2, F4 – BFS3S2; F5 – GWS4S5; F6 – GWS4S6; F7 – BFS4S1; B1 – 1E7; B2 – 1E6; B3 – 1E5; B4 – 1E4; B5 – 1E3; B8 – NTC.

Absolute quantification was performed using the Second Derivate Maximum method, which allows one to include an internal control when using this algorithm (Table 6.5). From the Cp values the *vanA* gene copy number could be calculated and found to range from 5.17E+02 to 1.14E+03 (note that the samples with E values of 01 have Cp values greater than 35 and as such are not within the dynamic range of the assay and should not be considered). From the amplification curve (Figure 6.25) it can be seen that the

eDNA samples have higher Cp values than the standards, which would imply that the amount of DNA in these samples are lower. This was expected, as the standards are purified target amplicon, while the experimental samples are total eDNA extracted from the greywater and biofilm samples. As such, it would include the genomic DNA from all organisms present. From this analysis, in future experiments the amount of input DNA for the eDNA samples will be increased.

Table 6.5: Cp values of *vanA* gene for eDNA samples

Sample	Sample Description	*vanA* Cp value	*vanA* gene copy numbers
	Greywater Site 3, Sample 4	25,79	1,14E+03
GWS3S5	Greywater Site 3, Sample 5	32,17	8,85E+01*
BFS3S2	Biofilm Site 3, Sample 2	25,92	1,07E+03
BFS3S2	Biofilm Site 3, Sample 2	27,53	5,17E+02
GWS4S5	Greywater Site 4, Sample 5	37,89	1,65E+01*

* Cp value for sample not within dynamic range of assay

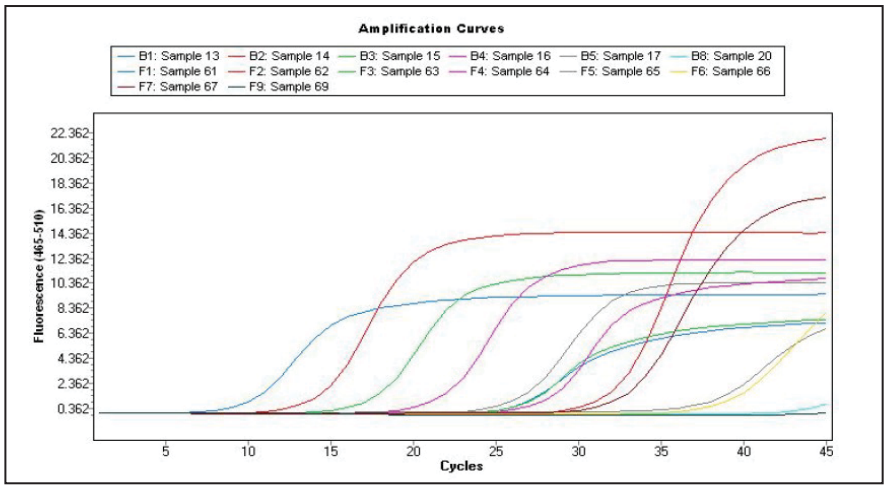

Figure 6.25: Amplification curve of the vanA gene primers generated for the Absolute Quantification analysis using the 2nd Derivative Max method. Samples F1 to F7 are eDNA samples extracted from greywater and biofilms, while samples B1 to H5 are standards made from purified amplicons generated with ampC primers with Enterobacter cloacae subsp. cloacae genomic DNA. F1 – GWS3S4, F2 – GWS3S5, F3 – BFS3S2, F4 – BFS3S2; F5 – GWS4S5; F6 – GWS4S6; F7 – BFS4S1; B1 – 1E7;B2 – 1E6; B3 – 1E5; B4 – 1E4; B5 – 1E3; B8 – NTC.

6.4 SUMMARY, CHALLENGES AND FUTURE WORK

The qPCR cycle and reaction parameters for all six primer sets have been optimised, and the specific amplicons have been cloned which were used to generate the standard curves. Preliminary testing using the *vanA* primers on eDNA samples was conducted to test the protocol on “mixed targets”. Although it appears that too little eDNA was used in the assay (which has been corrected in subsequent reactions) the *vanA* gene was detected in three of the 5 samples. Given that in Chapter 3 we reported

a high level of teicoplanin resistance, and studies have shown that strains resistant to this glycopeptide are frequently resistant to vancomycin this finding is not unexpected.

Despite the challenges encountered due to Covid (outlined below) considerable progress has been made in the qPCR analysis. As qPCR is so sensitive in order to obtain meaningful results it is critical that the PCR conditions for each primer set is optimised prior to analysis. One must ensure that only the specific amplicon is amplified and that the efficiency of reaction is between 90-100%. A considerable amount of time was spent optimising the qPCR.

The biggest challenge in completely this work was that due to Covid-related disruptions to the global supply chain. There were considerable delays in obtaining qPCR reagents and consumables as they are used in SARS CoV2 diagnostic kits. The specific white 96 well plates we need were ordered in May 2021 and the supplier was only able to provide the plates in January 2022 – we were eventually able to source similar plates which are specific for our instrument from an alternative supplier. The delivery of our SYBR Green Master 1 was also delayed and we had to use alternative mastermix for some of the analysis. These unforeseen delays were obviously never envisioned when the initial project workplan was designed.

CHAPTER 7: DETECTION AND QUANTIFICATION OF CLASS 1 INTEGRONS IN DOMESTIC GREYWATER SYSTEMS

7.1 INTRODUCTION

Integrons are bacterial genetic elements capable of capturing and expressing exogenous genetic material (typically gene cassettes) (Hall & Collis, 1995). These gene cassettes often carry genes which provide the microorganism with an adaptive advantage (Recchia & Hall, 1995). Integrons are composed of three key elements: (1) a gene encoding a site-specific integrase (*intI*), (2) an associated recombination site into which gene cassettes are inserted (*attI*) and (3) a promoter (*Pc*) capable of driving the expression of genes located in the cassettes inserted at *attI*. Integrons are extremely diverse and are classified into hundreds of classes which, together with their broad taxonomic distribution, suggests that they are ancient elements (Boucher *et al.,* 2007). All integrons of clinical relevance (classes 1 to 5) are found embedded in transposons, plasmids or integrative-conjugative elements, in contrast to sedentary chromosomal integrons that lack this mobility.

Class 1 integrons are the most intensely studied, mainly due to their well-established role in the acquisition and dissemination of antibiotic resistance genes in clinical environments, particularly in Gram-negative bacteria (Gillings *et al.* 2015). These integrons typically harbour one or more gene cassettes imparting resistance to a wide range of commonly used antibiotics (Hall & Collis, 1998). Several studies have investigated the abundance of *intI* genes in non-clinical environments such as rivers (Luo *et al.,* 2010), coastal waters (Koenig *et al.,* 2008), wastewater plants, aquatic biofilms (Gillings *et al.,* 2009) and even Antarctic soils (Ghaly *et al.*, 2019). Hardwick and co-workers screened for the presence of *intI* gene in creek sediments and the levels of the gene were found to correlate with the prevailing ecological conditions, implying that the integron provides selective advantages relevant to environmental pressures other than the use of antibiotics (Hardwick *et al.*, 2007). Early studies only looked at the presence of the integrons in culturable bacteria, and as such were only able to detect a fraction of the genes present in any environment. Later studies used molecular biology techniques, mainly PCR-based such as T-RFLP or qPCR, which have much greater sensitivity. Using these culture-independent methods researchers were able to detect significantly more integrons in environmental samples. A recent study investigated the diversity of integrons (both mobile and sedentary) in soil using next generation sequencing (NGS) and they detected between 4,000 and 18,000 unique cassettes per 0.3 grams of soil (Ghaly *et al.*, 2019). This finding would imply that previous studies likely under-estimated the abundance of integrons in the environment.

Studies have shown there is a strong association between the presences of integrons and multiple antibiotic resistance (MAR) phenotypes in bacteria (Leverstein-van-Hall *et al.* 2002). Integrons carrying four to five ARGs have been found in agricultural wastewater (Jacobs & Chenia 2007), urban wastewaters (Da Silva *et al.* 2007), and most concerning, water not recently exposed to antibiotics (Park *et al.* 2003). As integrons allow bacteria to adapt and evolve rapidly, their potential to spread ARGs in the natural environment is of increasing concern.

In this final experimental chapter, we describe the *intI* gene qPCR analysis of eDNA samples. The *intI* gene is used as a marker of horizontal gene transfer. This work specifically addresses Aim 5 “Screen for the presence of integrons as markers of horizontal gene transfer”.

Note: In the original application it was stated the detection of integrons would be performed via T-RFLP. However, during the inaugural reference group meeting several members of the reference group stated that due to the limitations of T-RFLP it would be preferable to rather use a quantitative method like qPCR. T-RFLP is still routinely used in studies which are focused on "gene diversity", however, despite being a PCR-based method T-RFLP may under-estimate the number of phylotypes present if two or more species have the same restriction site position within the target gene. Based on these limitations, it was decided to follow the recommendations of the reference group and to rather use qPCR for the detection and enumeration of the integrons.

7.2 METHODS AND MATERIALS

7.2.1 Optimisation of *intI* and 16S rRNA gene PCR

Conventional PCR was conducted to identify the optimal conditions for the target gene and reference primers (Table 7.1). PCR optimization entailed identifying the optimal annealing temperature using gradient PCR, as well as the optimal primer concentrations and genomic DNA for amplification.

Table 7.1: Real-Time PCR primer sets used in for integron PCR

Gene	Gene Description	Purpose	Primers	Size (bp)	Reference
IntI	*Class 1 Integrase* Involved in DNA Integration and DNA Recombination	Target Gene	qINT_3_MK qINT_4_MK	109	Paiva *et al*., 2015
16S rRNA	16S ribosomal RNA	Reference/ Housekeeping Gene	16S_MK01F 16S_MK01R	194	Pärnänen *et al*., 2019

Primers were tested against the following test strains: *E. coli* NCTC 13846, *E. cloacae* subsp. *cloacae* ATCC BAA-1143*, K. pneumoniae* subsp. *pneumoniae* ATCC 700603, *S. aureus* ATCC 29213 and *S. polyantibioticus* strain SPR^{T}. All bacterial strains, except for the *S. polyantibioticus,* were cultured in Luria Broth at 37°C, and the genomic DNA was extracted using the ammonium acetate method. *S. polyantibioticus* genomic DNA was kindly donated by Dr Marilize Le Roes-Hill (CPUT).

The above real-time PCR primer sets were first optimised using conventional PCR. Each reaction contained 0.5U Dream*Taq* Polymerase (Thermo Scientific), 0.2mM dNTP mix, 1X Dream*Taq* Buffer, 0.5µM forward and reverse primer and adjusted to a final volume of 25µl using PCR-grade water. Primers were tested using 10, 50 and 100ng genomic DNA. The amplification conditions are provided in the Table 7.2. PCR amplicons were electrophoresed on 2% (w/v) agarose gels. All gels contained 10µg/ml ethidium bromide and were electrophoresed in 1X TAE buffer at 90V for 1 hour.

