Microbial Communities across Water Treatment Plants: Fate of Coliforms and other Bacteria

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A thesis submitted to Newcastle University for the fulfilment of the requirements of the degree of Doctor of Philosophy within the Faculty of Science, Agriculture and Engineering

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September 2019

Declaration

I hereby certify that the work presented in this thesis is my original research work. Due reference is given to literature and any research collaborations where appropriate. No part of this thesis has been submitted previously for a degree at this or any university.

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Abstract

The production of clean drinking water is an essential process that greatly affects the health and prosperity of a community. Microbial content is a key parameter of water quality: the treatment process must remove all potentially harmful microorganisms. However, there is still much unknown about the microbial communities present in the water treatment process and their effect on the end quality of drinking water. In water treatment, coliform bacteria are used as 'indicator' organisms: the detection of these microorganisms signifies the water supply has been contaminated by faecal matter and other pathogens are likely to be present. Despite the proven efficacy of chlorine as a water disinfectant, treatment failures (i.e. detection of coliforms in final water samples) still occur at water treatment plants (WTPs). The possibility of chlorine resistance or tolerance in coliforms as a cause of treatment failures was explored and the chlorine tolerance of *Escherichia coli* isolated from different environments compared. Although chlorine tolerance was found to be higher in *E. coli* from a WTP environment than lab strain *E. coli,* coliform bacteria were found to be very sensitive to chlorine and no evidence of genetic resistance or tolerance was observed. In order to expand current knowledge of the overall microbial community of WTPs, a detailed sampling survey of two working WTPs was carried out and the biofilm and bulk water community was analysed across time, treatment stage and source water type using methods including next generation sequencing and quantitative PCR based on the 16S rRNA gene. Source water type was found to be the main determining factor of bulk water community composition, while treatment stage had greater influence on biofilm community composition.

Acknowledgments

I would like to thank my supervisor Professor David Graham for his generous guidance and support throughout the entirety of my PhD. I would also like to thank my industrial supervisor at Severn Trent, Dr Karen Heaton, for her advice and enthusiasm in supporting my PhD.

I'm very grateful to the many Severn Trent staff who have helped me over the last 4 years. My thanks go to Helen Pickett for sharing her technical knowledge and assistance and helping me to communicate my research to the wider company. I'm grateful to the Severn Trent staff on the treatment works who graciously gave up their time for assisting with sample collection – in particular, Andrea Smith, Emma Sharp, Jordan Kye-Arlott and Daisy King.

I would like to thank the STREAM IDC for funding, opportunities and helping me to develop as a researcher and as a person. Thank you to Justine Easten for her amazing support.

Advice and assistance from the technical team at Newcastle University was greatly appreciated, especially from Sarah Smith, David Race and Amy Bell.

I would like to say a big thank you to my friends in the PGR suit and research team who were always ready to help me deal with any obstacles, whether that meant research advice, a cup of tea and sympathy or a night off among friends.

Finally, I would like to thank my family for their unending and unconditional support throughout my entire life, but especially over the last 4 years. They are the foundation of all my achievements and I could not have made it to the end of this PhD without them.

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List of Abbreviations

ANOSIM: Analysis of similarities

ANOVA: Analysis of variance

CFU: Colony forming unit

CTC: 5-Cyano-2,3-ditolyl-tetrazolium chloride

DAF: Dissolved air flotation

DAPI: 4',6-diamidino-2-phenylindole

DNA: Deoxyribonucleic acid

ESBL: Extended spectrum beta lactamase

FISH: Fluorescent *in situ* hybridization

GAC: Granular activated carbon

GFP: Green fluorescent protein

HPC: Heterotrophic plate count

LB: Luria-Bertani agar/broth

MALDI-TOF-MS: Matrix Assisted Laser Desorption/Ionisation-Time of Flight-Mass Spectrometry

MLGA: Membrane lactose glucuronide agar

MRSA: Methicillin resistant *Staphylococcus aureus*

NCBI: National Centre for Biotechnology Information

NDM-1: New Delhi metallo-beta-lactamase

NTU: Nephelometric turbidity unit

OTU: Operational taxonomic unit

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PERMANOVA: Permutational analysis of variance

PMA: Propidium monoazide

QPCR: Quantitative polymerase chain reaction

RNA: Ribonucleic acid

SE: Standard error

THM: Trihalomethane

Tukey's HSD: Tukey's honest significant difference

UBC: University of British Columbia

UK: United Kingdom

UN: United Nations

USA: United States of America

UV: Ultraviolet

WHO: World Health Organisation

WTP: Water treatment plant

Chapter 1 – Introduction

The production and distribution of clean drinking water is an essential process that greatly affects the health and prosperity of a community (WHO, 2018). Microbial content is a key parameter of water quality: the treatment process must remove all potentially harmful microorganisms. It would be prohibitively time consuming and expensive to monitor water samples for all pathogens so water companies and health agencies rely on the use of so-called 'indicator' organisms to measure water quality (Bartram *et al.*, 2001). These organisms are groups or specific species of microorganisms that are relatively easy to culture in a laboratory, are potentially pathogenic themselves and derive from faecal sources (Edberg *et al.*, 2000). The detection of these microorganisms signifies the water supply has been contaminated by faecal matter and if these organisms are present, it is highly likely other pathogens are also in residence. Commonly used indicator organisms include coliforms, *Clostridia*, *Enterococci*, *Cryptosporidium* and bacteriophages (Bartram *et al.*, 2001). The exact organisms monitored depend on the regulations and practice of the country or state in question. For example, Severn Trent (a UK water company responsible for the water supply of the majority of the Midlands) routinely measures coliforms, *Escherichia coli, Clostridium perfringens* and *Enterococcus faecalis.*

In the event that a coliform or other indicator organism is detected in a final water sample (meaning a sample that has been through the treatment process and is now being distributed to customers), the repercussions are significant both for the general public and the water company in question. Customers must deal with a possible health risk, disruption caused by safety measures such as boiling water before use and also a loss of trust in their water supplier. The water company will face large regulatory fines, high costs involved in responding to the treatment failure and damage to their reputation.

The most important stage in drinking water treatment in terms of microbial control, is disinfection. There are a number of different methods utilised for water disinfection, the choice of process depending on factors such as cost, quality of source water, legal requirements or regulations, environmental considerations

and target organisms. The processes currently in use across the world include chlorination, chloramination, chlorine dioxide, ozonation and UV irradiation (Ngwenya *et al.*, 2013). Globally, chlorination is the predominant method of drinking water disinfection (Shannon *et al.*, 2008), as it is both highly effective and relatively inexpensive. If used in favourable conditions, chlorination should eradicate all microbial contaminants.

Despite the proven efficacy of chlorine as a water disinfectant, treatment failures (i.e. detection of coliforms in final water samples) still occur at water treatment plants (WTPs). A treatment failure can be caused by a vast number of scenarios. These include but are by no means limited to: failure to maintain the correct dose of chlorine; insufficient contact time between water and disinfectant; insufficient mixing during disinfection; a breach in the contact tank or pipework that allows ingress of bacteria into a treated water stream; contamination of the final water sample during collection; insufficient treatment of water prior to disinfection (e.g. high turbidity, high amount of organic carbon, high density of particulate matter). When a treatment failure occurs, WTPs endeavour to trace the incident to a root cause. However, there are a number of cases where the treatment process appears to be working to industry-recommended standards and there is no apparent source of contamination in any stage of the process.

The starting hypothesis in the genesis of the PhD project was the possibility of chlorine resistance in coliform bacteria as the root cause of treatment failures. Consequently, the first aim of this thesis is to determine the presence or absence of chlorine resistance in coliform bacteria in WTPs. In order to diagnose and solve a problem in a complex system such as a WTP, a thorough and in depth understanding of the system is required. Therefore, the second aim of this thesis is to expand current knowledge of the overall microbial community of working WTPs and produce a detailed description of the WTP microbiome.

Key literature relating to water treatment, water disinfection, chlorine resistance and the microbial communities of drinking water treatment plants and distribution networks is reviewed in Chapter 2.

The terminology of ‘resistance’ (as it relates to chlorine) is discussed in Chapter 2 and it is our belief that chlorine ‘tolerance’ is a more accurate and descriptive

term to be used in the context of these experiments. Chapter 3 describes a sampling survey carried out on five UK WTPs to search for coliforms displaying any sign of chlorine tolerance. An *E. coli* isolate obtained during this survey was observed to have a higher survival rate on exposure to chlorine than a lab strain *E. coli* isolate. This led to a further study of the differences in chlorine tolerance between *E.coli* isolates originating from three differing habitats (water environment, laboratory environment and human faeces).

Chapters 4 and 5 give a detailed account of the bacterial community dynamics throughout two working WTPs. Chapter 4 describes a sampling survey that encompassed two WTPs with differing source water (reservoir and river), five treatment stages (raw source water, post-clarification, post-filtration, post-granular activated carbon and post-chlorine contact tank) and two sample types (biofilm and bulk water) over a time period of 6 months. Chapter 4 illustrates the total number of bacteria (as determined by qPCR of the 16S rRNA gene) present in bulk water and biofilms at each treatment stage at both WTPs over 6 months sampling.

Chapter 5 describes the characterisation of the WTP microbiome by metagenomic analysis of samples collected in the survey described in Chapter 4. Bacterial community composition and dynamics of both WTPs are determined at each treatment stage and differences between source water and biofilm and bulk water communities are revealed.

The main hypotheses being tested in this thesis are as follows:

1) Coliforms have developed resistance or tolerance against chlorine.
2) Biofilms are a source of coliforms in WTPs.
3) The bacterial community composition of biofilms and bulk water is significantly different.
4) Bacterial communities in WTPs are affected by treatment stage.
5) Bacterial communities in WTPs are affected by the community of source water.

Chapter 2 – Review of literature

2.1 Drinking water treatment

The treatment of drinking water encompasses many different processes targeting various aspects of water quality, namely microbiological content, chemical content and aesthetic qualities such as taste, odour and general appearance. While the first instances of drinking water treatment can be traced back to ancient civilisations such as the Romans and ancient Greeks (Juuti *et al.*, 2007), real advances in treatment processes began around two hundred years ago (EPA, 2000). Slow sand filtration began to be used in the 1800s in Europe and the United States to remove particles and other debris (Huisman, 1974). Advances in bacteriology and the understanding of the role of microorganisms in disease by John Snow (Cameron and Jones, 1983) and Robert Koch (Blevins and Bronze, 2010) among others, revealed the role of drinking water treatment as a cornerstone of public health (Cutler and Miller, 2005).

In modern water treatment plants, the main stages of water treatment are: raw water screening; sedimentation or clarification; filtration; advanced treatment processes (e.g. fluoridation or the removal of volatile organic compounds by granular activated carbon) and disinfection (Twort, 2000). Smaller treatment plants may not have an advanced treatment process stage and the exact treatment technology used at each stage varies based on size of WTP, geographical location, laws and regulations of the country or region and individual preference and operating practises of the company or government running the plant.

Raw water screening is a simple process involving the use of bar screens or ‘band and drum’ screens (rotating drums of mesh screens) to remove large debris from water entering the treatment plant (Twort, 2000). The clarification process consists of three steps: chemical dosing with coagulants; coagulation and flocculation and finally, sedimentation. The most commonly used coagulant in water treatment is aluminium sulphate, however, ferric sulphate is also widely used (Edzwald, 1993). Upon addition to raw water, the coagulants form

aluminium or iron hydroxide complexes which then adsorb clay, turbidity and other particles, eventually forming large flocs of particulate material (Duan and Gregory, 2003). Constant mixing is required to ensure optimal rates of coagulation and flocculation. The final stage of clarification (i.e. sedimentation) involves the removal of flocs and any settled material. A number of different clarifier designs have been developed: simple horizontal flow clarifiers; sludge blanket or 'hopper bottomed' clarifiers and dissolved air flotation (DAF). In a horizontal flow clarifier, water simply flows slowly through the clarifier, allowing the higher density flocs to settle at the bottom of the tank (which are then periodically removed). In a hopper bottomed clarifier, water is pumped to the bottom of an inverted pyramidal tank and flows upwards (Figure 2.1). The higher density of flocs cause them to form a suspended layer or 'sludge blanket' at the point where the downward force of their weight is equal to the force of the upward water flow. Above this layer, clarified water is siphoned off and directed to the next treatment stage (Twort, 2000).

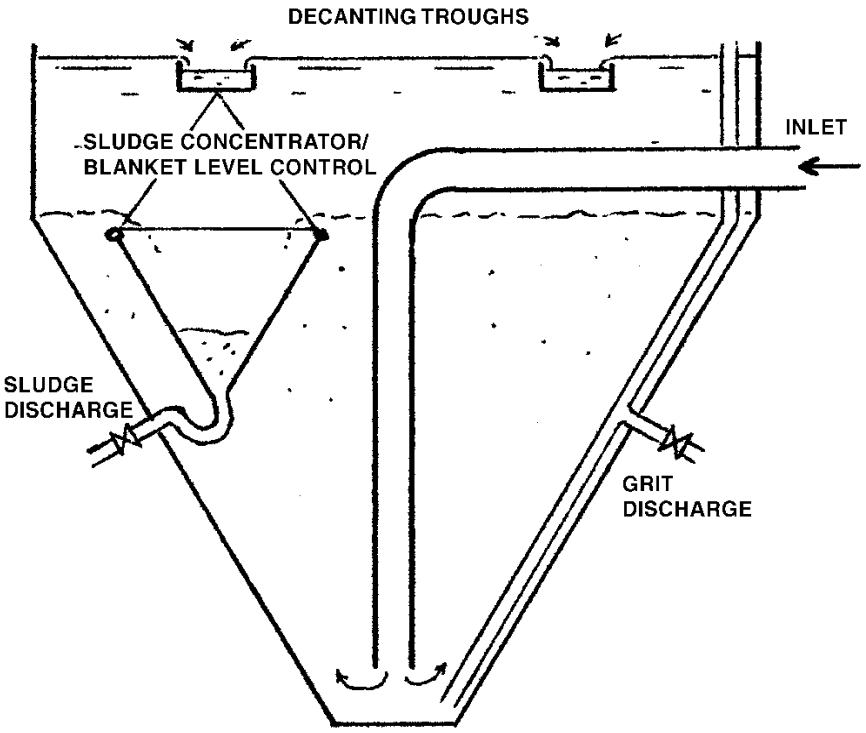


Figure 2.1 – *Hopper bottomed clarifier (Stevenson, 2003).*

Finally, dissolved air flotation operates by creating very small air bubbles in flocculated water. Flocs attach to the air bubbles, resulting in a layer of sludge on the surface of the tank and clarified water is removed from the bottom of the tank (Twort, 2000). DAF has been shown to be more effective in treating water with low turbidity and alkalinity and highly coloured water with a large algae presence (Zabel, 1985).

Filtration at modern WTPs is most commonly performed by rapid gravity filters. Slow sand filters are also in use, although they are less likely to be installed at new treatment plants due to the fact that they require a much larger area of land and more labour for cleaning and re-sanding than rapid gravity filters (Ellis and Wood, 1985). Despite these disadvantages, slow sand filters are very effective in removal of microbial organisms and produce water of a high bacteriological quality (Hijnen *et al.*, 2004; Bauer *et al.*, 2011). Rapid gravity filters typically consist of layers of anthracite, sand and gravel, with filter material increasing in grain size and density as water flows down the filter. Filtration, either by rapid gravity filter or slow sand filter, is the final stage of particle removal in the treatment process, as well as contributing significantly to microbial removal.

Source waters often contain organic compounds or contaminants (such as pesticides, pharmaceuticals, wastewater contaminants and micropollutants) that can have detrimental effects on drinking water quality even in trace amounts. Granular activated carbon is a highly effective advanced treatment process for removal of these compounds (Paune *et al.*, 1998; Ternes *et al.*, 2002; Stackelberg *et al.*, 2004; Kim *et al.*, 2007), as well as compounds that can result in an unpleasant taste or odour in the final drinking water product (Chen *et al.*, 1997). Consequently, GAC filters are used widely in WTPs.

The final stage, drinking water disinfection, is the most important treatment process in terms of the microbiological safety of the final water product and will be reviewed in Section 2.3.

2.2 Monitoring of water quality and indicator organisms

It would be impractical and uneconomical to test drinking water for all possible pathogens or microorganisms capable of causing disease in humans, therefore, ‘indicator’ organisms are used to monitor water quality. If these organisms are

present, it is an indication of faecal contamination and the presence of other pathogens is highly likely. *E. coli* and thermotolerant coliform bacteria are considered the best indicators of faecal contamination (Edberg *et al.*, 2000) and form the basis of most drinking water quality legislation (Inspectorate, 2010; WHO, 2011). The majority of drinking water standards in the UK and EU are based on the World Health Organisation's 'Guidelines for drinking-water quality', setting the limits for coliforms and *E. coli* at 0 per 100 ml water sample (WHO, 2011). Additional testing of other indicator organisms (namely *Clostridium perfringens* (Bisson and Cabelli, 1980) and *Enterococci*) is also routinely carried out by UK WTP operators as using a wider range of indicators is believed to give better representation of possible pathogens (Harwood *et al.*, 2005). Heterotrophic bacteria (i.e. bacteria able to be cultured in the lab at 22°C and 37°C) are also monitored in UK WTPs to give a general indication of water quality and provide a warning sign of large changes in microbial load (Sartory, 2004).

Microbial monitoring of UK WTPs currently relies almost exclusively on culture-based methods: membrane filtration of water samples followed by growth on a selective media (SCA, 2009). However, with the advancement of molecular biology techniques, new methods are being tested and considered for use in the water industry. Molecular methods such as polymerase chain reaction (PCR) and quantitative PCR (qPCR) are rapid (assays can be completed in hours as opposed to days with culture-based methods), sensitive and can be used on a wide range of microorganisms (Botes *et al.*, 2013). PCR and qPCR have been used to detect *Legionella pneumophila* (Bej *et al.*, 1991; Dusserre *et al.*, 2008), *Enterococci* (Shannon *et al.*, 2007; Lavender and Kinzelman, 2009; Haugland *et al.*, 2012), human adenoviruses (He and Jiang, 2005), Candida (Brinkman *et al.*, 2003), coliforms (Varma *et al.*, 2009; Martín *et al.*, 2010; Iv and Lowe, 2012; Soejima *et al.*, 2012; Maheux *et al.*, 2014; Hu *et al.*, 2016), *Helicobacter pylori* (Watson *et al.*, 2004; McDaniels *et al.*, 2005; Nayak and Rose, 2007), *Salmonella* (Ahmed *et al.*, 2009), *Campylobacter jejuni* (Ahmed *et al.*, 2009) and *Vibrio* (Wetz *et al.*, 2008; Liu *et al.*, 2009). While results from these studies show that molecular methods can be used successfully to detect and quantify pathogens in water environments and have many advantages over culture-based methods, a vast degree of standardisation is required before they can be implemented in

monitoring programmes at WTPs. Significant differences in quantification have been found when different protocols, primers and PCR conditions are used (Girones *et al.*, 2010).

Flow cytometry is another technology that has become of great interest to the water industry in recent years. While unsuited for monitoring specific organisms such as coliforms or *E. coli*, flow cytometry can provide rapid quantification of total bacteria passing through a treatment plant or water distribution network (Prest *et al.*, 2013). It is culture-independent and can be combined with a range of fluorescent dyes to characterise bacteria on the basis of viability, thus distinguishing between live and dead bacterial cells (Berney *et al.*, 2007). Flow cytometry has been shown to be more rapid and more sensitive than heterotrophic plate counts (Hoefel *et al.*, 2003). The development of online flow cytometers for continuous sampling is of particular interest to WTP operators as this technology could greatly improve knowledge of bacterial load and water quality throughout WTPs and distribution networks (Besmer *et al.*, 2014).

2.3 Water disinfection

Disinfection is the most crucial stage of drinking water treatment for the removal of microorganisms. There are five methods of disinfection in common practice in large-scale WTPs: chlorination, chloramination, use of chlorine dioxide, ozonation and ultraviolet (UV) radiation (Block, 2001).

Chlorine is the most widely used disinfectant globally (Ngwenya *et al.*, 2013); it is the predominant method used in South Africa (Genthe and Kfir, 1996), the USA (Committee, 2008), Canada (Canada, 2017), Australia (NHMRC, 2011) and most of Europe (Medema *et al.*, 2009). A notable exception is the Netherlands, where chlorine disinfection has not been used since 2006, instead relying on UV and ozonation (Medema *et al.*, 2009).

Upon addition to water, chlorine forms hypochlorous acid and hydrochloric acid (Equation 2.1). Hydrochloric acid dissociates into hydrogen ions and chloride ions (Equation 2.2), while hypochlorous acid partially dissociates into hydrogen and hypochlorite ions (Equation 2.3).

Equation 2.1 – The formation of hypochlorous acid from the addition of chlorine to water.

$$Cl_2 + H_2O \rightarrow HOCl + HCl$$

Equation 2.2 – Dissociation of hydrochloric acid.

$$HCl \rightarrow H^+ + Cl^-$$

Equation 2.3 – Partial dissociation of hypochlorous acid.

$$HOCl \leftrightarrow H^+ + OCl^-$$

Hypochlorous acid is the most bactericidal component and together with hypochlorite ions, makes up the 'free' chlorine concentration of a sample. 'Combined' chlorine consists of compounds formed from chlorine and ammonia and/or organic matter. While combined chlorine products still act as disinfectants, they are less effective than free chlorine. The ratio of hypochlorous acid to hypochlorite ions is largely determined by pH and temperature, as can been seen from Table 2.1, with lower pH of 6 – 7 favouring the domination of hypochlorous acid.

The efficiency of chlorine in water disinfection is greatly reduced by high turbidity, high concentration of metallic compounds and high concentration of organic matter (Powell *et al.*, 2000). High turbidity indicates a large density of particles, which can limit the penetration of chlorine to microorganisms. Metallic compounds such as iron and manganese are oxidised by chlorine, thereby leaving less chlorine available for bactericidal action. Organic matter produces combined chlorine products which are much less effective as disinfectants than free chlorine. The combined effect of these factors is known as the 'chlorine demand' of a water sample. Chlorine demand is equal to the difference between the chlorine concentration added to a water sample and the concentration of free chlorine detected afterwards.

Table 2.1 – *The percentage of hypochlorous acid at varying pH and temperature (Twort, 2000).*

	Percentage HOCl						
pH	0°C	5°C	10°C	15°C	20°C	25°C	30°C
6.0	98.5	98.3	98.0	97.7	97.4	97.2	96.9
6.25	97.4	97.0	96.5	96.0	95.5	95.1	94.6
6.5	95.5	94.7	94.0	93.2	92.4	91.6	91.0
6.75	92.3	91.0	89.7	88.4	87.1	86.0	84.8
7.0	87.0	85.1	83.1	81.2	79.3	77.5	75.9
7.25	79.1	76.2	73.4	70.8	68.2	66.0	63.9
7.5	68.0	64.3	60.9	57.7	54.8	52.2	49.9
7.75	54.6	50.5	46.8	43.5	40.6	38.2	36.0
8.0	40.2	36.3	33.0	30.1	27.7	25.6	23.9
8.25	27.4	24.3	21.7	19.5	17.6	16.2	15.0
8.5	17.5	15.3	13.5	12.0	10.8	9.8	9.1
8.75	10.7	9.2	8.0	7.1	6.3	5.8	5.3
9.0	6.3	5.4	4.7	4.1	3.7	3.3	3.0

The final determinant of chlorine efficiency is contact time. The WHO recommends a free chlorine concentration-contact time value (CT) value of 15 mg.min/l, resulting from contact of 0.5 mg/l free chlorine for 30 minutes (WHO, 2011). However, CT should be adapted to the quality of source water, with higher values used for waters with higher microbial loads. For example, many UK WTPs use a CT value of at least 30 mg.min/l; typical free chlorine concentrations in chlorination contact tanks are 1 – 2 mg/l for 30 minutes exposure.

In addition to low cost, high efficiency and ease of use, chlorine is able to retain a residual concentration in water distribution networks. This is essential for ensuring regrowth of bacteria or survival of bacteria entering through leaks in

pipework is kept to a minimum. The recommended chlorine residual in distribution networks is 0.2 – 0.5 mg/l (WHO, 2011), however, there are many factors that can cause variations in residual chlorine concentration at different points in a distribution network. Flow path, residence time, corrosion of pipe wall material and presence of biofilms have all been shown to influence chlorine decay rates (Clark *et al.*, 1993; Kiene and Levi, 1998).

The exact mechanism of action of chlorine on microbial cells has not been detailed, however, its bactericidal effects are believed to be due to its strong oxidising action. There is a substantial body of research showing chlorine (or hypochlorous acid) damages cellular components, including nucleic acids (Dennis *et al.*, 1979; Burrows and Muller, 1998; Prütz, 1998; Hawkins and Davies, 2001), lipids (Winterbourn *et al.*, 1992; Van den Berg *et al.*, 1993) and proteins (Thomas, 1979; Hawkins *et al.*, 2003). Chlorine can destroy membranes (Venkobachar *et al.*, 1977; Sips and Hamers, 1981; Phe *et al.*, 2005; Virto *et al.*, 2005), inhibit enzymes and metabolic processes (Albrich and Hurst, 1982; Barrette *et al.*, 1987; Barrette *et al.*, 1989; Hurst *et al.*, 1991; Hannum *et al.*, 1995; Estrela *et al.*, 2002) and produce hydroxyl radicals (Imlay and Linn, 1986; Dukan and Touati, 1996). Chlorine is also extremely fast acting, with cellular destruction or inactivation occurring in multiple components almost simultaneously (Albrich and Hurst, 1982; Dukan *et al.*, 1996).

Although chlorine is the disinfectant of choice in the majority of WTPs, there are a number of problems and limitations related to chlorine use. Chlorination can result in an unpleasant taste in the final water product, caused by overdosing or the presence of chlorinated phenols (Young *et al.*, 1996). One of the largest concerns is the formation of trihalomethanes (THMs) from the reaction of chlorine and organic matter. THMs have been linked to adverse birth outcomes (Gallagher *et al.*, 1998; Waller *et al.*, 1998; Dodds *et al.*, 1999) and cancer (Dunnick and Melnick, 1993; Hsu *et al.*, 2001; Tokmak *et al.*, 2004; Wang *et al.*, 2007; Panyakapo *et al.*, 2008). The recommended maximum concentration of chloroform (the most common THM in drinking water) is 300 ppb, however, guidelines state THM levels should be kept as low as is practically possible (WHO, 2011). THM formation can be limited by removing as much organic matter

as possible in treatment processes prior to disinfection; GAC filters can be useful in removing THM precursors (Vahala and Laukkanen, 1999; Yan *et al.*, 2010).

Chloramine is an alternative chlorine-based disinfectant, which is sometimes preferred as it produces much lower levels of THMs (Twort, 2000). Chloramine is formed from the reaction of chlorine and ammonia (Equation 2.4). Although it is not as bactericidal as chlorine, chloramine has some advantages in microbial control in distribution networks: it has been shown to be more effective against biofilms than chlorine (LeChevallier *et al.*, 1988b) and chloramine residuals last longer than chlorine residuals.

Equation 2.4 – Ammonia and chlorine react to form monochloramine and hydrochloric acid.

$$NH_3 + Cl_2 \rightarrow NH_2Cl + HCl$$

Ozonation is more effective than chlorine in eliminating viruses (Tyrrell *et al.*, 1995) and cysts containing *Giardia* and *Cryptosporidium* (Korich *et al.*, 1990; Betancourt and Rose, 2004). UV radiation is also very effective against *Cryptosporidium* (Linden *et al.*, 2001; Morita *et al.*, 2002), however, it requires water to have extremely low levels of turbidity and colour in order to be effective and does not provide residual disinfection in the distribution network.

2.4 Bacterial survival of chlorination

Despite the established efficacy of chlorine as a water disinfectant, bacteria are still present after disinfection in WTPs and distribution networks. On rare occasions, surviving bacteria are indicator organisms such as coliforms and it is essential to understand any ways in which such organisms could survive disinfection in WTPs.

There are four main adaptations or survival strategies that could allow bacteria to survive chlorination: genetic resistance; shielding from chlorine exposure within a biofilm; shielding within particles and shielding through ingestion and intracellular survival within protozoa.

2.4.1 Chlorine resistance

Genetic chlorine resistance in pathogenic bacteria in water environments could seriously compromise the effectiveness of WTPs in providing drinking water of safe microbiological standards.

As background, antibiotic resistance, while ancient and unrelated to anthropogenic activities in origin (D'Costa *et al.*, 2006), began to spread on a large scale following the introduction and widespread use of penicillin in the 1940s (Knapp *et al.*, 2010). Bacteria constantly evolve and adapt to stressful environments and combatting resistance is one of the greatest challenges facing the world today. As examples, methicillin resistant *Staphylococcus aureus* (MRSA), extended spectrum beta-lactamases (ESBLs) and New Delhi metallo-β-lactamase-1 (NDM-1) are among the most recent and concerning forms of resistance to emerge (Hawkey and Jones, 2009). NDM-1 protein is a carbapenemase that causes resistance to almost all known antibiotics – only colistin and tigecycline remain effective (Kumarasamy *et al.*, 2010).

Chlorine resistance, if it exists, has less immediate negative consequences than antibiotic resistance, however, it has a potentially massive impact on public health. Access to clean water was recognised as a human right by the UN General Assembly in 2010 and is considered by the World Health Organisation (WHO) to be a prerequisite for combatting poverty and child mortality among many other issues (World Health Organisation, 2014). Given that chlorine is the most widely used water disinfectant globally (Shannon *et al.*, 2008), bacteria that develop resistance to chlorine could compromise water quality and put the public at risk of infection with microbial contaminants, such as *Vibrio cholerae, Escherichia coli* (*E. coli*) and *Enterococcus faecalis.*

Many studies have investigated evidence of resistance to chlorine in a range of bacterial species. Chlorine resistance has been documented in *E. coli* (Lisle *et al.*, 1998; Inatsu *et al.*, 2010), *Mycobacterium* (Le Dantec *et al.*, 2002; Helbling and VanBriesen, 2007; Lee *et al.*, 2010; Chen *et al.*, 2012), *Vibrio cholerae* (Morris *et al.*, 1996; Yildiz and Schoolnik, 1999), *Methylobacterium* (Hiraishi *et al.*, 1995; Furuhata *et al.*, 2011), *Klebsiella* (LeChevallier *et al.*, 1988a)*, Legionella* (Furuhata *et al.*, 2014)*, Sphingomonas* (Sun *et al.*, 2013) and *Helicobacter pylori*

(Baker et al., 2002). The majority of results suggest that Gram(+) bacteria and *Mycobacteria* are among the most resistant to chlorine, while Gram(-) bacteria are more sensitive (Virto *et al.*, 2005; Helbling and VanBriesen, 2007; Chen *et al.*, 2012). It is believed intrinsic resistance resulting from the cell wall of Gram positive and *Mycobacteria* may play a role in greater chlorine resistance. An exception to this generalisation is the Gram(-) *Sphingomonas* TS001 isolated by Sun et. al (2013), which appeared to display unusually high levels of tolerance to chlorine (i.e., 240 min exposure to 4 mg/l chlorine resulted in only 5% inactivation). However, the underlying explanation for such high resistance is unknown, although a particular fatty acid not found in other genera was believed to have contributed to the phenomenon.

There are multiple theories regarding mechanisms of chlorine-related cellular defence, including: upregulation of intracellular compounds that protect against oxidative damage (Chesney *et al.*, 1996; Dukan and Touati, 1996); protection and survival within biofilms (Williams and Braun-Howland, 2003; Behnke *et al.*, 2011) and upregulation of multi-drug efflux pumps (Shi *et al.*, 2013; Karumathil *et al.*, 2014). Some papers have focused in more detail on specific genes related to increased chlorine defence; these include an integrative conjugative element believed to encode oxidative stress response genes (Flynn and Swanson, 2014) and chlorine-specific transcription factors (Gebendorfer *et al.*, 2012; Parker *et al.*, 2013).

In reviewing current findings on chlorine resistance, a key issue in the research question becomes apparent, which is: what is “resistance”? From a true genetic standpoint, resistance refers to a genetic, heritable trait – a gene or a group of genes that encode an enzyme, an efflux pump, an altered membrane protein or some other structure that confers resistance to a certain antimicrobial substance (Davies and Davies, 2010). When used in relation to antibiotics, resistance often refers to a certain threshold for bacterial survival – when an organism is able to survive a therapeutic dose of an antibiotic, it is considered resistant to that compound. In this context, resistance is a discrete characteristic – positive or negative, resistant or not resistant.

Chlorine resistance, on the other hand, is rarely described as a genetic mechanism in the literature. It is more often studied in terms of the phenotypic characteristics displayed by organisms in response to chlorine exposure. These include survival at different chlorine concentrations and contact times (Le Dantec *et al.*, 2002), cell morphology (Morris *et al.*, 1996) and enzyme activity (Gao and Liu, 2014). The mechanism of action of chlorine is still poorly understood and, unlike antibiotics which have specific targets, chlorine acts on multiple cellular structures as a strong oxidant almost simultaneously (Gray *et al.*, 2013a). Both of these factors may be a barrier to investigating genetic responses to chlorination. Furthermore, unlike antibiotics, chlorine has no 'therapeutic dose' to use as a benchmark for defining resistance. Chlorine concentrations used in water disinfection vary greatly depending on the type and quality of source water, the treatment process used prior to disinfection and the environmental regulations of the country or state in question. Chlorination is also sometimes used in wastewater treatment, which necessitates much higher doses than those used in drinking water treatment. In fact, in terms of a threshold for bacterial survival, there appears to be no clear consensus on what constitutes a "resistant" organism; i.e., there is no universal chlorine concentration above which organisms are considered resistant. For example, Chen *et al.* (2012) describes a strain of *Mycobacterium mucogenicum* with a concentration-time (CT) value of 99% inactivation of 29.6 mg.min/l and Furuhata *et al.* (2011) describe *Methylobacterium aquaticum* isolates with an average CT value of 99% inactivation of 0.89 mg.min/l. Despite the wide difference in CT values, both organisms are considered chlorine resistant, but using the term "resistant" is these contexts may be misleading. Unlike antibiotic resistance, chlorine resistance is described more on a scale of relative sensitivity rather than a discrete property that confers "absolute resistance". Therefore, due to the fact that genetic resistance to chlorine has not been definitively demonstrated and there is no established value for defining resistance, this thesis will refer to chlorine 'tolerance' rather than resistance as tolerance appears to be a more accurate and descriptive term in connection with chlorination.

2.4.2 Biofilms and chlorination

The effect of biofilm shielding on the efficacy of disinfectants and other antimicrobial compounds has been the subject of many studies and reviews (LeChevallier *et al.*, 1988a; De Beer *et al.*, 1994; Yu and McFeters, 1994; Srinivasan *et al.*, 1995; Xu *et al.*, 1996; Sanderson and Stewart, 1997; Sommer *et al.*, 1999; Momba *et al.*, 2000; Davies, 2003; Williams and Braun-Howland, 2003; Simões *et al.*, 2010; Behnke *et al.*, 2011). Bacteria growing in biofilms have been generally found more tolerant of chlorine than planktonic bacteria or small aggregates of bacterial cells (Behnke *et al.*, 2011). For example, Le Chevallier *et al.* (1988) observed a 150-fold increase in disinfection survival in biofilms consisting of *Klebsiella pneumoniae*. This is not surprising because chlorine diffusion through biofilms and chemical reactions with the biofilm matrix can results in chlorine concentrations in biofilms as low as 20% of the concentration present in the bulk water (De Beer *et al.*, 1994). There are a number of other factors that have been shown to increase the resilience of biofilms to disinfectants and biocides, including: the presence of multiple species rather than single species (Simões *et al.*, 2010; Behnke *et al.*, 2011; Miller *et al.*, 2015; Pang *et al.*, 2017); a more mature or well-established biofilm (Sommer *et al.*, 1999), a longer exposure time to disinfectants (Shen *et al.*, 2016), the production of extracellular polymeric substances (EPS) in response to low to moderate chlorination (Xue *et al.*, 2012; Liu *et al.*, 2017) and; the presence of abiotic particles (Srinivasan *et al.*, 1995). The result of which is that clinically relevant bacterial species, such as *Legionella pneumophila* and *E. coli*, have been detected in biofilms even after chlorination (Williams and Braun-Howland, 2003).

2.4.3 Particles and chlorination

Particle-associated bacteria have demonstrated increased survival in the presence of chlorine and other disinfectants compared to planktonic or unattached bacteria (LeChevallier *et al.*, 1984; Herson *et al.*, 1987; Berman *et al.*, 1988; Stewart *et al.*, 1990; Emerick *et al.*, 2000; Winward *et al.*, 2008). As well as providing physical shielding from chlorine, particles have also been found to provide a more advantageous environment for bacterial growth and reproduction compared to a planktonic environment (Li *et al.*, 2018). In a study by Li *et al.*

(2018), increased numbers of small-size particles was correlated with increased intact cell concentrations in drinking water distribution systems. The size and material of particles appears to have significant influence on their effectiveness as a defence against disinfection. Berman *et al.* (1988) found that particles of size >7 µm provided more protection against chlorine than <7 µm particles, and correlations between increasing particle size and decreasing chlorine efficacy were confirmed by Winward *et al.* (2008). However, the nature of the particles can influence the effects. As an example, goethite particles were found to provide no detectable protective effect in chlorination (Gauthier *et al.*, 1999), whereas granular activated carbon (GAC) particles appear to greatly inhibit the action of chlorine against particle-associated bacteria (LeChevallier *et al.*, 1984; Stewart *et al.*, 1990). Based on such data, mathematical models have been developed to describe the penetration and bactericidal action of chlorine and other disinfectants such as UV on particle-associated organisms (Emerick *et al.*, 2000; Dietrich *et al.*, 2003).

Beyond early studies that established that particles can provide physical shielding from chlorine, the extent of chlorine tolerance of particle-associated bacteria has been poorly characterised in the literature to date. The most recent research on particle-associated bacteria has focused on the effect of shielding on UV disinfection rather than chlorination (Kollu and Örmeci, 2011; Mattle and Kohn, 2012; Chahal *et al.*, 2016; Carré *et al.*, 2018).

2.4.4 Protozoa and chlorination

Background literature concerning bacterial ingestion and intracellular survival as a factor affecting survival in chlorination is more limited. An early study by King *et al.* (1988) found that laboratory strains of *Acanthamoeba castellanii* (an amoeba) and *Tetrahymena pyriformis* (a ciliate protozoa) were able to internalise a wide range of coliform bacteria and increased tolerance to chlorine by >50-fold. However, this study did not demonstrate the rate of bacterial ingestion and survival in situ or quantify the amount of protozoa typically present in drinking water, therefore more work is needed to verify the influence of protozoan hosts on coliform survival in WTPs. A later study sampled three working WTPs in the Netherlands for zooplankton and amoeba containing internalised *E.coli* and

Campylobacter jejuni (Bichai *et al.*, 2011). This study concluded that while intracellular bacteria were able to survive higher chlorine concentrations than free bacteria, the occurrence of internalised coliforms was less than one per 10^5 zooplankton and the resulting risk of infection by *E. coli* and *C. jejuni* was less than $5.9x10^{-5}$ from drinking water. *Legionella pneumophila* associated with the protozoon *Hartmannella vermiformis* has been found in high abundance in aquatic biofilms (Kuiper *et al.*, 2004).

2.5 Microbial communities of drinking water treatment plants and distribution networks

With the advances of next generation sequencing in recent years, in-depth metagenomic analysis has become a powerful tool in expanding current knowledge of drinking water treatment plant and distribution network microbial communities. Previous studies have characterised the bacterial community of WTP filters (Pinto *et al.*, 2012; Bai *et al.*, 2013; Lautenschlager *et al.*, 2014), WTP membranes (Chen *et al.*, 2004; Bereschenko *et al.*, 2008), bulk water in WTPs (Eichler *et al.*, 2006; Pinto *et al.*, 2012; Vaz-Moreira *et al.*, 2013; Yuanqing *et al.*, 2013; Chiao *et al.*, 2014; Lautenschlager *et al.*, 2014; Lin *et al.*, 2014) and biofilms in WTPs (Chen *et al.*, 2004; Emtiazi *et al.*, 2004; Lin *et al.*, 2014).

Bacterial communities in WTPs were significantly shaped by source water community (Emtiazi *et al.*, 2004; Bereschenko *et al.*, 2008; Pinto *et al.*, 2012), chlorination (Eichler *et al.*, 2006), chloramination (Chiao *et al.*, 2014) and filter material (Lautenschlager *et al.*, 2014).

Proteobacteria was the predominant bacterial phylum in the majority of WTP communities. Rapid sand, slow sand and GAC filter bacterial communities were found to consist of the same bacterial phyla (Proteobacteria, Planctomycetes, Acidobacteria, Bacteroidetes, Nitrospirae and Chloroflexi) however, each filter type had different proportions of each phyla (Lautenschlager *et al.*, 2014). Chloramination was found to increase the proportions of *Legionella, Escherichia, Mycobacterium* and *Sphingomonas* in bulk water communities post-filtration (Chiao *et al.*, 2014), while chlorination was found to promote the growth of nitrifying bacteria (Eichler *et al.*, 2006). Source water community composition was

found to be significantly different in different locations and was a strong determining factor of final treated water community composition (Eichler *et al.*, 2006). *Mycobacterium* and *Legionella* were detected in WTP biofilms directly after UV disinfection (Emtiazi *et al.*, 2004).

The effect of chlorine and chloramine on distribution network bacterial communities was investigated by Bal Krishna *et al.* (2013), Hwang *et al.* (2012) and Williams *et al.* (2005). Low concentrations of chloramine promoted prevalence of Solibacteres, Nitrospira, Sphingobacteria and Betaproteobacteria, while communities in distribution networks with high chloramine concentrations were dominated by Actinobacteria and Gammaproteobacteria (Bal Krishna *et al.*, 2013). In a distribution system that received alternating treatments of chlorine and chloramine, disinfectant was found to significantly affect the bacterial community composition. Cyanobacteria, *Methylobacteriaceae, Sphingomonadaceae*, and *Xanthomonadaceae* predominated when chlorine was used, whereas *Methylophilaceae, Methylococcaceae* and *Pseudomonadaceae* had the highest abundances under chloramination.

Source water community was found to shape distribution network communities as well as WTPs. Higher diversity and richness was found in communities in distribution networks receiving water from a surface water source as opposed to groundwater (Douterelo *et al.*, 2015).

Fewer studies have explored changes in drinking water distribution network bacterial communities over time. Lautenschlager *et al.* (2013) found 80% similarity in community composition in samples taken 2 years apart, although samples were taken during the same season (autumn of 2008 and 2010). A strong temporal effect was observed by Pinto *et al.* (2014); Betaproteobacteria were dominant in summer, wherease Alphaproteobacteria dominated in winter. Community richness was strongly inversely correlated with temperature and was observed to be lower in winter and spring than summer and autumn.

A small number of studies analysed both biofilm and bulk water communities in the distribution network (Martiny *et al.*, 2005; Henne *et al.*, 2012; Roeselers *et al.*, 2015) and the core community composition was found to be significantly different in each. For example, in the distribution network studied by Henne *et al.* (2012),

Alpha-, Beta- and Gammaproteobacteria, Candidate division TM6 and Chlamydiales had the highest abundances in biofilm communities, whereas the bulk water community was dominated by Bacteroidetes and Actinobacteria as well as Alpha- and Betaproteobacteria.

A key observation from the literature is that while certain bacterial phyla are ubiquitous in WTP environments (such as Proteobacteria), exact community composition varies widely and is shaped by the interaction of multiple factors. Geographical location, source water, growing environment (biofilm or planktonic), presence of absence of disinfectants, pipe materials and treatment process technology all appear to influence bacterial communities in WTPs.

2.6 Aims and objectives

The overall aim of this study is to characterise the bacterial community dynamics of water treatment plants, with particular focus on the survival and persistence of coliform bacteria.

The specific objectives of this study are:

1) To determine the extent of chlorine tolerance in coliforms in WTPs (Chapter 3, Section 3.2).
2) To determine the effect of treatment stage, source water type and growing environment on bacterial abundance throughout WTPs (Chapter 4, Section 4.2).
3) To determine the effect of treatment stage, source water type and growing environment on bacterial community composition throughout WTPs (Chapter 5.2).
4) To determine the extent to which coliforms contribute to biofilm and bulk water communities in WTPs (Chapter 5.2).

This thesis will address two main knowledge gaps in the current literature. Firstly, to my knowledge, chlorine tolerance in coliform bacteria isolated from UK WTPs has not been quantified or compared across different source waters. The literature to date is conflicting

Secondly, at the inception of the PhD project, there was a limited number of studies that have used next generation sequencing to determine the bacterial community of WTPs at multiple treatment stages. It is my hypothesis that treatment stage and source water type will exert selective pressure on the bacterial community of both biofilm and bulk water habitats. A number of recently published studies support the hypothesis that different treatment processes are associated with different bacterial communities (Li *et al.*, 2017; Lin *et al.*, 2017; Xu *et al.*, 2017; Hou *et al.*, 2018)**.**

Chapter 3 – Chlorine tolerance in coliforms and *E. coli*

3.1 Introduction

Chlorination is the most widely used method of water disinfection, combining low costs and high efficacy if used in the correct conditions. However, apparent "chlorine resistance" and the survival of coliform bacteria in water treatment and distribution systems has been observed for many years (Farkas-Himsley, 1964; LeChevallier *et al.*, 1988a; Mir *et al.*, 1997; Furuhata *et al.*, 2007; Chiao *et al.*, 2014). In an age where the emergence of genetic resistance to antibiotics and antimicrobials in bacteria has become a major threat to human health (Cosgrove, 2006; Johnson and Woodford, 2013), the possibility of genetically-encoded tolerance to chlorine must be considered, including as an explanation of coliform failures that occur in water treatment plants (WTPs).

The definition of chlorine tolerance or "resistance" is ill defined (Chapter 2, Section 2.4.1) and the literature concerning chlorine tolerant *E. coli* is conflicting. For example, Rice *et al.* (1999) and Inatsu *et al.* (2010) found that even after repeated exposure to sodium hypochlorite, *E. coli* O157:H7 did not display any increase in tolerance and was easily killed by chlorine. However, other studies have found it possible to increase chlorine tolerance in *E. coli* (Lisle *et al.*, 1998; Saby *et al.*, 1999).

Coliforms and Gram(-) bacteria have generally been found have the lowest levels of chlorine tolerance when compared to other bacterial species (Ridgway and Olson, 1982; Mir *et al.*, 1997; Le Dantec *et al.*, 2002; Helbling and VanBriesen, 2007; Lee *et al.*, 2010). However, chlorine-specific transcription factors and transcription repressors have been identified in *E. coli* (Gebendorfer *et al.*, 2012; Gray *et al.*, 2013b; Parker *et al.*, 2013) and, given the unparalleled ability of bacteria to adapt and evolve advantageous mutations, it is possible genetically-encoded chlorine tolerance could develop in coliform bacteria.

3.1.1 Aim and objectives

The aim of this chapter is to determine the extent of chlorine tolerance in WTPs. The hypothesis being tested is: coliforms entering WTPs are capable of surviving the water treatment process due to high chlorine tolerance.

The specific objectives are as follows:

1) To isolate any highly chlorine tolerant coliforms - any coliform bacteria capable of surviving high chlorine concentrations or long contact times would be suggestive of high chlorine tolerance and a possible cause of coliform failures in WTPs.
2) To determine whether *E. coli* isolated from raw water entering WTPs is more chlorine tolerant than *E. coli* isolated from other environments.

This chapter shows the level of chlorine tolerance present in coliforms and determines to what extent *E. coli* response to chlorine is affected by the environment from which it was isolated and also handling in the lab prior to testing for sensitivity to chlorine exposure.

3.1.2 Overview of experimental design

The diagram displayed in Figure 3.1 provides a summary of the three experiments carried out in this chapter. The methods for Experiment 1, 2 and 3 are described in Sections 3.2.2, 3.2.3 and 3.2.4, respectively. The results for Experiment 1, 2 and 3 are described in Sections 3.3.1, 3.3.2 and 3.3.3, respectively.

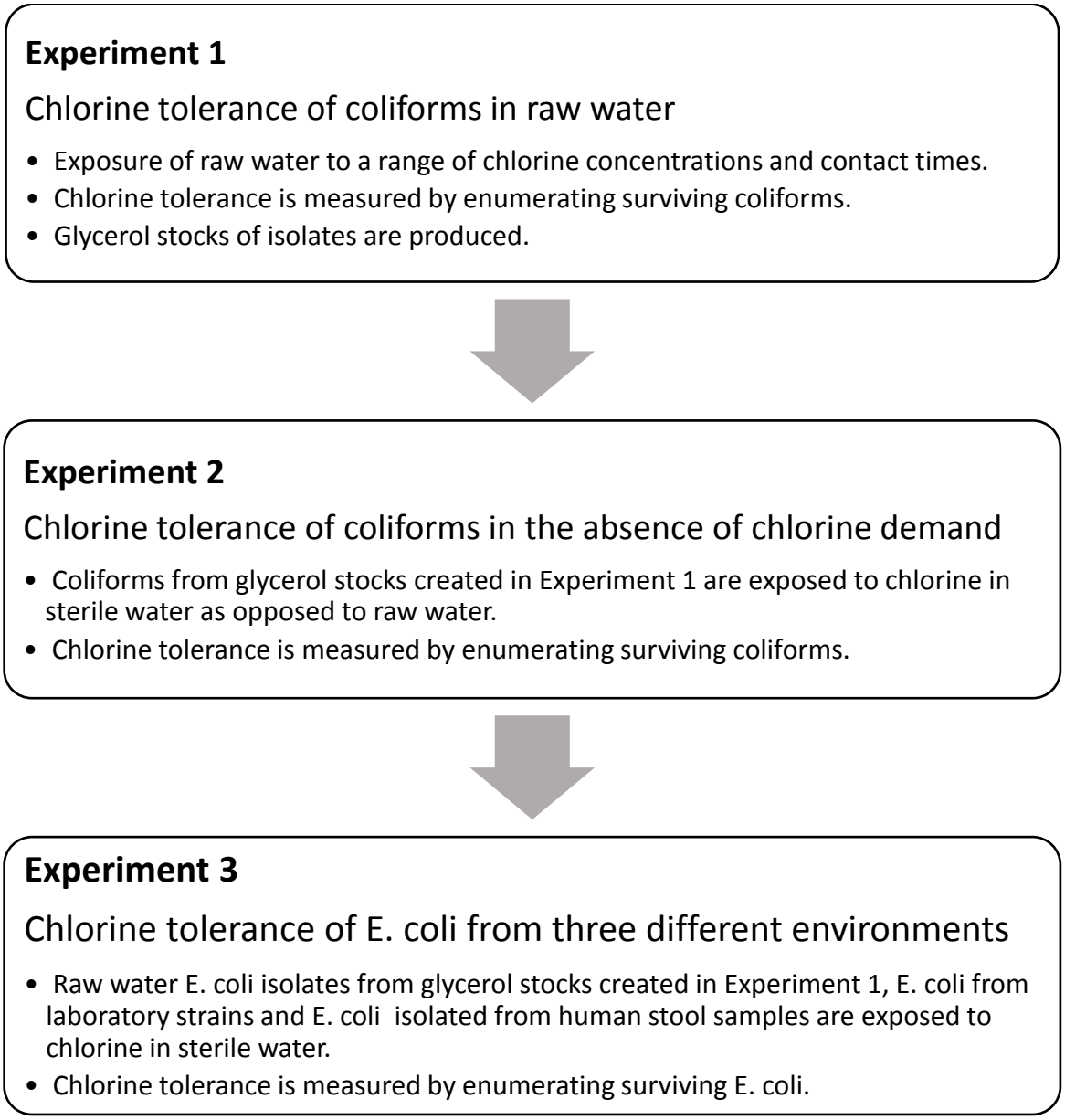


Figure 3.1 – *An overview of the three experiments described in this chapter. Chlorine tolerance was tested in coliforms in raw water and sterile, distilled water. Chlorine tolerance of E. coli isolated from WTP raw water was also compared to laboratory strain E. coli and E. coli isolated from human stool samples.*

3.2 Experiment 1 - Materials and Methods

3.2.1 Water treatment plants

Five UK WTPs were selected for use in the study. Four of the WTPs were selected due to historic problems with coliform failures, while WTP 2 was

included due to a history of a high level of chlorination. All WTPs used the same basic unit operations: clarification, followed by filtration through sand and anthracite filters, followed by passage through granular activated carbon filters and finally disinfection in a chlorination contact tank. There were some differences in the clarification process between plants: WTPs 1, 3 and 5 used ferric sulphate as the coagulant, while WTP 2 and 4 used aluminium sulphate. WTPs 2 and 3 used dissolved air flotation (DAF), whereas WTPs 4 and 5 used hopper bottomed clarifiers. WTP 1 used a combination of DAF and hopper bottomed clarifiers.

3.2.2 Sample collection

Eight litres of raw water (source water entering the water treatment plant) was collected from each of the five WTPs in October and November 2015. All samples were collected in sterile 500 ml Nalgene plastic bottles (VWR, Leicestershire, UK), transported in a cool box and processed (see below) within 24 hours of collection. pH and turbidity readings were taken using on-site monitors.

3.2.3 Chlorine exposure of coliforms in raw water

Each water sample was divided into 100 ml aliquots and placed in separate sterilized 250 ml glass Duran bottles (VWR, Leicestershire, UK) with closed lids. Samples were then exposed to chlorine doses calculated to produce free chlorine concentrations of 0.1, 0.2, 0.5, 1 and 2 mg/l for contact times of 1, 2, 5, 10 and 30 minutes. The appropriate volume of a stock solution of 350 mg/l sodium hypochlorite (NaOCl; VWR, Leicestershire, UK) was used to chlorinate the water samples and chlorine demand of the sample was not taken into account. After each designated contact time, sodium thiosulphate ($Na_2S_2O_3$; VWR, Leicestershire, UK) was used to terminate the chlorination reaction (2.7 mg of sodium thiosulphate quenches 1 mg of chlorine (Chiao *et al.*, 2014)). Three 100 ml replicates of raw source water from each WTP was left unchlorinated as a positive control to contrast microbial isolations.

3.2.4 Enumeration by culture methods

Membrane lactose glucuronide agar (MLGA; VWR, Leicestershire, UK) was used to enumerate and isolate strains under each exposure condition. MLGA was prepared according to the manufacturer's instructions, and triplicate 100 ml samples for each condition (e.g. 0.1 mg/l free chlorine exposure, 1 min contact time) were vacuum filtered through 0.2 µm-pore cellulose nitrate membranes (VWR, Leicestershire, UK). Membranes were removed with sterile forceps and placed on MLGA plates, followed by incubation at 37°C for 18-20hrs. As background, MLGA is a selective chromogenic agar: colonies with a yellow appearance were considered coliforms; green colonies were considered *E.coli* and pink colonies were considered Gram(-) non-coliform bacteria. Colonies were enumerated and recorded as the number of colony forming units (CFU) per 100 ml of water.

Relative survival of bacteria (i.e., chlorine tolerance) was defined as the percentage of bacterial cells killed during each chlorine exposure and was calculated using the formula shown in Equation 3.1.

Equation 3.1 – Calculation used to determine the percentage of bacterial cells killed, where N_0 = the number of cells at time 0, N_t = the number of cells at time t.

$$\frac{N_0 - N_t}{N_0} \times 100$$

A selection of coliform and *E. coli* isolates from each WTP were made into glycerol stocks for future study. Glycerol stocks were made by the addition of 500 µl of overnight culture to 500 µl 50% glycerol solution ($HOCH_2CH(OH)CH_2OH$; Sigma-Aldrich, Dorset, UK). All glycerol stocks were stored at -80°C.

3.3 Experiment 1 – Results and Discussion

3.3.1 Screening for high chlorine tolerance

Coliform and *E. coli* colony forming units (CFU) per 100 ml were recorded for each WTP raw water sample as a function of chlorine concentrations 0.1 mg/l, 0.2 mg/l, 0.5 mg/l, 1 mg/l and 2 mg/l for contact times of 1, 2, 5, 10 and 30

minutes. The positive control for each raw sample was the coliforms and *E. coli* detected at time 0, which is summarised in Table 3.1. WTP 4 had significantly higher number of coliforms and *E. coli* entering the plant in its raw water relative to the other WTPs in the study. This difference is most likely due to the fact that WTP 4 receives source water directly from a river, whereas all four other WTPs receive source water from surface reservoirs.

The pH and turbidity of raw source water collected from the five WTPs is detailed in Table 2. The pH was fairly consistent across the WTPs; the mean average was pH 8.28. Turbidity varied greatly across WTPs, with WTP 2 having the highest of 7.51 NTU as opposed to the lowest value of 1.87 NTU at WTP 3.

Table 3.1 – *The pH and turbidity of raw source water from each WTP, recorded on the day of sampling. Sampling took place between October and November 2015.*

WTP	Source type	pH	Turbidity (NTU)	Coliforms (CFU per 100ml)	*E. coli* (CFU per 100ml)
WTP 1	Reservoir	8.40	5.60	417 (± 45)	197 (± 40)
WTP 2	Reservoir	8.39	7.51	83 (± 3)	51 (± 3)
WTP 3	Reservoir	8.20	1.87	100 (± 20)	67 (± 21)
WTP 4	River	8.26	7.26	3467 (± 551)	933 (± 208)
WTP 5	Reservoir	8.16	2.03	187 (± 85)	87 (± 40)

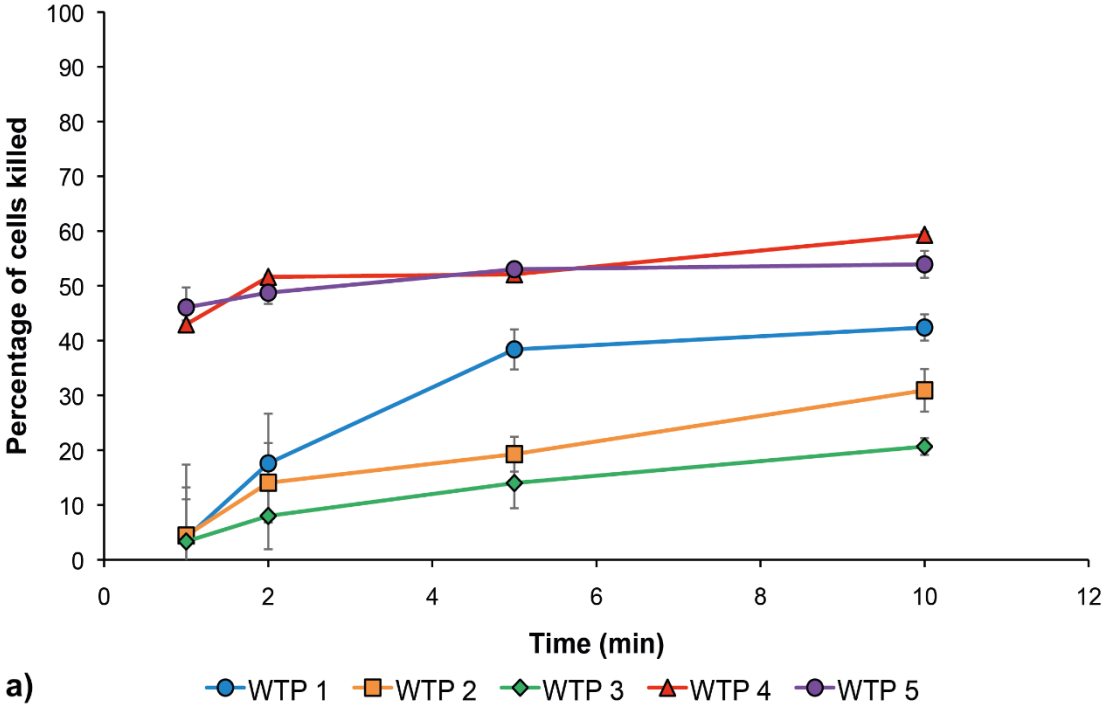
Turbidity in an important factor in chlorination efficacy as a higher turbidity indicates the presence of particles and other matter that will interact with chlorine (LeChevallier *et al.*, 1981). Particles are also capable of physically shielding microorganisms from exposure to chlorine (Herson *et al.*, 1987; Berman *et al.*,

1988). There has been some debate as to the accuracy of using turbidity as a measure of the quantity and nature of particles present in solution (McCoy and Olson, 1986). For example, low concentrations of large particles sized 5 µm and above can result in a low turbidity reading, while still having considerable interactions with chlorine and microbial communities (McCoy and Olson, 1986). Therefore turbidity could be a misleading measure of the interaction potential of a water sample and it may be preferable to use direct particle counts. Organic matter and ammonia react with chlorine to form combined chlorine products such as chloramine. Although combined chlorine products still function as disinfectants, combined chlorine is much less bactericidal than free chlorine. Chlorine is also lost through reaction with substances such as iron, manganese and iron sulphide. The combined effect of organic and inorganic compounds present in water generate the chlorine demand of that particular water sample. WTPs aim to take chlorine demand into account when deciding the optimal chlorine dose to use. Raw source water used in this study will have a high chlorine demand, much higher than water entering a chlorine contact tank in a working WTP. At that stage of the process, turbidity, particle density, amount of organic matter and bacterial cell density will be greatly reduced by previous treatment stages of clarification, filtration and passage through granular activated carbon. Although raw water is not representative of water at the pre-disinfection stage in a WTP, it was used in this study because it was the only sample with enough coliforms present to show large trends in reduction or survival. Experiment 1 was intended as an initial screening to generate an overall picture of coliform survival over a range of chlorine concentrations and contact times and to obtain isolates with high potential chlorine tolerance. Experiment 2, involving testing isolates at the same chlorine concentration in distilled water (in the absence of any of the interacting factors of raw water) acted as a measure of actual chlorine tolerance.

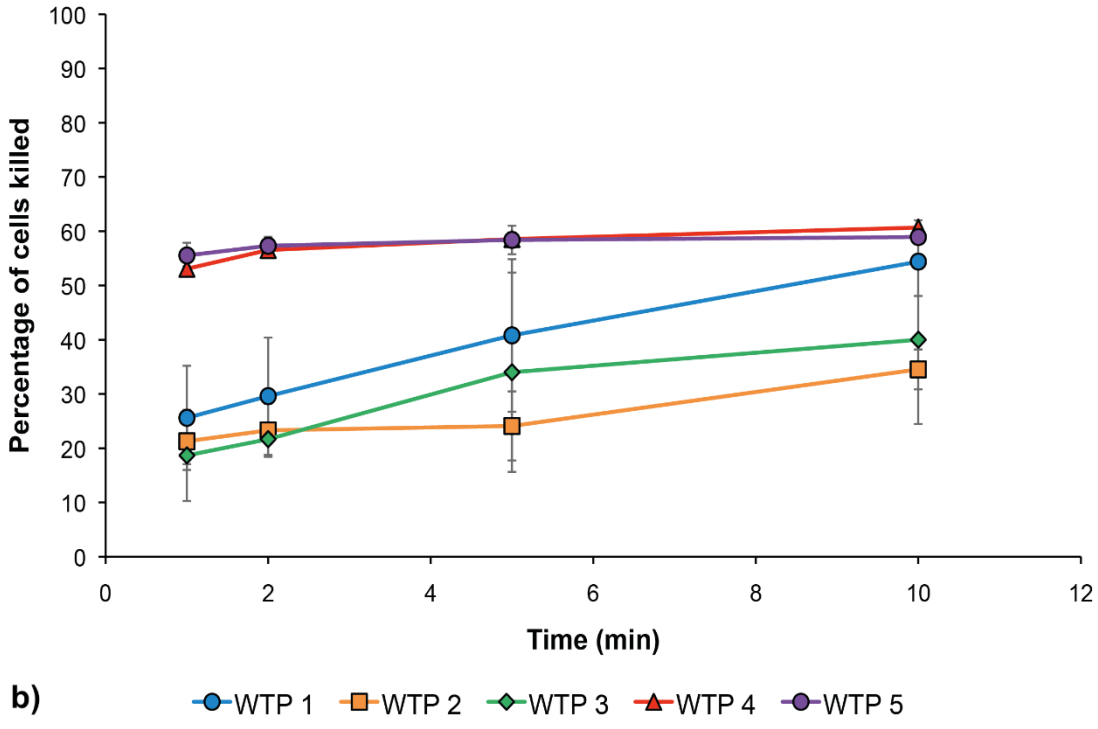
The percentage of cells killed during chlorine exposure for all samples is shown in Figure 3.2. Assays were run over 30 minutes, however as there was no significant change ($p > 0.05$) in percentage of cells killed from 10 to 30 minutes, the first 10 minutes only are shown in Figure 3.2 for better visualisation of the data. For all WTPs, the percentage of coliform bacterial cells killed increased with

increasing chlorine concentration and contact time. The percentage of cells killed at the end of chlorine exposure (i.e. at time 30 minutes) was compared for all WTP samples in a one-way ANOVA for each chlorine concentration. Significant differences were found between WTP samples at the lower chlorine concentrations of 0.1 mg/l, 0.2 mg/l and 0.5 mg/l (p values were all < 0.01). However, there was no significant observed difference between WTP samples at the higher chlorine concentrations of 1 mg/l and 2 mg/l (p values were 0.44 and 0.46, respectively). At the higher chlorine concentrations of 1 mg/l and 2 mg/l, all coliforms were inactivated after 30 minutes exposure. At 2 mg/l, 99% were killed within the first minute of exposure. The variation in percentage of cells killed observed between WTP samples at lower chlorine concentrations was most likely due to the differing chlorine demand of each water sample, which is a function of many factors such as turbidity, pH, and relative ammonia levels (Powell *et al.*, 2000).

As expected, higher chlorine concentrations and longer contact times resulted in greater bacterial cell death. The typical WTP chlorine contact tank doses of 1 mg/l and 2 mg/l free chlorine killed 100% of coliform bacteria after 30 minutes (which is the recommended contact time for drinking water treatment). Since this was observed in raw source water rather than more favourable clarified, filtered water present at the pre-chlorination treatment stage in a working WTP, the finding is even more significant. According to these results, coliforms should not be able to survive through a chlorine contact tank and cause a coliform failure because common doses as used here appear to be functionally lethal. This suggests factors other than chlorine resistance or tolerance in exposed bacteria are more likely responsible for coliform survival in these WTPs. However, it should be noted that a proportion of coliform bacterial cells were still alive after 30 minutes exposure to a slightly lower chlorine concentration of 0.5 mg/l in all WTP samples. To determine whether their survival was due to intrinsic chlorine tolerance or to interactions between source water and chlorine, isolates were tested again in sterile, distilled water. The results of Experiment 2 are discussed in Section 3.5.


Percentage of cells killed
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Time (min)
WTP 1
WTP 2
WTP 3
WTP 4
WTP 5

a)


Percentage of cells killed
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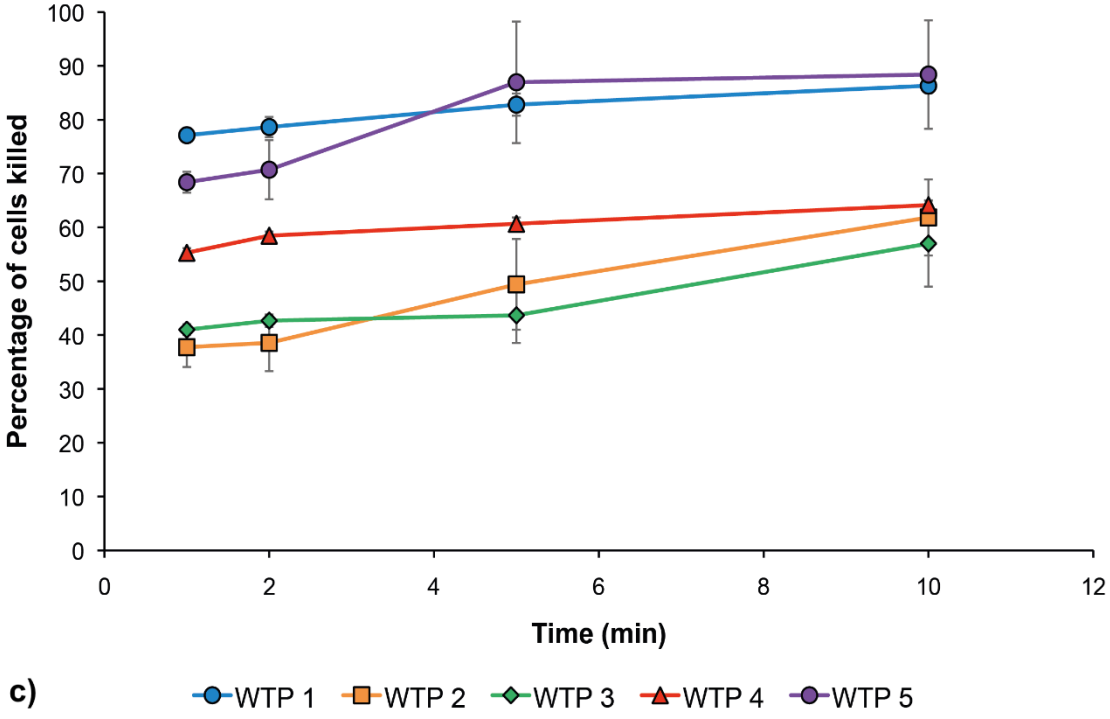
WTP 5

b)



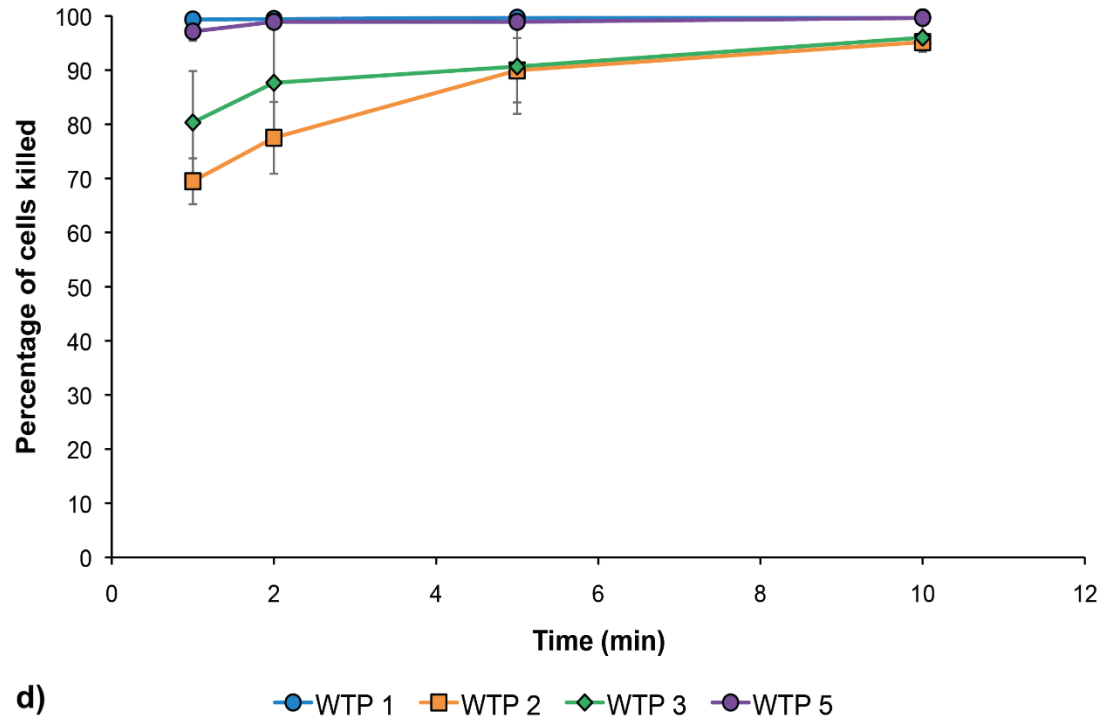


Figure 3.2 – *The percentage of coliform bacterial cells killed over 10 minutes exposure to free chlorine concentrations of* ***a)*** *0.1 mg/l,* ***b)*** *0.2 mg/l,* ***c)*** *0.5 mg/l,* ***d)*** *1 mg/l. Samples were taken from 5 WTPs. Values shown are the mean average of three replicate plate counts and standard error bars are shown.*

3.4 Experiment 2 – Materials and Methods

3.4.1 Chlorine exposure of coliforms in sterile, distilled water

Twenty-five coliforms isolates obtained in Experiment 1 were selected for use in Experiment 2. Five coliform isolates from each WTP (25 isolates in total) that survived in raw water with a chlorine concentration of 0.5 mg/l for 30 minutes were streaked onto LB agar (VWR, Leicestershire, UK) and stored at 4°C as representatives of more tolerant strains. Single colonies of isolates were suspended in 5 ml LB broth (VWR, Leicestershire, UK) in a 15 ml Falcon tube (VWR, Leicestershire, UK) and incubated at 37°C for 18hrs, shaking at 150rpm. The overnight culture was centrifuged at 4200 rpm for 5min and the supernatant was discarded. The pellet then was resuspended in 5-ml phosphate buffered saline (PBS; VWR, Leicestershire, UK). The sample was centrifuged and washed with PBS a further two times, in order to remove any residue of growth medium. The optical density of the sample was measured and adjusted to between OD_{600} 0.45 and 0.5 to ensure all samples had a similar starting bacterial concentration.

Sterile, distilled water (200 ml) was placed in a 250 ml Erlenmeyer flask (VWR, Leicestershire, UK) and adjusted to pH 7±0.05. The required volume of 350 mg/l sodium hypochlorite stock solution was added to the sterile water to generate a chlorinated solution with a residual concentration of 0.5 mg/l free chlorine. Free chlorine concentration was measured using the HI-701 Free Chlorine Checker (Hanna Instruments, Bedfordshire, UK).

PBS-washed bacterial culture (2 ml) was pipetted into the 200 ml chlorinated water under gentle, but constant mixing by magnetic stirrer (150 rpm). One ml aliquots of the solution were removed in triplicate at times 1, 2, 5, 10, 20 and 30 min, and pipetted into 1.5 ml Eppendorf tubes (VWR, Leicestershire, UK) containing an appropriate volume of sodium thiosulphate (500mg/l). In parallel, the assay was performed without the addition of chlorine (as a positive control) and without the addition of bacterial culture (as a negative control). All samples were serially diluted and 100 µl of each dilution was spread plated onto LB agar. Plates were incubated at 37°C for 24 hrs. Following overnight growth, plates with between 20-300 colonies were selected and the number of colonies at each time point was enumerated. The survival assay was repeated as described above for

each of the 25 isolates. The assay was also repeated for all isolates using a lower free chlorine residual concentration of 0.1 mg/l.

3.5 Experiment 2 – Results and Discussion

When exposed to a free chlorine concentration of 0.5 mg/l in sterile, distilled water, 100% of cells from all 25 isolates (five isolates from each WTP) were inactivated after only two minutes exposure. At the lower chlorine dose of 0.1 mg/l, 100% of cells from all 25 isolates were killed after five minutes. These results suggest that the coliform survival observed in the initial screening for high chlorine tolerance was due to interaction and inhibition of chlorine by raw source water rather than coliforms expressing tolerance to chlorine. Along with previous studies detailed in literature, these data suggest that increased tolerance to chlorine or “chlorine resistance” in coliforms has not emerged. Furthermore, it is my belief that chlorine resistance is unlikely to develop in the future due primarily to its mechanism of action and concentrations at which it is typically used. This is more clearly explained by comparing chlorine resistance to antibiotic resistance.

Antibiotics function by targeting specifics structures or processes in bacterial cells. For example, penicillins and other β-lactam antibiotics act by targeting the bacterial cell wall. Penicillin binding proteins (PBPs) catalyse the formation of peptidoglycan cross-links; by binding to these enzymes, β-lactam antibiotics prevent cross-links from being synthesised, thus weakening the cell wall and eventually leading to osmotic lysis. All antibiotics are specific in their targets, whereas chlorine is much more indiscriminate in its bactericidal action. Chlorine is a strong oxidising agent, which on exposure to bacterial cells, rapidly destroys the cell wall and membrane as well as structures and organelles inside the cell and DNA in the nucleus. To become resistant to an antibiotic, cells need only gain one gene or sustainable mutation that defends against specific targets. As such, to become resistant to chlorine, the cell would need genes that protect almost all cellular structures. Resistance is also easier to develop when low concentrations of an antimicrobial substance is used. Antibiotics have to be used at low doses due to the toxicity of higher amounts in the human body. Low concentrations increase the chance of small numbers of bacterial cells with

advantageous mutations (i.e. antibiotic resistance) surviving and replicating until the entire population is resistant. Chlorine on the other hand, is used at much higher concentrations for disinfection, limiting the chance of any surviving bacteria with the possible exception of within water distribution lines where chlorine residuals have become lower.