Table 7.2: PCR Cycling conditions used for the *intI* and 16S RNA gene primers

Gene	Primer	Sequence (5'-3')	Amplification conditions
intI	Forward (qINT3)	TGCCGTGATCGAAATCCAGATCCT	95°C for 3 minutes 40 cycles at 95°C for 1 minute, 60°C for 45 seconds, 72°C for 1 minute 5 minutes at 72°C
	Reverse (qINT4)	TTTCTGGAAGGCGAGCATCGTTTG	
16S rRNA	Forward (16S_MK01F)	CCTACGGGAGGCAGCAG	95°C for 3 minutes 40 cycles at 95°C for 30 seconds, 59°C for 30 seconds, 72°C for 1 minute 5 minutes at 72°C
	Reverse (16S_MK01R)	ATTACCGCGGCTGCTGGC	

7.2.2 Construction of plasmid control for qPCR

A plasmid containing the *intI* gene and 16S rRNA PCR products respectively, were constructed for use as a control for the qPCR assay. Based on conventional PCR optimization, PCR products containing the *intI* gene using *K. pneumoniae* subsp. *pneumoniae* ATCC 700603 DNA and 16S rRNA using *E. cloacae* subsp. *cloacae* ATCC BAA-1143 DNA was used for the construction of plasmid controls for qPCR. The PCR products were cloned into the pJET1.2 Cloning vector using the ThermoScientific Clone-Jet PCR Cloning Kit according to the manufacturer's instructions. The recombinant vectors were transformed into electro-competent *E. coli* DH5α cells using electroporation and 50µl of transformed culture was spread onto LB plates containing 100µg/ml ampicillin. Transformed colonies were picked and colony PCR was conducted to confirm positive transformants. Plasmid DNA was extracted from confirmed positive clones using the Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA was quantified using the Qubit HS DNA assay (Invitrogen) and quality was assessed by conducting gel electrophoresis and gene-specific PCR for *intI* and 16S rRNA to confirm the presence of the insert. The plasmids were designated *pJET_intI1* and *pJET_16S* respectively.

7.2.3 Construction of Standard Curves – *intI1* and 16S rRNA genes

The plasmids controls were used to construct a standard curve to evaluate RT-PCR efficiency. Plasmid DNA was quantified using the Qubit HS DNA Assay (Invitrogen) and the number of molecule per µl was determined using Equation 1 and a dilution series was prepared over the range of 10^8 to 10^3 for the *intI* gene primers, and 10^9 to 10^4 for the 16S rRNA primers using TE buffer (the dilution range was adapted from Hardwick *et al.,* 2008).

7.2.4 Quantitative PCR Assay of *intI1* gene

qPCR was performed using the Roche LightCycler 480 II (Roche Applied Sciences). The reactions were setup in white 96-well PCR plates (Roche Applied Sciences). All reactions were conducted using the Roche LightCycler 480 SYBR Green 1 Master as per the manufacturer's instructions. For the quantification of the 16S rRNA gene (serving as the reference gene), each reaction contained 2X SYBR Master Mix, 0.5µM of each primer and adjusted to a final volume of 7.5µl using Molecular Grade water. 2.5µl of template DNA was added for each reaction, for the standard curve the input DNA was the control plasmid pJET_16SrRNA and for the environmental samples ~1 ng of metagenomic DNA was

added. qPCR cycling conditions were as follows: initial activation step at 95°C for 2 mins, followed by 45 cycles of denaturation at 95°C for 10 sec and a combined 15 sec annealing/extension at 59°C for 16S rRNA gene primers. Melt curve analysis was performed from 58 to 97°C. Quantification was performed using the LC480 software and Excel.

For the quantification of the *intI* gene (serving as the target gene), each reaction contained 2X SYBR Master Mix, 0.7µM of each primer and adjusted to a final volume of 7.5µl using Molecular Grade water. 2.5µl of template DNA was added for each reaction, for the standard curve the input DNA was the control plasmid pJET_intI and for the environmental samples ~1 ng of metagenomic DNA was added. qPCR cycling conditions were as follows: initial activation step at 95°C for 2 mins, followed by 45 cycles of denaturation at 95°C for 10 sec and a combined 15 sec annealing/extension at 60°C for *intI* gene primers. Melt curve analysis was performed from 59 to 97°C. Quantification was performed using the LC480 software and Excel.

7.3 RESULTS AND DISCUSSION

7.3.1 eDNA extractions from greywater and biofilm samples

Table 7.3 lists the samples which were screened for the presence of *intI* gene. Where possible we tried to analyse a greywater and the corresponding biofilm sample (the samples would have been collected at the same time) – there are only two samples were we were not able to include the biofilm sample.

Table 7.3: Table of samples analysed in the *intI1* qPCR assay

	Greywater Samples	*intI1*	*16S rRNA RT*	Biofilm Samples	*intI1*	*16S rRNA RT*
Site 1	GWS1S1	✓	✓	BFS1S1	✓	✓
	GWS1S2	✓	✓	BFS1S2	✓	✓
	GWS1S3	✓	✓	BFS1S3	✓	✓
	GWS1S4	✓	✓	BFS1S4	✓	✓
Site 2	GWS2S1	✓	✓	BFS2S1	✓	✓
	GWS2S2	✓	✓			
	GWS2S3	✓	✓	BFS2S3	✓	✓
	GWS2S4	✓	✓	BFS2S4	✓	✓
Site 3	GWS3S1	✓	✓	BFS3S1	✓	✓
	GWS3S2	✓	✓	BFS3S2	✓	✓
	GWS3S3	✓	✓	BFS3S3	✓	✓
	GWS3S4	✓	✓			
	GWS3S5	✓	✓	BFS3S5	✓	✓
	GWS3S6	✓	✓	BFS3S6	✓	✓
	GWS3S7	✓	✓	BFS3S7	✓	✓
Site 4	GWS4S1	✓	✓	BFS4S1	✓	✓
	GWS4S2	✓	✓	BFS4S2	✓	✓
	GWS4S3	✓	✓	BFS4S3	✓	✓
	GWS4S4	✓	✓	BFS4S4	✓	✓
	GWS4S5	✓	✓	BFS4S5	✓	✓
	GWS4S6	✓	✓	BFS4S6	✓	✓
	GWS4S7	✓	✓	BFS4S7	✓	✓

It should be noted that the 16S rRNA primer set used in this analysis binds in a different region than the primer set used in Chapter 6, so required separate optimisation. Agarose gels of the environmental DNA (eDNA) extracted using the DNEasy PowerSoil Pro Kit for greywater and biofilm samples is presented in Figures 7.1-7.3. It can be seen that eDNA was successfully extracted from both sample types, although in general less eDNA could be extracted from the biofilm samples. However, this is to be expected as the amount of cell mass used to extract eDNA from these samples was very low (cell mass was collected using 2-3 swabs compared to the greywater samples where ~8 litres of water was processed per sample).

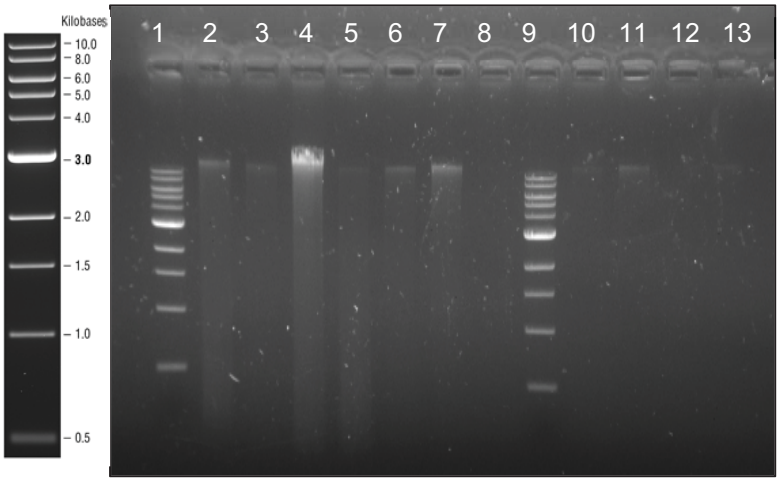

Figure 7.1: A 1% TAE gel representing environmental DNA extracted from greywater and biofilm samples using the DNEasy PowerSoil Pro Kit. Lane 1: 1Kb NEB Marker; Lane 2: GWS1S3; Lane 3: GWS2S3; Lane 4: GWS3S4; Lane 5: GWS4S5; Lane 6: GWS3S6; Lane 7: GWS3S5. Lane 9: 1kb NBE Marker; Lane 10: BFS1S4, Lane 11: BFS2S4; Lane 12: BFS3S7; Lane 13: BFS4S7.

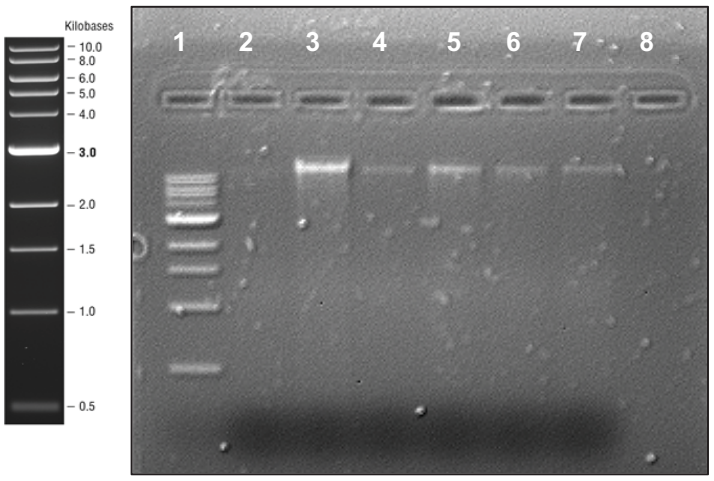

Figure 7.2: A 1% TAE gel representing environmental DNA extracted from greywater samples using the DNEAsy PowerSoil Pro Kit. Lane 1: 1Kb NEB Marker; Lane 2: GWS4S6; Lane 3: GWS4S7; Lane 4: GWS1S4; Lane 5: GWS2S4; Lane 6: GWS2S3; Lane 7: GWS3S7.

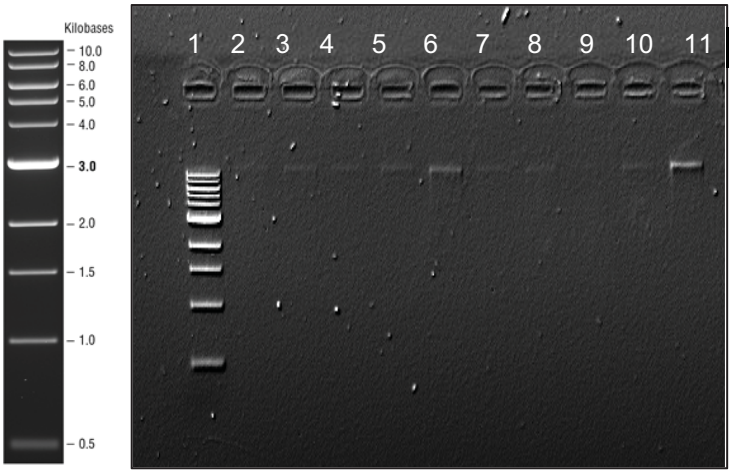

Figure 7.3: A 1% TAE gel representing Metagenomic DNA extracted from biofilm samples using the DNEAsy PowerSoil Pro Kit. Lane 1: 1Kb NEB Marker; Lane 2: BFS1S2; Lane 3: BFS2S3; Lane 4: BFS3S3; Lane 5: BFS3S5; Lane 6: BFS3S6; Lane 7: BFS4S3; Lane 9: BFS4S4; Lane 10: BFS4S5, Lane 11: BFS4S6

Before commencing with the qPCR assays, all the extracted eDNA was first tested via conventional PCR with 16S rRNA gene primers to confirm that the DNA was sufficiently free from inhibitors and was amplifiable. Figures 7.4 and 7.5 show the results from the PCR with biofilm and greywater eDNA, respectively. Intense bands were obtained for all the samples, even the biofilm samples even though the DNA concentration was very low for these samples. From these results it was decided that the extracted eDNA was of an acceptable quality for qPCR analysis.

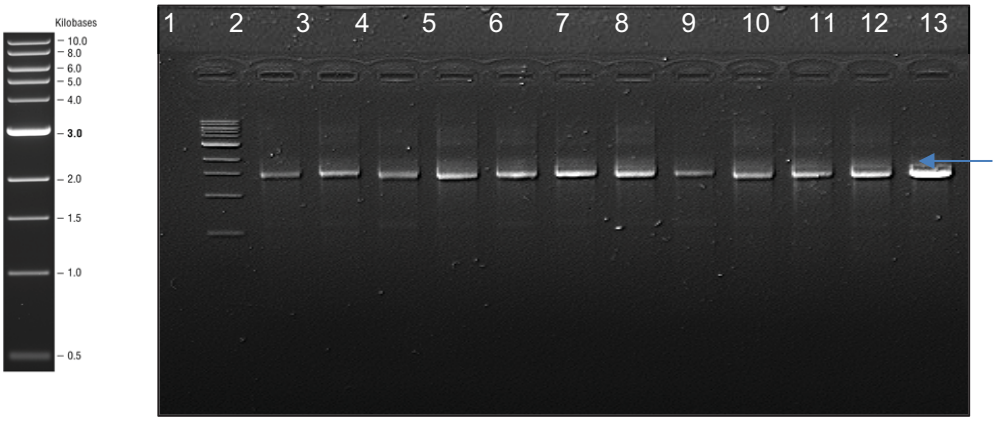

Figure 7.4: A 1% TAE gel depicting a 16S rRNA PCR of environmental DNA extracted from biofilm samples. Lane 1: 1Kb NEB Marker; Lane 2: BFS1S2; Lane 3: BFS2S3; Lane 4: BFS3S3; Lane 5: BFS3S5; Lane 6: BFS3S6; Lane 7: BFS4S3; Lane 8: BFS4S; Lane 9: BFS4S5; Lane 10: BFS4S6; Lane 11: BFS2S4; Lane 12: BFS4S7; Lane 13; *S. aureus* genomic DNA control.

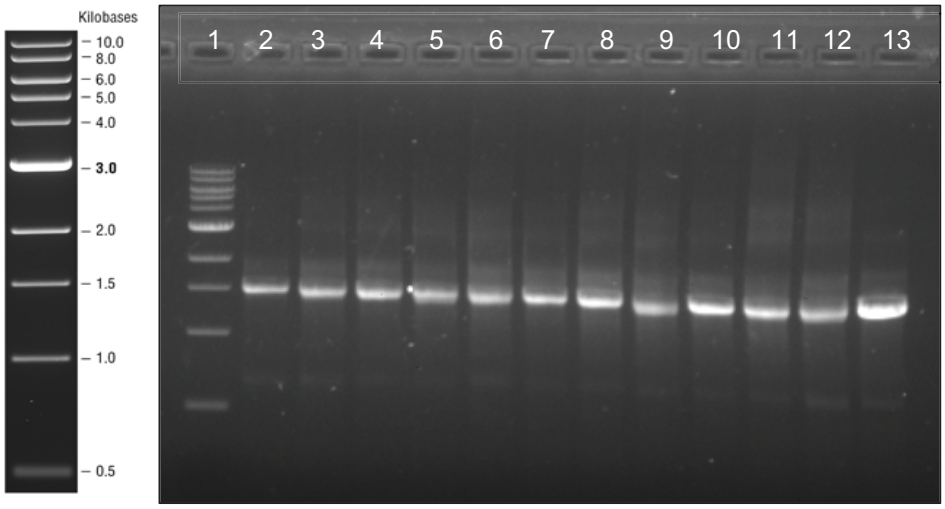

Figure 7.5: A 1% TAE gel depicting a 16S rRNA PCR of environmental DNA extracted from greywater samples. Lane 1: 1Kb NEB Marker; Lane 2: GWS1S1; Lane 3: GWS1S2; Lane 4: GWS2S2; Lane 5: GWS3S1; Lane 6: GWS3S2; Lane 7: GWS3S3, Lane 8: GWS4S1; Lane 9: GWS4S2; Lane 10: GWS4S3; Lane 11: GWS4S4; Lane 12: GWS1S3; Lane 13: *S. aureus* genomic DNA control.

7.3.2 PCR Optimization of the *intI* and 16S rRNA gene primers

The use of real-time qPCR on environmental samples requires careful optimization to ensure reliable and reproducible results. As SYBR Green I detects any double-stranded DNA generated during the PCR process, including primer-dimers and nonspecific products, the PCR cycling conditions must be optimised prior to commencing real time experiments. For this reason, considerable time was spent optimising the amplification process. Primers were tested at different annealing temperatures, at a range of primer concentrations, and using DNA from several test strains.

We were able to achieve relatively good and specific amplification (Figure 7.6) with the *intI* primers. The optimised annealing temperature of 60°C was significantly higher than the 57°C Tm provided on the synthesis report provided with the primers. Additionally, for the 16S rRNA gene which serves as the reference gene in the following study, the optimal annealing temperature was 59°C. A single defined bands was obtained for both primer sets after optimisation, as seen by the 109bp for *intI1* and 194bp for 16S rRNA (Figure 7.6).

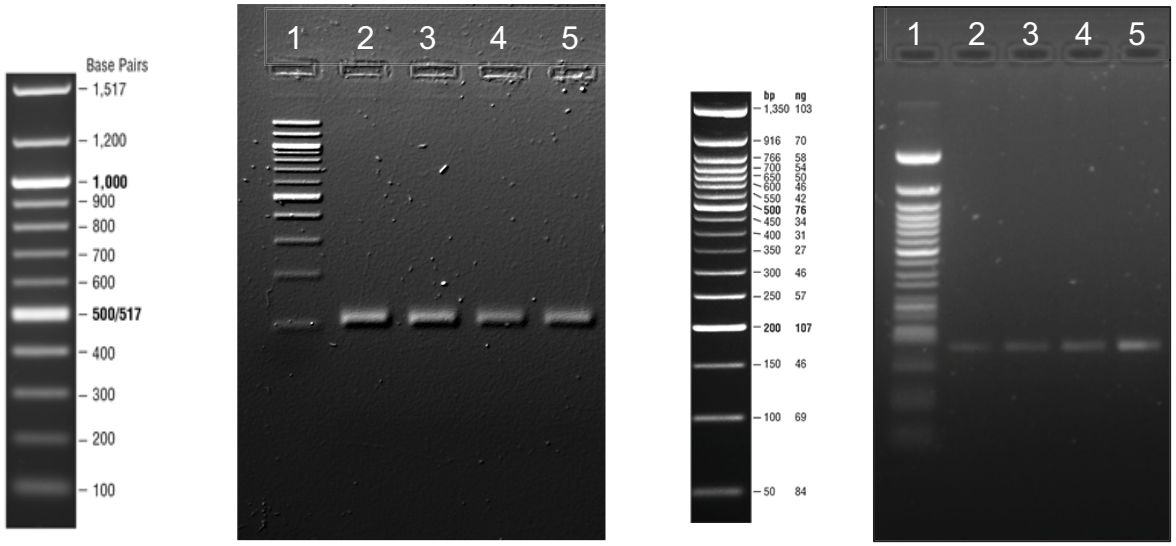

Figure 7.6: A 2% TAE gel depicting the results from the optimised *intI and* 16S rRNA gene PCR. Gel A: *intI* PCR using *K. pneumonaie* genomic DNA. Lane 1: 100bp NEB Marker; Lane 2-5: *intI* PCR products of 109bp. Gel B: 16S rRNA gene PCR using *E. cloacae* subsp. *cloacae* ATCC BAA-1143 genomic DNA. Lane 1: 50bp NEB Marker, Lane 2-5: 16S rRNA PCR products of 194bp.

For absolute quantification the recommendation is that you prepare standards using plasmid dilutions, not purified amplicon, as it has been shown to provide more accurate and consistent results. Therefore, once the PCR was optimised the correct sized fragments were excised from the gel, gel purified and cloned into pJET vector. PCR was then performed using pJET sequencing primers (Figure 7.7) and gene specific primers (Figure 7.8) to confirm that the correct amplicon had been cloned. Note that these two figures are gels which confirm that the constructs generated for all eight primer sets used for qPCR analysis were correct, including the six housekeeping and ARGs primer sets discussed in Chapter 6.

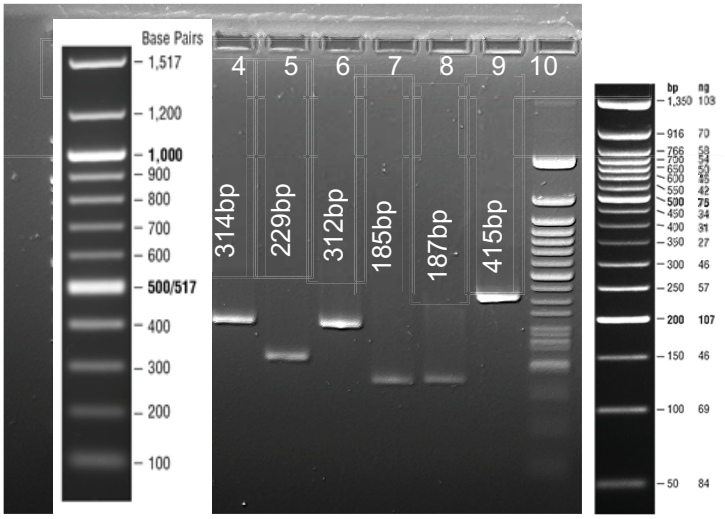

Figure 7.7: A 1% TAE agarose gel depicting the PCR confirmation of inserts using the pJET Sequencing Primers. Lane 1: 100bp NEB ladder; Lane 2: *gapA*; Lane 3: *mdh*; Lane 4: 16S rRNA RT; Lane 5: *intI1*; Lane 6: 16S rRNA Int; Lane 7: *vanA*; Lane 8: *ampC*; Lane 9: *aadA*; Lane 10: 50 bp NEB Ladder.

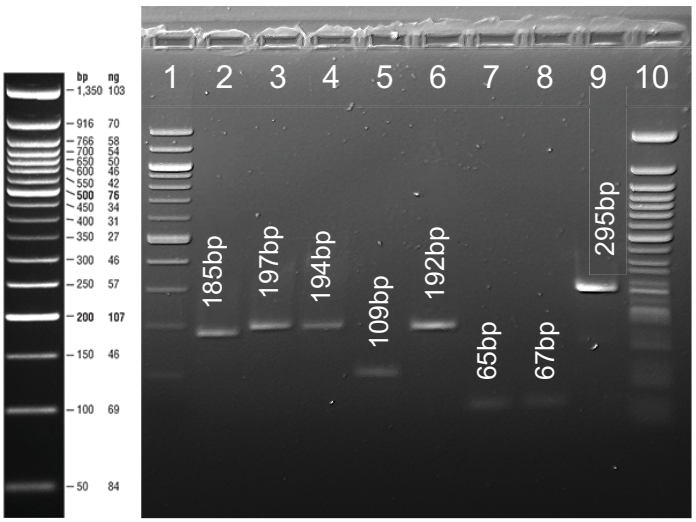

Figure 7.8: A 1% TAE agarose gel depicting the PCR confirmation of inserts using gene-specific primers. Lane 1: 100bp NEB ladder; Lane 2: *gapA*; Lane 3: *mdh*; Lane 4: 16S rRNA RT; Lane 5: *intI*; Lane 6: 16S rRNA Int; Lane 7: *vanA*; Lane 8: *ampC*; Lane 9: *aadA*; Lane 10: 50 bp NEB Ladder.

7.3.3 Generation of Standard Curves for the 16S rRNA and *intI* gene qPCR

Standard curves were generated using the plasmids constructed for the *intI* and 16S rRNA gene primers, respectively. As stated in the MIQE guidelines, between 5-7 dilutions were used to generated the standard curves. Melt curve analysis revealed that the *intI* primers were highly specific as only a single sized amplicon was generated (Figure 7.9). The efficiency of the PCR was found to be within the accepted range of 90-100% (as the slope of the standard curve was -3.296) and the R^2 = 0.9954 (Figure 7.10). Similarly, the 16S rRNA gene primers were specific (Figure 7.11) and the efficiency (the slope) and R^2 were -3.3897 and 0.9987 (Figure 7.12), respectively.