3.6 Experiment 3 – Materials and Methods

3.6.1 Selection of raw water E. coli isolates for use in chlorine tolerance comparison

Twenty *E. coli* isolates obtained in Experiment 1 were selected for use in Experiment 3. Four glycerol stocks of *E.coli* from each of the five WTPs were chosen (20 isolates in total) as representative. For each WTP, the E. coli isolate that had survived exposure in raw water at the highest chlorine concentration and contact time was selected for use. Each isolate was streaked onto MacConkey agar (VWR, Leicestershire), followed by incubation at 37°C for 24hrs. Isolates that grew as non-mucoid pink colonies with a red halo on MacConkey agar were considered to be *E. coli*. Presumptive *E. coli* isolates were further confirmed using MALDI-TOF-MS analysis. The final five raw water WTP *E. coli* isolates selected are listed in Table 3.2.

Table 3.2 *– E. coli isolates selected for use in the chlorine tolerance comparison study. The chlorine concentration and contact time that each E. coli isolate previously survived in raw chlorinated water is also listed.*

Isolate name	WTP	Chlorine concentration (mg/l)	Contact time (min)
E1	1	1	10
E2	2	0.1	10
E3	3	0.1	30
E4	4	1	30
E5	5	0.1	10

3.6.2 Selection of laboratory and human-derived E. coli isolates for use in chlorine tolerance comparison

Fifteen *E. coli* isolates were used in the comparison study (see Table 3.3). Lab strain *E. coli* were kindly donated by Prof John Perry at the Freeman Hospital in Newcastle-upon-Tyne. Human faecal *E. coli* isolates were donated by a local hospital and were obtained from anonymous patient stool samples. Raw water *E. coli* were isolated from five different water treatment plants (WTPs) as in Section 3.6.1.

Table 3.3 *– A summary of the type and source of all E. coli isolates used. Lab isolates are all variants of the K12 strain, apart from E. coli NCTC 13125, which is a strain of O157:H7.*

Isolate name	Isolate type	Source
L1	Lab	E.coli NCTC 9001
L2	Lab	E.coli NCTC 10418
L3	Lab	E.coli NCTC 12486
L4	Lab	E.coli MG1655
L5	Lab	E.coli NCTC 13125
E1	Raw water	WTP 1, UK
E2	Raw water	WTP 2, UK
E3	Raw water	WTP 3, UK
E4	Raw water	WTP 4, UK
E5	Raw water	WTP 5, UK
F1	Faecal	Human sample
F2	Faecal	Human sample
F3	Faecal	Human sample
F4	Faecal	Human sample
F5	Faecal	Human sample

3.6.3 Chlorine exposure of E. coli

E. coli isolates from 15 glycerol stocks (five lab strain, five raw water and five faecal) were streaked onto LB agar plates and stored at 4°C. The chlorine exposure assay as described in Section 3.4.1 was carried out with one change in protocol: a free chlorine residual concentration of 0.05 mg/l was used instead of 0.5 mg/l. This was to assess how lower concentrations of chlorine impacted the strains and to more sensitively assess how "tolerance" was displayed in isolates from difference sources. The same chlorine exposure assay was carried out on all 15 isolates.

3.6.4 Measurement of specific growth rate of E. coli strains and data analysis

Cultures of all 15 isolates were set up in duplicate in 5 ml LB broth in 15 ml Falcon tubes and incubated at 37°C for 18hrs, shaking at 150rpm. After overnight incubation, 5 µl of each culture was added to 50 ml fresh LB broth, then re-incubated at 37°C, 150 rpm. Aliquots (2 ml) were removed every hour for 12 hrs and the optical density was measured at 600 nm as a representative of bacterial growth.

All statistical analysis was performed in Microsoft Excel and R Studio. Graphs were generated in Microsoft Excel and edited using Adobe Illustrator. Unless otherwise noted, statistical significant was defined as 95% confidence in observed differences ($p < 0.05$).

3.7 Experiment 3 – Results and Discussion

3.7.1 Chlorine tolerance of E. coli strains isolated from different habitats

The survival of lab-strain, WTP and faecal *E. coli* were compared upon challenge by 0.05 mg/l free chlorine for 30 minutes. Surviving *E. coli* CFUs were enumerated and the proportion of inactivated bacterial cells calculated as a function of *E. coli* origin. In summary, all isolates were sensitive to chlorine and over 90% of cells were killed within the first five minutes of exposure for all strains. However, lab strains reached 99.9% inactivation within the first minute,

whereas environmental and faecal isolates did not achieve 99.9% inactivation until 10 minutes and 20 minutes, respectively (see Figure 3.3), suggesting increased chlorine tolerance ranged from faecal > WTP > lab strains.

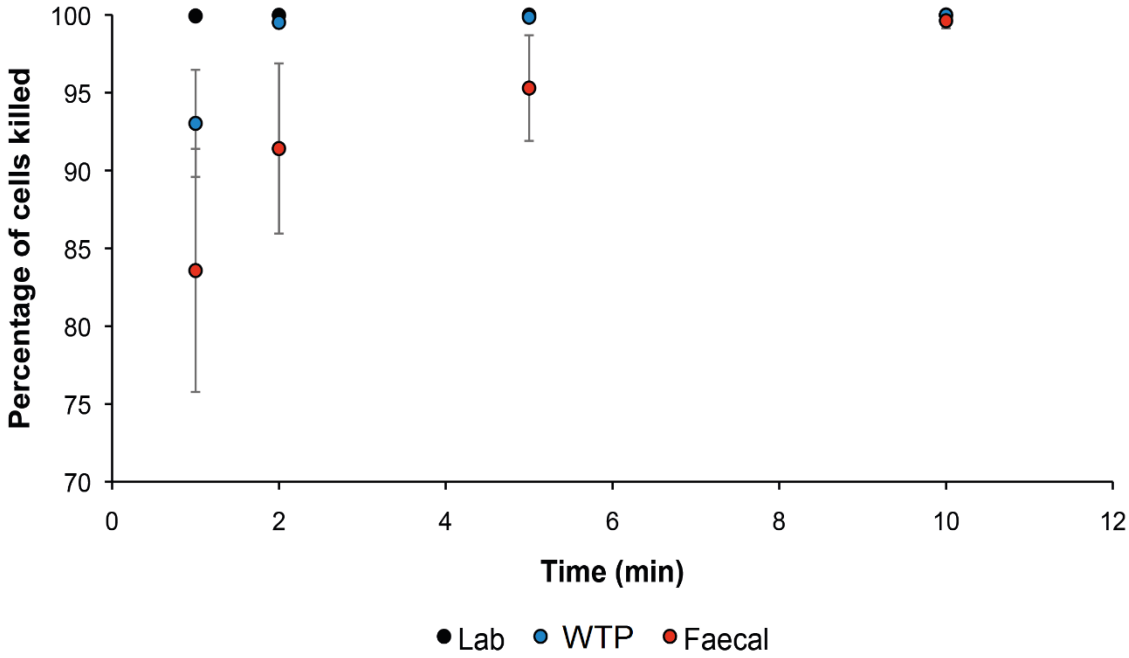


Figure 3.3 - *The proportion of E. coli cells killed or inactivated during 10min exposure to 0.05mg/l free chlorine. At 1min, lab strain E. coli reached 99.93% inactivation. At 10min, WTP E. coli reached 99.98% inactivation. At 20min, faecal E. coli reached 99.93% inactivation while lab and environmental were 100% inactivated. At 30min, all strains were 100% killed or inactivated. The first ten minutes only are shown as this allows for better visualisation of the main inactivation period. Standard error (S.E.) bars are shown.*

To understand this pattern, one must consider the nature of tolerance in Gram(-) bacteria and *E. coli*, which are among the most chlorine-sensitive bacterial species (Ridgway and Olson, 1982). Here we assessed their survival at very low chlorine concentrations (such as 0.05 mg/l), which allows one to observe altered inactivation of different strains that is not visible at higher concentrations. After the initial one minute exposure to chlorine, an average of 99.9% lab strain, 93.0% environmental and 83.6% faecal E. coli were killed or inactivated. These data suggest that there may be a subset of bacterial cells in each population that have increased tolerance to chlorination and that inactivation actually occurs in two

stages: the initial large-scale destruction of cells followed by slower inactivation of more tolerant cells. This more tolerant subset does not appear to be present in the lab strain population.

To place numbers to these data, -logN/N_0 was calculated for all isolates, which is summarised in Table 3.4. N_0 represents the number of cells at time 0 and N represents the number of cells at time t. The average values of the five isolates of each group were plotted against each other (see Figure 3.4) and a statistically significant difference in inactivation was observed across *E. coli* source (two-way ANOVA analysis resulted in a p-value of $2x10^{-16}$). Tukey's honest significant differences test (Tukey's HSD) revealed that each *E. coli* type was significantly different to the other two (WTP vs. lab and WTP vs. faecal produced $p < 0.01$, while lab vs. faecal resulted in $p < 0.001$). Lab strain *E. coli* are significantly less chlorine tolerant than WTP isolates, which are in turn less tolerant than faecal isolates.

The Chick-Watson law (Chick, 1908; Watson, 1908) is the simplest and most conventional model of disinfection kinetics. It is expressed as the formula shown in Equation 3.2.

Equation 3.2 – The Chick-Watson law, where N represents the number of bacterial cells at time t, N_0 is the number of bacterial cells at time 0, k is the decay rate constant, C is concentration of disinfectant, n is the coefficient of dilution (assumed to be 1) and t is the contact time.

$$log\frac{N}{N_0} = -kC^n t$$

Using this equation, k values were calculated for all isolates (see Table 3.5). k values were plotted against CT (the product of chlorine concentration and contact time) and the coefficient of each line (using power regression) was calculated to determine the inactivation rate constant (k_i) for each *E. coli* group (see Figure 3.5). The lab strain k_i was 6.17; the environmental k_i was 5.46 and the faecal k_i was 3.48.

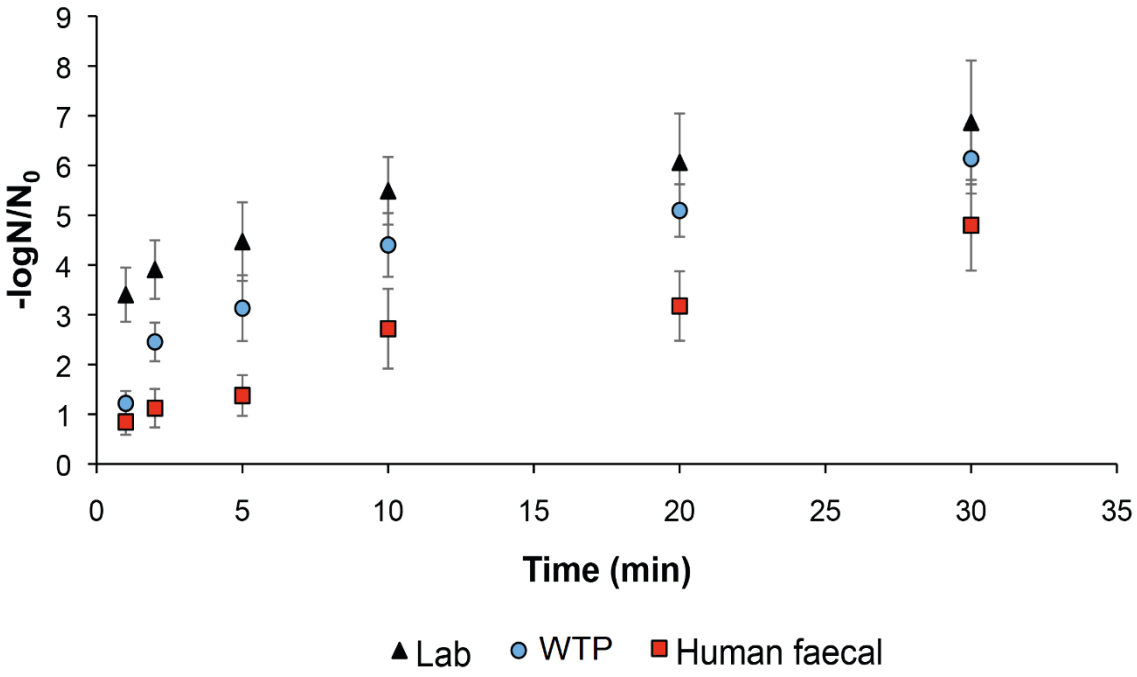


Figure 3.4 – *Inactivation of E. coli strains during 30min exposure to 0.05mg/l free chlorine. Each data point represents the mean average of triplicate counts from 5 isolates and standard error (S.E.) bars are shown. The gradient values for lab, environmental and faecal type are 0.1114, 0.1479 and 0.1298 respectively. R^2 values are 0.922, 0.8793 and 0.962 for lab, WTP and faecal type.*

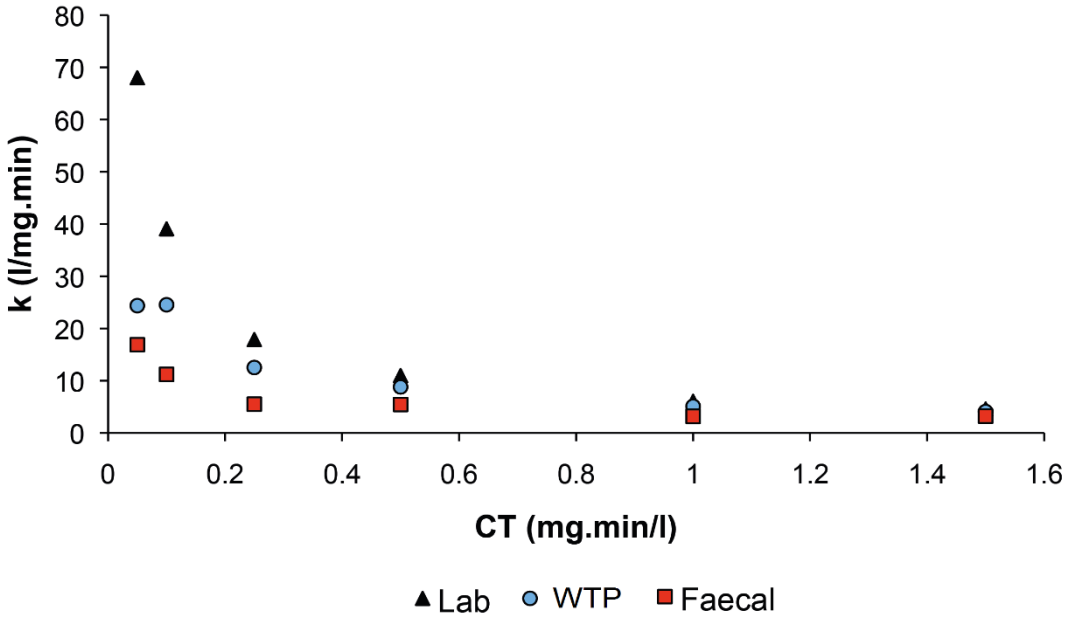


Figure 3.5 - *The mean average decay rate of lab, WTP and faecal E. coli at CT 0.05–1.5mg.min/l.*

Table 3.4 *- -logN/N_0 values for all 15 E. coli isolates at six time points over 30 minutes. The gradient and R^2 values are listed.*

Isolate	1min	2min	5min	10min	20min	30min	Inactivation rate (slopes)	R^2
L1	4.32	4.27	5.82	6.31	7.47	7.90	0.125	0.944
L2	3.34	3.82	4.04	5.04	5.39	5.82	0.134	0.747
L3	3.21	3.55	4.10	5.06	5.45	6.04	0.144	0.848
L4	3.19	3.34	3.80	4.83	5.25	6.04	0.160	0.874
L5	2.94	4.80	5.05	6.05	6.74	8.53	0.163	0.885
E1	1.13	2.03	2.57	3.16	4.22	5.21	0.125	0.880
E2	1.43	3.10	4.36	4.82	5.82	6.20	0.0805	0.881
E3	1.18	2.46	2.87	4.59	5.10	5.93	0.0924	0.896
E4	1.18	2.27	2.96	4.73	4.93	6.50	0.0966	0.938
E5	1.17	2.42	2.90	4.71	5.15	6.55	0.155	0.881
F1	0.607	0.616	0.909	1.57	2.17	3.48	0.0970	0.986
F2	1.11	1.46	1.50	3.51	3.51	5.17	0.133	0.902
F3	0.695	0.859	1.08	2.41	3.45	5.24	0.156	0.988
F4	1.04	1.43	1.58	3.32	3.59	2.07	0.312	0.929
F5	0.763	1.24	1.82	2.77	3.15	5.03	0.131	0.950

Table 3.5 *– k values of each isolate at CT values from 0.05-1mg.min/l (CT values calculated as the product of chlorine concentration (0.05mg/l) and contact time).*

Isolate	0.05mg.min/l	0.1mg.min/l	0.25mg.min/l	0.5mg.min/l	1mg.min/l	1.5mg.min/l
L1	86.5	42.7	23.3	12.6	7.47	5.27
L2	66.7	38.2	16.2	10.1	5.39	3.88
L3	64.2	35.5	16.4	10.1	5.45	4.02
L4	63.9	33.4	15.2	9.66	5.25	4.03
L5	58.8	48.0	20.2	12.1	6.74	5.69
E1	22.7	20.3	10.3	6.32	4.22	3.47
E2	28.7	31.0	17.5	9.65	5.82	4.13
E3	23.6	24.6	11.5	9.17	5.07	3.96
E4	23.5	22.7	11.8	9.47	4.93	4.32
E5	23.3	24.2	11.6	9.42	5.15	4.37
F1	12.2	6.16	3.6	3.15	2.17	2.32
F2	22.3	14.6	5.99	7.02	3.51	3.45
F3	13.9	8.59	4.32	4.83	3.45	3.50
F4	20.9	14.3	6.30	6.65	3.59	3.38
F5	15.3	12.4	7.26	5.54	3.15	3.35

The proportion of cells killed and inactivation rate constants have shown lab strain *E. coli* isolates to have a significantly lower chlorine tolerance than *E. coli* isolated from the environment of a WTP or the human gut. Reference or lab strain bacteria have been repeatedly grown and sub-cultured in environments completely devoid of stressors. Environmental or host-associated bacteria have to contend with limited nutrient availability (Morita, 1988), competition for nutrients and growth space from other microbial species (Hibbing *et al.*, 2010), host defence mechanisms, antimicrobials in the form of disinfectants or antibiotics and osmotic, heat and oxidative stress from their environment (Roszak and Colwell, 1987). Given these vast differences in external pressures, it is likely that lab versus environmental bacteria have different responses to stress. In particular, I suggest lab strains may have lost stress defence traits that still prevail in environmental strains. A more detailed understanding of differences between the genomes and transcriptomes of these strains could yield an explanation of variation in chlorine tolerance and is an interesting area of future study.

Faecal *E. coli* isolates were found to have a higher level of chlorine tolerance than WTP isolates. The faecal isolates used in this study were collected from hospital patients, therefore it is possible that some selection bias occurred. Hospital patients are more likely to have taken antibiotics recently which would increase the possibility that any surviving enteric bacteria possess antibiotic resistance genes. There are reports of co- or cross-resistance between antibiotics and other antimicrobials (McMurry *et al.*, 1998; Chuanchuen *et al.*, 2001; Braoudaki and Hilton, 2004), which could explain the increased chlorine tolerance observed in the study. Reference strain *E. coli* NCTC 13125 (isolate L5) was a variant of O157:H7 and was no more chlorine tolerant than the K-12 reference strains. This supports previous work by Rice *et al.* (1999) which found increased virulence did not lead to increased survival in chlorine.

The growth rate of each isolate was measured by monitoring optical density of bacterial culture over 12 hours (see Figure 3.6). It was hypothesised that fitness cost of adaptations to increase chlorine tolerance may result in reduced growth rate in environmental or faecal type *E. coli.* WTP and faecal *E. coli* isolates display very similar growth characteristics. In contrast, the lab strains averaged

much lower growth rates, although there was greater variation between individual isolates than seen in the other two groups. This difference was not statistically significant ($p > 0.05$), although we suspect this is because of the small size.

The findings of papers on chlorine efficacy and the inactivation of microorganisms by chlorine are often used as a basis for calculating the CT values needed for adequate microbial removal at working water treatment plants. They are also used in the generation of mathematical models that aim to predict treatment plant failure rates and risk of infection from waterborne pathogens. Therefore, the accuracy and applicability of the CT values reported in literature have huge importance in real-world situations.

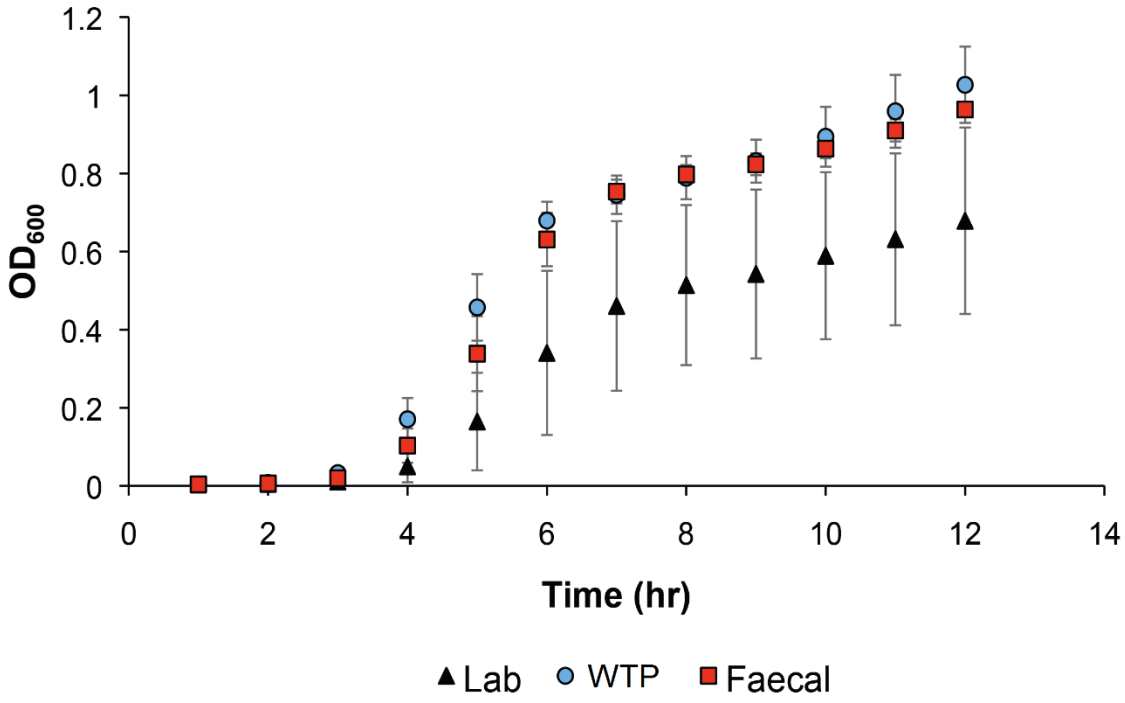


Figure 3.6 *– The growth rate of lab, WTP and faecal strains in 50ml LB broth over 12 hrs. Each data point represents duplicate readings of 5 isolates and standard error (S.E.) bars are shown. The gradient values for lab, environmental and faecal groups are 0.0725, 0.1056 and 0.1038 respectively.*

A number of chlorination studies use reference strain bacteria as the test subject (LeChevallier *et al.*, 1988a; Stewart and Olson, 1992; Lisle *et al.*, 1998; Momba *et al.*, 1999; Saby *et al.*, 1999; Baker *et al.*, 2002; Helbling and VanBriesen, 2007; Phe *et al.*, 2009; Lee *et al.*, 2010; Gao and Liu, 2014). Lab strain bacteria are

useful for a number of reasons: they are easily obtainable; they have been thoroughly characterised and the genome is often available; they are relatively safe or less harmful than other strains and they allow comparisons to other studies using the same standard reference types. For these reasons, reference strains are used as a proxy for environmental bacteria. However, there is an inherent assumption in using reference strains in chlorination studies: that reference types are representative of environmental types of the same species. Findings from this study suggest that is an unreliable assumption.

There are a number of mathematical models used to represent inactivation kinetics, the majority of which are derived from or based on the Chick-Watson equation. Further models have been proposed by Hom (1970), Majumdar *et al.* (1973) and Haas and Karra (1984) among others. Inactivation kinetics are affected by a large number of variables, not all of which can be easily defined, and each model has varying levels of success in representing experimental data (Block, 2001). The organism in question (bacteria, viruses, protozoa), the experimental set-up (batch reactors, flowing networks), the disinfectant (chlorine, chloramine, ozone) and many other factors determine which model best fits the experiment results.

Regardless of the success of a model or its level of complexity, the findings of this paper suggest that experimental data using lab strains to validate the CT model is not valid because it is not representative of environmental (WTP) bacteria. This severely limits the usefulness of any results or predictions from the model for real world applications such as water treatment plants. Any inactivation or chlorination efficacy model designed for practical use by a water company is recommended to use data based on environmental isolates, ideally from the local environment the model will be describing.

This work further shows that genetic chlorine resistance does not likely exist or even chlorine tolerance at a practical level. Coliform failures at WTPs still occur, but the extreme sensitivity of coliforms shown here, especially in *E. coli*, suggests such failures are not related to the bacteria themselves. Implicitly failures are, therefore, more likely to be explained by other factors that compromise the disinfection process. Biological factors could include protection from chemical

disinfection of coliforms within biofilms or shielding whilst in suspension by attachment to particles. Operational factors could include insufficient mixing or contact time within the chlorination tank or ingress of bacteria into the contact tank or treated water stream. Inaccurate tank dimensions, poor flow efficiency calculations and ingress occurring towards the end of the contact tank will all increase the likelihood of a coliform passing through without being inactivated or killed. Therefore, focus at a given WTP should be on optimising operations as a strategy to reduce failures because if chlorination is performed correctly, data here suggest even tolerant coliforms will not survive.

3.7.2 Potential for regrowth of E. coli

Enteric bacteria such as *E. coli* are not well suited for the low temperatures and oligotrophic conditions of water environments. However, the putative ability of *E. coli* and other potentially pathogenic bacteria to survive and regrow in water distribution systems is central to determining the risk these bacteria pose to effective water treatment. While *E. coli* detection at a WTP has serious repercussions for the water company, a number of studies suggest that *E. coli* is not able to regrow in water distribution systems and is not a major component of water-associated biofilms (Fass *et al.*, 1996; Lehtola *et al.*, 2007; Jjemba *et al.*, 2010; Thayanukul *et al.*, 2013a; Abberton *et al.*, 2016). There is a body of evidence showing that biofilms in water environments can act as a reservoir for potential pathogens (Angles *et al.*, 2007; Wingender and Flemming, 2011; Chaves Simes and Simes, 2013; Koh *et al.*, 2013) and some studies have detected *E. coli* in biofilms (Banning *et al.*, 2003; Juhna *et al.*, 2007; Farkas *et al.*, 2012). While there are some conditions that can have been shown to increase *E. coli* growth such as iron availability (Appenzeller *et al.*, 2005) and assimilable organic carbon (Vital *et al.*, 2010; Thayanukul *et al.*, 2013b), the literature consensus suggests that while *E. coli* may have been detected in water distribution biofilms, the abundances were extremely low and *E. coli* are not able to regrow to significant numbers.

3.8 Conclusions

This study investigated water taken from working WTPs for highly chlorine tolerant coliform bacteria that could survive the disinfection process to cause a treatment failure. The main findings of this study are as follows:

1. No evidence of high chlorine tolerance or chlorine resistance was observed in any coliform isolate.
2. *E. coli* is very sensitive to chlorination and typical CT values used in working WTPs are more than adequate for complete inactivation.
3. Failures in coliform removal in WTPs are not believed to be related to increased tolerance to disinfection of the bacteria themselves.
4. Lab strain *E. coli* are less chlorine tolerant than WTP or faecal type.
5. Models designed to measure and evaluate chlorine efficacy in WTPs are recommended to use data from studies based on environmental isolates.
6. Coliform failures occur in WTPs, but data herein suggest it is very unlikely such failures result in intrinsic chlorine tolerance and are more likely explained by operational problems within a given WTP.

Chapter 4 – Quantification of 16S rRNA genes in biofilms and bulk water in WTPs

4.1 Introduction

Genetic chlorine resistance or elevated bacterial tolerance is not likely to be the main cause of coliform failures in WTPs (Chapter 3). Therefore, one must consider other possible sources of coliform bacteria and other mechanisms that might allow these bacteria to survive or avoid treatment and disinfection processes in WTPs. There are three main survival strategies detailed in the literature that could result in genetically chlorine-sensitive coliform bacteria being able to survive water disinfection by avoiding direct exposure to chlorine (LeChevallier *et al.*, 1988a). The strategies are shielding within biofilms; shielding through attachment to particles within the processes; and shielding by ingestion and intracellular survival within zooplankton, protozoa or other possible host organisms. Literature regarding the interaction of disinfection and biofilms, particles and protozoa was reviewed in Chapter 2, Sections 2.4.2, 2.4.3 and 2.4.4, respectively.

Of the three strategies discussed above, biofilm shielding was chosen here as the most likely explanation for unexplained coliform failures at WTPs. While ingestion and intracellular survival of bacteria is an interesting area of study, particularly in view of the limited number of previous experiments, the current literature would suggest it is a minimal risk factor for coliform failures (Bichai *et al.*, 2011). Particle-associated coliforms, on the other hand, would appear to significantly increase the risk of a coliform failure (LeChevallier *et al.*, 1984; Herson *et al.*, 1987; Stewart *et al.*, 1990). However, this fact has already been well established in the literature and relatively simple interventions could greatly mitigate the risk. For example, current practices of ensuring water entering the disinfection stage has a turbidity of less than 1 NTU go some way to combatting the presence of particle-associated bacteria. Further strategies could include the installation of particle counters at points in the treatment process after passage through GAC filters and prior to chlorination, to allow for more accurate

monitoring or the installation of a post-filter on GAC filters to reduce the amount of carbon fines being released.

In terms of the possibility of biofilms acting as reservoirs of coliform bacteria, relatively little is known about the dynamics and composition of bacterial communities in working WTPs. The majority of research focuses on biofilms in the distribution network, rather than the treatment plant itself (Berry *et al.*, 2006; Eichler *et al.*, 2006; Hong *et al.*, 2010; Henne *et al.*, 2012; El-Chakhtoura *et al.*, 2015). It is also possible that bacterial communities will be highly individual to the local environment, meaning findings may not transfer across countries or regions. Routine testing at WTPs in the UK encompasses a small number of indicator organisms only, including coliforms, *E. coli, Clostridium perfringens*, *Enterococcus faecalis* and heterotrophic plate counts. Also, this testing only focuses on bacteria present in bulk water.

4.1.1 Aims and objectives

The aim of this chapter is to determine the effect of treatment stage, source water and growing environment on the number of total bacteria (as estimated by quantification of 16S rRNA genes). The hypotheses being tested are: firstly, the number of 16S rRNA genes will decrease with advancing treatment stage; secondly, river source water WTP will have higher numbers of 16S rRNA genes than reservoir source water WTP.

The specific objectives are as follows:

1) To quantify the 16S rRNA gene abundance in biofilms at five treatment stages in two WTPs.
2) To quantify the 16S rRNA gene abundance in bulk water at five treatment stages in two WTPs.
3) To compare the 16S rRNA gene abundances of a reservoir source water WTP with a river source water WTP.

In Chapter 4, the total number of bacteria (as evidenced by 16S rRNA gene abundances) in both biofilms and bulk water samples is compared across multiple treatment stages, over time, and between differing sources of raw water. Coliforms, *E. coli* and Gram(-) non-coliforms also were quantified for bulk water

samples to contrast with bacterial estimates from 16S rRNA gene data. Community composition generated by amplicon sequencing will be reported for all samples in greater detail in Chapter 5.

4.2 Materials and Methods

4.2.1 Design and installation of biofilm collectors

Biofilm collectors consisted of frosted glass slides (VWR, Leicestershire, UK) placed in an adapted plastic slide holder (VWR, Leicestershire, UK). Stainless steel wire was used to fasten the slide holder into a stainless steel mesh cage (160 mm x 110 mm x 110 mm). Stainless steel cages were constructed by DP Structures Ltd (Lancashire, UK). The cages were suspended by stainless steel metal chains (Falcon Workshop Supplies Ltd, Greater Manchester, UK) into the water stream at multiple stages throughout WTP A (reservoir source water) and WTP B (river source water). Figure 4.1 shows a complete biofilm collector prior to installation. Glass slides were selected as the surface to harvest biofilms rather than sand or other WTPs materials. This was because glass slides would provide a consistent growing environment at each treatment stage, meaning bacterial community composition or 16S rRNA gene abundance would not be influenced by different surface material. Glass slides were also preferred because sterilised glass is an acceptable material to introduce to the water supply at any stage of treatment. For example, the placement of any biofilm collector containing sand in a chlorinated contact tank supplying water to customers would not have been acceptable. The effect of surface material on bacterial growth and biofilm community composition is an interesting area of research, however, it was not within the scope of this study.

As previously noted, biofilm collectors were installed in two WTPs at five locations within in the water treatment process (Figure 4.2).

- Stage 1: Raw water entering the treatment works
- Stage 2: Post-clarification with ferric sulphate
- Stage 3: Post-filtration through sand and anthracite filters
- Stage 4: Post-passage through granular activated carbon

- Stage 5: Chlorine contact tank

WTP 3 and WTP 4 (see Chapter 3), received raw water from reservoir and river sources, respectively. Due to the slightly different physical layout of WTP 4, I was unable to attach a biofilm collector at stage 4 without potentially compromising the treatment process. Therefore, WTP 4 biofilm samples were collected from stages 1, 2, 3 and 5 only. Biofilm collectors were installed at WTP 3 and 4 in August and September 2016, respectively.

4.2.2 Catchment supplying WTP 3 and WTP 4

WTP 3 abstracts water from the River Dove and water is then pumped into two surface water reservoirs which supply the WTP. There are some known point source pollution risks in the catchment, including an upstream sewage treatment works, a road haulage depot with bulk diesel storage, a mushroom farm, an industrial estate with a food processing factory and finally, surface drainage from the M42 motorway. The land surrounding the surface water reservoirs is largely used for agricultural purposes, particularly upland livestock farming (Trent, 2019). WTP 4 relies on direct river abstraction from the River Severn. The catchment receives average annual rainfall of 856 mm and the land use is mainly agriculture and forestry, with some industrial development in the east of the catchment (Trent, 2019).

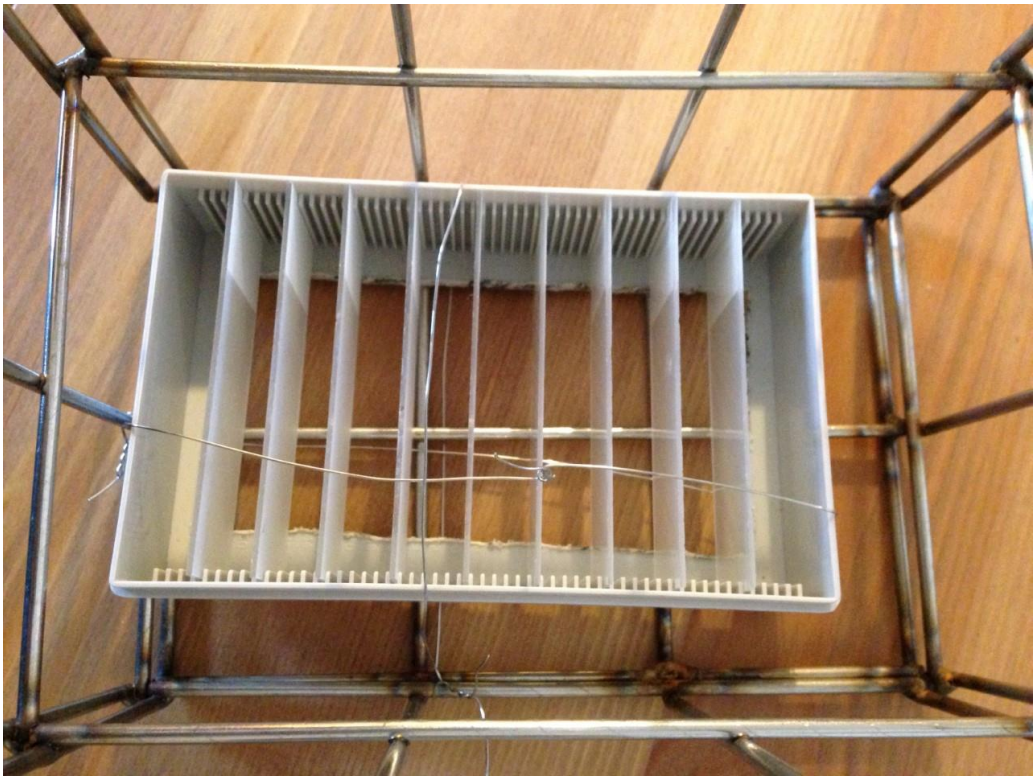


Figure 4.1 *– Biofilm collectors were constructed from stainless steel mesh cages containing nine frosted glass slides held in place by a plastic slide holder, secured with stainless steel wire. The cages were suspended and secured using stainless steel chains.*

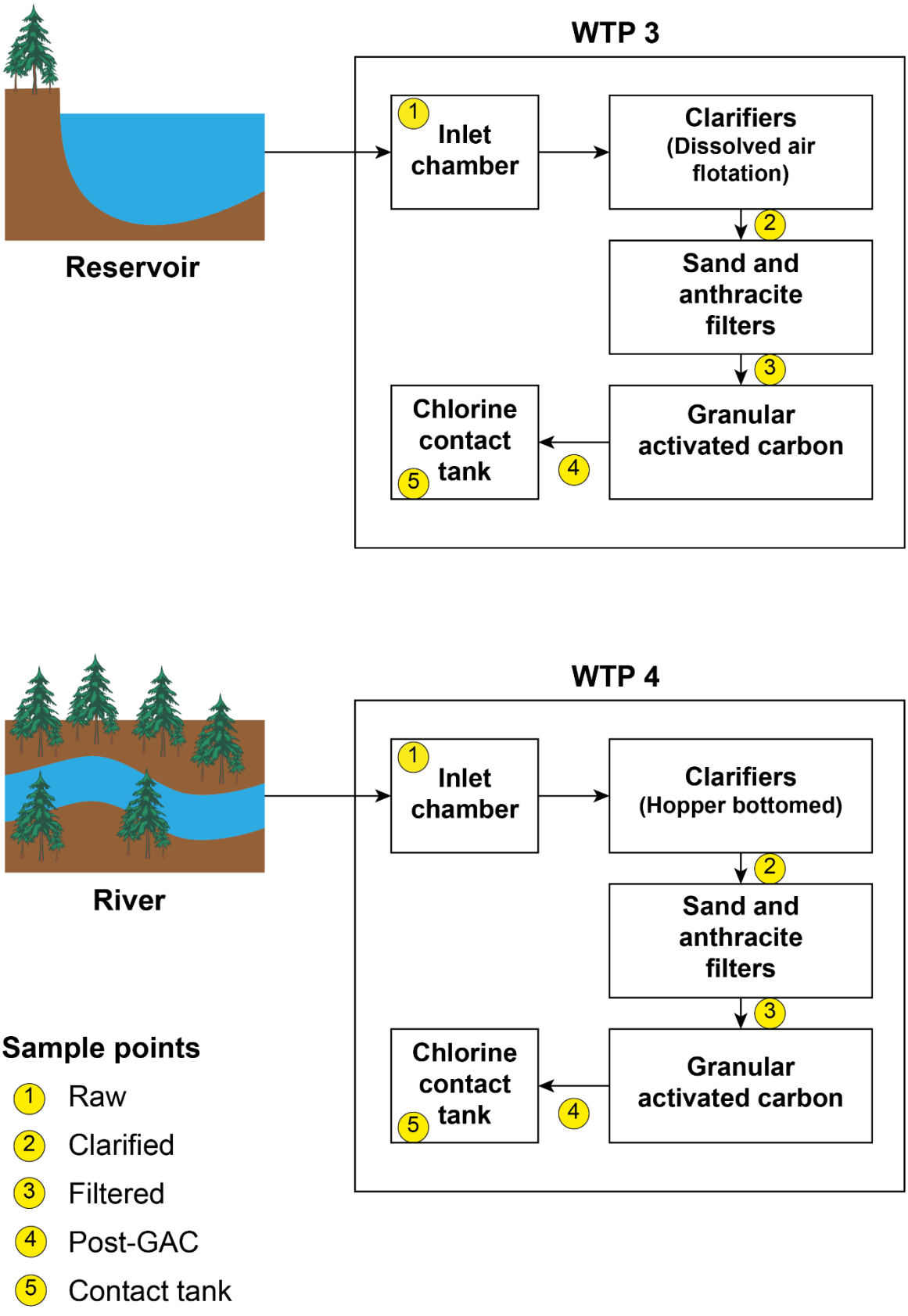


Figure 4.2 – *Overview schematic diagrams of WTP 3 and 4. Locations of biofilm collectors are marked in yellow. Bulk water samples were collected from sample taps as close as possible to the biofilm collector locations.*

4.2.3 Collection of biofilm and bulk water samples

The sampling program was performed between August 2016 and April 2017, and included both biofilm and bulk water sampling. Bulk water samples were collected from WTP sample taps at the following treatment stages: raw water, post-clarification, post-filtration, post-GAC and post-contact tank. Volumes of 500 ml were collected from the raw and clarification stages, 1 L was collected from the filtration and post-GAC stages and 2-3 L was collected from the post-contact tank stage. All samples were collected in sterile Nalgene plastic bottles (VWR, Leicestershire, UK).