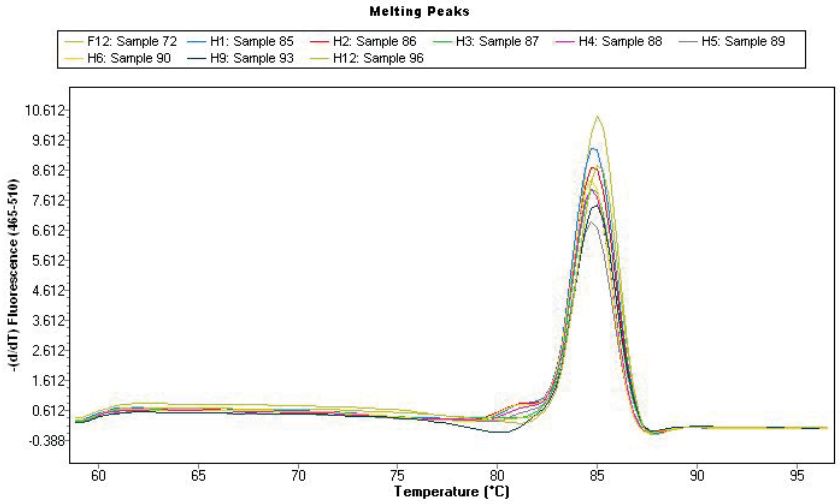

Figure 7.9: Melt Curve of *intI* gene amplicons generated using a dilution series of control plasmids. Cp values for these amplicons were used to construct the standard curve used for subsequent absolute quantification experiments.

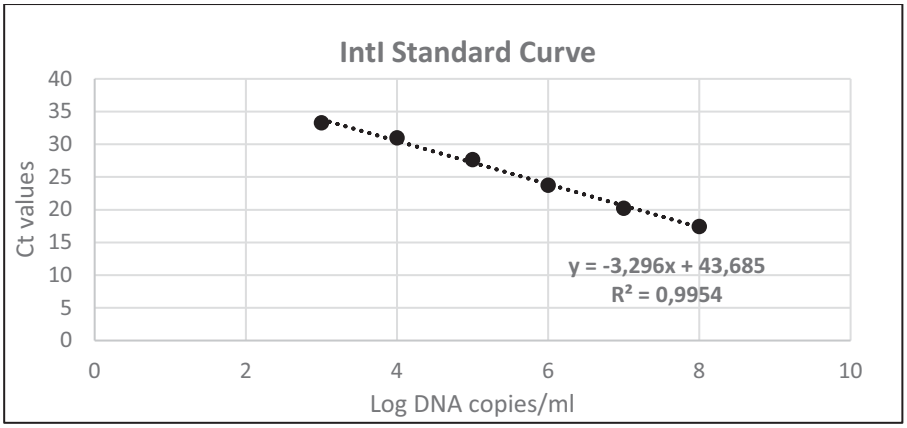

Figure 7.10: Standard curve for the *intI* primers of the log molecules/µl versus the Cp value.

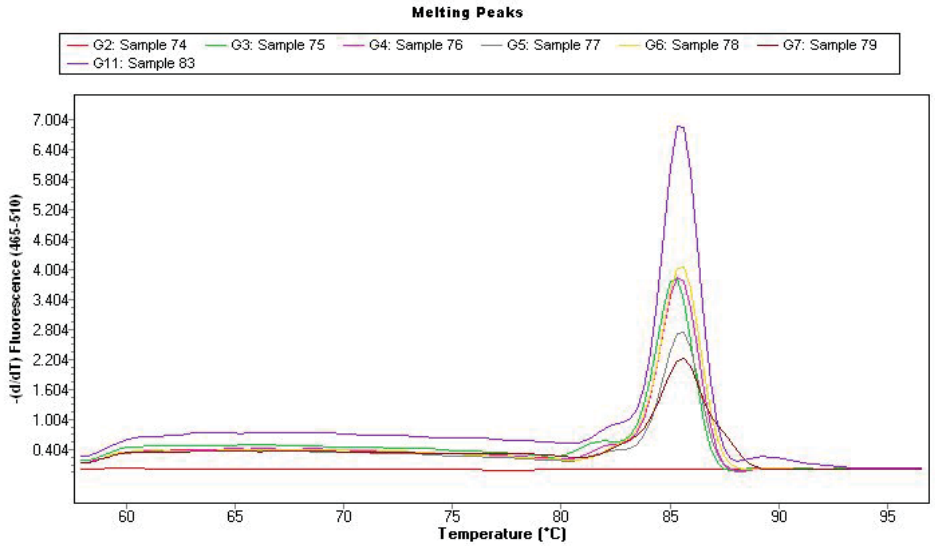

Figure 7.11: Melt Curve of 16S rRNA gene amplicons generated using a dilution series of control plasmids. Cp values for these amplicons were used to construct the standard curve used for subsequent absolute quantification experiments.

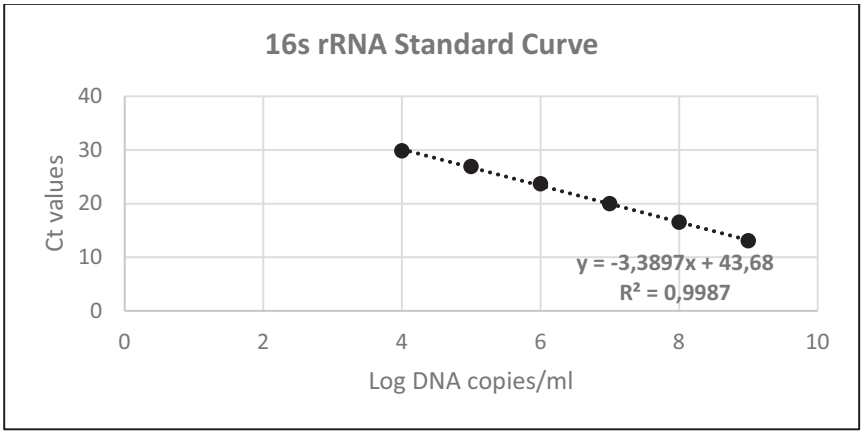

Figure 7.12: Standard curve for the 16S rRNA gene primers of the Log molecules/µl versus the Cp value.

7.3.4 Quantification of the *intI1* gene in greywater and biofilm samples

In order to simplify the analysis process and to keep track of which samples had been analysed the eDNA samples were grouped (1-6) each containing greywater and biofilm samples as shown in Table 7.4. The optimized qPCR conditions were used to evaluate eDNA samples extracted from greywater and biofilms, respectively. As seen in Figures 7.13-7.15 from the melt curve analysis of the *intI1* gene primers only the specific band of interest (at temperature of ~85°C) was amplified in metagenomic samples.

Table 7.4: Layout of reactions for the Absolute Quantification of the *intI1* gene – Integron Assay

Greywater Samples	*IntI1*	16S rRNA	Biofilm Samples	*IntI1*	16S rRNA	Groups
GWS1S1	✓	✓	BFS1S1	✓	✓	Group 1
GWS1S2	✓	✓	BFS1S2	✓	✓	Group 2
GWS1S3	✓	✓	BFS1S3	✓	✓	Group 3
GWS1S4	✓	✓	BFS1S4	✓	✓	Group 4
						Group 5
GWS2S1	✓	✓	BFS2S1	✓	✓	Group 6
GWS2S2	✓	✓				
GWS2S3	✓	✓	BFS2S3	✓	✓	
GWS2S4	✓	✓	BFS2S4	✓	✓	
GWS3S1	✓	✓	BFS3S1	✓	✓	
GWS3S2	✓	✓	BFS3S2	✓	✓	
GWS3S3	✓	✓	BFS3S3	✓	✓	
GWS3S4	✓	✓				
GWS3S5	✓	✓	BFS3S5	✓	✓	
GWS3S6	✓	✓	BFS3S6	✓	✓	
GWS3S7	✓	✓	BFS3S7	✓	✓	
GWS4S1	✓		BFS4S1	✓	✓	
GWS4S2	✓	✓	BFS4S2	✓	✓	
GWS4S3	✓	✓	BFS4S3	✓	✓	
GWS4S4	✓	✓	BFS4S4	✓	✓	
GWS4S5	✓	✓	BFS4S5	✓	✓	
GWS4S6	✓	✓	BFS4S6	✓	✓	
GWS4S7	✓	✓	BFS4S7	✓	✓	

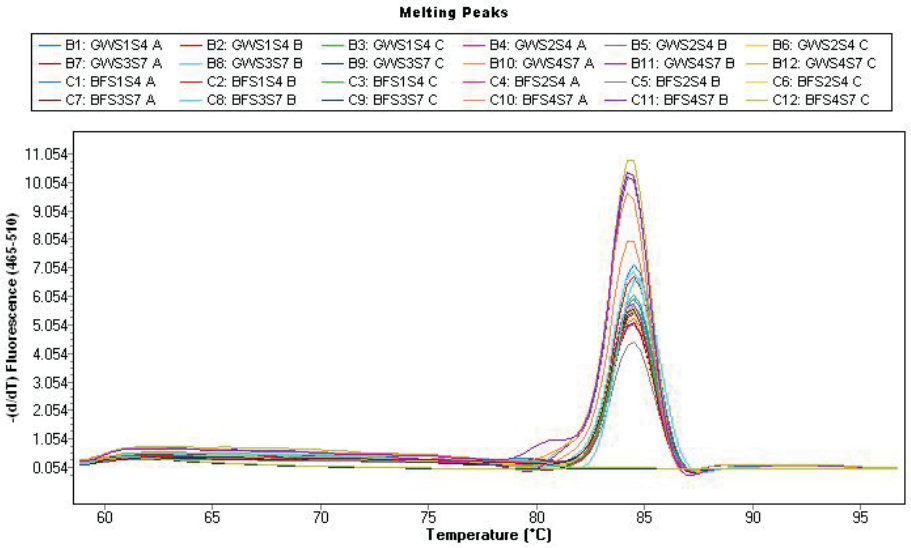

Figure 7.13: Melt Curve analysis of *intI* primers in group 1 samples

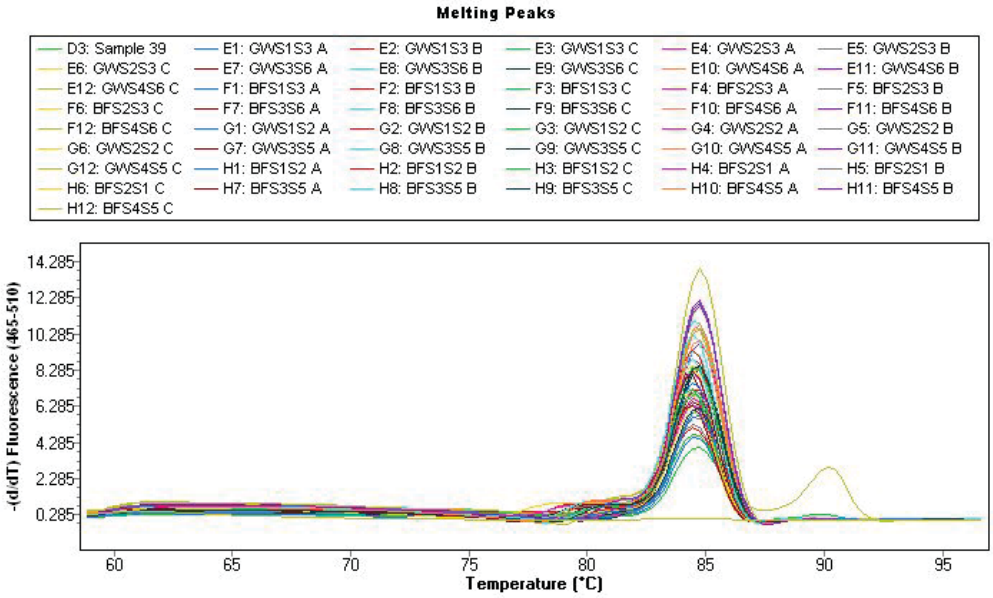

Figure 7.14: Melt Curve analysis of *intI* primers in group 2 and group 3 eDNA samples.

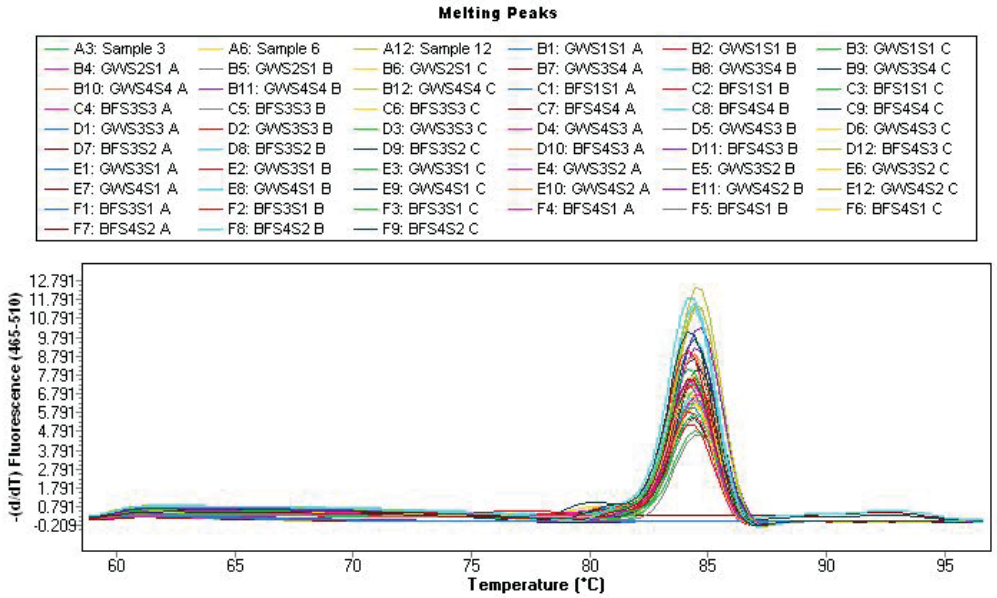

Figure 7.15: Melt Curve Analysis of *intI* primers in group 4, 5 and 6 eDNA samples.

Absolute quantification was performed using the Second derivate method, as the software allows one to include internal controls when using the algorithm. Each sample was assessed in triplicate and the mean Ct value and copy number was derived from the standard curve.

qPCR revealed that the *intI* gene was detected in all 22 greywater samples and 19 biofilm samples analysed. The copy numbers of both the *intI* and 16S rRNA genes were calculated for each sample by plotting on the respective standard curve as seem in Figures 7.16 and 7.17. Table 7.5 represents the mean Ct values and their corresponding mean copy numbers of *intI* gene in greywater samples. As shown in the table all gene copy numbers fell within the range of the standard curve, this can also be seen in Figure 7.16. For the greywater samples the mean copy numbers ranged from 2.38E+04 (spring sample, GWS3S6) to 1.73E+08 (summer sample, GWS3S4).

Table 7.5: *intI1* Mean Cp values and Mean Copy Numbers of greywater samples

Site	Collection	Sample	Collection Season	Mean Ct value	Mean Copy Number
1	1	GWS1S1	Spring	27,48	7,10E+04
	2	GWS1S2	Winter	26,51	9,31E+04
	3	GWS1S3	Summer	23,733	5,92E+05
	4	GWS1S4	Autumn	24,10333	5,40E+05
2	1	GWS2S1	Spring	20,86	6,82E+06
	2	GWS2S2	Spring	27,49666	4,05E+04
	3	GWS2S3	Summer	27,87	3,20E+04
	4	GWS2S4	Autumn	25,94	1,59E+05
3	1	GWS3S1	Spring	19,22	1,77E+07
	2	GWS3S2	Summer	26,0733	1,81E+05
	3	GWS3S3	Spring	29,24	2,26E+04
	4	GWS3S4	Summer	15,775	1,73E+08
	5	GWS3S5	Winter	26,95666	5,52E+04
	6	GWS3S6	Spring	28,21	2,38E+04
	7	GWS3S7	Autumn	26,54	1,06E+05
4	1	GWS4S1	Summer	28,42	3,87E+04
	2	GWS4S2	Autumn	25,75666	2,26E+05
	3	GWS4S3	Autumn	26,9	1,04E+05
	4	GWS4S4	Spring	25,69	1,11E+05
	5	GWS4S5	Summer	25,286666	1,66E+05
	6	GWS4S6	Winter	28,13	3,35E+04
	7	GWS4S7	Spring	28,07	3,86E+04

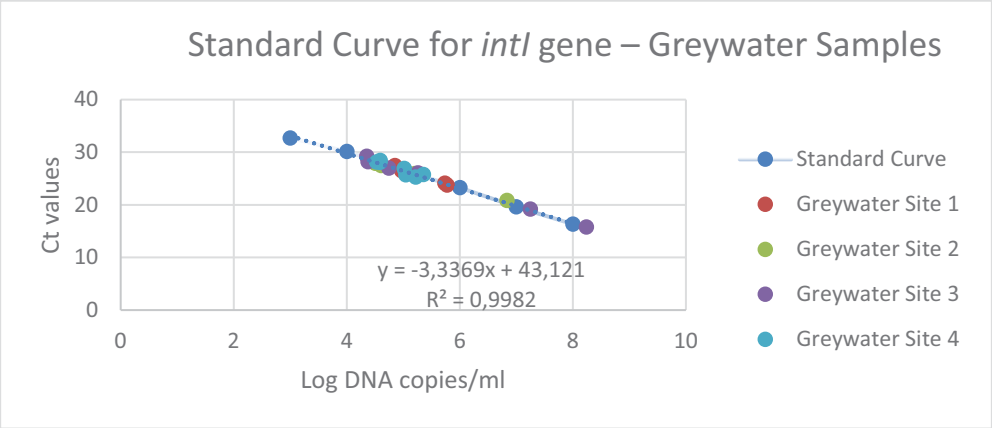

Figure 7.16: Standard Curve with Greywater Samples present on graph for *intI* gene

Table 7.6 represents the mean Ct values and their corresponding mean copy numbers of the *intI* in biofilm samples. Based on the copy numbers, it is identified that the range is between 5.77E+03 and 9.07E+05, which is significantly lower than that of the greywater sample of which a mean copy number of 1.77E+07 was encountered for the sample GWS3S1. As for greywater samples, all biofilm samples fell within the range of the standard curve as seen in Figure 7.17. As stated earlier, the lower copy number observed for the biofilm samples is a reflection on the amount of starting material.

Table 7.6: Mean Ct values and Mean Copy Numbers for Biofilm Samples

Site	Collection	Sample	Collection Season	Mean Ct value	Mean Copy Number
1	1	BFS1S1	Spring	25,656	1,68E+05
	2	BFS1S2	Winter	26,976	5,82E+04
	3	BFS1S3	Summer	28,976	1,45E+04
	4	BFS1S4	Autumn	27,546	2,26E+05
2	1	BFS2S1	Spring	27,533	3,76E+04
	2				
	3	BFS2S3	Summer	28,85	1,53E+04
	4	BFS2S4	Autumn	26,083	1,45E+05
3	1	BFS3S1	Spring	27,825	5,68E+04
	2	BFS3S2	Summer	29,8	1,52E+04
	3	BFS3S3	Spring	28,78	2,98E+04
	4		Summer		
	5	BFS3S5	Winter	27,6066	3,56E+04
	6	BFS3S6	Spring	27,88	2,92E+04
	7	BFS3S7	Autumn	30,98	5,77E+03
4	1	BFS4S1	Summer	32,203	2,63E+03
	2	BFS4S2	Autumn	23,66	9,07E+05
	3	BFS4S3	Autumn	28,933	2,69E+04
	4	BFS4S4	Spring	28,405	3,95E+04
	5	BFS4S5	Summer	26,465	7,47E+04
	6	BFS4S6	Winter	28,52	1,90E+04
	7	BFS4S7	Spring	28,795	2,38E+04

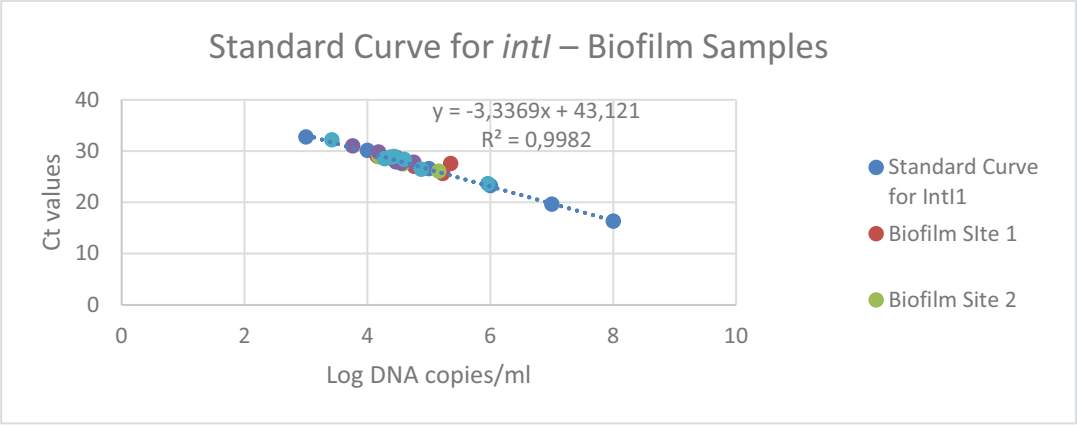

Figure 7.17: Standard Curve with Biofilm Samples present on graph for *intI*

The same process was completed for the 16S rRNA gene analysis. From the Melt curve analysis of the 16S rRNA primer set on eDNA samples was not as refined and elegant as that of the *intI1* primer set. Melting peaks with a left hand side "shoulder" (encircled in Figure 7.19), well as multiple melting peaks were detected for many samples (Figures 7.18-7.23). Based on the melting peaks present in the standard curve, most eDNA samples peaked at 85°C, however many samples had a peak at slightly higher temperature. This may be indicative that there is variation in the microbial diversity of the eDNA

sample, hence different melting temperatures. This is supported by the fact that the primers are for the variable region of the 16S ribosomal RNA gene as seen when the primer set is subjected to a Primer-BLAST analysis.

As we were analysing total environmental DNA which contains the 16S rRNA genes for all the organisms present in a sample this result is not unsurprising, as one would expect different species to have difference in the sequence of their 16S rRNA genes, which would ultimately result in a different melting temperature. One would expect to see more sequence differences in the variable region of the 16S gene amplified with this primer set compared to the *intI* gene primers which looks at a highly conserved region. The 16S primers specifically targets a variable region and as such distantly related species would have several differences in their gene sequences (there may also be slight differences in size of the resulting amplicon). This left hand side shoulder is common in 16S melt curves and does not influence the quantification calculations.

One can also see a slight left shoulder in the amplification curve. The one possible reason for this shoulder is “short” primers, however, as these curves were generated with PAGE purified primers one would not expect there to be residual short primers present. Another reason for the shoulder is poor primer design resulting in primer dimers which could be rectified by using less primers in future experiments. However, as the automatic noise threshold excluded this slight shoulder it was not expected to influence the Ct values obtained.