Biofilm collectors (see Section 4.2.1) were submerged in the water stream for 1 month prior to first sampling. After 1 month, the collectors were retrieved and 3 slides were selected at random from each collector at each treatment stage. The slides were placed into 50 ml Falcon tubes (VWR, Leicestershire, UK) containing 40ml sterile phosphate buffered saline (PBS; VWR, Leicestershire, UK). The position of the slides in the slider holder was noted, then fresh slides were placed in the holder and the collector re-submerged in the water stream. Biofilm and bulk water samples were transported in a cool box, stored at 4°C and processed (see below) within 30 hours of collection.

Sample collection was repeated as described above at 2, 3 and 6 months. Due to the collector containing only 9 slides, the 6 month sample slides were placed in the collector after previous month samples were collected, meaning the study was actually carried out over eight months (see Table 4.1).

Table 4.1 – *Biofilm samples were collected from WTP 3 and 4 over eight months between August 2016 and April 2017. 1, 2, 3 and 6 month-old biofilms were obtained.*

WTP	Age of biofilm samples	Time period
3	1 month	August – September 2016
	2 months	August – October 2016
	3 months	August – November 2016
	6 months	October 2016 – April 2017
4	1 month	September – October 2016
	2 months	September – November 2016
	3 months	September – December 2016
	6 months	October 2016 – April 2017

4.2.4 Membrane filtration of samples

Biofilm growth was removed from a defined surface area on the glass slides by scraping with a sterile nylon brush (VWR, Leicestershire, UK) into the 50 ml Falcon collection tube containing 40 ml sterile PBS. The resulting suspension was filtered by vacuum-pump membrane filtration through 0.2 µm-pore cellulose nitrate membranes (VWR, Leicestershire, UK). Sterile forceps were used to fold the membrane and transfer into a 2 ml sterile Eppendorf tube (VWR, Leicestershire, UK). Membrane samples were stored at -20°C.

Water samples were filtered through sterile 0.2 µm-pore cellulose nitrate membranes (VWR, Leicestershire, UK). Sterile forceps were used to fold and

transfer the membranes into 2 ml Eppendorf tubes (VWR, Leicestershire, UK) which were stored at -20°C for further analysis. Due to the varying concentration of bacteria (and other particulate matter) in water samples, different volumes of water were filtered for different treatment stages; the volumes of water filtered are shown in Table 4.2.

Table 4.2 – *The volume of bulk water filtered for each treatment stage sample.*

Treatment stage	Volume filtered (ml)
Raw	500
Post-clarification	500
Post-filtration	1000
Post-GAC	1000
Post-contact tank	2000

4.2.5 Extraction of DNA

DNA extraction was carried out using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). All materials and solutions were sterile. Membrane filters were thawed from -20°C and transferred with sterile forceps into Lysing Matrix E tubes (MP Biomedicals, Santa Ana, CA, USA). Sodium Phosphate Buffer (978µl) and 122 µl of MT Buffer (components of the FastDNA Spin Kit for Soil) were added to each Lysing Matrix E tube containing filter samples. All samples were then placed in the FastPrep-24 Instrument and homogenized for 40 seconds at speed setting 6.5. The samples were then rested at 4°C for 5 min. The cycle of 40 seconds homogenization followed by 5 min rest was repeated a further two times. The protocol of the FastDNA Spin Kit for Soil was then followed according to manufacturer's instructions. Extracted DNA samples were stored at -20°C.

4.2.6 Microbial culturing

Plate count data was kindly provided by the WTP operators and was obtained using the following methods.

Membrane lactose glucuronide agar (MLGA) was produced in order to selectively culture coliforms and *E. coli* and was prepared according to manufacturer's instructions. Water samples (100 ml) were filtered by vacuum-pump membrane filtration through 0.45 µm nitrocellulose membrane filters (Sartorius, Goettingen, Germany). Membranes were transferred with sterile forceps onto a MLGA plate then incubated at 30°C for 4 hrs, followed by 14 hrs at 37°C. The total number of water samples taken over the study period at WTP 3 and 4 was 565 and 1296, respectively. Samples were taken every working day (Monday-Friday), where possible.

4.2.7 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA gene and create gene products suitable for use as standards in quantitative PCR (qPCR). Forward primer 331bF: 5'–TCCTACGGGAGGCAGCAGT–3' and reverse primer 797R: 5'–GGACTACCAGGGTATCTAATCCTGTT–3', designed by Nadkarni *et al.* (2002) to amplify 16S rDNA from a broad range of bacteria, were used (produced by ThermoFisher Scientific; Waltham, MA, USA). This set of primers amplifies the 466 bp region between residues 331 and 797 on the *E. coli* 16S rRNA gene. FastStart Taq DNA Polymerase dNTPack (Sigma-Aldrich, Dorset, UK) reagents were used; they consisted of a thermostable Taq DNA polymerase, PCR reaction buffer and a PCR nucleotide mix (dNTPs). Reactions were carried out in 50 µl volumes containing 1 µl dNTP mix, 5 µl PCR reaction buffer, 0.5 µl Taq DNA polymerase, 40.5 µl UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher Scientific, Waltham, MA, USA), 1 µl forward primer, 1 µl reverse primer and 1 µl template DNA. *E. coli* MG1655 was used as the template DNA and was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions. PCR reaction conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Reaction products were stored at -20 °C.

4.2.8 Agarose gel electrophoresis

PCR products were visualised using agarose gel electrophoresis. Agarose (1.5%) gels were prepared by melting 1.5 g agarose (Sigma-Aldrich, Dorset, UK) in 100

ml 1 x TAE buffer (Sigma-Aldrich, Dorset, UK) and stained with 20 µl Nancy-520 DNA Gel Stain (Sigma-Aldrich, Dorset, UK). Gels were loaded with 5 µl of DNA reference marker (GeneRuler 50bp DNA Ladder; ThermoFisher Scientific, Waltham, MA, USA) in the first well. DNA Gel Loading Dye (2 µl; ThermoFisher Scientific, Waltham, MA, USA) was added to 5 µl of each PCR product, mixed thoroughly and loaded into the gel. Agarose gels were run at 100 V for 1hr.

Gels were visualized under UV illumination using the E-Gel Imager (ThermoFisher Scientific, Waltham, MA, USA).

4.2.9 2nd PCR and purification of DNA products

Successfully amplified PCR products (as determined by agarose gel electrophoresis) were diluted 1 in 100 with UltraPure DNase/RNase-Free Distilled Water. A second PCR was carried out using the reaction mix and conditions detailed in section 4.2.5, with the diluted PCR product used as template DNA instead of *E. coli* MG1655. Resulting samples then were visualised by agarose gel electrophoresis as described in the previous section.

PCR products were purified using the Agencourt AMPure XP PCR Purification system (Beckman Coulter, High Wycombe, UK) according to the manufacturer's instructions.

4.2.10 Quantification of DNA and preparation of qPCR standards

DNA was quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. To be used as standards in the qPCR assay, purified 16S rRNA gene products needed to be diluted to create a dilution series of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies per µl. Copies/µl were calculated according to Equation 4.1 and 4.2. For Equation 4.2, fragment length was equal to 466 bp and the weight of bp was 1.02×10^{-21} g/molecule.

Equation 4.1 – Calculation used to determine copies per µl in DNA samples.

$$Copies\ per\ \mu l = \frac{Concentration\ (g/\mu l)}{Total\ weight\ of\ fragment\ (g/molecule)}$$

Equation 4.2 – Calculation used to determine total weight of 16S rRNA gene fragment.

$$Total\ weight\ of\ fragment = bp\ weight\ (g/molecule)\ \times\ fragment\ length\ (bp)$$

Once copies/µl had been determined, samples were diluted with UltraPure DNase/RNase-Free Distilled Water to create a dilution series of the 16S rRNA gene from 10^8 to 10^2 copies/µl. These samples were used in the qPCR to generate a standard curve for gene abundance, allowing quantification of 16S rRNA gene abundances in the WTP samples.

The standard curve generated by the primers had an efficiency of 95.7%, r^2 value of 0.99 and a *y*-intercept value of 36.71. The efficiency was considered acceptable (above 90%) and the low y-intercept value indicated the primers had good sensitivity (Smith *et al.*, 2006). The cycle threshold point (Ct) of the no template control was 33.67 ± 1.02, while samples containing 10^2 copies/µl had a Ct of 30.43 ± 0.99. Any samples with Ct value at or above the no template control were removed from the data set as the results could not be distinguished from the background fluorescence of the control.

4.2.11 Quantitative PCR of 16S rRNA gene

qPCR of the 16S rRNA gene was carried out on all biofilm and bulk water samples collected from WTP 3 and 4 at 1, 2, 3, and 6 month time periods. The assay was carried out in 96-well plates (Bio-Rad, Hertfordshire, UK) and all samples and standards were tested in triplicate. Each well contained 0.5 µl forward primer, 0.5 µl reverse primer, 2 µl DNA sample or DNA standard, 2 µl UltraPure DNase/RNase-Free Distilled Water and 5 µl SsoFast EvaGreen Supermix (Bio-Rad, Hertfordshire, UK). A negative control of UltraPure DNase/RNase-Free Distilled Water was tested in triplicate on each plate. Microseal B PCR Plate Sealing Film (Bio-Rad, Hertfordshire, UK) was used to seal the plate after loading of samples. qPCR was carried out using a CFX96 Real-Time System thermal cycler (Bio-Rad, Hertfordshire, UK). The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

4.2.12 Data analysis and visualisation

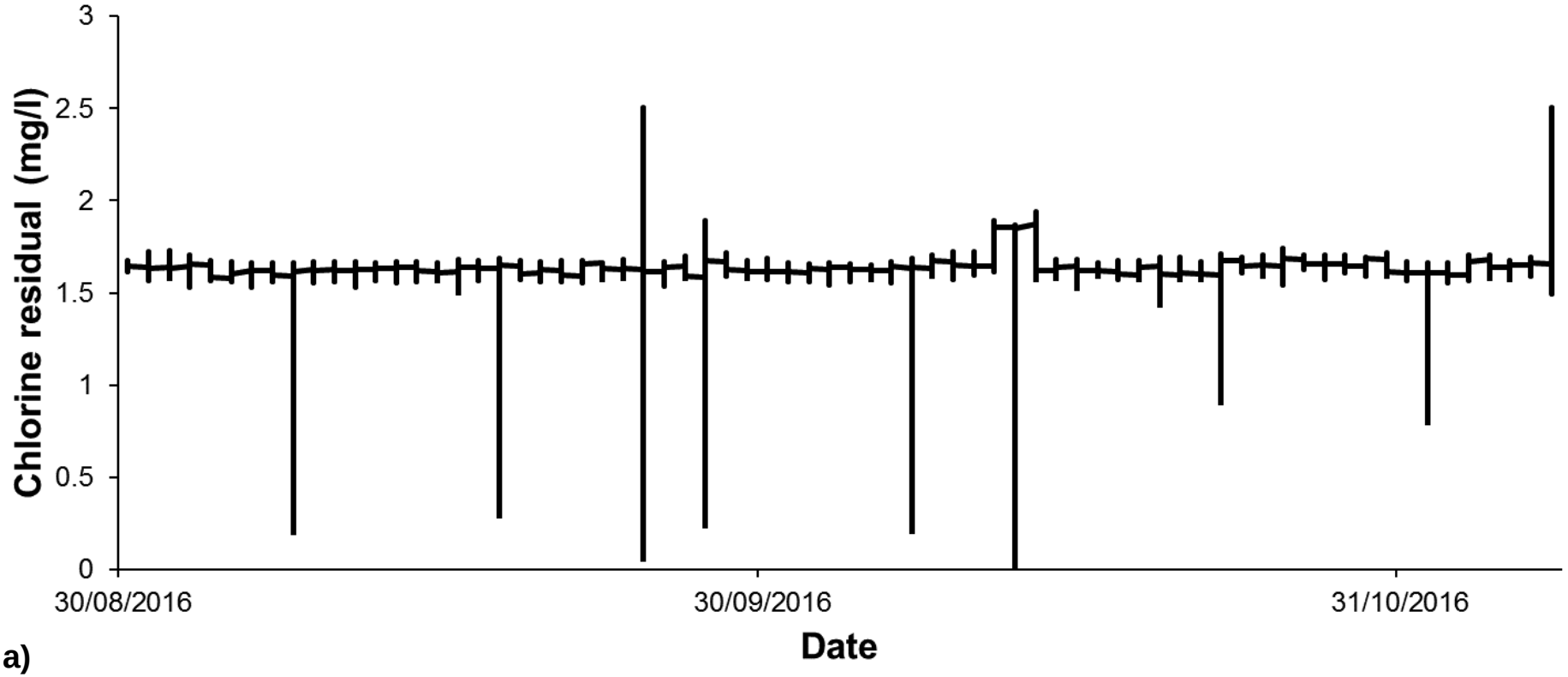
All statistical analysis (including ANOVA, Tukey's HSD and independent t-tests) was performed in Microsoft Excel and R Studio. Graphs were generated in Microsoft Excel and edited using Adobe Illustrator. Unless otherwise noted, statistical significance was defined as 95% confidence in observed differences ($p < 0.05$).

4.3 Results and Discussion

4.3.1 Quantification of 16S rRNA genes in biofilms over 6 month's development

Biofilms were successfully grown and harvested from collectors placed at the first four stages of treatment; however, no consequential biofilm could be recovered from collectors placed in the contact tank in either WTP. The amount of available DNA in the contact tank samples was always below the level of detection (i.e. <20 ng/ml), even after 6 months. This suggests that chlorine concentration, residence time and mixing conditions in the contact tanks of both WTPs were sufficient to prevent the establishment and growth of bacterial biofilms.

The real-time residual chlorine concentrations in each contact tank are shown in Figure 4.3, which spans the entire sampling period. These data were collected by on line telemetry at the WTPs and kindly provided to the project, although due to access issues, there are a few gaps in the data communication; most notably at WTP 3, where data is only shown for the first three and half months of the study's duration. The mean residual chlorine concentration in the contact tank in WTP 3 and 4 were 1.62 ± 0.15 mg/l and 1.83 ± 0.10 mg/l, respectively, which are typical of chlorination systems operated in the UK.


Chlorine residual (mg/l)
3
2.5
2
1.5
1
0.5
0
30/08/2016
30/09/2016
31/10/2016
Date

a)

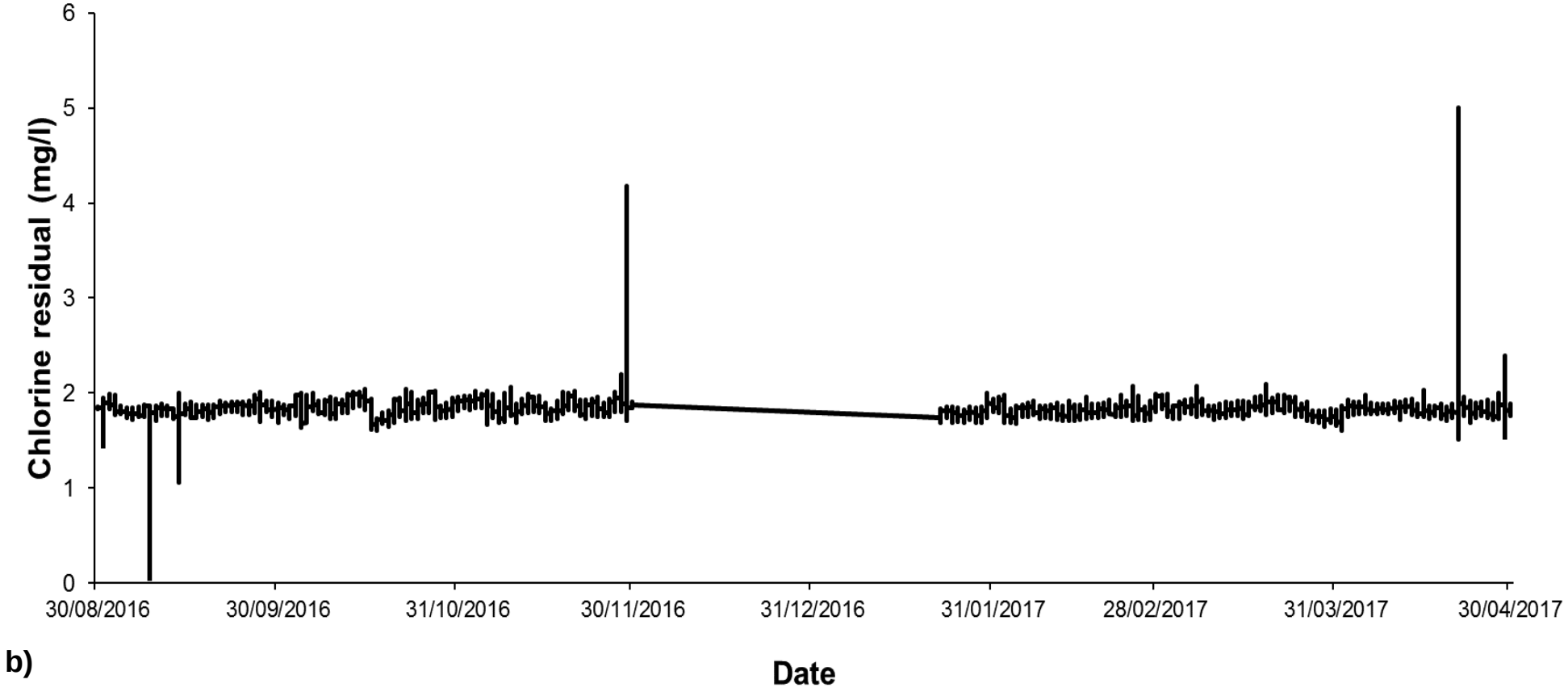


Figure 4.3 – *The residual chlorine concentration in the contact tank of **a)** WTP 3 between 30/08/2016 and 07/11/2016 and **b)** WTP 4 between 30/08/2016 and 30/04/2017. A chlorine concentration measurement was recorded every minute of every day in the sampling period shown.*

Total bacteria (calculated as the number of copies of the 16S rRNA gene per unit volume) in biofilms at each treatment stage of WTP 3 and 4 are summarised in Figure 4.4. At most of the treatment stages in both WTPs, the total bacterial abundances in biofilm samples was fairly consistent over time. However, one-way ANOVA analysis within each treatment stage showed that the difference between the quantities of 16S rRNA gene between monthly samples at the majority of stages was statistically significant ($p<0.04$, see Figure 4.4). The quantity of 16S rRNA genes dropped noticeably in each successive sampling after clarification in WTP 3. Given the flow rate through the clarifier remained relatively constant throughout the study period (see Table 4.3), reductions in total bacteria in biofilms at this stage are not likely due to changes in shear (i.e., aggregates of bacteria being released from the biofilm due to pressure from the water flow). Considering this, a change or reduction in the availability of nutrients and/or organic carbon is a more likely explanation (Chandy and Angles, 2001). Unfortunately, no measurements of carbon, phosphorus, nitrogen or other nutrients could be taken at the time of sampling, therefore this is speculation. There is also a large reduction in the number of 16S rRNA genes at WTP 3 post-GAC stage at 3 months. The quantity of 16S rRNA genes at this stage is significantly lower ($p<0.01$) in the 3 month samples than samples collected at all other times.

Overall, the data suggest that one month is sufficient time for detectable biofilms to establish under WTP conditions. The mean total biofilm bacteria (log copies of the 16S rRNA gene per cm^2 biofilm) over the study time period at WTP 3 were 0.324 ± 0.02, 0.258 ± 0.06, 0.240 ± 0.02 and 0.222 ± 0.05 at the raw, clarified, filtered and post-GAC treatment stages, respectively, which indicate bacterial abundances in biofilms steadily decrease with treatment stage. One-way ANOVA analysis reveals a significant difference ($p <0.001$) between quantity of 16S rRNA genes over treatment stage, although Tukey's HSD (honest significant difference) test indicates the number of total biofilm bacteria after filtration is not significantly different to clarified or post-GAC biofilms. However, total bacteria quantities at all other stages are significantly different from each other ($p < 0.01$).

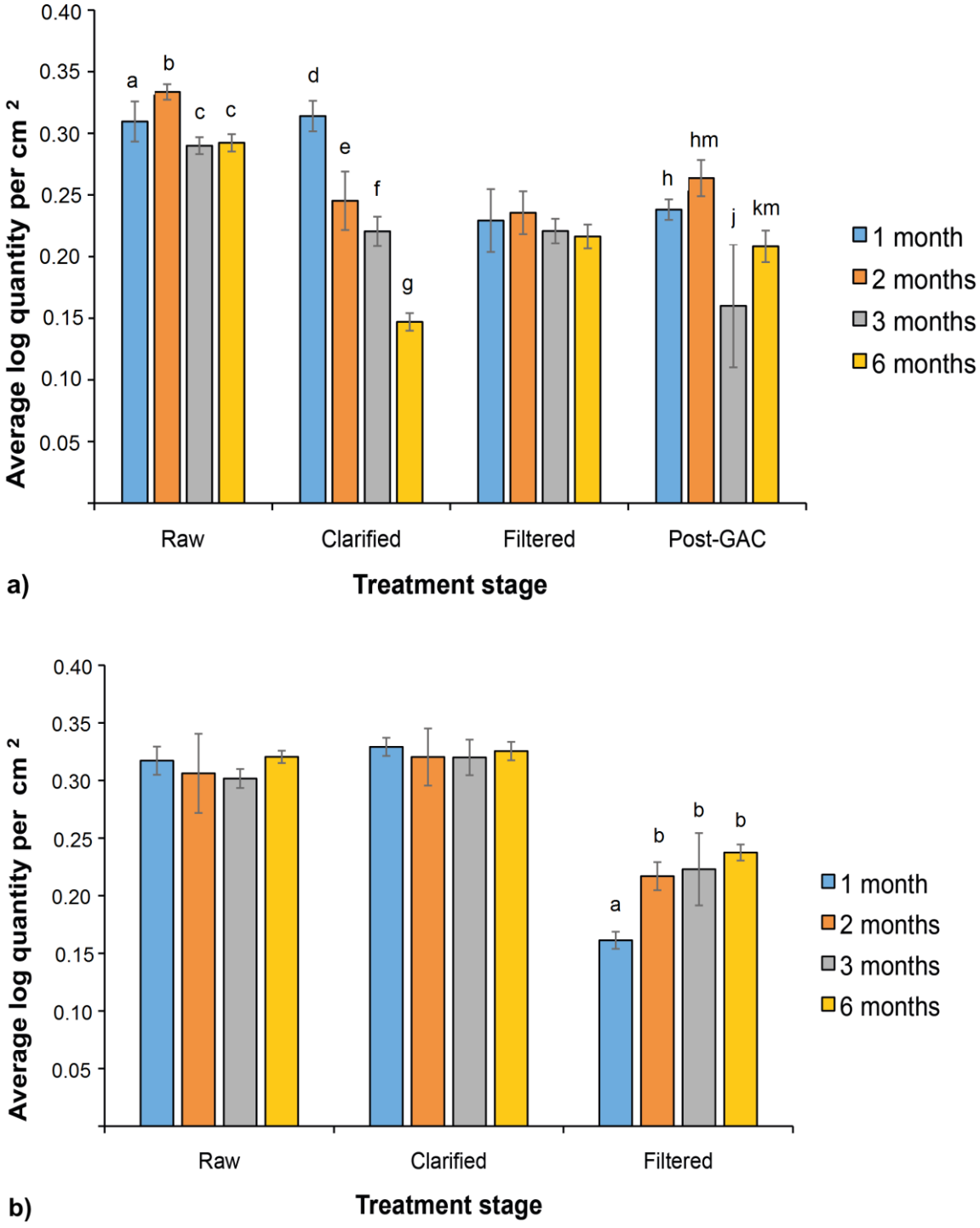


Figure 4.4 – *Total bacteria over time and sampling stage presented as log copies of 16S rRNA gene per cm² of biofilm surface for **a)** WTP 3 (reservoir source) and **b)** WTP 4 (river source). Values represent the mean values of triplicate qPCR runs of triplicate samples for each treatment stage. Standard error bars are shown. Means with different letters are significantly different (Tukey's HSD, p<0.04, compared within treatment stage only)*.

Table 4.3 – *Mean flow rate and, where applicable or available, turbidity, chlorine concentration, pH, concentration of dissolved organics and temperature at WTP 3 (30/08/2016 – 07/11/2016, data for the remaining time period was not available) and WTP 4 (30/08/2016 – 30/04/2017). *The low flow rate seen here is due to the water stream being split into four separate groups of clarifiers, this flow rate representing the amount of water passing through one group of hopper bottomed clarifiers.*

WTP	Treatment stage	Flow (Ml/day)	Turbidity (NTU)	Chlorine (mg/l)	pH	Dissolved organics (mg/l)	Temperature (°C)
3	Raw	208 ± 27	2.92 ± 2.69	-			
	Clarified	147 ± 21	1.69 ± 2.18	-			
	Filtered	149 ± 24	0.11 ± 0.03	-			
	Post-GAC	186 ± 30	0.06 ± 0.03	1.85 ± 0.19	7.19 ± 0.17		10.1 ± 2.3
	Contact tank	53 ± 33	0.11 ± 0.45	1.62 ± 0.15	7.56 ± 0.34		10.1 ± 3.2
4	Raw	147 ± 10	17.5 ± 29.3	-	7.97 ± 0.23	13.8 ± 11.9	
	Clarified	19 ± 2*	0.44 ± 0.43	-			
	Filtered	146 ± 14	0.06 ± 0.06	-		5.70 ± 1.33	
	Post-GAC	142 ± 17	0.05 ± 0.08	2.05 ± 0.15	6.89 ± 0.16		10.7 ± 3.9
	Contact tank		0.06 ± 0.07	1.83 ± 0.10	7.53 ± 0.13		9.3 ± 4.1

At WTP 4, mean total biofilm bacteria abundances (log copies of the 16S rRNA gene per cm^2 biofilm) over the study time period were 0.334 ± 0.02, 0.345 ± 0.01 and 0.224 ± 0.04 at the raw, clarified and filtered treatment stages, respectively. There appears to be a small increase in 16S rRNA gene numbers in the biofilm from raw to clarified treatment stages before a larger reduction at the filtered stage. One-way ANOVA and Tukey's HSD test reveal a significant difference between 16S rRNA gene abundance at the filtered treatment stage to levels seen at the raw and clarified stages ($p<0.001$), whereas there was no significant difference in abundances between raw and clarified biofilm samples.

Interestingly, the quantity of 16S rRNA genes at the filtered stage increased over time, which was not observed in the raw and clarified biofilms (although the difference was only statistically significant comparing the 1 month biofilm, $p<0.001$). This could be due to lower concentrations and availability of organic carbon in water after filtration, which might lead to slower growth of bacterial biofilms at this stage. It is also possible the biofilm collector was in a more sheltered position from the water flow compared to previous stages, reducing the relative exposure to shear and allowing greater biofilm mass to accumulate over time. This is speculation because in no case was it possible to visualise the actual placement of the samplers, although most of the bacterial data from samplers are similar at each stage with time, suggesting their placement probably was consistent.

Metadata surrounding sampling of the biofilms, which could affect biofilm growth and development, were obtained from the WTP operators and is summarised in Table 4.3. Mean flow rate, turbidity, pH, temperature, chlorine concentration and concentration of dissolved organics was measured or calculated for each treatment stage, where possible.

Flow rate throughout both WTPs was fairly consistent over the study period, which is likely a contributing factor to the stability of total bacterial numbers in biofilm samples over time: i.e., consistent shear conditions would create pseudo-stable biofilm mass and depth, rather than varying due to fluctuations in flow rate. In contrast, water turbidity was highly variable at all treatment stages at both WTPs, with the greatest ranges seen in raw water at WTP 4 (see Figure 4.5).

Since WTP 4 receives raw water from a river source, higher variability in turbidity as compared to WTP 3 would be expected.

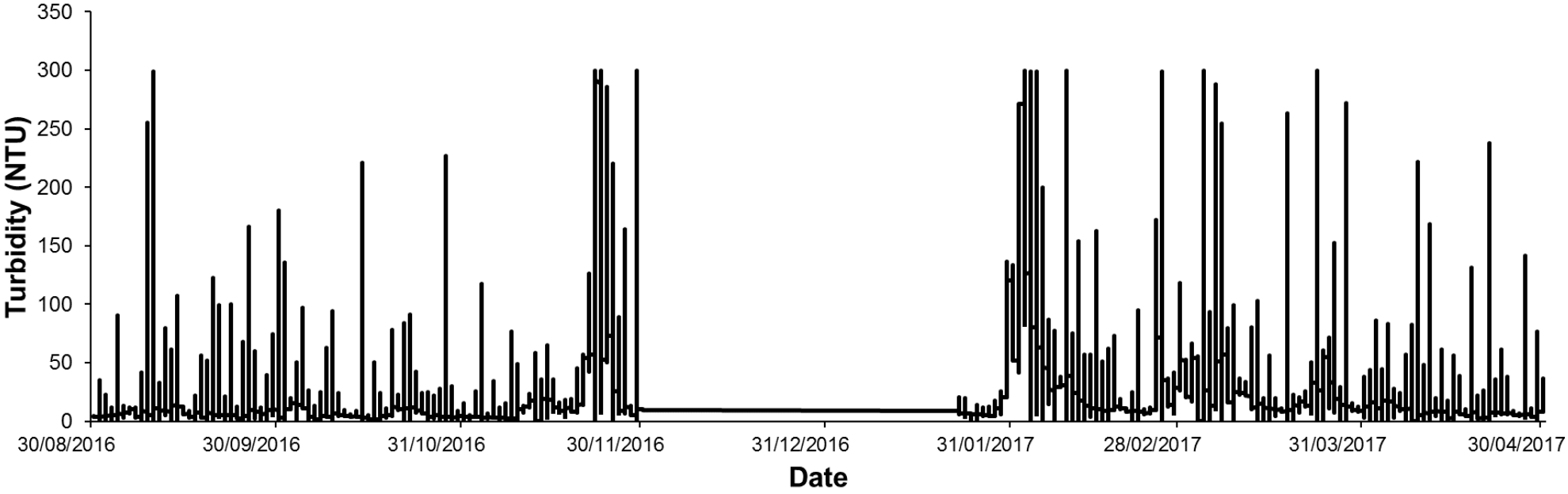


Figure 4.5 - *Turbidity of raw water entering WTP 4 over the study time period. Turbidity measurements were taken every minute, every day. The mean turbidity was 17.5 ± 29.3 with maximum and minimum values ranging from below detection to 299 NTU.*

4.3.2 16S rRNA gene quantity in bulk water over eight months sampling

Bulk water samples were collected from WTP 3 and 4 on the same day that biofilm samples were collected. Unlike the biofilm samples, which represent the total bacteria accumulated over each time period from one to six months, the bulk water samples are a measurement of bacterial levels on the specific sampling day, which is used as reflective of that period. A better representation would have required more frequent sampling for total bacteria (as measured by 16S qPCR) throughout each month. However, physical logistics limited in depth sampling to once a month. Plate counts of specific bacterial groups (coliforms, *E. coli* and Gram(-) non-coliforms) using traditional plating methods (see section 4.2.5; n = 565 at WTP 3, n = 1296 at WTP 4) were produced and data are summarised in Tables 4.4 and 4.5. Total colony forming units (CFU) of Gram(-) bacteria (coliforms and non-coliforms) obtained from culture-based plate counts were plotted against total bacterial abundance (represented by the log number of copies of the 16S rRNA gene obtained from qPCR analysis) and is displayed in Figure 4.6. Results from the culture and non-culture based methodologies correlated well, with Pearson correlation coefficient (r) values of 0.847 and 0.892 for WTP 3 and 4, respectively.