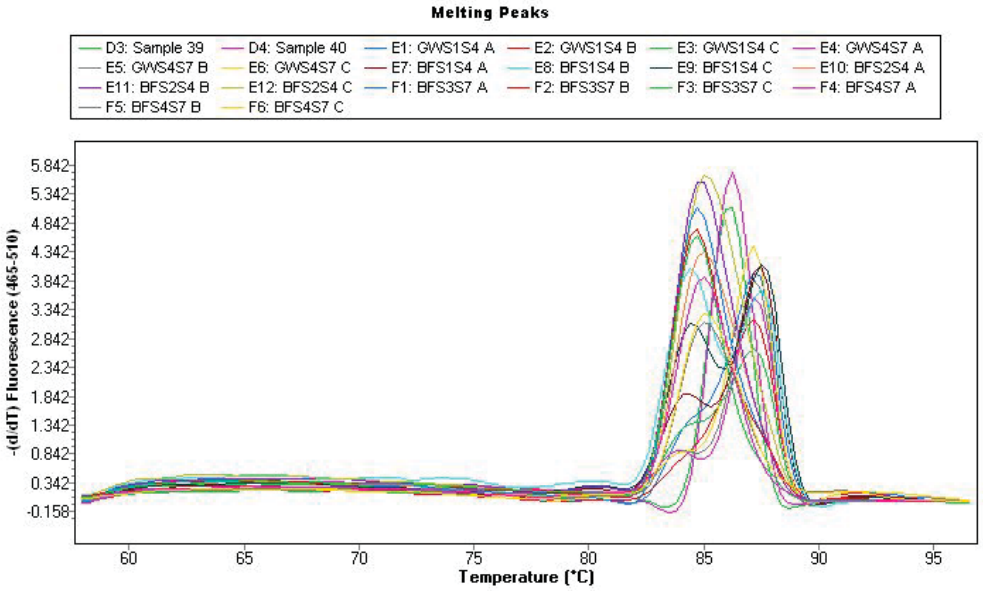

Figure 7.18: Melt Curve Analysis of 16S primers in group 1 eDNA samples.

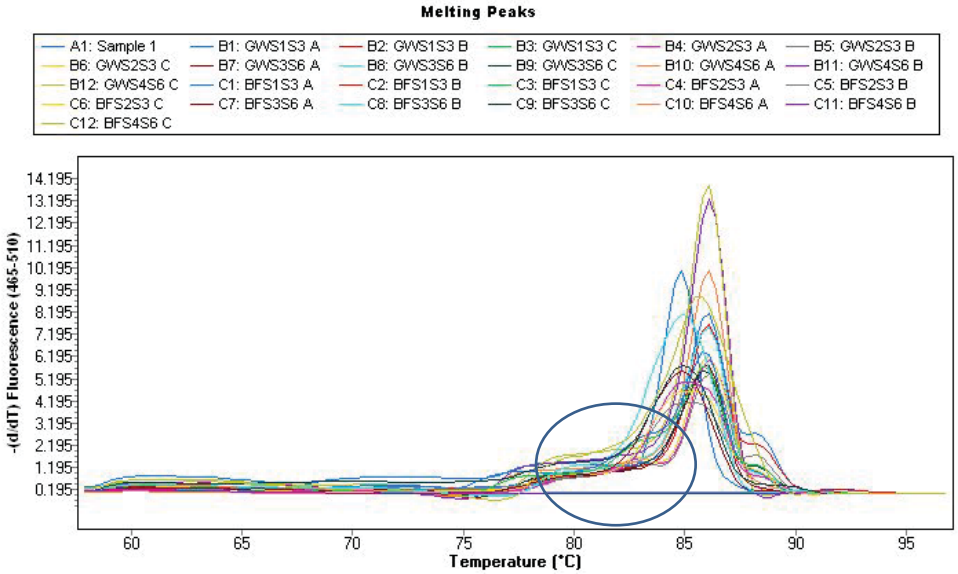

Figure 7.19: Melt Curve Analysis of 16S primers in group 2 eDNA samples.

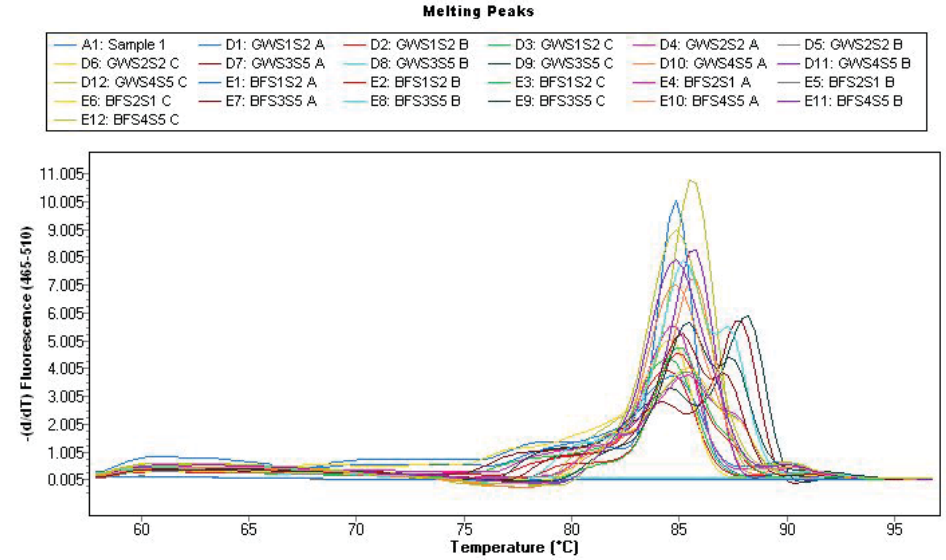

Figure 7.20: Melt Curve Analysis of 16S primers in group 3 eDNA samples.

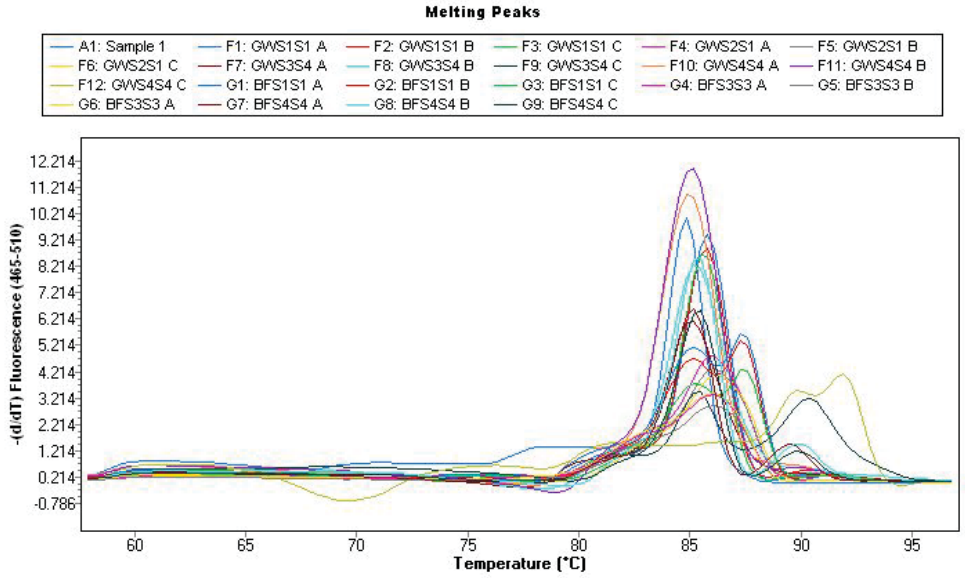

Figure 7.21: Melt Curve Analysis of 16S primers in group 4 eDNA samples.

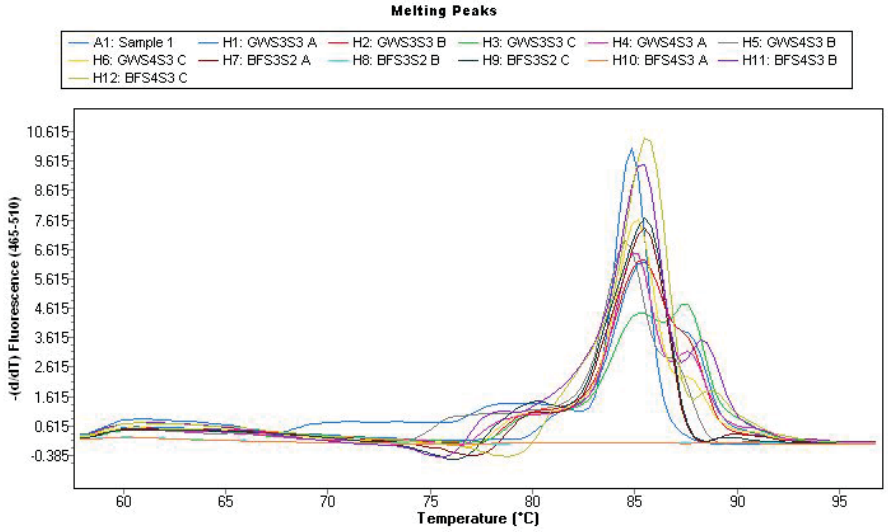

Figure 7.22: Melt Curve Analysis of 16S primers in group 5 eDNA samples.

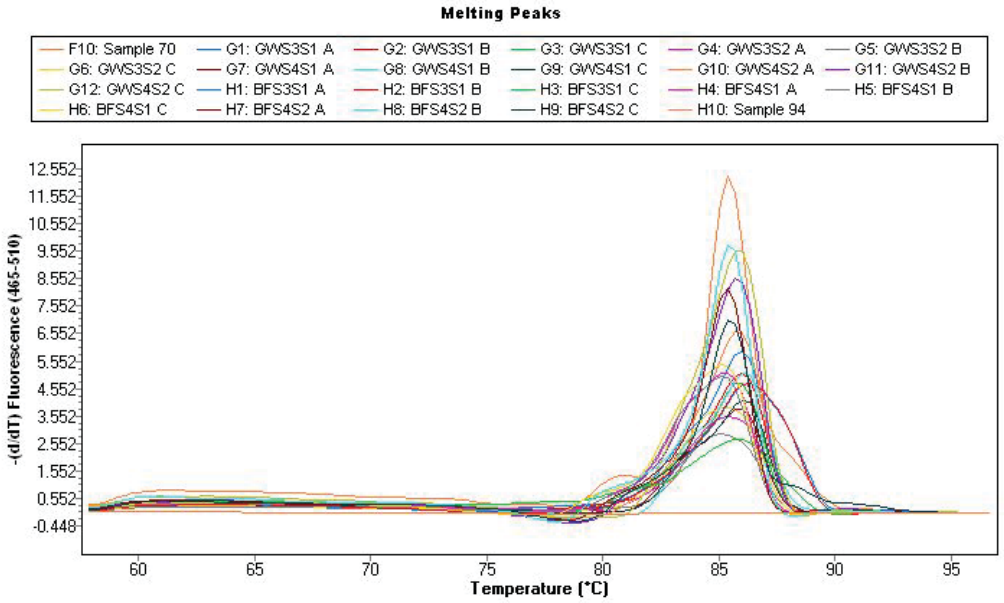

Figure 7.23: Melt Curve Analysis of 16S primers in group 6 eDNA samples.

The mean Ct values and Mean copy numbers of the 16S rRNA gene are presented in the Tables 7.7 (greywater) and 7.8 (biofilm). Based on initial analysis it is identified that many samples from greywater and biofilm samples fall out of the linear range produced by the standards as shown in the graphs below This may be due to the high concentration of 16S rRNA genes, however even when normalized as shown in the table below copy numbers still exceed the 10^9 range. This may be corrected by further diluting the metagenomic DNA samples as well as setting up a wider dilution range to take into consideration such high copies.