Table 4.4 – *Mean colony forming units (CFUs) of coliforms, E. coli and Gram(-) non-coliforms at WTP 3 (with standard errors). BDL = below detection level (<1 CFU per 1 L).*

Month	Bacteria group	Colony forming units (CFU) per 1L				
		Raw	Clarified	Filtered	Post-GAC	Contact tank
September 2016	Coliforms	1640 ± 848	493 ± 217	145 ± 62	10 ±12	BDL
	E. coli	864 ± 493	299 ± 128	90 ± 47	8 ± 10	BDL
	Non-coliforms	87600 ± 16100	46300 ± 17100	9570 ± 759	805 ± 225	BDL
October 2016	Coliforms	4480 ± 3930	33 ± 75	2 ± 4	BDL	BDL
	E. coli	1440 ± 459	9 ± 14	1 ± 3	BDL	BDL
	Non-coliforms	25300 ± 24900	115 ± 265	6750 ± 15900	735 ± 375	BDL
November 2016	Coliforms	7110 ± 5030	1070 ±1310	240 ± 291	3 ± 5	BDL
	E. coli	2620 ± 886	433 ± 572	100 ± 121	BDL	BDL
	Non-coliforms	16900 ± 14900	2850 ± 5790	1023 ± 1300	68 ± 13	BDL
April 2017	Coliforms	982 ± 917	33 ± 116	26 ± 48	7 ± 12	BDL
	E. coli	351 ± 348	21 ± 77	5 ± 18	BDL	BDL
	Non-coliforms	12500 ± 25200	40 ±136	117 ± 135	3 ±5	BDL

Table 4.5 - *Mean colony forming units (CFUs) of coliforms, E.coli and Gram(-) non-coliforms at WTP 4. Numbers in brackets represent standard error. BDL = below detection level (<1 CFU per 1 L).*

Month	Bacteria group	Colony forming units (CFU) per 1L				
		Raw	Clarified	Filtered	Post-GAC	Contact tank
October 2016	Coliforms	54100 ± 48100	932 ± 700	308 ± 177	81 ± 56	BDL
	E. coli	10300 ± 17100	137 ± 135	36 ± 33	8 ± 10	BDL
	Non-coliforms	60500 ± 37000	921 ± 329	363 ± 287	132 ± 90	BDL
November 2016	Coliforms	116000 ± 157000	1410 ± 2070	761 ± 930	285 ± 285	BDL
	E. coli	46100 ± 96600	419 ± 856	175 ± 291	52 ± 96	BDL
	Non-coliforms	99000 ± 122000	949 ± 649	598 ± 645	266 ± 289	BDL
December 2016	Coliforms	45300 ± 18900	488 ± 324	159 ± 148	55 ± 46	BDL
	E. coli	7820 ± 5630	89 ± 65	22 ± 24	4 ± 6	BDL
	Non-coliforms	48300 ± 32400	531 ± 350	187 ± 244	82 ± 95	BDL
April 2017	Coliforms	34100 ± 22100	357 ± 191	169 ± 67	41 ± 31	BDL
	E. coli	3250 ± 1770	21 ± 14	12 ± 10	3 ± 6	BDL
	Non-coliforms	50600 ± 31200	649 ± 311	218 ± 147	43 ± 46	BDL

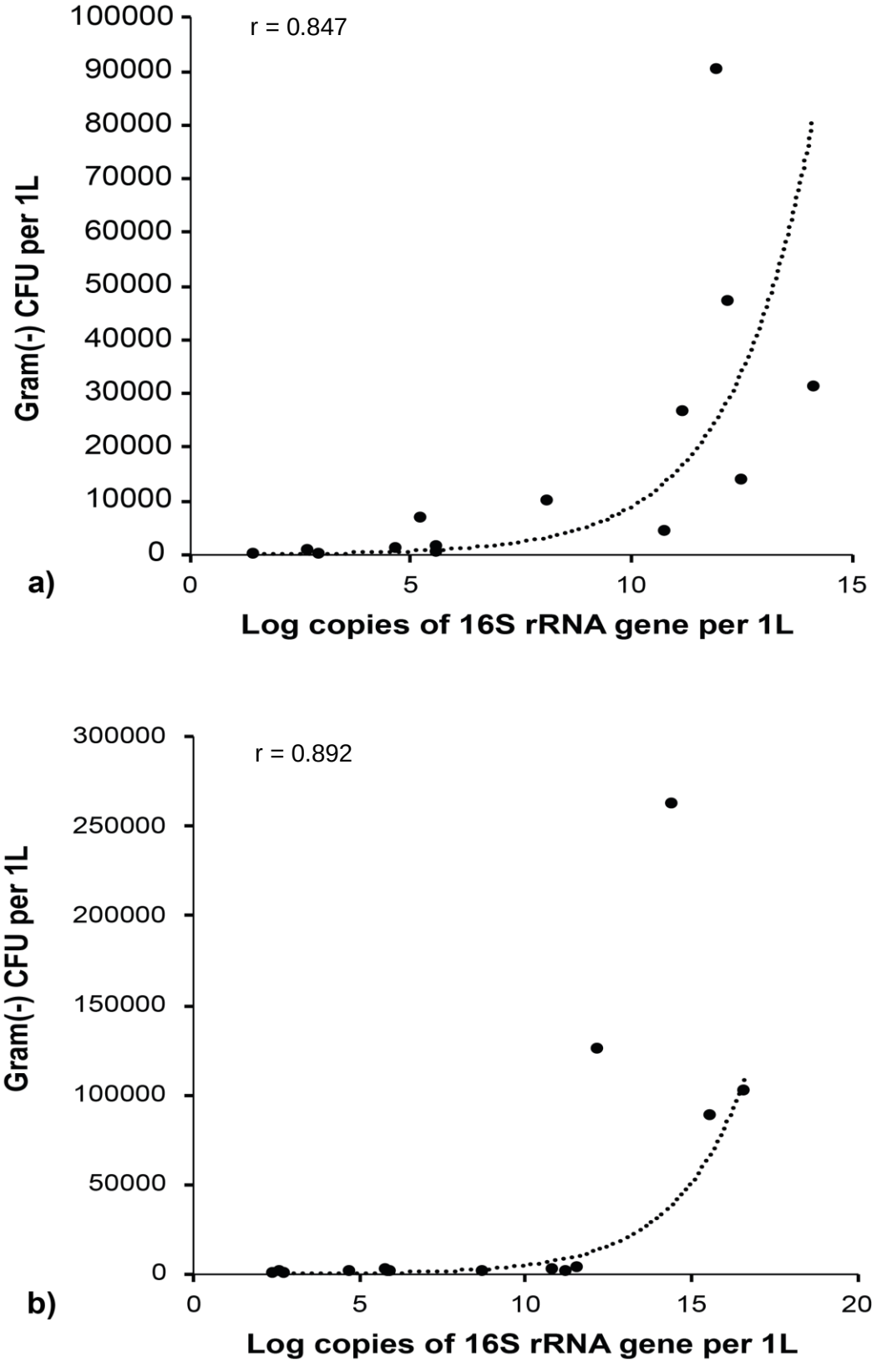


Figure 4.6 – *Total Gram(-) bacteria abundance (as measured by plate counts) was positively correlated to total bacteria abundance (as measured by qPCR) at* ***a)*** *WTP 3 and* ***b)*** *WTP 4. Pearson correlation coefficient (r) values are shown.*

Total bacteria (as estimated from 16S rRNA gene copy data) in bulk water over the four sampling time points is shown in Figure 4.7. At both WTPs, 16S rRNA gene numbers decreased with advancing treatment stage, with the greatest reduction (prior to disinfection) seen after filtration. One-way ANOVA analysis within treatment stage showed that there were some statistically significant differences in the quantity of 16S rRNA genes in samples collected in different months (see Figure 4.7). However, these differences were not consistent and the time of year did not clearly correlate with an increase or decrease in 16S rRNA gene numbers across all treatment stages. For example, the highest total bacterial concentrations entering WTP 3 were in October samples (14.1 log copies of the 16S rRNA gene per L), which is between lower levels in September (11.9 log copies/L) and November (11.2 log copies/L) samples. Total bacteria in April (12.5 log copies) was within the same range as September to November samples.

In contrast, 16S rRNA gene numbers at the WTP 4 intake increased by about 2.2 log/L with each successive month from October to December. The quantity observed in April (15.6 log copies/L) was similar to December (16.6 log copies/L). Results from WTP 4 suggest total bacteria entering the works (i.e. bacteria contained in raw water) may increase over the winter months, however, this was not seen at WTP 3. Interestingly, an increase in coliforms and *E. coli* entering the works over the winter months was observed at WTP 3 (see Table 4.4). Overall, these data are to be considered with caution, especially the genetic estimates, because sampling frequency was limited. The increase in coliform bacterial counts over the winter supports previous studies on the effect of season on indicator bacteria (Van Donsel *et al.*, 1967; Hirn *et al.*, 1980; Carter *et al.*, 1987).

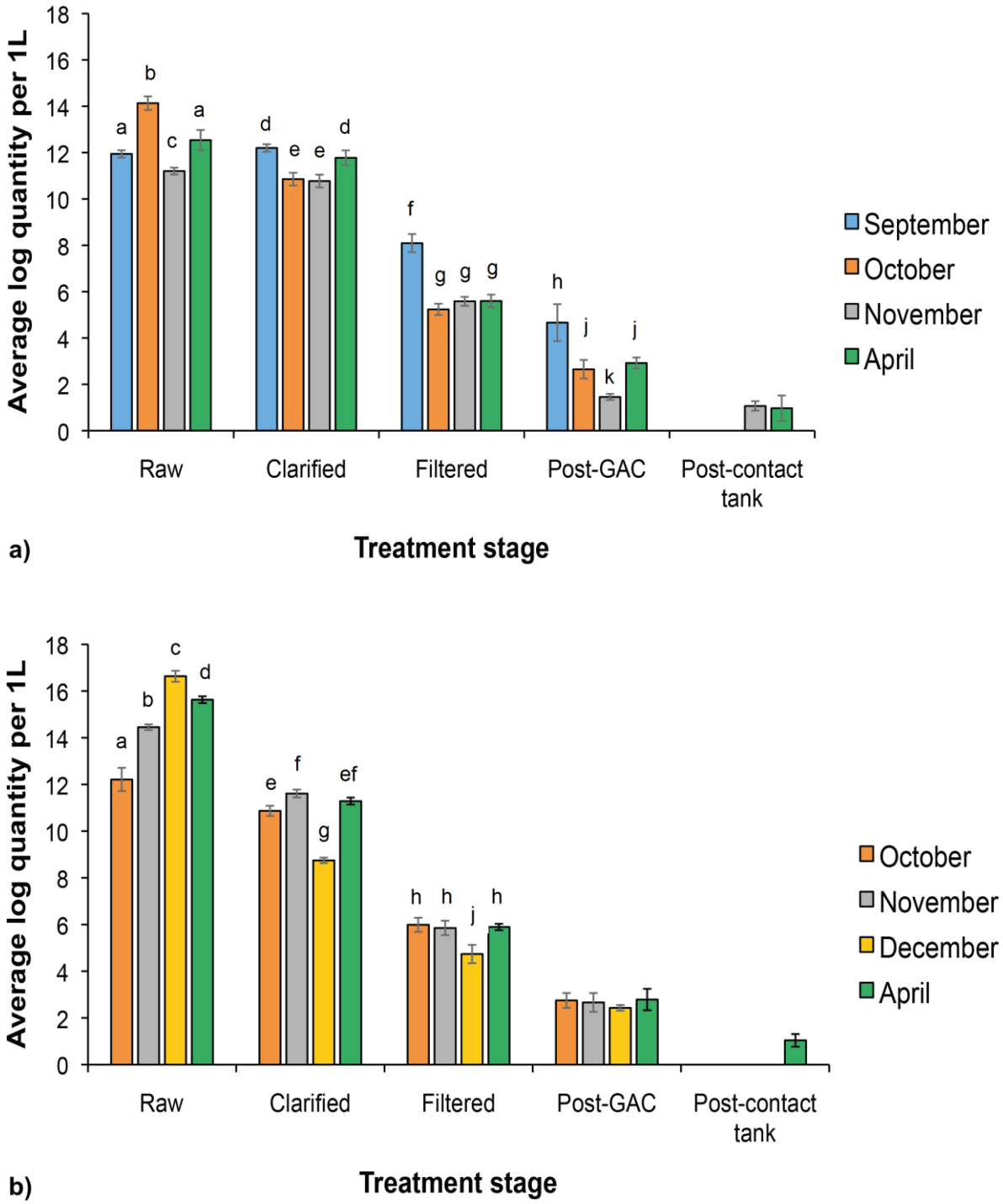


Figure 4.7 - *Total bacteria, represented as log copies of the 16S rRNA gene per 1L of water, at **a)** WTP 3 (reservoir source) and **b)** WTP 4 (river source). Values represent the mean average of triplicate qPCR results of triplicate samples for each treatment stage and standard error bars are shown. Means with different letters are significantly different (Tukey's HSD, p<0.05, compared within treatment stage only)*.

4.3.3 Comparison of 16S rRNA gene quantities between source water types

To assess whether the water source (i.e. reservoir or river) impacted the number of total bacteria passing through and/or becoming established in biofilms in the WTPs in this study, Tables 4.6 and 4.7 were compiled. Table 4.6 compares 16S rRNA gene qPCR results over time in biofilm samples between WTPs 3 and 4. Table 4.7 compares 16S rRNA gene qPCR results between WTPs 3 and 4 in the bulk water for the three months where sampling overlapped (i.e. October, November and April).

In biofilm samples, a significant difference was found in total bacteria between reservoir and river sources (ANOVA, $p < 0.05$). In general, WTP 4 (river) biofilms contained higher numbers of total bacteria per surface area than WTP 3 (reservoir) for all biofilm samples, although there were three exceptions to this finding. Two-way ANOVA showed a significance level of $p < 0.05$ in total bacteria abundance at the raw treatment stage and $p < 0.001$ at the clarified and filtered stages. Independent t-tests showed total bacteria in 1 and 2 month raw water biofilms and biofilms after 3 months after filtration were not significantly different, however, all others samples significantly differed. This pattern (i.e., source water effects) is most apparent after the water clarification stage between the two WTPs (see Figure 4.8), with differences over time being more apparent in reservoir-sourced biofilms.

Table 4.6 – *Log copies of the 16S rRNA gene per cm^2 in biofilms over 6 months sampling period at **a)** WTP 3 (reservoir) and **b)** WTP 4 (river). Values represent the mean of triplicate qPCR results of triplicate samples for each treatment stage. Standard errors are indicated.*

	1 month		2 months		3 months		6 months	
Treatment stage	**WTP 3**	**WTP 4**	**WTP 3**	**WTP 4**	**WTP 3**	**WTP 4**	**WTP 3**	**WTP 4**
Raw	0.330 ± 0.014	0.338 ± 0.013	0.356 ± 0.007	0.327 ± 0.037	0.309 ± 0.007	0.322 ± 0.009	0.312 ± 0.008	0.342 ± 0.006
Clarified	0.335 ± 0.013	0.351 ± 0.008	0.262 ± 0.052	0.342 ± 0.040	0.235 ± 0.027	0.341 ± 0.014	0.157 ± 0.049	0.347 ± 0.005
Filtered	0.245 ± 0.027	0.172 ± 0.08	0.251 ± 0.067	0.231 ± 0.041	0.235 ± 0.034	0.238 ± 0.017	0.231 ± 0.066	0.253 ± 0.005
Post-GAC	0.254 ± 0.009		0.281 ± 0.065		0.171 ± 0.038		0.222 ± 0.075	

Table 4.7 – *Log copies of the 16S rRNA gene per 1L of water in bulk water samples from October and November 2016 and April 2017 at **a)** WTP 3 (reservoir) and **b)** WTP B (river). Values represent the mean of triplicate qPCR results of triplicate samples for each treatment stage. Standard errors are shown.*

	October		November		April	
Treatment stage	**WTP 3**	**WTP 4**	**WTP 3**	**WTP 4**	**WTP 3**	**WTP 4**
Raw	14.1 ± 0.29	12.2 ± 0.50	11.2 ± 0.15	14.4 ± 0.12	12.5 ± 0.44	15.6 ± 0.15
Clarified	10.9 ± 0.28	10.9 ± 0.22	10.8 ± 0.28	11.6 ± 0.17	11.8 ± 0.32	11.3 ± 0.15
Filtered	5.23 ± 0.24	5.99 ± 0.30	5.58 ± 0.19	5.85 ± 0.31	5.60 ± 0.27	5.89 ± 0.14
Post-GAC	2.65 ± 0.40	2.75 ± 0.32	1.46 ± 0.13	2.67 ± 0.40	2.92 ± 0.24	2.79 ± 0.46
Post-contact tank			1.07 ± 0.20		0.97 ± 0.55	1.04 ± 0.27

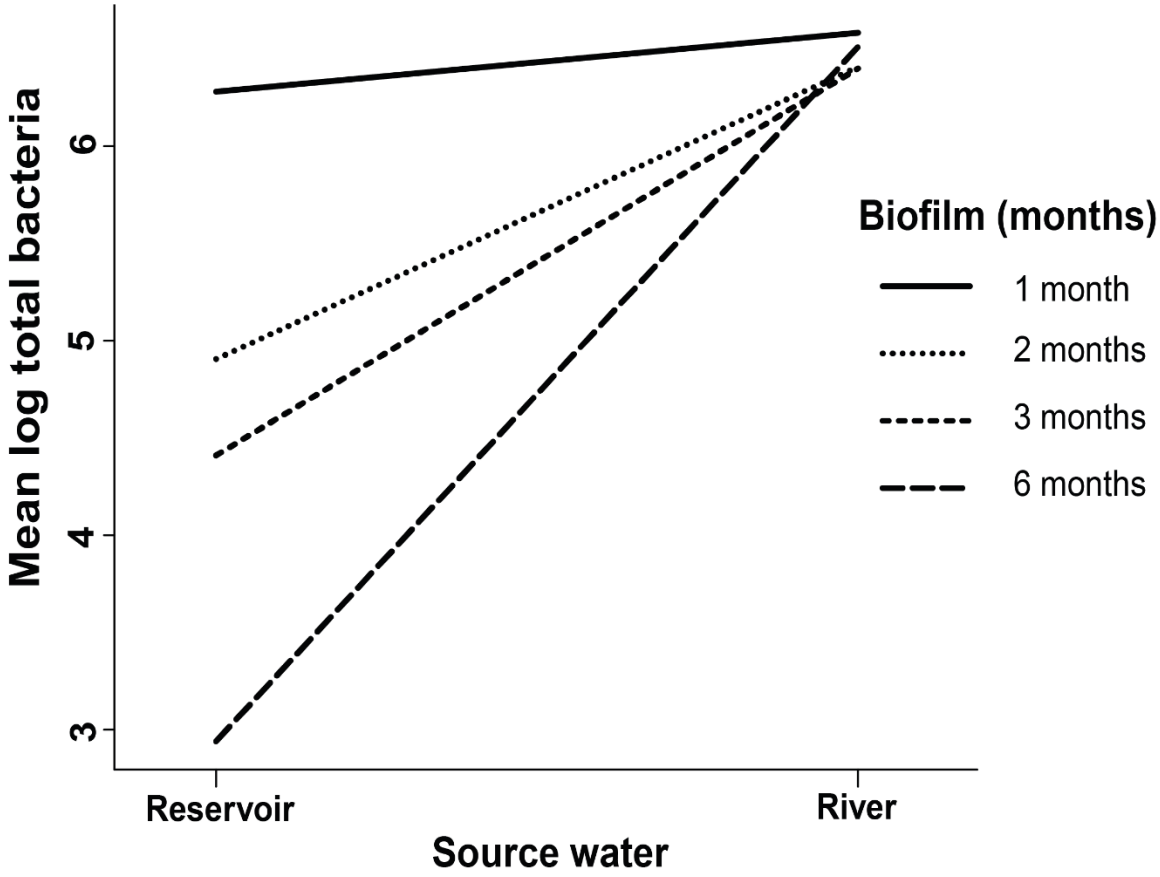


Figure 4.8 - *Interaction plot of the mean log total bacteria (number of copies of the 16S rRNA gene per 18.75 cm^2) quantified in clarified stage biofilm samples from WTP 3 (reservoir) and WTP 4 (river). River WTP biofilms contained more total bacteria than reservoir WTP biofilms.*

It was expected that higher numbers of total bacteria would be found in raw bulk water entering the treatment plant at WTP 4 compared to WTP 3, due to it being a river source. Although two-way ANOVA analysis produced a significance level of $p < 0.05$, independent t-tests showed that the total bulk water bacteria concentrations at WTP 4 were significantly higher in November ($p < 0.001$) only. No significant difference in total bacterial concentrations was found in bulk water after clarification in any month or in post-GAC bulk water in the months of October or November. Total bacteria in bulk water after filtration was significantly higher at WTP 4 ($p < 0.05$) than WTP 3 in October, November and April.

Although total bacteria was not significantly different between WTPs in half of the months and stages tested, Tables 4.4 and 4.5 show that the number of coliforms, *E. coli* and Gram(-) non-coliforms was consistently and significantly higher in

WTP 4 compared to WTP 3 at all stages in all months. This implies that river and reservoir source water WTPs may have similar quantities of total bacteria, but river source has a higher proportion of coliforms and other 'indicator' organisms than the reservoir source.

Overall, clarification and subsequent stages appear to reduce differences in total bacterial numbers between WTPs, although WTP 4 did have significantly more total bacteria after filtration than WTP 3. It is likely that factors other than source water type, such as the efficacy of the sand and anthracite filters in each WTP in removing bacteria, are responsible for this difference. The mean log removals of total bacteria by filtration at WTP 3 and 4 were 5.67 and 5.35, respectively. The slightly lower removal rate in WTP 4 combined with slightly higher initial bacteria concentrations entering the filtration stage may explain the disparity in total bacteria concentrations post-filtration.

4.3.4 Discussion of quantitative bacterial data in the context of wider literature

The majority of recent studies using qPCR to quantify microorganisms in water treatment and distribution have targeted specific groups or species, rather than total bacterial or microbial numbers. For example, the quantification of *Legionella pneumophila* from cooling towers and hot systems (Chen and Chang, 2010; Yáñez *et al.*, 2011), adenoviruses in water treatment plants (Albinana-Gimenez *et al.*, 2009) and polyomaviruses in source waters (McQuaig *et al.*, 2009). In recent studies, qPCR has also been used to provide complimentary quantitative data to qualitative sequencing or community data, however, once again, specific groups rather than total abundance is more commonly measured. For example, in their study of the microbial community of a drinking water treatment plant, Li *et al.* (2017), used qPCR to quantify *Mycobacterium* and *Legionella* at multiple treatment stages. Similarly, *Legionella*, *Mycobacterium*, *Naegleria, Acanthamoeba and Hartmanella* were quantified in an earlier study using pyrosequencing to investigate WTP community composition (Lin *et al.*, 2014). Both of these studies showed that the copy numbers of the species tested were lower with each successive treatment stage in bulk water. This finding supports the decreasing trend observed in total bacteria (as estimated by 16S rRNA gene

quantity) in WTP 3 and WTP 4 bulk water samples with advancing treatment stage (Section 4.3.2 and Figure 4.7). It is more difficult to compare total bacterial numbers (as estimated by 16S rRNA gene copies) in biofilms because species such as M*ycobacteria* and *Legionella* are particularly well-adapted for growth in water-associated biofilms (Falkinham *et al.*, 2001; van Der Kooij *et al.*, 2017) and therefore are not representative of the typical reduction in bacterial quantity.

Flow cytometry is a widely used method of quantifying total bacterial load in water treatment plants and distribution systems. Although flow cytometry uses completely different parameters (total cell counts as opposed to 16S rRNA gene copies for qPCR) and methodology to measure bacterial load, both techniques can be used to draw conclusions on the effect of treatment stage on bacterial quantity. Hammes *et al.* (2008) monitored bacterial load through a pilot WTP and observed significant regrowth after passage through the GAC filter. This observation does not support results described in Section 4.3.2 and Figure 4.7. Namely, bulk water from WTP 3 and WTP 4 did not display an increase in 16S rRNA gene numbers after the GAC filter. Findings from this study actually show a decrease in bacterial load (as estimated by 16S rRNA gene copies) in post-GAC bulk water. The difference between these findings is likely due to the difference in methodologies, however, it could also be related to the specific biological activity and microbial community of the individual GAC filters.

4.3.5 Critical review of 16S qPCR methodology

There are many methods available to monitor and quantify total bacteria in environmental samples, each with its own advantages and disadvantages. This section will briefly review the existing methods, compare the qPCR methodology selected for use in this study with other techniques and comment on its suitability for routine monitoring and process performance assessment.

Quantification methods can be divided into three main categories: culture-based (such as heterotrophic plate counts and membrane filtration combined with selective agars), epifluorescence microscopy (combined with the use of stains such as DAPI and CTC; more advanced techniques include FISH (fluorescent *in situ* hybridization)), flow cytometry and non-culture-based molecular methods (such as qPCR and multiplex PCR).

Heterotrophic plate counts (HPCs) involve the use of media such as R2A, m-HPC and yeast extract agar to culture a wide range of bacteria from water or other environmental samples. In the UK water industry, HPCs are used as a general indicator of treatment efficacy and a large change in the number of microorganisms recovered is a warning sign of potential problems in the treatment process (Sartory, 2004). HPC agar plates for water samples are usually incubated at 22°C and 37°C with an incubation time of 2-3 days required for microbial growth to develop. HPCs are simple and low-cost, however, due to the fact that less than 2% of environmental bacteria are culturable in laboratory conditions (Wade, 2002), they are also severely limited in providing a "complete" estimate of total bacterial numbers in a sample. In view of the long time for generation of results (up to 3 days versus less than 3 hours for qPCR) and the relatively rudimentary quality of data produced, HPCs were ruled out as a quantification method for this study.

In FISH, fluorescent labelled oligonucleotide probes and an epifluorescence microscope are used to identify and quantify specific microorganisms or microbial groups (Douterelo *et al.*, 2014). This method is more rapid than plate counts, however, due to the highly specific nature of the oligonucleotide probes, it seems to be more suited to analysing specific groups of bacteria rather than enumerating total bacteria in environmental samples. It is also a very laborious method in terms of staff time. A number of studies have developed and validated probes for a range of bacterial species (Franks *et al.*, 1998; Harmsen *et al.*, 2002), however, our final conclusion was that none detected a sufficiently wide range of bacterial groups for reliable total bacteria quantification. FISH may have been a useful additional technique for analysis of the biofilm samples, in particular, for the detection of coliforms or other pathogenic bacteria.

Flow cytometry has successfully been used to enumerate total bacteria in water samples (Hammes *et al.*, 2008) and is a viable alternative to PCR-based technologies. However, flow cytometry is less exact when used on biofilm samples or samples containing aggregates of bacterial cells or other particulates (Kooij and Wielen, 2014). It was desirable to have a quantification method that was equally suitable for the biofilm and bulk water samples, therefore, this drawback was the main factor in deciding not to use flow cytometry.

Quantitative PCR or real-time PCR has been the focus of much interest and development as a rapid, molecular based alternative to culture-based plate counts in the water industry and beyond (Girones *et al.*, 2010). As well as quantification of total bacteria (Nadkarni *et al.*, 2002), qPCR has been used to detect and quantify many microorganisms, including *Legionella pneumophila* (Dusserre *et al.*, 2008), pathogenic Candida cells (Brinkman *et al.*, 2003), Bacteroides spp. (Okabe *et al.*, 2007; Converse *et al.*, 2009), Nitrosomonas and Nitrospira spp (Dionisi *et al.*, 2002), Enterococci (He and Jiang, 2005), denitrifying bacteria (Henry *et al.*, 2004) and coliforms (McDaniels *et al.*, 2005; Varma *et al.*, 2009; Martín *et al.*, 2010; Soejima *et al.*, 2012; Hu *et al.*, 2016). Quantitative PCR is highly sensitive, rapid and accurate. However, using qPCR based on the 16S rRNA gene for quantification of total bacteria has two main disadvantages: the unknown copy number of the 16S rRNA gene in the bacterial species contained in environmental samples and the inability to distinguish between live and dead bacteria. In this study, one copy of the 16S rRNA gene was assumed to represent one bacterial cell. While this is true for many species, some bacterial species can contain up to 15 copies of the 16S rRNA gene (Kembel *et al.*, 2012). Therefore, it is possible that the abundance of total bacteria has been overestimated by using this method. Secondly, the inability to distinguish between live and dead bacteria is a common disadvantage of all DNA-based technologies. It is possible to overcome this by the addition of substances such as propidium monoazide (PMA), which select for live cells on the basis of membrane integrity. PMA is able to permeate into cells with damaged membranes and then binds with DNA to prevent it being amplified by PCR (Nocker *et al.*, 2007; Varma *et al.*, 2009). A final point of concern in using qPCR for gene copy number quantification is that it can be difficult to reliably compare qPCR data with other studies in the literature. The final copy number quantity can be significantly affected by the assay used, as well as the preparation method of the standards, primers and probes (Smith *et al.*, 2006; Botes *et al.*, 2013).

After due consideration, quantitative PCR was selected as the most suitable method of total bacteria quantification as it would allow a large number of

samples to be analysed in a reasonable amount of time and provide more accurate results than culture methods.

4.4 Conclusions

This chapter describes the collection of biofilm and bulk water samples from two WTPs with differing sources of water and compares total bacteria between treatment stage, age of biofilm, time of year of bulk water sampling and source water type. The main findings are as follows:

1. Chlorine concentration and conditions in the contact tank at both WTPs was sufficient to prevent biofilm growth over 6 months.
2. Stable biofilms were established after 1 month.
3. Total bacteria in biofilms at WTP 3 (reservoir source) decreased with advancing treatment stage.
4. Total bacteria in biofilms at WTP 4 (river source) decreased only after the filtration stage.
5. Total bacteria in bulk water at both WTPs decreased with advancing treatment stage.
6. No clear seasonal effect on total bacteria concentrations in bulk water was apparent, although this may be an artefact of sampling frequency.
7. Numbers of coliforms, *E. coli* and Gram(-) non-coliforms increased in the winter months.
8. No significant difference in total bacteria entering in raw bulk water was found between river and reservoir source water WTPs over time.

Chapter 5 – Characterization of microbiomes between and across two water treatment plants

5.1 Introduction

Having isolated and quantified bacteria in biofilms and bulk water from multiple treatment stages of two WTPs with differing source waters (Chapter 4), Chapter 5 provides deeper analysis of the composition and spatial differences in bacterial communities across the two WTPs. The goal is to determine whether WTP source water or unit operations within a WTP more influence resident biofilm and bulk bacterial communities. To do this, amplicon sequencing was used to characterize the microbiomes in the WTPs to identify exactly which bacterial groups are present in both biofilms and bulk water, their proportions and how those proportions change throughout the treatment process.

Many previous studies have assessed different characteristics of drinking water distribution systems in a range of countries, including Germany (Schmeisser *et al.*, 2003; Emtiazi *et al.*, 2004; Eichler *et al.*, 2006; Henne *et al.*, 2012), the Netherlands (Bereschenko *et al.*, 2008; Liu *et al.*, 2014; El-Chakhtoura *et al.*, 2015; Roeselers *et al.*, 2015), Switzerland (Lautenschlager *et al.*, 2013; Lautenschlager *et al.*, 2014), Portugal (Vaz-Moreira *et al.*, 2013), South Korea (Lee *et al.*, 2005), Singapore (Chen *et al.*, 2004), China (Yuanqing *et al.*, 2013; Lin *et al.*, 2014) and the USA (LeChevallier *et al.*, 1987; Hong *et al.*, 2010; Hwang *et al.*, 2012; Pinto *et al.*, 2012; Pinto *et al.*, 2014). However, less attention has been focused on the “whole” microbiome of drinking water treatment plants, including spatial variations. Instead, most studies view WTPs more narrowly (e.g., single sample points), most commonly focusing on raw source water or final treated water leaving the plant. Considering the vast changes in physical, chemical and biological conditions occurring throughout a WTP, single sample points are extremely unlikely to capture an accurate representation of WTP communities, including how and why different WTPs differ.

A relatively smaller number of studies have investigated the communities of WTPs in more detail. Emtiazi *et al.* (2004), Bereschenko *et al.* (2008) and (Vaz-

Moreira *et al.*, 2013) used PCR and denaturing gradient gel electrophoresis (DGGE) to identify changing patterns in bacterial populations throughout WTPs. A proportion of bacterial groups appeared to be ubiquitous in samples taken from different stages in the treatment plant and distribution system (Emtiazi *et al.*, 2004), however band patterns were significantly different at different stages. For example, the reverse osmosis membrane filter biofilm community in the treatment plant studied by Bereschenko *et al.* (2008) was unique compared with other locations and different to the bulk water community. Proteobacteria were found to be the dominant bacterial phyla of the WTPs (Emtiazi *et al.*, 2004; Bereschenko *et al.*, 2008; Vaz-Moreira *et al.*, 2013), however due to the limitations of DGGE, broad coverage of bacterial phyla was lacking in these studies.

Later studies were able to use more advanced sequencing techniques, such as 454-pyrosequencing and Illumina next generation sequencing, to provide greater depth to the taxonomic composition of WTPs and distribution systems (Eichler *et al.*, 2006; Pinto *et al.*, 2012; Yuanqing *et al.*, 2013; Lautenschlager *et al.*, 2014; Lin *et al.*, 2014). Proteobacteria, Bacteroidetes, Actinobacteria, Candidate division OD1 and Nitrospira were found to be predominant phyla in the drinking water microbiome (Eichler *et al.*, 2006; Pinto *et al.*, 2012), along with Acidobacteria and Chloroflexi (Lautenschlager *et al.*, 2014). Further, the community composition of source water was found to have a significant influence on the composition of the final drinking water microflora (Eichler *et al.*, 2006) and specific treatment processes also were found to influence community composition to differing extents (Pinto *et al.*, 2012; Yuanqing *et al.*, 2013; Lautenschlager *et al.*, 2014; Lin *et al.*, 2014).

In the four years since the PhD project began, there have been a number of papers published focusing directly on microbial community changes across WTPs (Li *et al.*, 2017; Lin *et al.*, 2017; Xu *et al.*, 2017; Hou *et al.*, 2018) as well as water distribution systems (Li *et al.*, 2016; Revetta *et al.*, 2016; Vanessa *et al.*, 2019). The four studies based on WTPs are based exclusively on treatment plants in China, while the water distribution system experiments also took place in North America. The main consistent findings from these publications are: Proteobacteria was the most dominant phyla across water treatment and distribution; disinfection causes large scale changes to bacterial community

composition; and biofilm and bulk water communities have significantly different proportions of bacterial phyla.

To my knowledge, the work described in this chapter is the first to determine the microbiome of both biofilms and bulk water at multiple stages of WTPs in the UK using next generation sequencing. Recent studies also have not collected biofilm samples at every treatment stage, instead just sampling from sand and GAC filters (Li *et al.*, 2017; Hou *et al.*, 2018). By sampling two WTPs with differing types of source water (reservoir and river), novel comparisons and conclusions can be made regarding the selection pressure of treatment stage as opposed to the incoming microbial community of raw water. Findings from this chapter will contribute to the growing body of knowledge of the microbiome of water treatment processes.

5.1.1 Aims and objectives

The aim of this chapter is to determine the forces that shape bacterial communities in WTPs. The hypotheses being tested are: firstly, the bacterial community composition of biofilms and bulk water is significantly different; secondly, bacterial community composition in both biofilms and bulk water are influenced by treatment stage; and finally, bacterial community composition in both biofilms and bulk water are influenced by the community of the incoming source water.

The specific objectives of this chapter are as follows:

1) To determine the bacterial community composition of two WTPs at five treatment stages in biofilm and bulk water.
2) To determine to what extent coliforms and other groups of potentially clinically relevant organisms are present in biofilm and bulk water communities in WTPs.

5.2 Materials and Methods

5.2.1 Sample collection

Biofilm and bulk water samples were collected as described in Chapter 4, Section 4.2.3. DNA samples were obtained by membrane filtration of biofilm or bulk water (Chapter 4, Section 4.2.4) followed by DNA extraction (Chapter 4, Section 4.2.5).

DNA was quantified using the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Biofilm and bulk water DNA samples collected from each WTP after three months development were selected for microbiome analysis. A full summary of samples is shown in Table 5.1.

Table 5.1 – *Samples selected for metagenomic analysis. Biofilm samples were collected after 3 months development with bulk water samples taken the same day (November and December 2016 at WTP 3 and 4, respectively).*

WTP	Sample type	Treatment stage	Number of replicates
3	Biofilm	Raw	3
3	Biofilm	Post-clarification	3
3	Biofilm	Post-filtration	3
3	Biofilm	Post-GAC	3
3	Bulk water	Raw	3
3	Bulk water	Post-clarification	3
3	Bulk water	Post-filtration	3
3	Bulk water	Post-GAC	2
3	Bulk water	Post-contact tank	2
4	Biofilm	Raw	3
4	Biofilm	Post-clarification	3
4	Biofilm	Post-filtration	3
4	Bulk water	Raw	3
4	Bulk water	Post-clarification	3
4	Bulk water	Post-filtration	3
4	Bulk water	Post-GAC	3

5.2.2 Sequencing analysis

Sequencing analysis and all work described in this section (5.2.2) was carried out by LGC Genomics (Berlin, Germany). Briefly, forward primer (341F) 5'–CCTACGGGNGGCWGCAG–3' and reverse primer (785R) 5'–GACTACHVGGGTATCTAAKCC–3' were used to amplify 16S rDNA from all samples by touchdown PCR (10 cycles with annealing temperature decreasing 0.6°C per cycle, 61 – 55°C) followed by 2-step PCR (26 cycles, combined annealing and extension temperature 55°C). Primers 341F and 785R are universal primers producing 464 bp amplicons covering hypervariable regions 3 – 4 of the 16S rRNA gene and have been experimentally validated as providing good coverage of a wide phylum spectrum (Klindworth *et al.*, 2013).

Agarose gel electrophoresis was used to confirm successful amplification. Library preparation, consisting of tagging, equimolar mixing and clean up, was performed prior to 16S rDNA amplicon sequencing using Illumina MiSeq V3 (2 x 300 bp).

5.2.3 Bioinformatics and statistical analysis

Bioinformatics analysis was carried out by LGC Genomics (Berlin, Germany) and consisted of: demultiplexing and sorting of reads by amplicon inline barcodes; clipping of sequencing adapter remnants; 16S pre-processing and OTU (operational taxonomic unit) picking from amplicons using Mothur. Putative species level annotation of OTUs was completed using NCBI BLAST and OTU diversity analyses were carried out with QIIME.

Statistical analysis was carried out by myself as described in the following paragraph. Alpha and beta diversity were calculated in QIIME. Plots of phylum composition, observed and Chao1 OTU values and Shannon's and Simpson's diversity index values were produced in Microsoft Excel and edited in Adobe Illustrator. ANOVA, Tukey's honest significant difference test and independent t-test were carried out in R Studio. Predominant OTU bubble plots were created in Microsoft Excel and edited in Adobe Illustrator. PRIMER 7 (PRIMER-E, Plymouth, UK) was used for analysis of beta diversity, specifically for principal coordinate analysis (PCO), permutational analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) based on Bray-Curtis similarity (after

square root transformation of OTU abundance data) and weighted UniFrac distances.

5.3 Results: bacterial diversity within WTP 3

5.3.1 Biofilm community alpha diversity

The relative abundance of bacterial phyla in biofilm samples taken from WTP 3 is shown in Figure 5.1. The amplicon sequencing data was consistent between triplicate samples at all stages, with only slight variations in proportions of different phyla.

The biofilm community of WTP 3 was distinct at each progressive treatment stage. In the raw biofilm, the predominant bacterial phyla were Proteobacteria (32.2%), Nitrospirae (14.9%), Planctomycetes (12.8%), Chloroflexi (11.5%) and Bacteriodetes (10.7%). After initial clarification, the proportion of Proteobacteria was similar (35.7%), whereas Actinobacteria greatly increased (28.4%). Bacteriodetes also increased in post-clarification biofilms (16.0%). The predominant phyla in the biofilms post-filtration were much less diverse, consisting mainly of Planctomycetes (43.2%) and Proteobacteria (42.2%). Post-GAC biofilms were dominated by a single bacterial phylum: Proteobacteria (90.9%). The relative abundance percentages reported here in brackets are the mean value of triplicate samples.

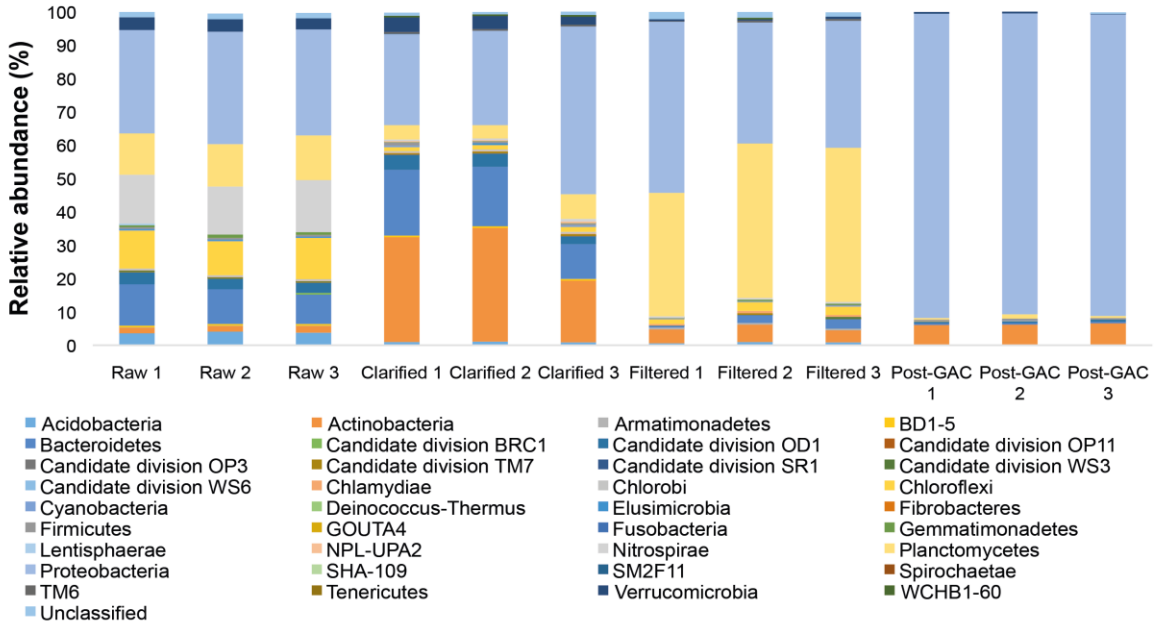


Figure 5.1 – *Bacterial phyla present in triplicate biofilm samples taken from four treatment stages at WTP 3. Proteobacteria was ubiquitous throughout the WTP, however, each treatment stage displayed a unique community composition.*

Bacterial community data from triplicate samples were combined for each treatment stage and diversity analysis carried out as described below. Community richness was estimated by calculating the observed species (count of unique OTUs in each sample) and Chao1 index values (an estimation of richness based on the abundance of rare OTUs). Figure 5.2 (c) shows a general trend of decreasing richness with treatment stage. One-way ANOVA and Tukey's HSD reveals significant differences in observed OTUs at all four treatment stages ($p < 0.001$, except for clarified vs filtered where $p < 0.01$). The same trend is displayed by the Chao1 index values, with community richness decreasing from raw > clarified > filtered > post-GAC biofilms. The Chao1 values of the clarified and filtered biofilms were not significantly different, however when compared with Tukey's HSD, all other treatment stages were significantly different from each other ($p < 0.001$).

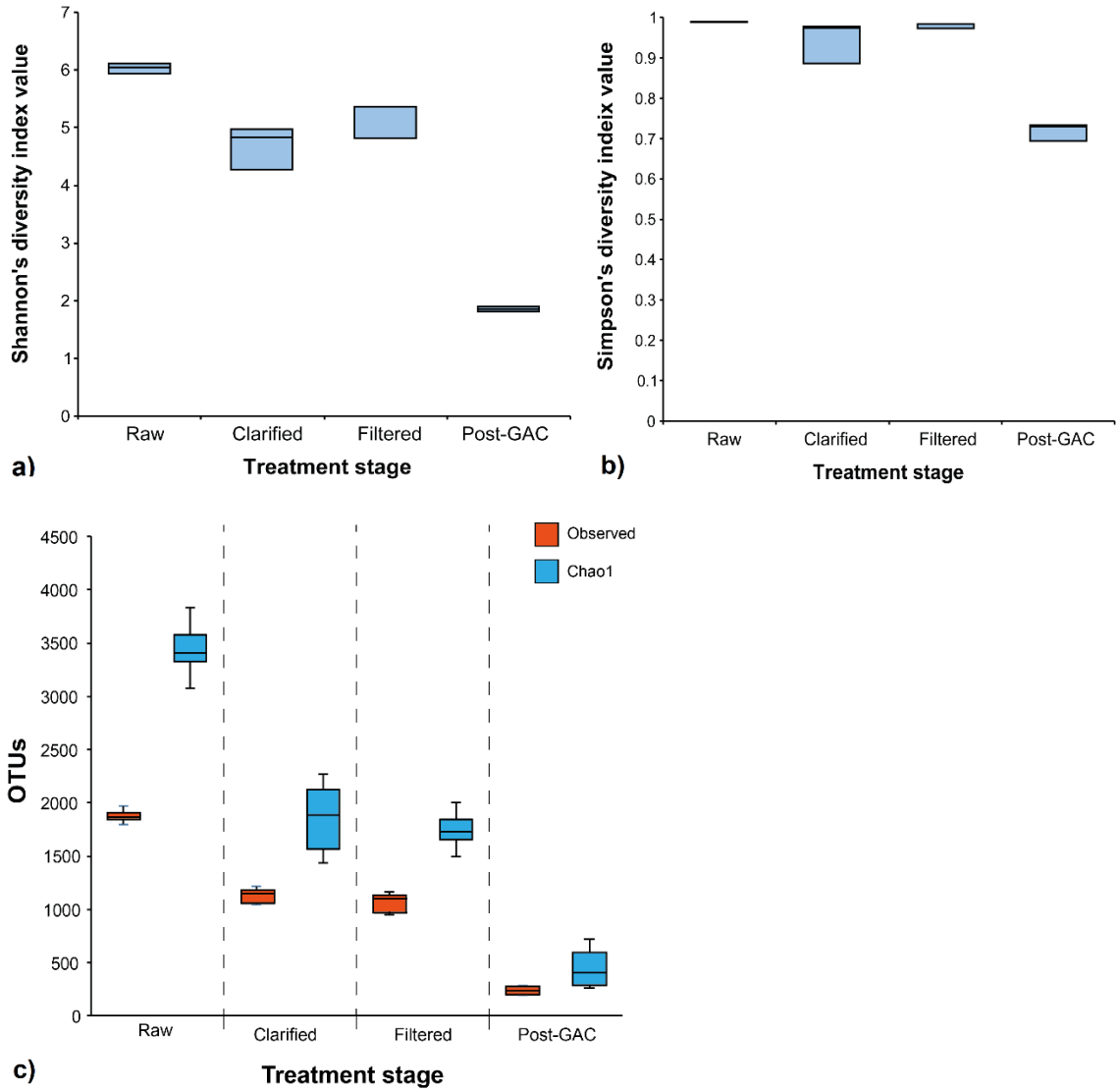


Figure 5.2 – *Diversity within the biofilm community of WTP 3 as represented by boxplots of* ***a)*** *Shannon's diversity index values,* ***b)*** *Simpson's diversity index values and* ***c)*** *observed species and Chao1 values.*

Shannon's and Simpson's indices both quantify diversity by combining measures of richness and evenness. Shannon's diversity index is more influenced by the presence of rare OTUs, whereas the abundance of OTUs has greater influence on Simpson's diversity index (Nagendra, 2002). Diversity indices of the biofilm communities of WTP 3 are shown in Figure 5.2 (a, b). According to Shannon's index, the raw biofilm community had the highest level of diversity (5.9 - 6.1), whereas post-GAC biofilms had by far the lowest indices (1.8 - 1.9). ANOVA and Tukey's HSD showed significant differences between all stages ($p < 0.001$), except post-clarification and post-filtration. As seen in the Chao1 values, clarified

and filtered biofilms did not show significantly different levels of diversity to each other. According to Simpson's index, the first three stages of treatment showed high levels of diversity (raw = 0.99, clarified = 0.87 – 0.98 and filtered = 0.97 – 0.98). The post-GAC biofilm community was significantly less diverse than all other stages ($p < 0.001$), although there were no significant differences among raw, post-clarification and post-filtration biofilm Simpson's index values.

The predominant OTUs (61 OTUs that had ≥0.8% relative abundance at one or more treatment stages) of WTP 3 biofilms are summarised in Figure 5.3. Taxonomic classification level is noted in brackets, where P = phylum, C = class, O = order, F = family and G = genus. OTUs were classified to genus level where possible. As would be expected from the phylum composition previously described, different OTUs predominated at each treatment stage.

Raw water biofilms contained a high relative abundance of *Nitrospira* (OTUs 23, 28, 34 and 108) and Chloroflexi belonging to the *Caldilineaceae* family (OTUs 52, 101 and 119). In clarified biofilms, Proteobacteria made up 35.7% of bacterial phyla and predominant OTU analysis reveals that *Undibacterium* (OTU 3) is responsible for 13.3% of this group. Bacteroidetes (16.0% relative abundance) predominantly consisted of *Pseudarcicella, Flavobacterium, Fluviicola* and *Sediminibacterium,* while Actinobacteria (28.4%) was comprised of two families: *Acidimicrobiaceae* (OTUs 4, 20, 40 and 65) and *Sporichthyaceae* (OTUs 5 and 14). Filtered biofilms were dominated by Proteobacteria and Planctomycetes. The most abundant Proteobacteria OTUs belonged to *Sphingomonadaceae* (OTUs 19, 25, 21, 116 and 118), with this family comprising 11.9% of the filtered biofilm community. *Arenimonas* (OTUs 36 and 41) is also notable, contributing 10.2% relative abundance. With the exception of *Phycisphaera* and *Phycisphaera SM1A02,* predominant Planctomycetes OTUs all belonged to the *Planctomycetaceae* family (combined relative abundance of 15.4%), with *Schlesneria* (OTUs 30 and 86)*, Gemmata* (OTUs 54 and 111) and *Planctomyces* (OTUs 68 and 145) identified to genus level.

Biofilms from post-GAC treatment contained 90.9% Proteobacteria and predominant OTU analysis reveals this relative abundance is attributable to three main genera: *Porphyrobacteria* (OTU 12; 21.2%), *Unibacterium* (OTU 3; 44.4%) and *Methylotenera* (OTU 13; 18.5%).

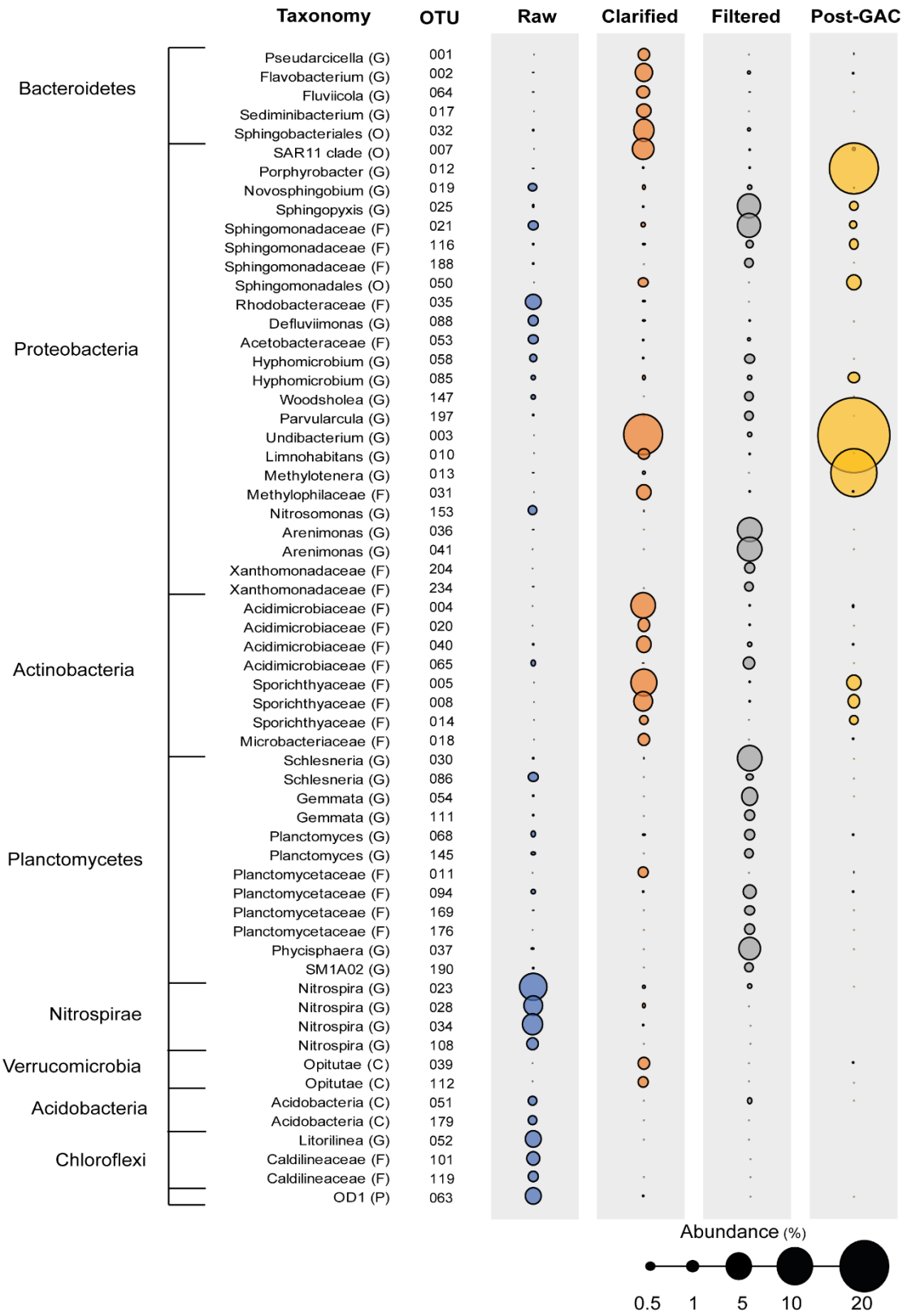


Figure 5.3 – *Predominant OTUs in WTP 3 biofilm samples (≥0.8% relative abundance) at raw, clarified, filtered and post-GAC treatment stages. OTUs are classified to genus level (G) where possible. Relative abundance of OTUs is represented by area of the bubbles.*

Bacterial genera that contain species of potential clinical significance (i.e. able to cause infection in humans) are summarised in Table 5.2 and their relative abundance in biofilms at each treatment stage are listed. All groups, including coliforms, represent extremely small proportions of the bacterial community.

Table 5.2 – *Relative abundance (%) of bacterial genera in WTP 3 biofilms that contain species of potential clinical significance.*

Genus	Raw	Clarified	Filtered	Post-GAC
Coliforms (*Enterobacteriaceae*)	0.006	0.006	0.001	BLD*
Escherichia	0.001	BLD	BLD	BLD
Acinetobacter	0.063	0.012	0.006	<0.001
Bacillus	0.036	0.111	0.005	0.025
Burkholderia	0.001	0.023	0.009	0.009
Clostridium	0.015	0.016	0.007	0.002
Enterococcus	BLD	BLD	0.002	BLD
Legionella	0.017	0.080	0.112	0.002
Mycobacterium	0.004	0.017	0.020	<0.001
Prevotella	0.001	0.004	BLD	<0.001
Pseudomonas	0.009	0.012	0.004	0.003
Rickettsia	0.016	0.040	0.016	BLD
Streptococcus	0.002	0.013	0.002	BLD

*BLD = below level of detection

5.3.2 Bulk water community alpha diversity

In accord with biofilm samples, considerable consistency existed in community composition among triplicates of bulk water samples for most of the treatment stages. An exception to this can be seen post-contact tank (see Figure 5.4), where there was a notable difference in the relative abundance of different phyla between the two samples taken.

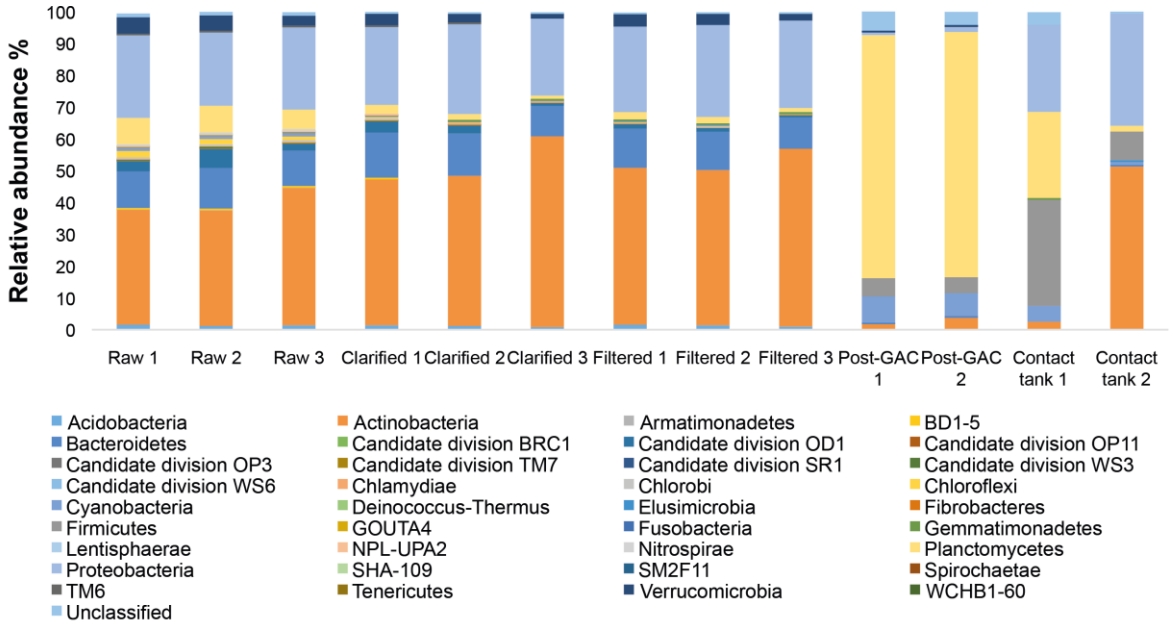


Figure 5.4 – *Bacterial phyla present in triplicate bulk water samples taken from five treatment stages in WTP 3. Bulk water community composition is very similar through the first three stages of treatment before a marked change after passage through the GAC filter.*

Unlike biofilm communities, bulk water community composition was very similar across the first three treatment stages. The same three phyla were dominant in raw water, post-clarification and post-filtration stages: i.e., Actinobacteria (38.9%, 51.2% and 51.3%, respectively), Bacteroidetes (11.8%, 12.4% and 11.4%, respectively) and Proteobacteria (25.4%, 25.9% and 27.8%, respectively). Treatment stage did not appear to pose a significant selective pressure until the post-GAC stage, where the bulk water composition changed massively, becoming dominated by Planctomycetes (76.8%). Cyanobacteria and Firmicutes also increased at post-GAC to 7.7% and 5.6% respectively. Duplicate contact tank bulk water samples contained fairly similar relative abundances of Proteobacteria (27.3% and 35.5%), however, the proportions of Firmicutes, Planctomycetes and Actinobacteria were considerably different in the two samples. Contact tank sample 1 was more similar to post-GAC samples, with Firmicutes (33.7%) and Planctomycetes (27.1%) in high relative abundance. Contact tank sample 2, however, showed a high proportion of Actinobacteria (51.3%), similar to levels seen previously in raw, clarified and filtered bulk water.

Bulk water community richness (as measured by observed species and Chao1 values) decreased with progressing treatment stage (Figure 5.6(c)), which mirrors the trend seen in biofilm samples.

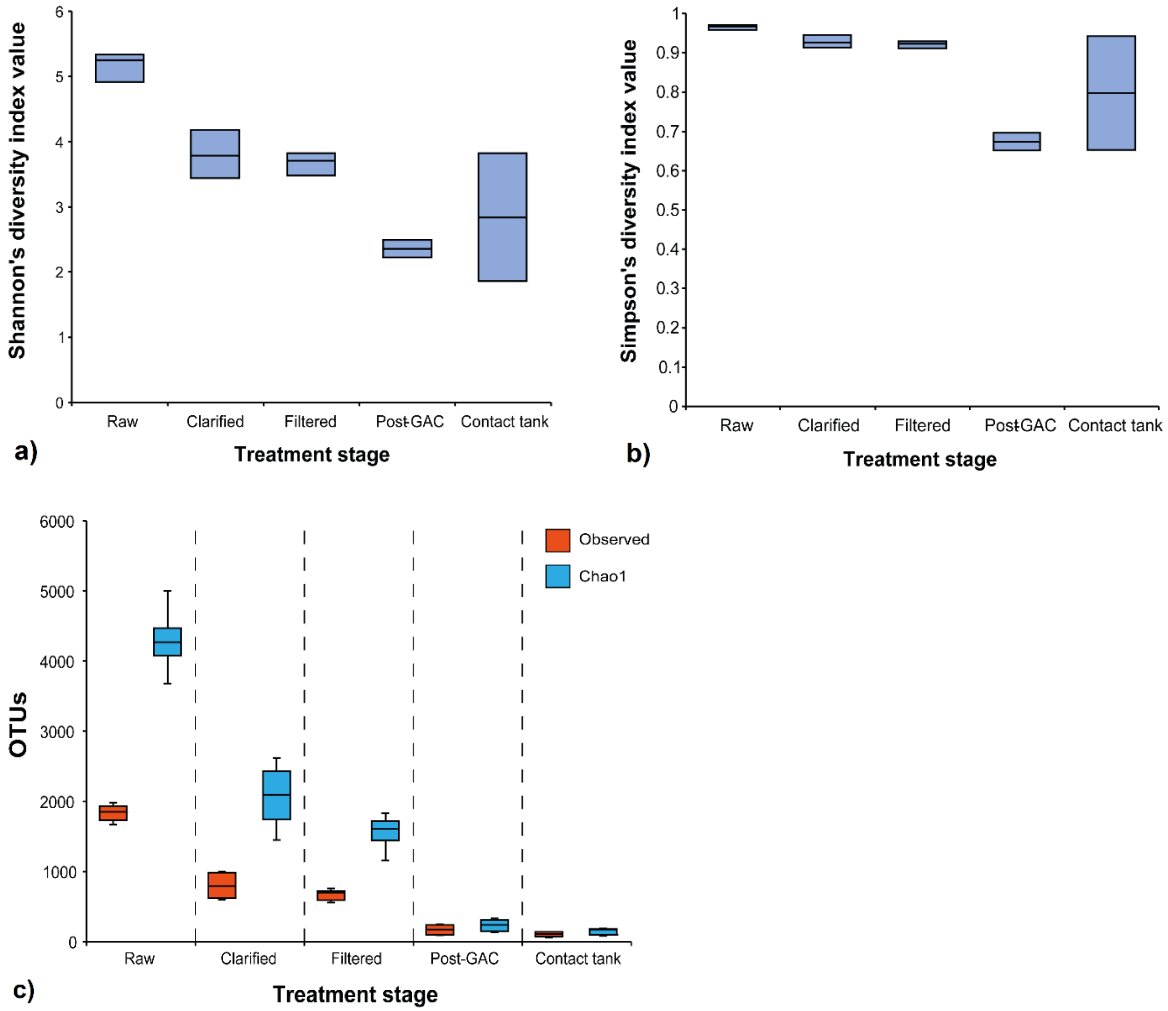


Figure 5.5 – *Diversity within the bulk water community of WTP 3 as represented by boxplots of **a)** Shannon's diversity index values, **b)** Simpson's diversity index values and **c)** observed species and Chao1 values.*

The community richness of bulk water at each advancing treatment stage was significantly lower ($p < 0.001$) than the previous stage, in the order of raw > clarified > filtered > post-GAC. Post-GAC and contact tank bulk water samples were not significantly different in either observed species or Chao1 values.

As measured by Shannon's diversity index, raw bulk water community is significantly more diverse ($p < 0.05$) than all other treatment stages. However, there was no significant difference in Shannon's diversity index value between

clarified, filtered, post-GAC and contact tank bulk water communities. Simpson's diversity index values were comparatively high for the first three stages of treatment: 0.96 – 0.97, 0.91 – 0.95 and 0.91 – 0.93 for raw, clarified and filtered bulk water, respectively. According to Simpson's diversity index values, post-GAC bulk water community was significantly less diverse ($p < 0.05$) than raw, clarified and filtered bulk water, whereas no significant difference in diversity was observed between any other stages.

Predominant OTU analysis (Figure 5.6) shows the 60 most abundant OTUs (OTUs with ≥0.5% relative abundance at one or more treatment stages) in bulk water passing through WTP 3. In accordance with phylum composition analysis, bulk water at the raw, clarified and filtered stage was dominated by the same OTUs and no significant change is seen until after passage through the GAC filter.

Notable groups with high relative abundances were: Alphaproteobacteria SAR11 clade (OTU 7), comprising 5.7%, 12.6% and 13.6% relative abundance at raw, clarified and filtered, respectively); *Acidimicrobiaceae* (OTUs 4, 20, 40 and 62) comprising 14.5%, 18.8% and 22.1% at raw, clarified and filtered, respectively, and *Sporichthyaceae* (OTUs 5, 8, 14, 38, 46, 48, 66, 80 and 87) comprising 20.8%, 29.4% and 26.2% at raw, clarified and filtered, respectively. Post-GAC bulk water was dominated by the *Planctomycetaceae* family (72.0%), particularly by OTU 11 with a relative abundance of 56.3%. Notable groups found in contact tank bulk water included Burkholderiales (OTU 122; 21.1% relative abundance), *Propionibacterium* (OTU 61; 25.9%) and *Planctomycetaceae (*OTUs 11, 71, 98, 170, 280 and 343; 13.6% combined relative abundance).

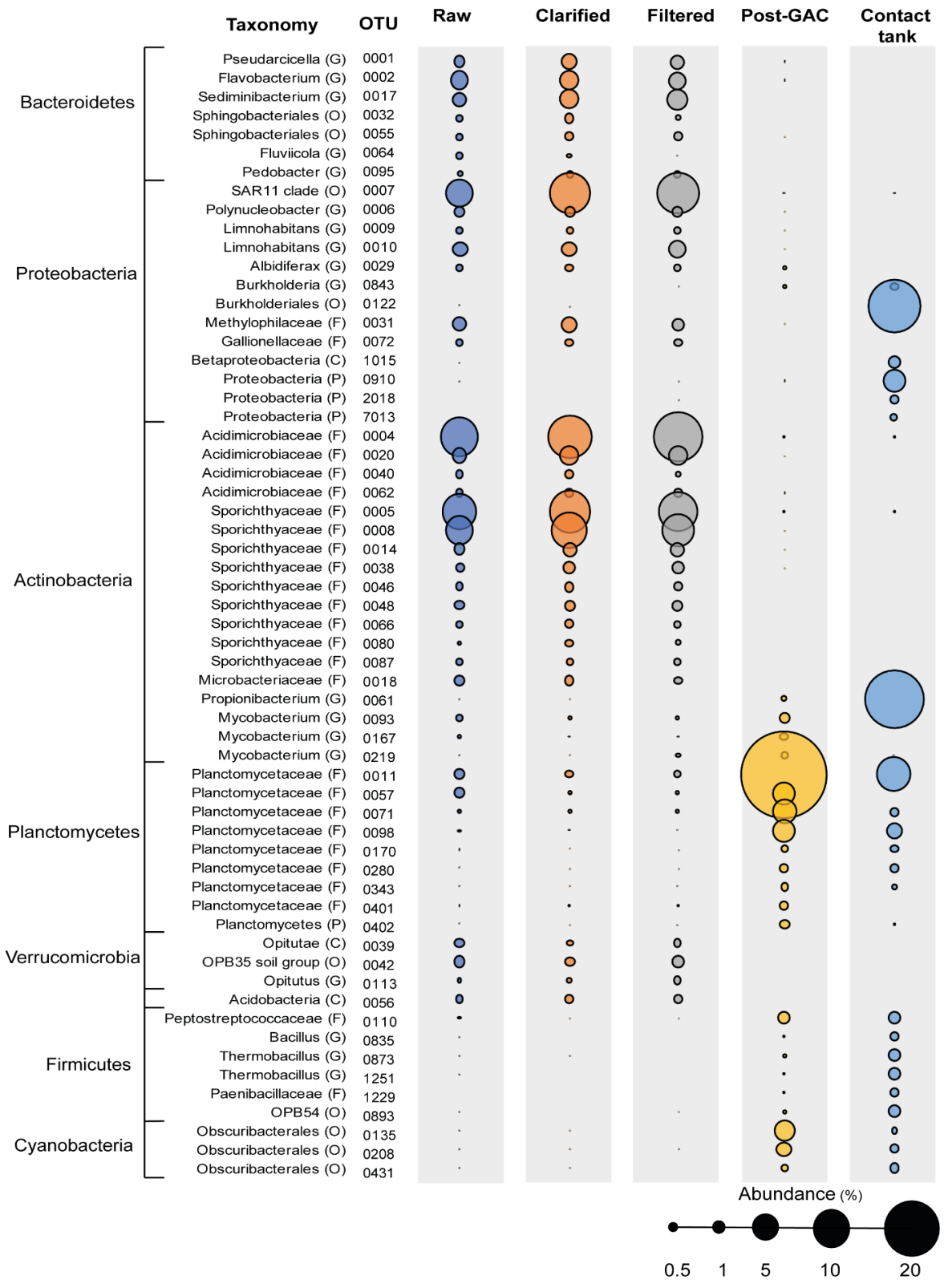


Figure 5.6 – *Predominant OTUs in WTP 3 bulk water samples (≥0.5% relative abundance) at raw, clarified, filtered, post-GAC and contact tank treatment stages. OTUs are classified to genus level (G) where possible. Relative abundance of OTUs is represented by area of the bubbles.*

The relative abundance of bacterial groups containing species of potential clinical significance are shown in Table 5.3. As with WTP 3's biofilm communities, the abundances of these groups tend to be very small. *Mycobacterium* is the most abundant genera of potential significance at each treatment stage, most notably, ~2.4% relative abundance in post-GAC bulk water. It is interesting to note that coliforms were detected as comprising 0.008% of the bulk water community after passage through the contact tank on the particular day of sampling.

Table 5.3 - *Relative abundance (%) of bacterial genera in WTP 3 bulk water that contain species of potential clinical significance.*

Genus	Raw	Clarified	Filtered	Post-GAC	Post-contact tank
Coliforms (*Enterobacteriaceae*)	0.013	0.006	<0.001	0.014	0.008
Escherichia	0.007	BLD	BLD	0.001	BLD*
Acinetobacter	0.110	0.031	0.013	BLD	0.054
Bacillus	0.076	0.002	0.002	0.393	1.99
Burkholderia	BLD	BLD	0.002	0.332	0.985
Clostridium	0.114	0.007	0.006	0.301	0.302
Corynebacteria	0.003	BLD	BLD	BLD	BLD
Enterococcus	0.001	0.001	<0.001	BLD	BLD
Legionella	0.068	0.047	0.029	0.051	0.163
Mycobacterium	0.687	0.239	0.535	2.378	0.016
Mycoplasma	0.002	BLD	BLD	BLD	BLD
Nocardia	0.003	BLD	BLD	BLD	BLD
Prevotella	0.004	0.002	BLD	BLD	BLD
Pseudomonas	0.069	0.017	0.016	BLD	BLD
Rickettsia	0.041	0.013	0.022	0.052	0.163
Staphylococcus	0.001	BLD	BLD	0.065	0.054
Streptococcus	0.008	0.002	<0.001	BLD	BLD

*BLD = below level of detection

5.4 Results: bacterial diversity within WTP 4

5.4.1 Biofilm community alpha diversity

Phylum composition of the biofilm community of WTP 4 is shown in Figure 5.7. As was observed with samples taken from WTP 3, community composition was very consistent across triplicate samples at each treatment stage. Raw, post-clarification and post-filtration biofilms each had distinct patterns of community composition, indicating treatment stage has selection pressure in biofilm establishment, composition and development.

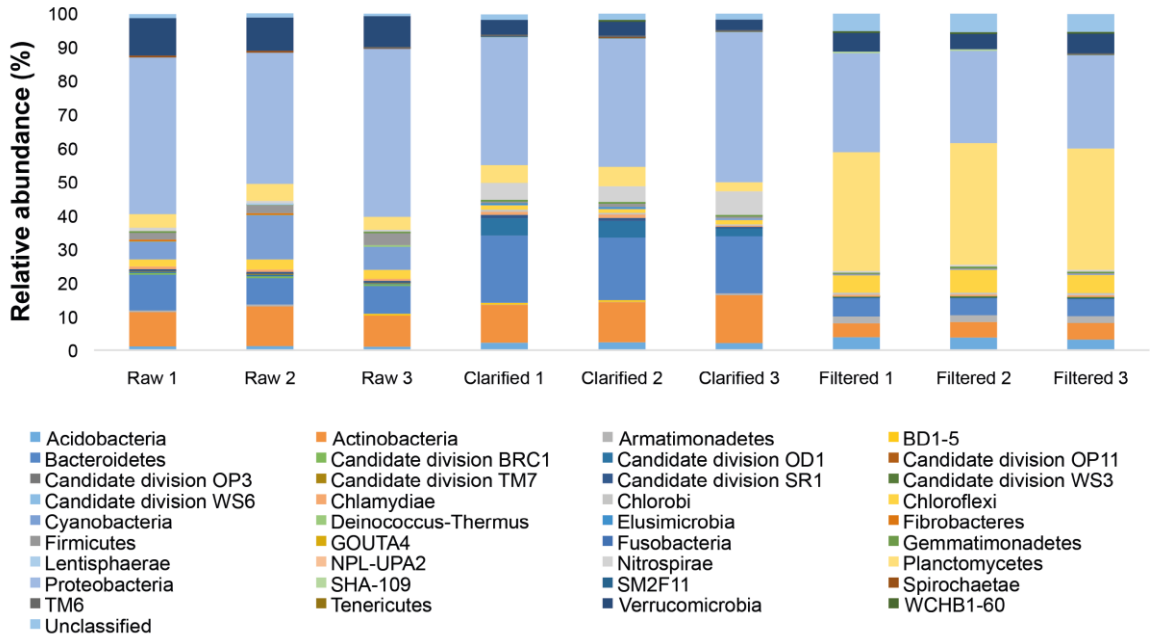


Figure 5.7 – *Bacterial phyla present in triplicate biofilm samples taken from three treatment stages at WTP 4. Proteobacteria, Planctomycetes, Bacteroidetes and Actinobacteria were ubiquitous throughout, however each treatment stage shows different proportions of each phyla.*

Proteobacteria was the major constituent of biofilm communities at all three stages with mean relative abundances of 45.5%, 40.6% and 28.3% in raw, clarified and filtered biofilms, respectively. Raw biofilms also contained notable proportions of Actinobacteria (10.8%), Verrucomicrobia (10.0%), Bacteroidetes (9.3%), Cyanobacteria (8.8%) and Planctomycetes (4.4%). In post-clarification biofilms, the proportion of Bacteroidetes almost doubled to 18.5%, while Cyanobacteria and Verrucomicrobia greatly decreased to 0.1% and 4.0%,

respectively. Actinobacteria (12.8%) and Planctomycetes (4.6%) remained at similar ratios. Post-filtration biofilm communities showed a huge increase in Planctomycetes (35.8%). Actinobacteria (4.6%), Verrucomicrobia (5.8%) and Chloroflexi (5.7%). Relative abundances reported here in brackets are the mean values of triplicate samples.

Community diversity analysis by observed species, Chao1, Shannon's and Simpson's values is shown in Figure 5.8. For the purpose of analysis, data from triplicate samples at each treatment stage were combined.

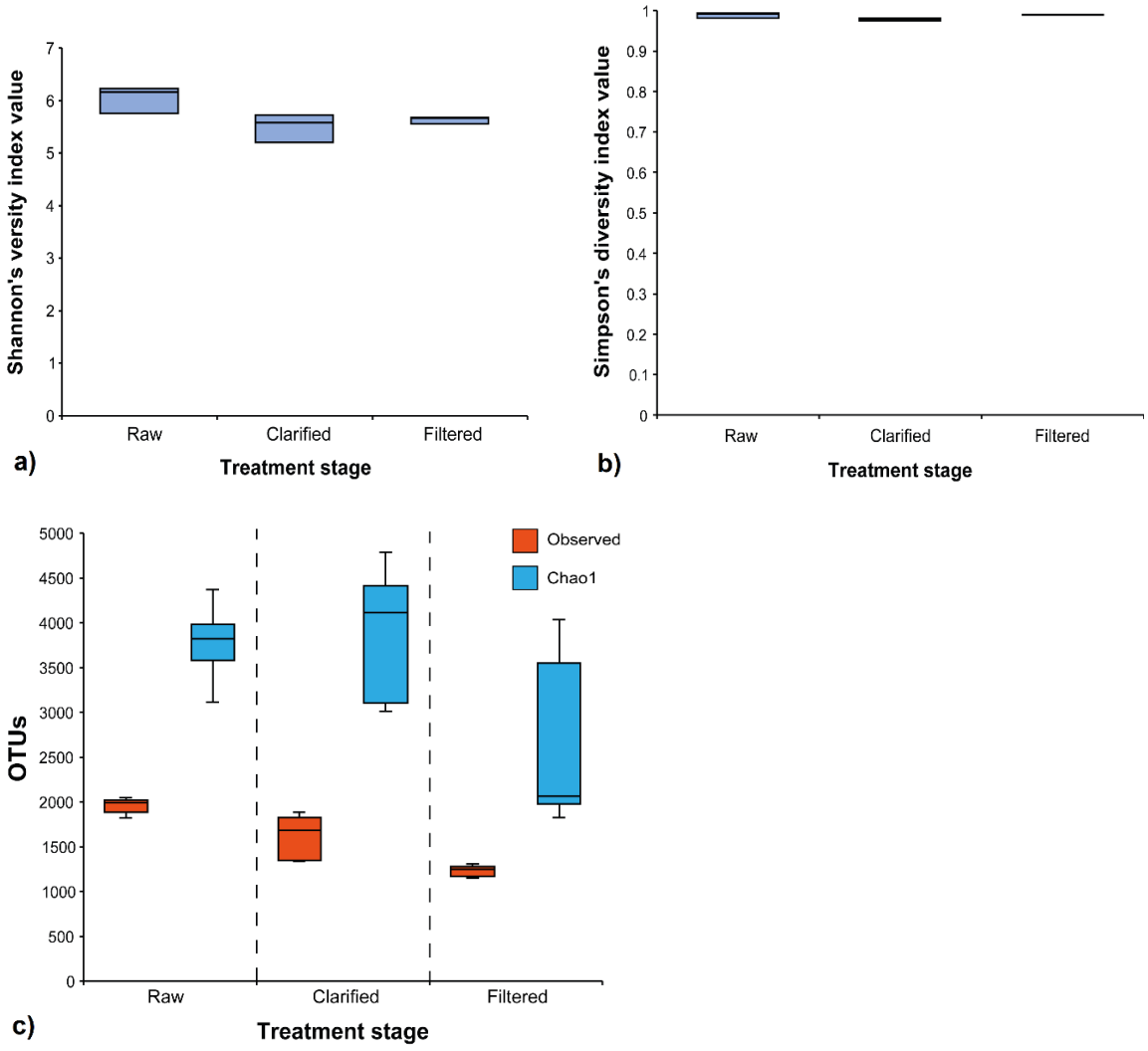


Figure 5.8 – *Diversity within the biofilm community of WTP 4 as represented by boxplots of* ***a)*** *Shannon's diversity index values,* ***b)*** *Simpson's diversity index values and* ***c)*** *observed species and Chao1 values.*

Community richness as measured by observed species was significantly different ($p = 0$) at the raw, clarified and filtered stages. Chao1 values also showed a

decrease in richness from raw > clarified > filtered, although raw and clarified Chao1 values were not significantly different. Both measures show a trend of decreasing richness with progressing treatment, which also was observed in biofilm communities in WTP 3.