Table 7.7: Mean Ct values and Mean Copy Numbers for 16S – Greywater Samples

Site	Collection	Sample	Collection Season	Mean Ct	Mean Copy Number	Normalized 16S rRNA
1	1	GWS1S1	Spring	19,3766	6,02E+13	1,51E+13
	2	GWS1S2	Winter	31,91667	2,13E+10	5,33E+09
	3	GWS1S3	Summer	19,66	5,21E+13	1,30E+13
	4	GWS1S4	Autumn	13,17	8,16E+08	2,04E+08
2	1	GWS2S1	Spring	16,458	2,64E+14	6,60E+13
	2	GWS2S2	Spring	34,215	1,91E+09	4,78E+08
	3	GWS2S3	Summer	23,96	3,00E+12	7,50E+11
	4	GWS2S4	Autumn	9,153	1,24E+10	3,10E+09
3	1	GWS3S1	Spring	12,703	1,64E+09	4,10E+08
	2	GWS3S2	Summer	13,13667	1,18E+09	2,95E+08
	3	GWS3S3	Spring	15,98666	3,10E+14	7,75E+13
	4	GWS3S4	Summer	20,7366	7,69E+15	1,92E+15
	5	GWS3S5	Winter	26,52	5,30E+11	1,33E+11
	6	GWS3S6	Spring	24,3033	2,55E+12	6,38E+11
	7	GWS3S7	Autumn	9,96	7,17E+09	1,79E+09
4	1	GWS4S1	Summer	10,0133	1,22E+10	3,05E+09
	2	GWS4S2	Autumn	12,68	1,61E+09	4,03E+08
	3	GWS4S3	Autumn	20,91	1,90E+14	4,75E+13
	4	GWS4S4	Spring	23,423	5,33E+14	1,33E+14
	5	GWS4S5	Summer	16,003	3,00E+14	7,50E+13
	6	GWS4S6	Winter	27,77	2,65E+11	6,63E+10
	7	GWS4S7	Spring	10,84	3,69E+09	9,23E+08

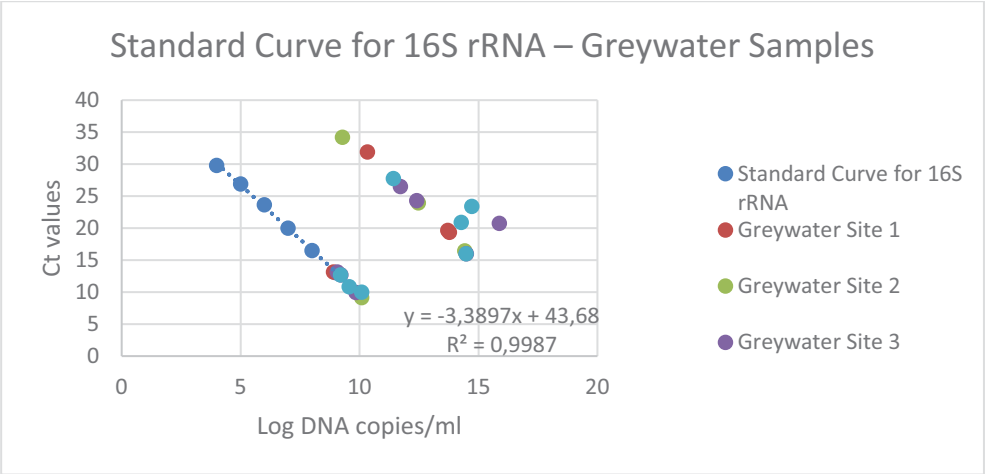

Figure 7.24: Standard Curve with greywater samples present on graph for the 16S rRNA gene

Table 7.8: Mean Ct values, Mean Copy Number for 16S rRNA – Biofilm Samples

Site	Collection	Sample	Collection Season	Mean Ct	Mean Copy Number	Normalized 16S rRNA
1	1	BFS1S1	Spring	19,933	3,84E+13	9,60E+12
	2	BFS1S2	Winter	2,935	2,19E+13	5,48E+12
	3	BFS1S3	Summer	30,77	2,55E+10	6,38E+09
	4	BFS1S4	Autumn	15,023	2,44E+08	6,10E+07
2	1	BFS2S1	Spring	23,22	4,59E+12	1,15E+12
	2		Spring			
	3	BFS2S3	Summer	27,83	3,06E+11	7,65E+10
	4	BFS2S4	Autumn	13,386	7,12E+08	1,78E+08
3	1	BFS3S1	Spring	18,956	3,15E+07	7,88E+06
	2	BFS3S2	Summer	25,7	1,41E+12	3,53E+11
	3	BFS3S3	Spring	22,543	6,55E+12	0,00E+00
	4		Summer			
	5	BFS3S5	Winter	19,53667	4,81E+13	1,20E+13
	6	BFS3S6	Spring	29,046	4,61E+11	1,15E+11
	7	BFS3S7	Autumn	18,63	2,40E+07	6,00E+06
4	1	BFS4S1	Summer	15,0266	3,28E+08	8,20E+07
	2	BFS4S2	Autumn	21,7733	3,60E+06	9,00E+05
	3	BFS4S3	Autumn	23,205	6,45E+12	1,61E+12
	4	BFS4S4	Spring	18,7966	8,91E+13	2,23E+13
	5	BFS4S5	Summer	20,09	3,17E+13	7,93E+12
	6	BFS4S6	Winter	29,46	4,20E+10	1,05E+10
	7	BFS4S7	Spring	14,8866	2,67E+08	6,68E+07

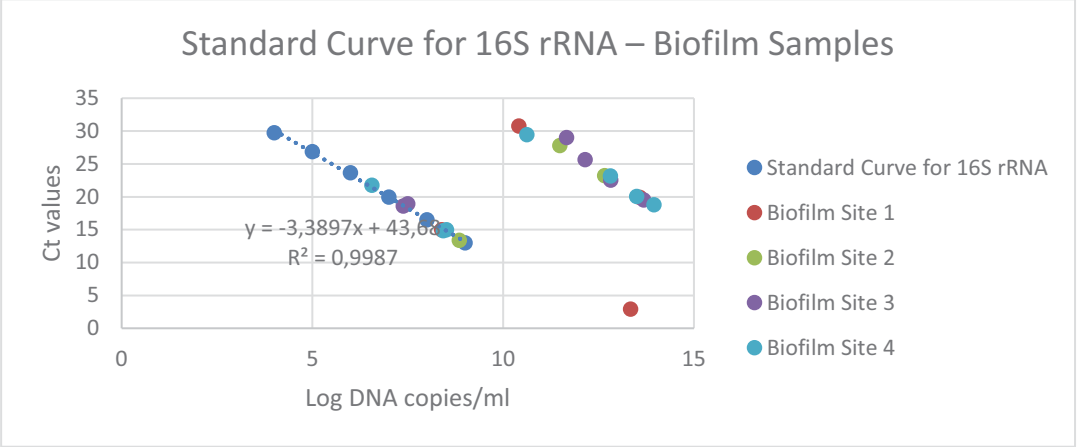

Figure 7.25: Standard Curve with biofilm samples present on graph for the 16S rRNA gene

Based on the preliminary analysis, the microbial population appears to be larger in the summer than in the colder winter months. For the greywater samples the average normalised 16S rRNA gene copy number in summer was 2.22E+13 compared to 1.88E+10 in winter (three-fold higher in summer). Other researchers have also reported that in aquatic systems higher microbial numbers are detected in the warmer summer months (Luo *et al.*, 2010). Interestingly, the similar numbers were detected in the biofilm samples, with the average gene copy number in summer being 2.09E+12 and 1.33E+12 for the winter samples. Several studies have reported that the seasonal fluctuations in population sizes is smaller in biofilm/sediment compared to the water column (Luo *et al.*, 2010). This is likely due to several

factors, such as a more constant food supply available in a biofilm and that the EPS provides protection from external environment.

The *intI* gene was detected in all eDNA samples analysed, with higher levels (at least two-fold differences) detected in the greywater (2.38E+04 to 1.73E+08) compared to the biofilm samples (5.77E+03 to 9.07E+05). This finding could be attributed to the fact that the *intI* gene is normally associated with human activity (such as water used for hand washing/bathing), compared to a more "stable" population within the biofilms. It is interesting to note that the relatively large increase in microbial population size observed in the summer samples (based on 16S rRNA gene analysis) was not as pronounced when looking at the *intI* gene. The average *intI* gene copy number for greywater samples in the summer was 2.43E+5 compared to 8.52E+4 in the winter (only a one-fold increase). The *intI* gene levels within the biofilms did not seem to fluctuate seasonally, as the average gene copy number was 6.54E+4 in the winter, and 2.99E+4 in the summer. This would support our hypothesis that the biofilms are colonised by stable microbial communities throughout the year

7.4 SUMMARY, CHALLENGES AND FUTURE WORK

The biggest challenge in conducting this analysis was finding the correct amount of eDNA to use in the assays. Initially, we used the same amount of template DNA as was used for the other qPCR experiments. However, for the 16S qPCR analysis the Cp values were all out of the dynamic range for the assay implying that too much DNA was used. Experiments with an extended dilution range were conducted and the Cp values (while still high) are within the dynamic range of the assay.

In the future, once the phenotypic and genotypic screening is completed it will be interesting to see whether this site differs from the other three sites (i.e. does it have lower levels of resistance genes? where the strains isolated from this site resistant to the antibiotics screened against?). Ideally, once the data set is complete a comparison between seasons as well as collection sites will be conducted. We will also be able to determine whether there is any direct correlation between the presence of integrons and the levels of antibiotic resistance genes. This will also be compared to the results from the phenotypic study.

CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

The growing global climate crisis is going to require governments and individuals to look at alternative water sources. During the 2017-2019 drought WC residents experienced first-hand the effects of climate change and preparations were made for “Day Zero” – the day the traps would run dry. One approach that was widely adopted by residents was using greywater for activities which do not require fresh water and many households had greywater systems installed which harvest wastewater generated in the kitchen and bathrooms. While greywater use is a viable option in households, given the nature of greywater if improperly used/stored there are potential health risks. These systems are known to harbour bacteria, including pathogens, and for this reason consumers are advised to use the water within 24 hours if it is untreated. However, many systems (especially the smaller DIY systems) do not have any form of treatment and/or automated timed release. As such, it is likely that in many households untreated greywater is allowed to sit for longer than 24 hours before being used. Applications such as irrigation and washing cars are of particular concern as any harmful bacteria present in the water would be released directly into the environment.

Numerous studies have investigated greywater systems and looked at their potential to harbour pathogenic bacteria. However, an often-overlooked risk is whether these systems could act as a reservoir for antibiotic resistance genes. The conditions within greywater systems are ideal to support microbial growth, particularly in the form of biofilms and the presences of sub-lethal doses of antimicrobial agents and chemicals can drive the development of resistance.

Based on this knowledge, the main hypothesis of this study was that domestic greywater systems are a potential source of antibiotic resistance organisms, resulting in high levels of ARG exchange. In particular, the biofilms which form on the inside walls of the tanks will likely be colonised by bacteria and conditions within these biofilms will support the development and spread of ARGs. The primary aim of the study was to identify and enumerate ARGs within domestic greywater systems. Key findings of the study were:

- Culturing experiments revealed that there is an abundance of microorganisms within these systems, but the species diversity is low. Similar organisms were detected in all four systems studied and at the different time points. This would imply that many of the organisms that were detected (especially in the greywater) are not transient (i.e. they do not just enter the system, get retained for a short time and are then released with the water when the tank is emptied), but rather that some form of stable microbial communities are present – this could be in the biofilm, but also attached to large particulates within the water column itself. The low species diversity also supports this hypothesis, as species diversity has been shown to decrease during storage. The presence of these “stable” communities is also likely due to the fact that the tanks are not emptied completely and at least a quarter of the volume is retained within the tanks.

- From phenotypic resistance assays, it was found that ~64% of the strains obtained in pure culture were resistant to ampicillin and streptomycin, of which 57.14% were biofilm isolates and 42.86% were GW isolates. The higher resistance observed for the biofilm isolates was expected and also supports the hypothesis that stable communities have developed in the systems. The

biofilms would provide an opportunity for structured populations to form, which would allow for the development and spread of antibiotic resistant determinants.

- Preliminary qPCR assays using the *vanA* primers on eDNA samples revealed that the *vanA* gene was detectible in three of the five samples analysed (albeit at low levels). This presence of vancomycin resistance determinants aligns with the culture-based study which also isolated vancomycin resistant strains.

- qPCR analysis revealed that the microbial numbers in the greywater samples are high compared to the biofilm. Cell numbers within the biofilm samples were relatively stable, again supporting the hypothesis that the biofilms house permanent microbial communities compared to the greywater systems which may contain both "resident" and "transient" organisms. Microbial numbers were higher in summer compared to the colder winter months.

- Class 1 integrons are mobile genetic elements associated the exchange of antibiotic resistance genes. In this study the *intI* gene was detected in all samples analysed (all four sites, sampled during all four seasons) which is to be expected as the presence of integrons is typically associated with human activities (such as bathing, washing, and food preparation). From this finding, it is likely that the exchange of resistant genes via horizontal gene transfer is occurring within these systems.

- From our findings, we can conclude that domestic greywater systems are a source of ARGs and resistant organisms, which may be a source of environmental contamination.

8.2 RECOMMENDATIONS

We have made recommendations based on the findings for each experimental chapter individually, as it makes it easier to link a recommendation to a specific conclusion and research aim. Our recommendations are outlined below.

Work presented in Chapter 3 focused on culture-based screening for antibiotic resistance (Aim 1). Based on the findings of Chapter 3 and the conclusions above the following recommendations are made:

- There is an urgent need for increased public awareness about the potential health hazards associated with improper greywater use. Clear "best practise" guidelines should be developed to inform the public how to safely collect and use greywater. These guidelines need to include information for all greywater collection strategies – including bucketing and DIY systems. Ideally, this information should be made available in different formats (information booklets, videos, etc.) and be made available on different platforms (social media platforms), websites and TV.
- Companies which install these systems should be required to inform customers how to correctly use the systems, especially that water must not be stored for periods longer than 24 hours. Larger systems must have some form of treatment (filtration, etc.) if the water is to be stored.
- Information should be provided to hardware stores which sell DIY systems (or the components to make a system) so they would be in a better position to inform their customers about the accepted best practises.
- These findings also highlight the importance of good antibiotic stewardship and public education related to the correct means of discarding unused antibiotics – pouring antibiotics

down the kitchen/bathroom sink which is connected to a greywater system would create the perfect conditions for the development of resistance.

Chapter 4 addressed Aim 4, namely the development of a novel assay for the *in vivo* detection of β-lactamase activity. While the development of the meropenem assay is not yet completed, our recommendations are:

- Most antibiotic resistance assays require expensive substrates, which have limited stability and due to the cost are not suitable for routine or high throughput testing. A way to circumvent this problem is for researchers to develop their own assays, ideally ones which use substrates which can easily be synthesised in the laboratory. Development of these assays requires strong collaborations and research teams which would include microbiologists, molecular biologists and chemists/pharmacists. Funding and networking opportunities should be created which foster such collaborative projects.
- As there are well established culture-based assays, scientists in the water sector may initially be reluctant to include new types of assays. However, there are excellent training courses (in FACS, fluorescent microscopy, etc.) offered at local Universities and many Universities offer the use of their instruments as a service. Scientists working at water treatment plants or in government, so should be encouraged to attend these courses/workshops to learn the basic theory, sample preparation, data collection and interpretation.

Chapter 5 outlined the development of a protocol that could reproducibly extract high quality DNA and RNA from greywater and biofilms samples. Every step from sample pre-processing, to extraction, QC and cDNA synthesis was optimised. Using these protocols DNA and RNA was extracted from the samples which was subsequently analysed in Chapters 6 and 7. As such, this chapter aligns with Aim 2 and 5. Based on the optimisation process outlined in Chapter 5, our recommendations are as follows:

- For successful extraction of nucleic acids, where possible, a large sample volume (at least 10 litres) should be collected – this should be increased for environments where the expected microbial abundance is lower than the systems described here.
- Every water source is unique, with different components and characteristics which will influence the extraction process. While the described protocol works well for the samples collected from the greywater systems, they may not work on other water samples. However, they can serve as an excellent starting point when developing a new protocol.
- While the optimization process outlined here was laborious, due to the sensitivity of qPCR it is critical that high quality RNA is extracted, as the quality of the RNA is integral to the success of the experiment. In future proposed studies at least 6 (ideally 12) months should be allocated to this optimisation process if protocols for that sample type have not been established.

Chapter 6 described the optimisation of the qPCR assays which will be used to enumerate the levels of ARGs in the greywater systems (Aim 2).

- This study only looked at three ARGs, namely *vanA, ampC* and *aacA*. Given the high levels of these genes detected, future work should include an extension of this study to look at other resistance genes, particularly those which are of the most concern in hospital settings or linked to treatment failure.
- As qPCR provides you with actual gene copy numbers (compared to culturing and DNA based assays) the protocols developed here could be used to investigate whether different operational parameters/system types influence the level of ARGs. For instance, it could be used to address questions like: What form of pretreatment is the most effective at reducing the levels of resistant microbes? Does kitchen wastewater or bathroom wastewater contribute more to the pool of resistance? How much does the season influence the levels of resistance?

The final experiment section, Chapter 7, described the qPCR assay used to screen for integrons in the greywater systems (Aim 5). As one cannot directly test for the occurrence of horizontal gene transfer, the presence of integrons can be seen as a marker of HGT. Class 1 integrons are associated with the transfer of ARGs. The presence of integrons in all greywater and biofilm samples is worrisome as it implies that HGT is occurring, ultimately resulting in the spread of resistance into the surrounding environment. As such, our recommendations are as follows:

- If a household has “high risk” residents (people who are immunosuppressed or new-borns) the greywater generated from systems without any treatment steps should be limited to activities were there is limited human contact. These households must ensure that their greywater is not used in a way that can contaminate the environment with ARGs or resistant microbes or directly expose the high-risk residents to the untreated water. Special care should be taken when using the greywater for indoor activities (such as toilet flushing) and any greywater that is spilled on surfaces must be cleaned with proper detergent.
- When the greywater is used for irrigation of gardens (particularly home-grown vegetables/herbs) residents must wash their hands after handling the plants/soil, and any produce must be properly washed before consumption.

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APPENDIX A: SUPPLEMENTARY TABLES AND FIGURES

Table S1. Quantitation and analysis of DNA extracted from grey water and biofilm samples using the PSC-B method.

Sample	Concentration (ng/µl)	A260/A280	A260/A230
GW1	2.80	1.18	0.90
GW2	1.60	1.51	1.20
BF1	0.20	0.12	0.40
BF2	0.50	0.50	0.35

Table S2. Quantitation and analysis of DNA extracted from non-filtered, sand filter grey water and biofilm samples using the PowerSoil DNA Isolation Kit.

Sample	Concentration (ng/µl)	A260/A280	A260/A230
Non-filtered GW 1A	14.5	1.69	**0.75**
Non-filtered GW 1B	11.6	2.10	**1.12**
Sand filtered GW 2A	8.2	2.30	**1.39**
Sand filtered GW 2B	6.9	2.29	**0.97**
Biofilm 1	5.4	1.97	**1.60**
Biofilm 2	**2.7**	**2.28**	**1.21**

Table S3. Quantitation and analysis of DNA extracted from preserved grey water samples using the PowerSoil DNA Isolation Kit.

Sample	Concentration (ng/µl)	A260/A280	A260/A230
GW FDL 1A	1.8	1.50	2.13
GW FDL 1B	2.0	1.70	0.38
GW LFG 2A	10.7	1.98	2.49
GW LFG 2B	12.7	1.87	2.48

Table S4. Quantitation and analysis of RNA extracted from a pellet of centrifuged grey water sample using the RNeasy PowerWater Kit.

Sample	Concentration (ng/µl)	A260/A280	A260/A230
GW 1	65.2	1.92	1.1
GW 2	39.6	2.22	1.6
Tap water (negative control)	66.8	1.5	0.6

Table S5. A comparison of the quantitative analysis of RNA extracted using the RNeasy PowerWater Kit from filters following filtration of grey water samples using the Nanodrop and the DeNovix

		Concentration (ng/µl)	A260/A280	A260/A230
Nanodrop	A *	5.0	2.96	0.66
	B **	3.5	1.09	0.45
	C1***	13.8	2.04	1.27
	C2 ****	19.0	1.76	0.97
DeNovix	A *	5.29	2.012	0.569
	B **	0.91	2.632	0.191
	C1***	14.4	1.956	1.121
	C2 ****	11.1	1.889	1.121

**: 430 ml of grey water was filtered using a 20µm filter; **:430 ml of grey water was filtered using a 10µm filter;*
****: 110 ml of grey water was filtered using a 0.45µm filter; ****: 60 ml of grey water was filtered using a 0.45µm filter.*

Table S6. A comparison of the quantitative analysis of DNA extracted using the PowerSoil DNA Isolation Kit from filters following filtration of grey water samples using the Nandrop and the DeNovix.

	Sample	Concentration (ng/µl)	A260/A280	A260/A230
Nanodrop	A *	-0.4	0.23	-0.29
	B **	1.6	1.22	0.53
	C1 ***	24.9	1.88	1.44
	C2 ****	0.3	-0.45	0.12
DeNovix	A*	1.261	1.08	0.29
	B **	1.617	1.18	0.41
	C1***	22.896	1.82	1.80
	C2 ****	1.316	1.19	0.73

**: 430 ml of grey water was filtered using a 20µm filter; **:430 ml of grey water was filtered using a 10µm filter;*
****: 110 ml of grey water was filtered using a 0.45 µm filter; ****: 60 ml of grey water was filtered using a 0.45 µm filter.*

Table S7. The Kirby Bauer disc diffusion method used to determine the susceptibility or resistance of bacteria obtained from previously conducted microbial analysis using grey water samples (in mm).

Antibiotics	Class of Antibiotics	Plate 1A	Plate 1B	Plate 2A	Plate 2B	Plate 3A	Plate 3B	Plate 4A	Plate 4B
Ampicillin (AMP10)	Penicillin/ β-lactams	0	0	-	-	0	0	-	-
Cefpodoxime (CPD10)	Cephalosporins/ β-lactams	-	-	11 (R)	17 (R)	-	-	11 (R)	13 (R)
Cephalothin (KF30)	Cephalosporins	-	-	0	0	-	-	0	0
Streptomycin (S10)	Aminoglycosides	15 (S)	13 (I)	-	-	10 (R)	10 (R)	-	-
Teicoplanin (TEC30)	Glycopeptides	0 (R)	0 (R)	-	-	0 (R)	0 (R)	-	-

(S) = susceptible (R) =resistant and (I) = intermediate

Table S8. The Kirby Bauer disc diffusion method used to determine the susceptibility or resistance of bacteria obtained from previously conducted microbial analysis using biofilm samples (in mm).

Antibiotics	Class of Antibiotics	Plate 1A	Plate 1B	Plate 2A	Plate 2B
Ampicillin (AMP10)	Penicillin/ β-lactams	15 (I)	19 (S)	19 (S)	13 (R)
Cefpodoxime (CPD10)	Cephalosporins/ β-lactams	30 (S)	23 (S)	30 (S)	26 (S)
Cephalothin (KF30)	Cephalosporins	20 (S)	21 (S)	20 (S)	20 (S)
Streptomycin (S10)	Aminoglycosides	12 (I)	12 (I)	10 (R)	10 (R)
Teicoplanin (TEC30)	Glycopeptides	0 (R)	0 (R)	0 (R)	0 (R)

(S) = susceptible (R) =resistant and (I) = intermediate