According to the Shannon's diversity index (Figure 5.8b), raw biofilm communities were significantly more diverse than post-clarification communities ($p < 0.05$), however, no significant difference was found between any other stages. When measured using Simpson's diversity index, the clarified biofilm community was significantly less diverse than raw and filtered biofilms ($p < 0.05$). However, all three stages produced generally high Simpson's diversity index values: raw (0.98-0.99), post-clarification (0.97-0.98) and post-filtration (0.99).

Predominant OTU analysis focusing on 57 OTUs with ≥ 0.8% relative abundance at one or more treatment stages is shown in Figure 5.9. As might be expected from the high diversity values previously calculated, biofilm communities were not as dominated by single OTUs or groups of related genera as was seen in biofilm communities from WTP 3. Instead, there was a greater spread of smaller relative abundances over predominant OTUs.

Raw biofilms were dominated by Proteobacteria (Figure 5.9), and OTU analysis reveals the highest proportions of this phyla belong to the order of Pseudomonadales, specifically *Psychrobacter* (OTUs 59, 132 and 156; 6.1%) and *Pseudomonas* (OTU 97; 0.8%). Two families of Alphaproteobacteria, *Sphingomonadaceae (*OTUs 19, 25 and 21) and *Rhodobacteraceae* (OTUs 35 and 84) were also notable, with 4.6% and 2.7% relative abundance, respectively. Other genera of note include *Arthrobacter* (OTU 16; 1.1%) and *Microbacterium* (OTU 27; 1.9%) belonging to Actinobacteria and *Chamaesiphon* (OTU 81; 3.4%) and *Pleurocapsa* (OTU 255; 1.0%) belonging to Cyanobacteria.

The predominant OTUs found in post-clarification biofilm communities belong to Bacteroidetes, specifically *Flavobacterium* (OTUs 2, 24 and 157) and *Pseudarcicella* (OTU 1) with 12.2% and 2.5% relative abundance, respectively. *Microbacteriaceae* (OTUs 18, 27 and 44) family of Actinobacteria also contributed a high relative abundance of 7.4% (Figure 5.9). Clarified biofilms contained the

highest relative abundance of *Nitrospira* (OTUs 23 and 28; 5.2%) found in WTP 4.

Finally, predominant OTUs analysis of the filtered biofilm community showed the highest relative abundances were found in *Sphingomonadaceae* (OTUs 19, 25 and 21) and *Planctomycetaceae* (OTUs 30, 33, 67, 96, 126, 47, 49, 92, 141, 143, 150, 75, 77 and 125) at 7.5% and 21.5%, respectively. *Planctomycetaceae* consisted of five genera: *Schlesneria* (1.4%), *Pirellula* (7.8%), *Planctomyces* (8.1%), *Singulisphaera* (1.6%) and *Gemmata* (2.5%).

The relative abundance of coliforms and other bacterial genera of potential clinical significance in the biofilm communities of WTP 4 decreased with progressing treatment stage (Table 5.4). The highest abundances of note were *Acinetobacter* (1.25%) and *Pseudomonas* (1.73%) in raw water biofilm communities.

Table 5.4 - *Relative abundance (%) of bacterial genera in WTP 4 biofilms that contain species of potential clinical significance.*

Genus	Raw	Clarified	Filtered
Coliforms (*Enterobacteriaceae*)	0.125	0.036	0.002
Escherichia	0.002	0.001	0.001
Acinetobacter	1.250	0.158	0.001
Bacillus	0.283	0.048	0.004
Burkholderia	0.002	BLD	0.001
Clostridium	0.215	0.074	0.003
Corynebacteria	0.055	0.013	BLD
Enterococcus	0.021	0.006	BLD
Legionella	0.200	0.101	0.109
Mycobacterium	0.325	0.262	0.163
Nocardia	0.013	0.002	0.002
Prevotella	0.008	0.022	0.002
Pseudomonas	1.730	0.946	0.015
Rickettsia	0.054	0.019	0.059
Staphylococcus	0.005	0.002	0.001

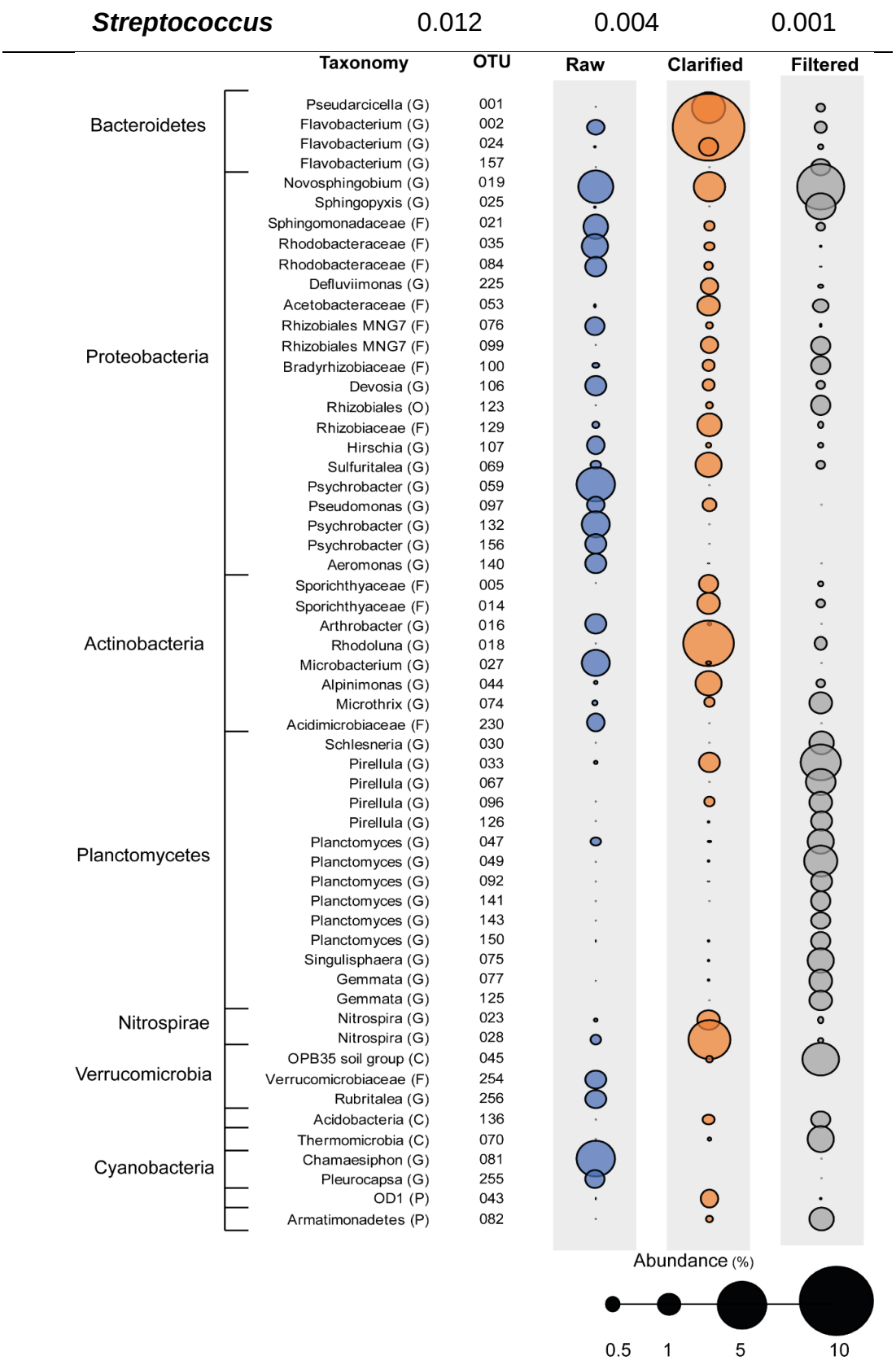


Figure 5.9 - *Predominant OTUs in WTP 4 biofilm samples (≥0.8% relative abundance) at raw, clarified and filtered treatment stages. OTUs are classified to*

genus level (G) where possible. Relative abundance of OTUs is represented by area of the bubbles.

5.4.2 Bulk water community alpha diversity

The community composition of bulk water passing through WTP 4 was very similar at each stage of treatment and the bacterial phyla are shown in Figure 5.10. Again, triplicate samples were consistent at all stages. From these data, it would appear that treatment stage did not exert a significant selection pressure on bulk water community composition.

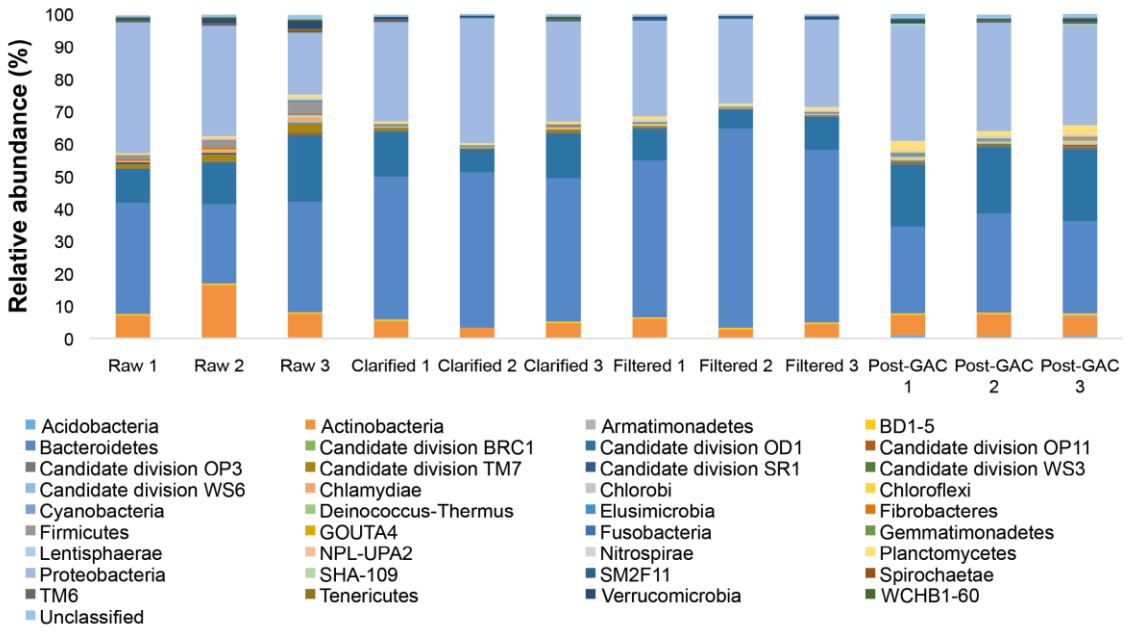


Figure 5.10 – *Bacterial phyla present in triplicate bulk water samples taken from four treatment stages at WTP 4. Community composition is similar throughout the treatment plant, with Bacteroidetes, Proteobacteria, Candidate division OD1 and Actinobacteria ubiquitous.*

The dominant phyla throughout WTP 4 were Bacteroidetes, Proteobacteria and Candidate division OD1 (Figure 5.11). Bacteroidetes constituted 30.9%, 45.4%, 54.3% and 28.6% mean relative abundance from raw, clarified, filtered and post-GAC treatment stages, respectively. Proteobacteria were responsible for 14.9%, 11.7%, 8.7% and 20.5% of raw, clarified, filtered and post-GAC bulk water, respectively. Finally, Candidate division OD1 comprised 31.5%, 33.6%, 27.7% and 33.6% of raw, clarified, filtered and post-GAC bulk water community, respectively. Actinobacteria was also present at all stages: 10.5%, 4.7%, 4.6%

and 6.7% at raw, clarified, filtered and post-GAC, respectively. Unlike the bulk water community of WTP 3, there was no dramatic change in composition after passage through the GAC filter; the proportions of Bacteroidetes decreased and OD1 increased, however, these changes are small compared to the domination of Planctomycetes observed at WTP 3.

The diversity of WTP 4's bulk water community is described in Figure 5.11. These results differ from the observations of bulk water communities in WTP 3, in that the post-GAC bulk water appears to have higher community richness and diversity than clarified and filtered bulk water from earlier stages in the treatment process.

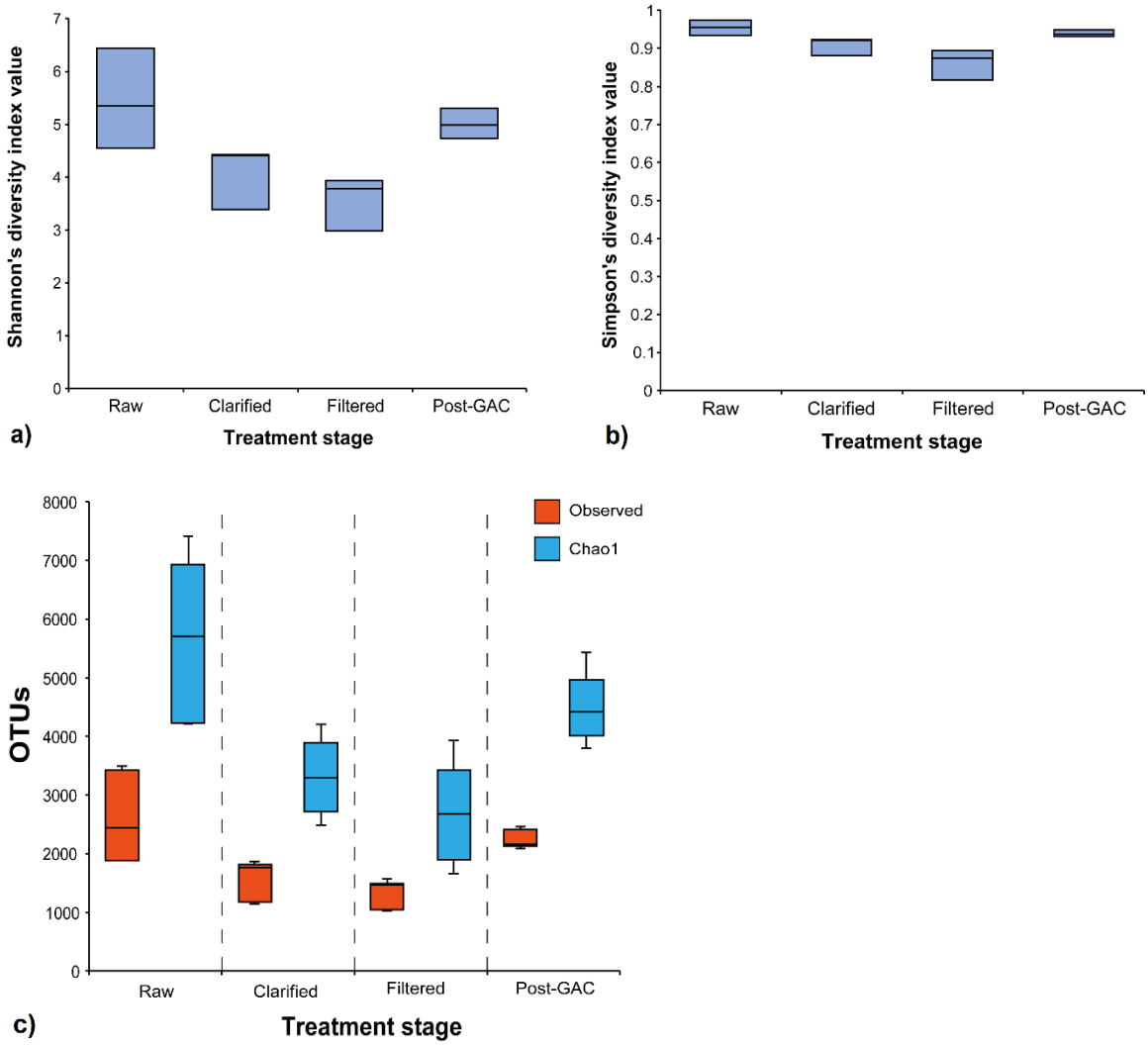


Figure 5.11 – *Diversity within the bulk water community of WTP 4 as represented by boxplots of* ***a)*** *Shannon's diversity index values,* ***b)*** *Simpson's diversity index values and* ***c)*** *observed species and Chao1 values.*

As measured by observed species, community richness decrease in the order of raw > post-GAC > clarified > filtered (Figure 5.12). This difference is statistically significant ($p < 0.001$) apart from clarified vs filtered, where $p = 0.06$. ANOVA and Tukey's HSD analysis of community richness measured using Chao1 values shows each treatment stage is significantly different to every other stage ($p < 0.05$).

Raw bulk water was found to be significantly more diverse than filtered bulk water ($p < 0.05$); however, Shannon's diversity index showed no other significant difference in diversity between treatment stages. According to Simpson's diversity index, the filtered bulk water community was significantly less diverse ($p < 0.05$) than raw and post-GAC bulk water. Both raw and post-GAC had high Simpson's diversity values of 0.93 – 0.97 and 0.93 – 0.95, respectively.

In accordance with phyla composition and diversity analysis, the predominant 47 OTUs (≥0.25% relative abundance at one or more treatment stage) of WTP 4 bulk water community were extremely similar at all treatment stages and are shown in Figure 5.12.

Two genera belonging to Bacteroidetes were highly prevalent in bulk water throughout WTP 4: *Pseudaricella* (OTU1) and *Flavobacterium* (OTUs 2, 24, 78, 103, 162, 224, 239, 240 and 268) (Figure 5.13). *Pseudaricella* increased from 5.7% in raw bulk water, to 20.7% in clarified, then to 29.8% in filtered, before decreasing to 16.6% relative abundance in post-GAC bulk water. *Flavobacterium* remained at a fairly constant proportion in the first three treatment stages (19.4%, 19.6% and 20.2% at raw, clarified and filtered, respectively) before decreasing to 9.0% in the post-GAC community.

The other predominant phyla in WTP 4 bulk water was Proteobacteria and two genera in particular were present at high relative abundances at all treatment stages (Figure 5.13). *Polynucleobacter* (OTU 6) increased through the treatment works, with relative abundances of 2.7%, 8.8%, 9.1% and 14.3% in the raw, clarified, filtered and post-GAC communities, respectively. *Limnohabitans* (OTUs 9, 10 and 79) remained fairly constant and comprised 8.7%, 13.0%, 10.6% and 7.9% of the raw, clarified, filtered and post-GAC bulk water communities, respectively.

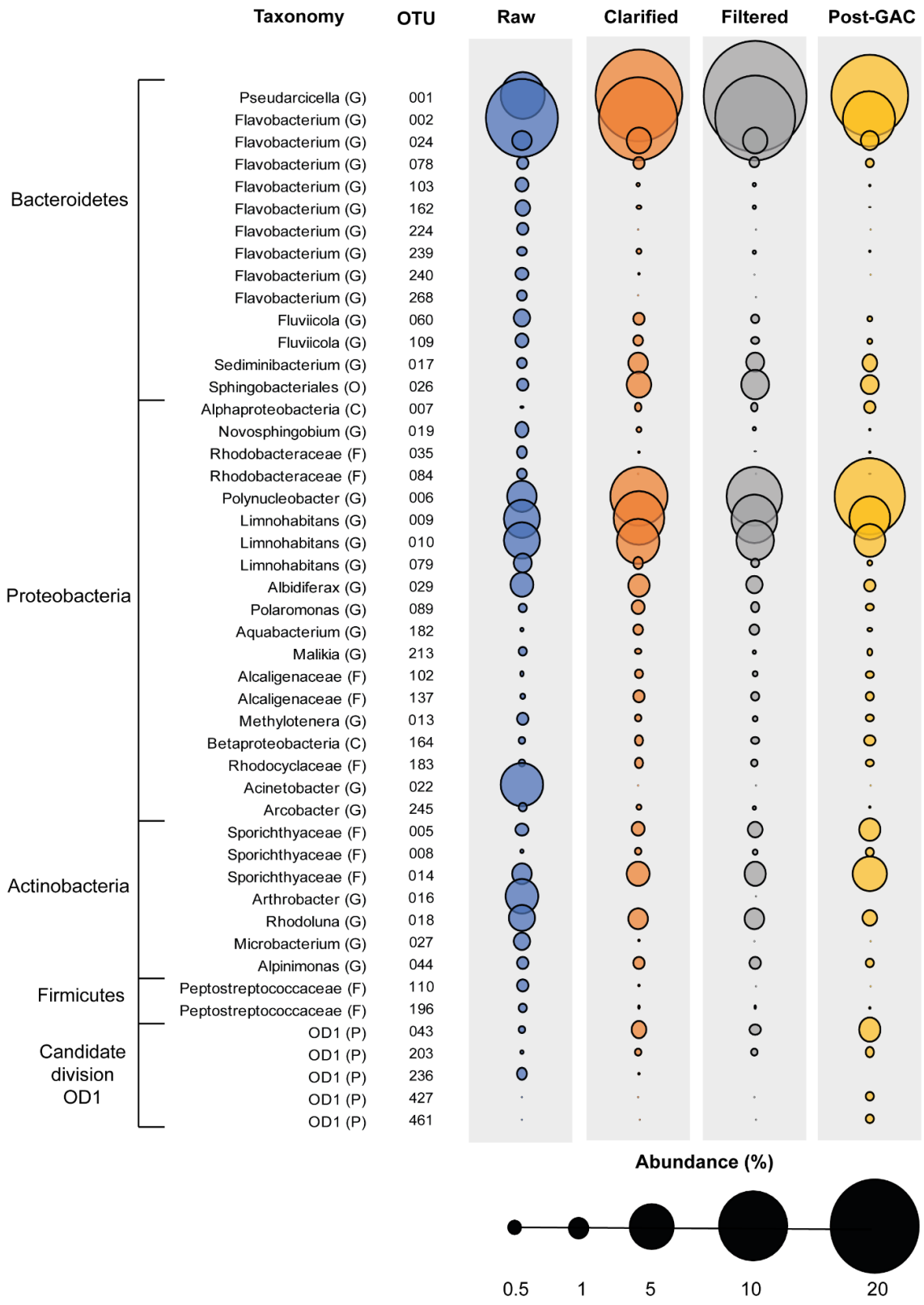


Figure 5.12 - *Predominant OTUs in WTP 4 bulk water samples (≥0.25% relative abundance) at raw, clarified, filtered and post-GAC treatment stages. OTUs are classified to genus level (G) where possible. Relative abundance of OTUs is represented by area of the bubbles.*

The relative abundance of potentially significant “clinical” bacterial genera in WTP 4 bulk water is described in Table 5.5. Coliforms and the majority of other groups of concern are a very small proportion of the overall community. A notable exception to this is *Acinetobacter*, which comprises 5.5% of raw bulk water.

Table 5.5 - *Relative abundance (%) of bacterial genera in WTP 4 bulk water that contain species of potential clinical significance.*

Genus	Raw	Clarified	Filtered	Post-GAC
Coliforms (*Enterobacteriaceae*)	0.042	0.007	0.001	0.008
Escherichia	BLD	BLD	BLD	BLD
Acinetobacter	5.530	0.025	0.004	0.007
Bacillus	0.190	0.019	<0.001	<0.001
Burkholderia	0.001	0.003	BLD	0.001
Clostridium	0.227	0.030	0.004	0.002
Corynebacteria	0.073	0.001	BLD	BLD
Enterococcus	0.005	0.003	BLD	BLD
Legionella	0.065	0.081	0.050	0.055
Mycobacterium	0.196	0.105	0.074	0.048
Nocardia	0.009	BLD	BLD	BLD
Prevotella	0.041	0.013	0.011	0.004
Pseudomonas	0.339	0.144	0.048	0.046
Rickettsia	0.017	0.012	0.015	0.026
Staphylococcus	0.012	0.003	<0.001	0.003
Streptococcus	0.005	0.001	BLD	BLD

5.5 Comparison of bacterial communities across WTPs

Having compared bacterial community composition across treatment stages within each WTP in Sections 5.3 and 5.4, this section focuses on differences in phylum composition and community diversity between WTPs. Figure 5.13 displays the phylum composition of biofilm and bulk water samples at the raw, clarified and filtered treatment stages that have been described in earlier results sections. However, here samples from each WTP are shown directly next to each

other, thus allowing clearer visualisation and comparison between source water types at the two WTPs.

As can be seen in Figure 5.13 (a, b), raw water biofilm and bulk water communities have noticeably different community compositions from WTP 3 and 4. WTP 3 raw biofilms contain high relative abundances of Nitrospirae and Chloroflexi, which in contrast, are present in very low abundances in WTP 4 raw biofilms. Conversely, WTP 4 raw biofilm contain high relative abundances of Actinobacteria, Verrucomicrobia and Cyanobacteria, whereas this is not seen in WTP 3 biofilms. The main phyla of raw bulk water are Proteobacteria, Bacteroidetes and Actinobacteria, however, WTP 3 and 4 contain different proportions of each phyla. WTP 4 raw bulk water communities also contain a high relative abundance of Candidate division OD1 (31.5%), which is only 4.1% in WTP 3.

Conversely, bulk water from post-clarification and post-filtration stages contain the same predominate phyla (Proteobacteria, Bacteroidetes, Actinobacteria and Candidate division OD1) at each WTP, but relative proportions of Actinobacteria and Candidate division OD1 are vastly different in WTP 3 and WTP 4 at both stages.

WTP 3 and 4 biofilm communities from the clarified treatment stage appear to be more similar to each other than was observed in the raw biofilm communities or bulk water communities from any stage. For example, clarified biofilms from each WTP have similar proportions of Proteobacteria (16.0% and 18.5%), Bacteroidetes (35.7% and 40.6%), Planctomycetes (5.2% and 4.6%) and Verrucomicrobia (4.0% at both plants). The major difference at this treatment stage is a relative abundance of 5.6% Nitrospirae at WTP 4, whereas WTP 3 contains only 0.5%.

Filtered stage biofilms from WTP 3 and WTP 4 contain the same relative abundance of Actinobacteria (4.6%), however, proportions of all other predominate phyla are slightly different. Biofilms from WTP 3 filtered treatment stage contain higher abundances of Proteobacteria (42.2%) and Planctomycetes (43.2%) than WTP 4 (28.3% and 35.8%, respectively). Conversely, WTP 4 contains larger proportions of Bacteroidetes (5.8% as opposed to 2.2% at WTP

3), Chloroflexi (5.7% compared to WTP 3's 5.7%) and especially Verrucomicrobia (5.8% as opposed to 0.6% at WTP 3).

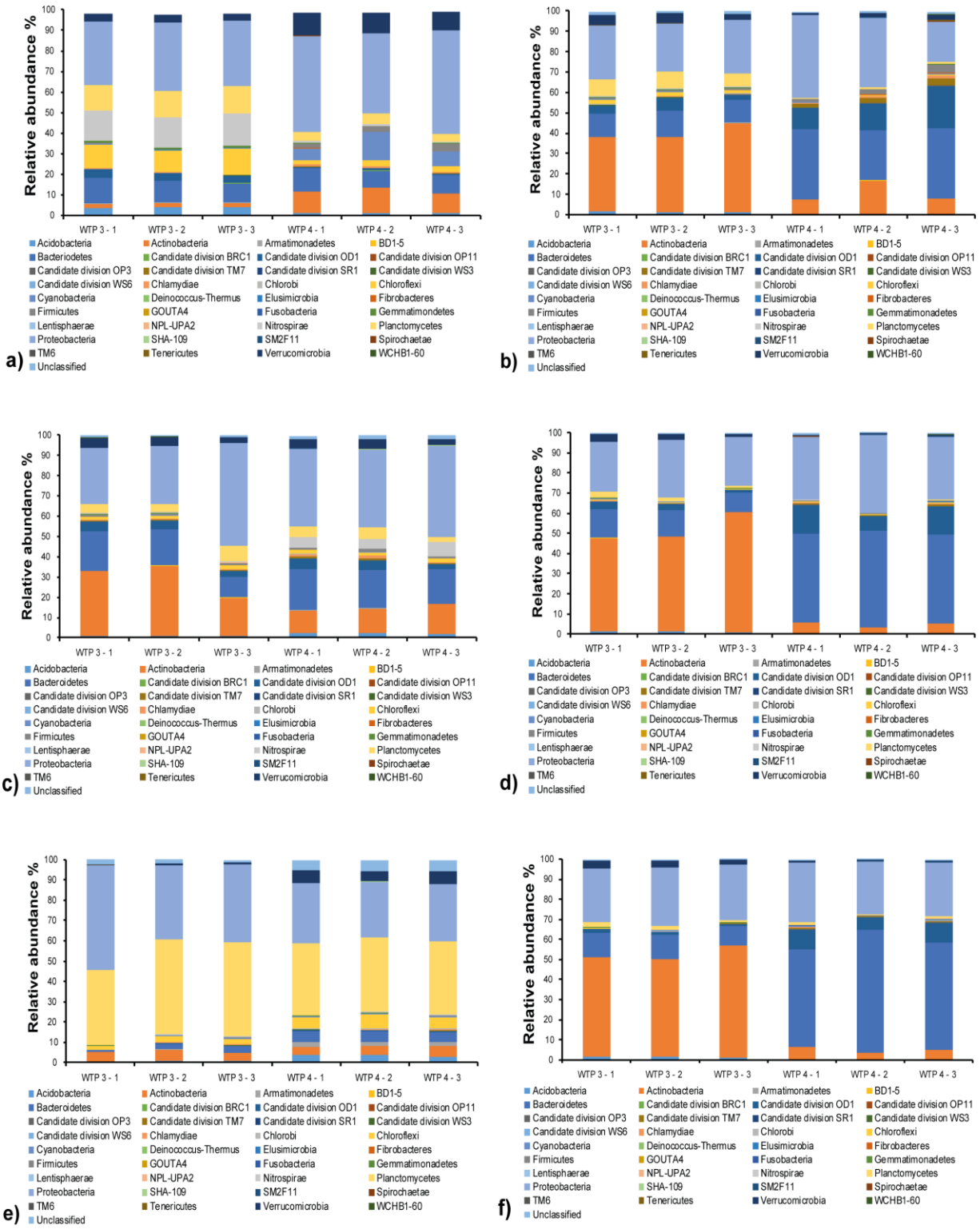


Figure 5.13 – *Phylum composition of WTP 3 and 4 in* ***a)*** *raw biofilms,* ***b)*** *raw bulk water,* ***c)*** *clarified biofilms,* ***d)*** *clarified bulk water,* ***e)*** *filtered biofilms and* ***f)*** *filtered bulk water.*

Since post-GAC biofilms could not be collected at WTP 4, only the bulk water communities are shown in Figure 5.14. The most dramatic difference in community composition between the two WTPs is seen at this treatment stage. WTP 3 bulk water has high relative abundances of Planctomycetes (76.8%), Cyanobacteria (7.7%) and Firmicutes (5.4%), whereas WTP 4 post-GAC bulk water community consists of Bacteroidetes (54.3%), Proteobacteria (27.7%) and Candidate division OD1 (8.7%).

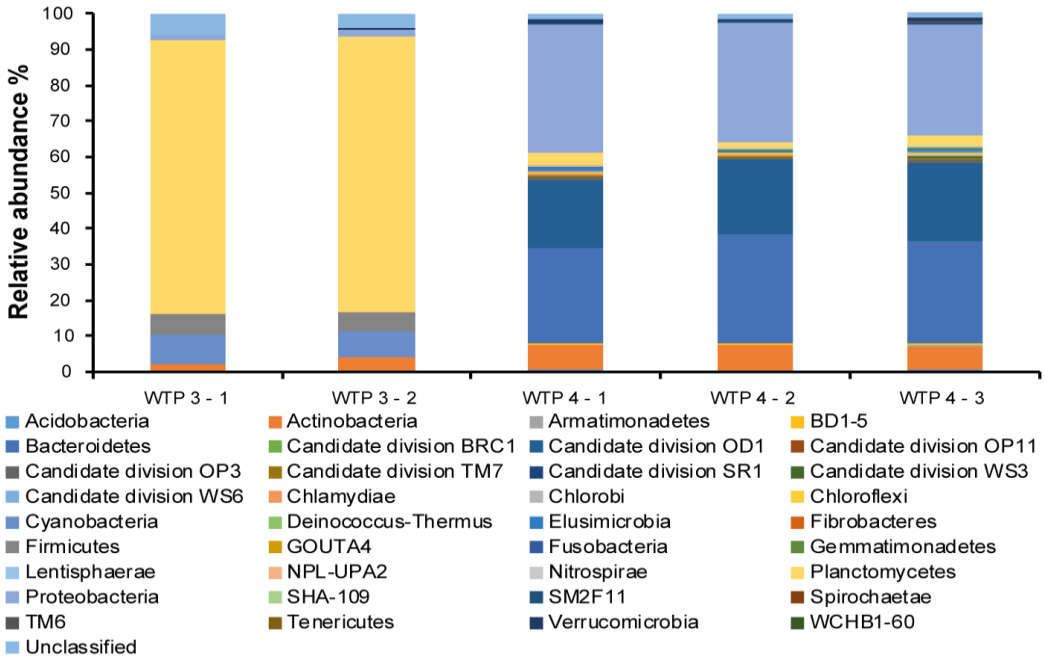


Figure 5.14 – *Phylum composition of WTP 3 and WTP 4 in post-GAC bulk water.*

Observed species, Chao1, Shannon's diversity index and Simpson's diversity index values previously calculated were compared at each treatment stage and sample type by independent t-test to determine whether there are significant differences in community richness and diversity between WTP 3 and WTP 4 (Table 5.6). The bacterial communities of WTP 4 were found to have significantly higher richness values than WTP3 at all treatment stages, in both biofilms and bulk water. However, when evenness was also taken in account in the Shannon's and Simpson's diversity indices, WTP 4 was significantly more diverse than WTP 3 in the clarified biofilm community and post-GAC bulk water community only.

Table 5.6 – *Significance (as measured by p-values resulting from independent t-test) of differences between WTP 3 and WTP 4 biofilm and bulk water communities. Significant values are highlighted in bold.*

	Raw		Clarified		Filtered		Post-GAC
	Biofilm	Bulk	Biofilm	Bulk	Biofilm	Bulk	Bulk
Observed species	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Chao1 value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Shannon's index	0.92	0.60	**0.04**	0.5	0.12	0.78	**0.001**
Simpson's index	0.89	0.46	0.40	0.31	0.14	0.12	**0.04**

5.6 Beta diversity between WTPs

In order to determine whether source water (WTP 3 reservoir or WTP 4 river), sample type (biofilm or bulk water) and treatment stage cause significant differences in overall community composition, beta diversity was analysed by calculating Bray-Curtis similarity (Bray and Curtis, 1957) and weighted UniFrac distances (Lozupone and Knight, 2005) between samples.

Principal coordinate analysis of Bray-Curtis similarities are shown in Figure 5.15. As can be seen in Figure 5.15(a), samples from each WTP clustered clearly into two separate groups, indicating the microbial community of the source water is a much stronger factor in determining community composition throughout a treatment plant than treatment processes. When labelled with both WTP and sample type (Figure 5.15(b)), the samples from WTP 4 form two distinct clusters of biofilm and bulk water samples. At WTP 3, however, four clusters are recognizable – the biofilm samples form one clearly defined cluster and another cluster which overlaps with bulk water samples and vice versa. It appears that the biofilm and bulk water communities are more similar at WTP 3 than at WTP 4.

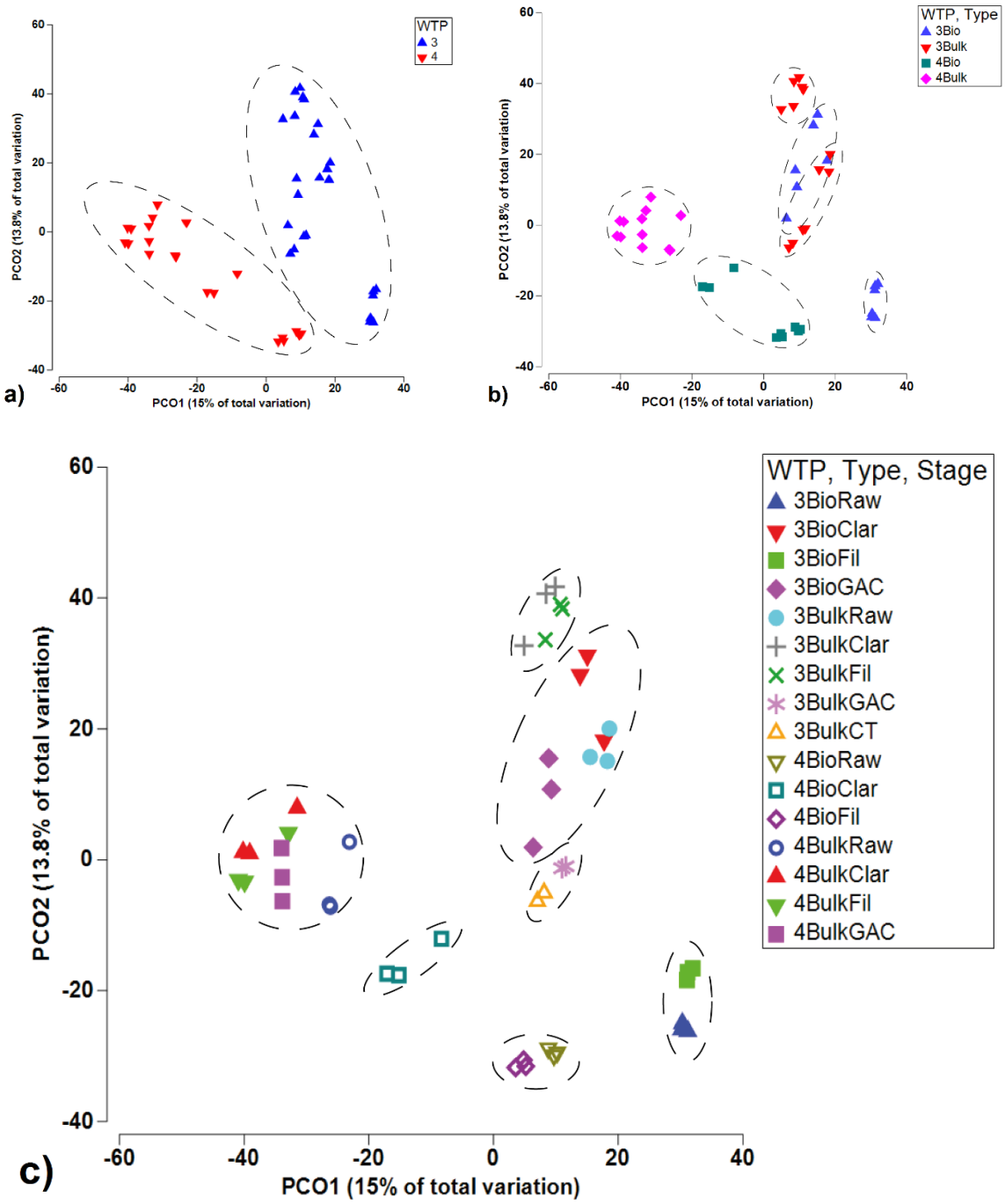


Figure 5.15 – *PCOs of Bray Curtis similarities, highlighted by **a)** WTP, **b)** WTP and sample type and **c)** WTP, sample type and treatment stage.*

The final panel in Figure 5.15 shows the coordinates of samples as labelled by WTP, sample type and treatment stage and shows the majority of triplicate samples cluster together closely. Interestingly, at both WTPs the raw and filtered biofilm samples form a group (at the bottom right of the graph) and are more similar to each other than to post-clarification biofilms. The post-GAC biofilm community samples from WTP 3 are more similar to the clarified samples than

raw or filtered biofilms. Bulk water samples from WTP 3 are separated into three clusters: clarified and filtered; post-GAC and contact tank; and raw samples (the community of which is more similar to clarified and post-GAC biofilms than bulk water at other treatment stages). This is quite different to the bulk water communities of WTP 4, which cluster together closely, apparently irrespective of treatment stage. Raw bulk water samples slightly separate from samples from later treatment stages.

The significance of beta diversity was tested by ANOSIM and PERMANOVA (Table 5.7). Source water (i.e. WTP 3 or 4), sample type (biofilm or bulk water) and treatment stage were all found to have a significant effect on community composition (0.1% significance level as measured by ANOSIM, p = 0.001 as measured by PERMANOVA). The effect of the interaction of all three factors was also found to be significant.

Table 5.7 – *Beta diversity (as calculated by Bray-Curtis similarity) significance of WTP, sample type (biofilm or bulk water) and treatment stage as measured by ANOSIM and PERMANOVA. The significance of the interaction between different combinations of the three factors was also tested by PERMANOVA.*

Factor	ANOSIM		PERMANOVA	
	Global R	Significance level	p-value	Square root of ECV
WTP (W)	1.0	0.1	0.001	29.9
Sample type (S)	0.95	0.1	0.001	24.8
Treatment stage (T)	0.749	0.1	0.001	28.6
W x S	-	-	0.001	24.7
W x T	-	-	0.001	29.5
S x T	-	-	0.001	30.0
W x S x T	-	-	0.001	23.3

Beta diversity was also analysed by comparison of weighted UniFrac distances between samples (Figure 5.16). As when measured by Bray-Curtis similarity, the bacterial communities of WTP 3 and WTP 4 form two separate groups with no overlap (Figure 5.16(a)). Figure 5.16(b, c) show that bulk water samples from

WTP 4 cluster together closely (again supporting results based on Bray-Curtis similarity). At WTP 3, bulk water communities from the clarified, filtered and GAC treatment stages are closely related (Figure 5.16(c)), while raw bulk water samples are markedly more distant. The biofilm communities of WTP 3 form distinct groups according to treatment stage and are spaced well apart, indicating a low level of relatedness. The same result is seen in the biofilm communities of WTP 4 (Figure 5.16(c)). The effect of WTP source water, sample type and treatment stage on bacterial community composition was found to be significant when measured by ANOSIM and PERMANOVA of weighted UniFrac distances (Table 5.8)

Table 5.8 – *Beta diversity (as calculated by weighted UniFrac distances) significance of WTP, sample type (biofilm or bulk water) and treatment stage as measured by ANOSIM and PERMANOVA. The significance of the interaction between different combinations of the three factors was also tested by PERMANOVA.*

Factor	**ANOSIM**		**PERMANOVA**	
	Global R	Significance level	p-value	Square root of ECV
WTP (W)	1.0	0.1	0.001	0.23
Sample type (S)	0.988	0.1	0.001	0.19
Treatment stage (T)	0.947	0.1	0.001	0.21
W x S	-	-	0.001	0.19
W x T	-	-	0.001	0.24
S x T	-	-	0.001	0.23
W x S x T	-	-	0.001	0.20

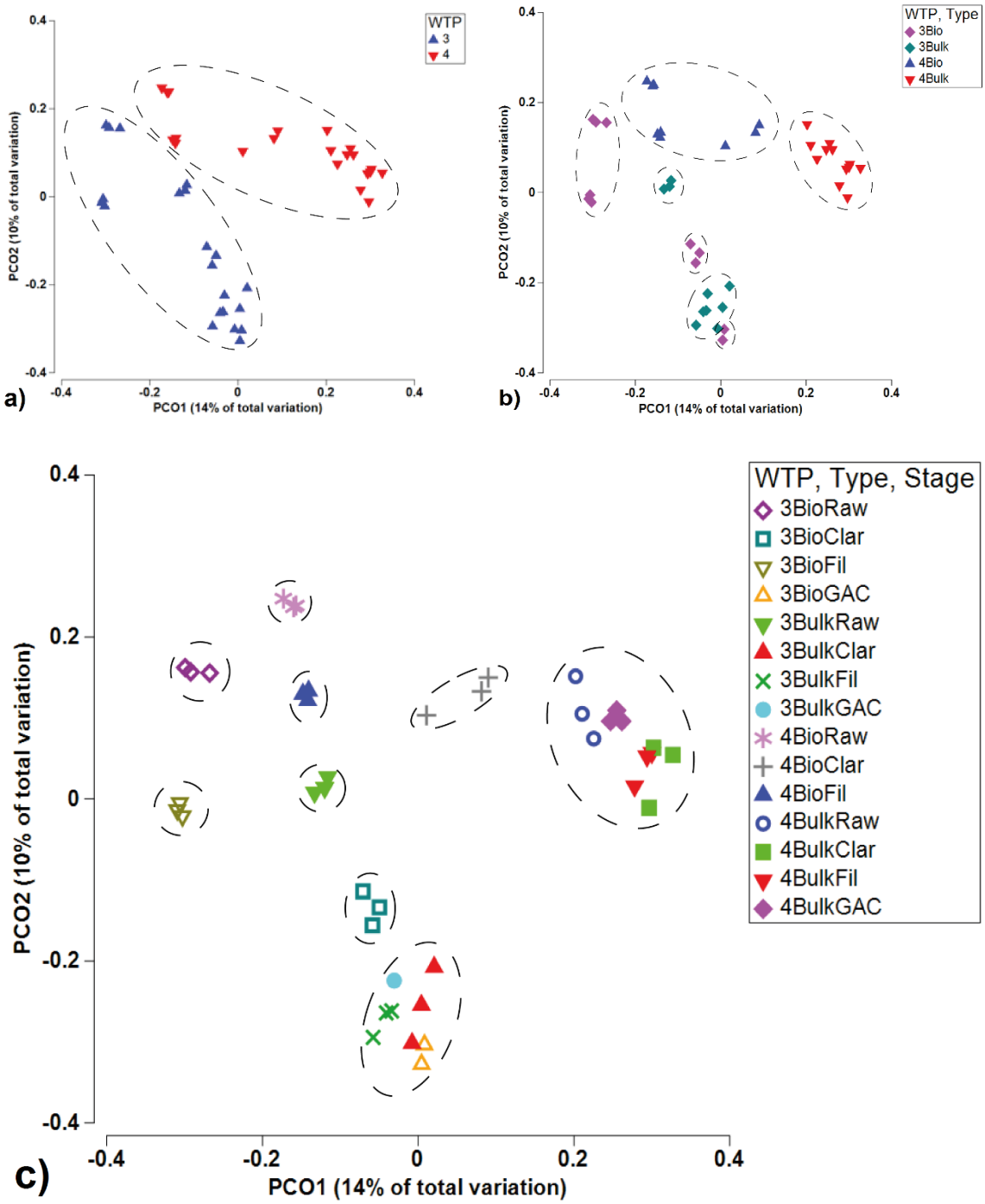


Figure 5.16 – *PCOs of weighted UniFrac distances, highlighted by **a)** WTP, **b)** WTP and sample type and **c)** WTP, sample type and treatment stage.*

5.7 Discussion

The most ubiquitous phyla of the WTP microbiome was Proteobacteria and this is consistent with previous research into drinking water microbiomes

(Lautenschlager *et al.*, 2013; Lin *et al.*, 2014; Liu *et al.*, 2014; Pinto *et al.*, 2014; Li *et al.*, 2016; Li *et al.*, 2017; Lin *et al.*, 2017; Hou *et al.*, 2018; Vanessa *et al.*, 2019). The phylum composition of WTP 3 and WTP 4 would suggest that the bulk water community across a WTP is not greatly impacted by treatment process. At both WTP 3 and 4, community composition appeared similar in raw, clarified and filtered bulk water and beta diversity analysis showed bulk water samples from the first three treatment stages clustered together. This supports the findings of Li *et al.* (2017), who observed a very stable community composition in treatment stages prior to passage through activated carbon and disinfection. In WTP 4, this trend continued (i.e. post-GAC bulk water had very similar community composition and clustered with all other WTP 4 bulk water samples). However, the community in post-GAC bulk water in WTP 3 was vastly different, with a huge increase in Planctomycetes. It would appear that the GAC filter at WTP 3 is producing a selective pressure for this phylum, however, the mechanism of action is unknown. Future study could include sampling the biofilm community of the filter material itself and chemical analysis of GAC influent and effluent.

Biofilm communities, on the other hand, had distinctly different community compositions at each treatment stage in WTP 3 and WTP 4. Beta diversity as calculated by weighted UniFrac distances (Figure 5.17(c)) shows this most clearly: each set of biofilm triplicates cluster together separately by treatment stage. These results suggest that treatment stage is a stronger determining factor of community composition in biofilms than bulk water. Considering the vastly different exposure time periods of planktonic bacterial cells passing through in bulk water compared to bacteria established in a fixed biofilm, this makes sense as treatment stage (i.e. the chemical and physical environment produced by the treatment process) would be more likely to shape biofilm communities than bulk water that is actively flowing through the WTP.

The community composition of biofilms was different to that of bulk water at every treatment stage, in both WTPs. This finding supports previous work by Lin *et al.* (2014) and Bereschenko *et al.* (2008). Biofilm communities were also found to have higher richness and diversity values at each treatment stage compared to bulk water communities; the only exception to this was at WTP 4 at the raw treatment stage, where bulk water had a richer community (as measured by

observed species and Chao1 value) than the biofilm community, although biofilm was more diverse when evenness was also taken into account. This is would suggest that river source water entering WTP 4 contains a higher number of unique or rare species than the biofilm community established from it. River water is likely to be the most changeable type of source water in terms of microbial community since environmental factors affecting the community can be highly variable over time: nutrient availability (Rubin and Leff, 2007), chemical composition (e.g. pH, temperature, dissolved organic carbon), heavy metals (Zhu *et al.*, 2013), pesticides (Pesce *et al.*, 2008; Ricart *et al.*, 2010) and wastewater treatment plant discharges (Wakelin *et al.*, 2008) have all been shown to influence microbial community structure and composition.

Community richness and diversity in biofilm communities decreased significantly with progressing treatment stage at both WTPs, showing the drinking water treatment process results in dominance of fewer species (mainly those belonging to Proteobacteria or Planctomycetes) in later treatment stages. Community richness in bulk water communities also decreased with progressing treatment stage at both WTPs, however, diversity was only significantly different in the raw bulk water. Although differences in diversity in later stages are present, they were not always statistically significant.

WTP 4 bacterial communities had higher values of community richness than WTP 3 communities, in both biofilm and bulk water samples. This probably is a direct reflection of a richer source-water bacterial community (i.e., river water) as opposed to reservoir water; the influence of the source water community appears to persist through the entire treatment process. However, according to diversity index values, only the bulk water community post-GAC and clarified stage biofilm community were significantly more diverse in WTP 4 than WTP 3, suggesting community evenness is very similar at the two WTPs and treatment processes have a normalising effect on resident WTP communities.

The biggest divergence between WTPs in seen in post-GAC bulk water: WTP 4 bulk water community is vastly richer and more diverse (observed species and Shannon diversity values were 172 ± 71 and 2.4 ± 0.2 at WTP 3 and 2238 ± 140 and 5.0 ± 0.3 at WTP 4). Community composition (Figure 5.15) is also vastly

different between WTPs. The huge difference seen between WTP 3 and WTP 4 at this stage is most likely due to factors associated with the granular activated carbon filter: the microbial community on the filter, its material and-or the removal of chemicals and organic compounds (or the combined effect of both) on the unique community composition of each WTP could be responsible for both the huge decrease in diversity and dominance of Planctomycetes at WTP 3 and the increase in diversity at WTP 4. As previously stated, it would be highly interesting to sample the microbial community of each GAC filter's material, focussing on the presence of fungi, macroinvertebrates and protozoa as well as bacteria. It would also be beneficial to carry out detailed chemical analysis of the bulk water pre- and post-GAC and determine whether environmental changes can be linked to changes in the microbial community.

Source water had the greatest effect on the microbiome of the WTP. As can be seen in the PCO plots of beta diversity, samples from WTP 3 and WTP 4 formed two completely separate clusters. If treatment stage produced a strong homogenising pressure on either the biofilm or bulk water community, some overlap between these two groups would be expected. Phylum community composition shown in Figure 5.14 demonstrates the different relative abundances in source water at the raw stage (in biofilm and bulk water) and how these differences between WTPs persist throughout the treatment process. This supports the findings of studies in water distribution systems, where source water was the main determining factor of community composition (Li *et al.*, 2016; Revetta *et al.*, 2016).

Predominant OTU analysis revealed the most commonly occurring bacterial groups of WTPs. Belonging to Proteobacteria, *Unibacterium* was highly prevalent in WTP 3 clarified and post-GAC biofilms; *Sphingomonadaceae* was highly abundant in WTP 4 raw biofilms and filtered biofilms at both WTPs; and Burkholderiales were predominant in WTP 3 contact tank bulk water and WTP 4 bulk water from all stages (*Polynucleobacter* and *Limnohabitans* were classified to genus level in contact tank bulk water). Bacteroidetes, namely *Pseudarcicella* and *Flavobacterium* were abundant in clarified biofilms at both WTPs and WTP 4 bulk water at all treatment stages. Two families of Actinobacteria (*Acidimicrobiaceae* and *Sporichthyaceae*) had high relative abundances in WTP

3 clarified biofilms and bulk water from raw, clarified and filtered stages. *Planctomycetaceae* were highly prevalent in WTP 3 in post-GAC and contact tank bulk water, as well as filtered biofilms from both WTPs. In the filtered biofilm communities, *Schlesneria, Gemmata* and *Planctomyces* were classified to genus level. Finally, *Nitrospira* was abundant in WTP 3 raw and WTP 4 clarified biofilms. *Nitropsira* has been previously found to be a dominant species in biologically active GAC filters (Lapara *et al.*, 2015; Lin *et al.*, 2017). Since biofilms were grown on glass slides rather than being sampled directly from GAC media, it's likely that any species which is highly unique or adapted to the filter material will not have been captured.

Coliforms were found to be a constant, but very small component of the WTP microbiome. Coliforms were detected in biofilms at the raw, clarified and filtered treatment stages, although they were below the limit of detection in the post-GAC biofilm in WTP 3 (it was not possible to collect a biofilm post-GAC sample at WTP 4). The highest relative abundance of coliforms (0.125%) in this study was found in the raw biofilm at WTP 4. Coliforms were also present in the community of bulk water at all treatment stages in both WTPs. The presence of coliforms is not unexpected at earlier treatment stages, however, the finding of 0.008% coliforms in the post-contact tank bulk water community at WTP 3 was surprising. No evidence of chlorine tolerance in coliforms has been found (Chapter 3) and biofilms in the contact tank did not develop to a detectable level of DNA even after 6 months establishment (Chapter 4) so it is highly improbable coliform bacteria were able to survive chlorination either by genetic resistance or biofilm shielding. Therefore, the most likely explanation is a breach in the water stream allowing ingress of coliform bacteria at a point somewhere between contact tank and sample point. It is possible biofilm shielding might still play a role in coliform survival: coliforms contained in an aggregation of other microbial cells detaching from a biofilm earlier in the process due to shear pressure might theoretically give enough physical protection from chlorine for the duration of contact time. Physical shielding from chlorine by attachment to particles is another possibility, however, more data is needed to develop an estimation of actual risk. It would also be expected that bacterial survival strategies such as shielding within biofilms or particles are occurring at roughly the same rate in all WTPs, therefore, persistent

treatment failures at individual WTPs are more likely to be due to operational factors.

Data discussed here show that the microbiome of WTPs is spatially dynamic; community composition is a product of complex interactions between the incoming microbial community of source water, treatment stage and growing environment (biofilm or planktonic). There are also many further variables in working WTPs that will have a great influence on community composition that were not within the scope of this study: namely, the effect of seasonal fluctuations in temperature; pH variations; biotic and abiotic particle density; chemical properties of the water environment (e.g. dissolved organic carbon, concentration of nitrogen and phosphorous); operational events such as plant shut-downs, the effect of pipe materials and the presence or absence of other microorganisms (such as fungi, protozoa and viruses) in biofilms and bulk water.

Having characterized the microbiome of two typical UK WTPs in terms of bacterial community composition and change, the next step would be to focus further on the forces responsible for shaping the community: this study determined ‘who’ is present, the next step is to find out ‘why’ they are present. A second sampling survey incorporating measurement of the factors listed above (such as chemical parameters of water quality) would provide valuable insight into the drivers of biofilm and bulk water microbial composition in WTPs and highlight any variables that could be controlled in order to produce a WTP microbiome for optimal water treatment performance.

5.8 Conclusions

This chapter characterised the microbiome of two WTPs across treatment stage in both biofilm and bulk water environments. The main findings are as follows:

1. Predominant phyla of the WTP microbiome are Proteobacteria, Planctomycetes, Bacteroidetes, Candidate division OD1, Actinobacteria, Nitrospirae, Chloroflexi, Cyanobacteria and Firmicutes.
2. Source water community is the main determining factor of WTP community composition.
3. Treatment stage exerts a significant pressure on biofilm community composition.

4. Biofilm and bulk water communities are significantly different to each other throughout WTPs.
5. In general, biofilm communities are more diverse than bulk water communities.
6. River source water produces bacterial communities with higher richness and diversity values than reservoir source water.
7. Coliforms are a small but persistent proportion of biofilm and bulk water WTP bacterial communities.

Chapter 6 – Conclusions and recommended future work

6.1 Conclusions

The core purpose of this thesis was to develop a detailed understanding of the bacterial communities of WTPs and thus provide greater knowledge of the possible fate of coliform bacteria in the drinking water treatment process. Coliforms isolated from WTPs were investigated for chlorine tolerance and the chlorine tolerance of *E. coli* in particular was studied in detail (Chapter 3). Two WTPs were thoroughly characterised by quantification and identification of bacterial communities across spatial and temporal parameters (Chapters 4 and 5).

The following hypotheses were stated in the introduction to this thesis:

1) Coliforms have developed resistance or tolerance against chlorine.
2) Biofilms are a source of coliforms in WTPs.
3) The bacterial community composition of biofilms and bulk water is significantly different.
4) Bacterial communities in WTPs are affected by treatment stage.
5) Bacterial communities in WTPs are affected by the community of source water.

All three chapters contributed to the main aim of the thesis by increasing microbial knowledge of WTPs and providing information that can be used to inform operating practice.

The first experimental chapter describes a survey of coliform bacteria from five WTPs for high levels of chlorine tolerance (Chapter 3). The key finding of this chapter was that no coliform isolate showed evidence of significant chlorine tolerance. When tested in the absence of any interfering environmental conditions (i.e. when survival in chlorine could only be caused by genetically-encoded increased tolerance), all coliforms were killed or inactivated after 2 minutes exposure to 0.5 mg/l free chlorine residual. This indicates coliforms are highly sensitive to chlorine and have not developed significant chlorine tolerance. The lack of evidence of genetic chlorine tolerance in coliforms is an important

finding because it makes the possibility of genetic resistance as a cause of coliforms failures highly unlikely. This is useful knowledge for WTP operators as in the event of a coliform failure, efforts can be focused on more likely explanations or interventions without the question of whether a genetic trait of the bacteria themselves is responsible for the failure. Considering the huge implications and potential risk to drinking water safety if chlorine tolerance or resistance *was* developing in coliforms, it is important to have investigated the possibility.

Chapter 3 also concluded that while *E. coli* was very chlorine-sensitive, small but significant differences in chlorine tolerance can be observed in strains isolated from different environments. Lab strain *E. coli* was significantly less tolerant than *E. coli* isolated from WTPs, which in turn was less tolerant than *E. coli* isolated from human faecal samples of hospital patients. This finding raises some fundamental questions about the practice of using lab strain bacteria as proxies for environmental strains. At least in the case of *E. coli* response to chlorine, lab strain bacteria were not representative of strains from natural or wild environments. In terms of practical advice for WTPs, any chlorination efficacy models being designed or commissioned are highly recommended to use data based on environmental isolates. Although the sample size was insufficient to definitively prove genetic chlorine tolerance in coliforms has not developed, the findings of Chapter 3 provide evidence for the rejection of hypothesis 1.

The remaining experimental chapters aimed to characterise the microbiome between and across two WTPs by quantifying total bacteria in bulk water and biofilms at five treatment stages over 6 months (Chapter 4) and by metagenomic sequencing of biofilm and bulk water communities at five treatment stages (Chapter 5).

Chapter 4 provided useful knowledge on biofilm formation in WTPs: biofilms were not established to detectable levels in the chlorine contact tank after 6 months sampling and at the majority of other treatment stages, biofilms reached stable levels of total bacteria after 1 month’s growth. While biofilm growth in the high chlorine concentrations present in contact tanks was unlikely, it is again important to confirm this as if biofilms *had* developed, the microorganisms in question

would be extremely chlorine tolerant and potentially dangerous as protection and reservoir for pathogenic organisms. Other conclusions of Chapter 4 state total bacteria in bulk water decreased with advancing treatment stage. The same effect was seen in biofilms at WTP 3, however, total bacteria abundance in biofilms was not reduced until after filtration at WTP 4. No significant difference in total bacteria entering the treatment plants was found between the reservoir (WTP 3) and river (WTP 4) source. This was surprising since much higher counts of coliforms and *E. coli* are found in routine monitoring at WTP 4 than WTP 3. This suggests that while total bacteria abundances are similar, river source water has higher proportions of indicator organisms and possibly pathogens.

The predominant phyla of bacterial communities in the two WTPs were identified as Proteobacteria, Planctomycetes, Bacteroidetes, Candidate division OD1, Actinobacteria, Nitrospirae, Chloroflexi, Cyanobacteria and Firmicutes (Chapter 5). Source water was found to be the main determining factor of WTP community composition, with treatment stage also exerting a significant pressure on biofilm communities. Biofilm and bulk water communities had significantly different community composition to each other at every treatment stage. Based on these findings, hypotheses 3, 4 and 5 can be accepted. Further conclusions state that biofilm communities were more diverse than bulk water in the majority of samples and that river source water produces greater richness and diversity in bacterial communities than reservoir source water.

Genera that contain species with potential clinical significance were identified in the majority of samples, however, generally at very low proportions. Coliforms were present (although at very low relative abundances) throughout biofilm and bulk water communities at both WTPs. Insofar as coliforms were detected in biofilm samples, hypothesis 2 can be accepted. However, the relative abundances were not above expected and biofilms are certainly not being dominated by coliform bacteria. Unfortunately, the nature of coliform treatment failures as rare and unlikely events means that shielding within a biofilm of other microorganisms cannot be ruled out as a root cause. It is theoretically possible for coliforms to be released from biofilms pre-disinfection within an aggregate of cells and survive chlorination by avoiding physical exposure. However, it would be expected that these events occur at roughly equal rates in similar WTPs,

therefore, high frequencies of failures at one WTP in particular are more likely due to another cause.

6.2 Future work

There are many possible directions future research could follow in order to expand on the findings detailed in this thesis. Two main topics are suggested: further investigation into possible causes of coliform failures and additional research into the microbial communities of UK WTPs.

Firstly, it would be highly beneficial to quantify the number of coliforms present in biofilm samples obtained in this survey. Relative abundances provide useful knowledge related to overall community composition, but exact numbers could be more easily used by WTP operators in estimations of risk. FISH or qPCR could be used to detect and quantity coliforms and *E. coli.* qPCR based on the lacZ gene for coliforms and rodA gene for *E. coli* was attempted during the course of this thesis, however, reliable qPCR standards were not successfully made in the time frame of study. Future study could complete this work and allow comparisons in coliform numbers between biofilm and bulk water as well as qPCR data and culture-based routine monitoring. Quantification of coliforms in biofilms could also allow the use of modelling to predict the likelihood of biofilm detachments containing coliforms, although a great deal of data on biofilm formation and behaviour in WTPs would be required.

Secondly, the installation of particle counters at the post-GAC pre-chlorination stage would provide information on the amount and size of particles entering the contact tank. Turbidity is not always a good indicator for particle density and particle counters would provide more accurate data. Coliforms can be shielded from chlorine by attachment to and within particles, therefore, it would be highly useful to know whether persistent treatment failures can be correlated with influxes of a large amount of particles or the presence of particles of a particular size.

Future work concerning expansion of the knowledge of bacterial WTP communities described in this thesis could focus on a number of questions. An

area of major improvement in future work would be the collection of chemical and environmental data to determine whether changes in bacterial community composition can be correlated with environmental factors. Discovering the main drivers of community composition would be the first step in identifying possible interventions to shape the bacterial community as desired for optimal water treatment.

Having characterised the bacterial community, the entire microbiome would be revealed by sequencing for eukaryotes such as protozoa and fungi as well as viruses. Bacterial community composition is highly likely to be influenced by the presence of these microorganisms and interaction effects could be determined.

Metagenomic sequencing (Chapter 5) was based on DNA, specifically the rRNA gene. The community composition based on RNA, representing ‘active’ bacterial groups, could be significantly different and future work could compare these two datasets. It would be of interest to establish whether there is a greater proportion of ‘active’ bacteria in biofilms or bulk water.

Finally, greater or more representative knowledge of WTP biofilm communities could be obtained by sampling directly from filter material in the sand and anthracite filters and GAC filters. If glass slides used in the biofilm collector could be replaced with materials matching the pipe or tank walls of the treatment stage being studied, it would mitigate the influence of a different surface material on biofilm formation and composition.

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Appendix A

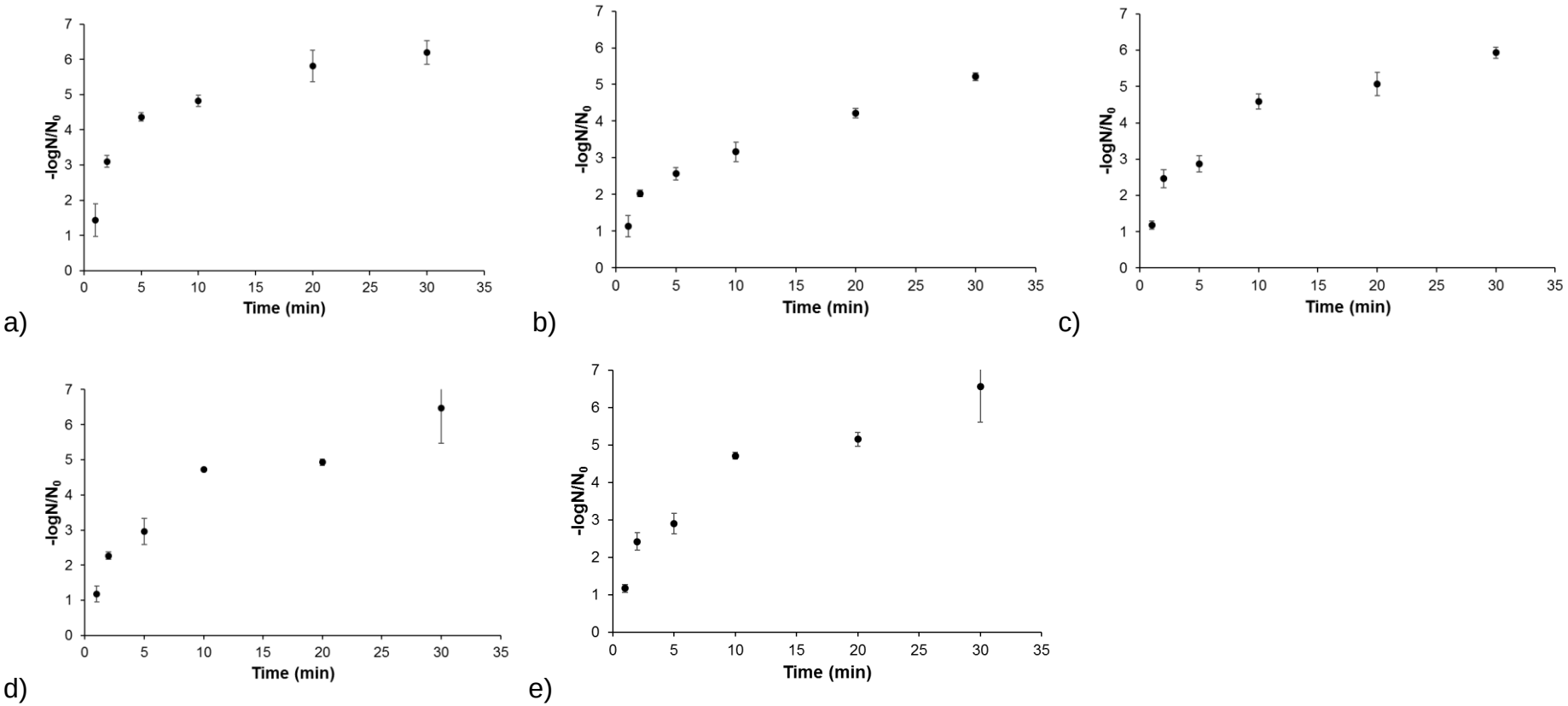

Figure A.1 – *Inactivation of E. coli isolates a) WTP 1, b) WTP 2, c) WTP 3, d) WTP 4 and e) WTP 5, in response to 0.05 mg/l free chlorine over 30 minutes contact time.*

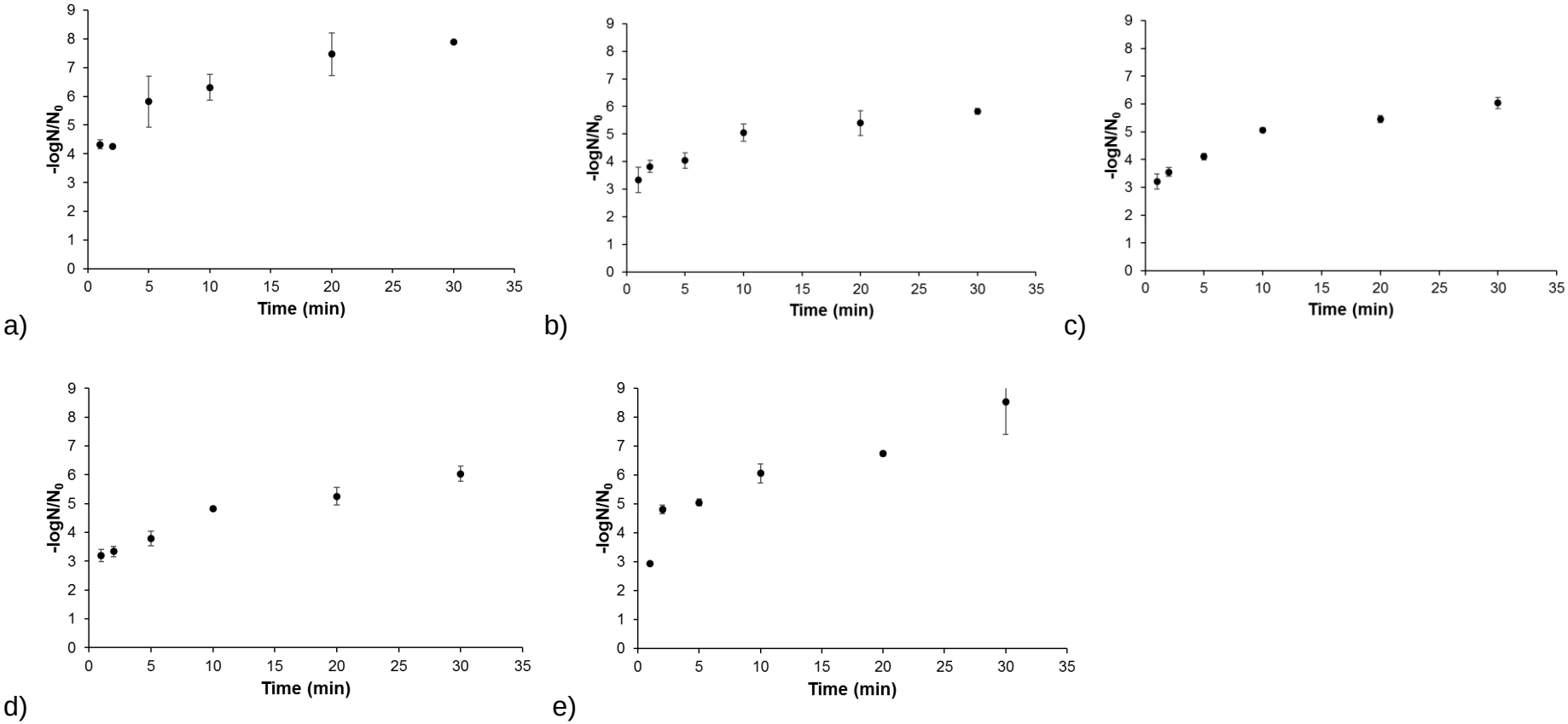

Figure A.2 – *Inactivation of E. coli isolates a) L1 (9001), b) L2 (10418), c) L3 (12486), d) L4 (MG1655) and e) L5 (13125), in response to 0.05 mg/l free chlorine over 30 minutes contact time.*

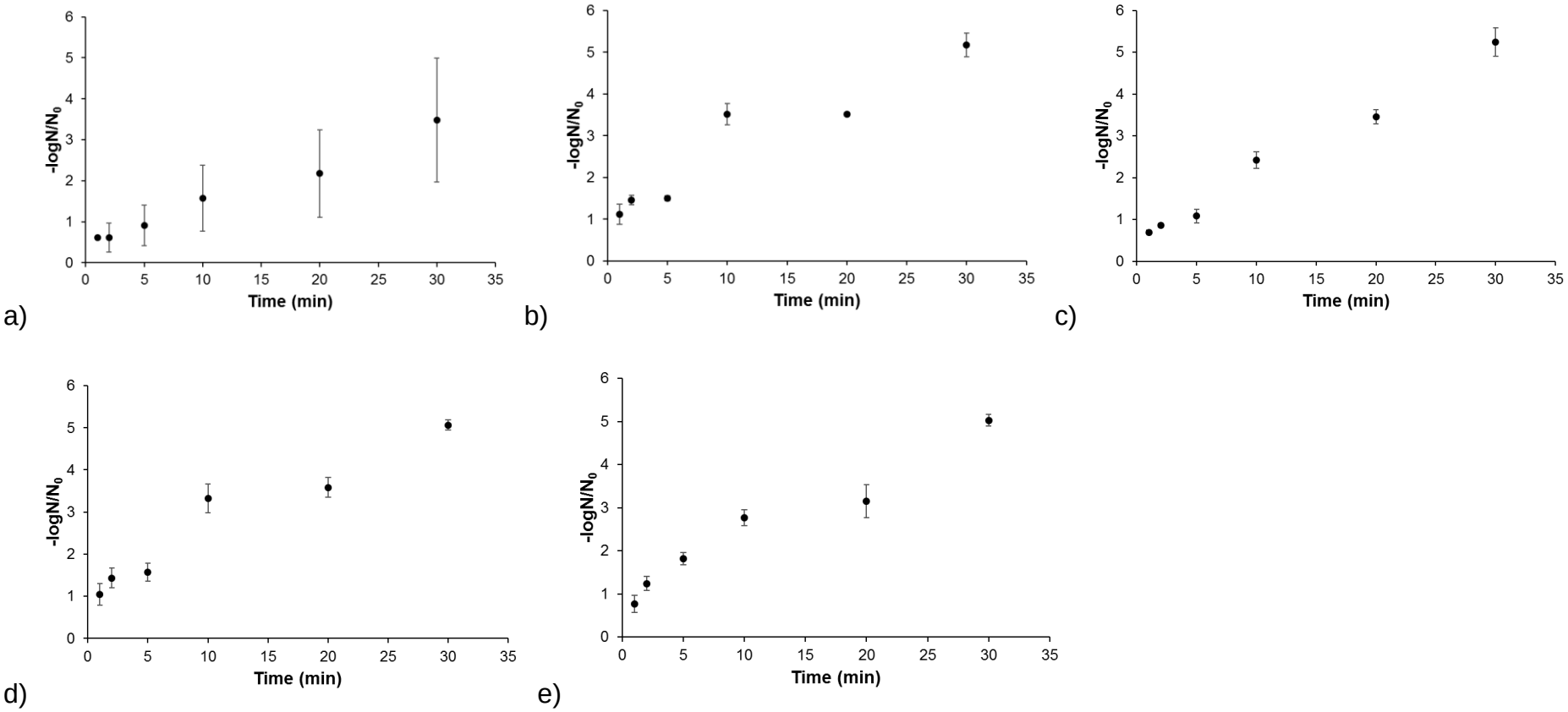

Figure A.3 – *Inactivation of E. coli isolates a) F1, b) F2, c) F3, d) F4 and e) F5, in response to 0.05 mg/l free chlorine over 30 minutes contact time.*