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# Epidemiology of *Acanthamoeba* species in water treatment works in England

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# Dedication

For grandma and grandpa Kanapathipillai

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# Abstract

*Acanthamoeba* spp. are often described as emerging opportunistic protozoan pathogens. Infection is associated with two main clinical diseases: *Acanthamoeba* keratitis (AK), and infection of the central nervous system in immunocompromised individuals, granulomatous amoebic encephalitis (GAE). The risk factors for AK are contaminated water and wearing contact lenses. Although the occurrence of *Acanthamoeba* spp. in tap water is well documented, its original source, whether from the public water supply or more locally, within domestic properties, is unknown.

This thesis describes studies of the prevalence of *Acanthamoeba* spp. in raw water from ground and surface sources, through water treatments works (WTW), to the tap water supplied to customers. The four WTW studied had different processes, depending on their raw water source. *Acanthamoeba* spp. were isolated from water samples using membrane filtration, cultured on non-nutrient agar (NNA) seeded with *E.coli* and incubated for 14 days. Light microscopy was used to observe trophozoites and cysts of typical morphology, and isolation was confirmed by PCR using genus-specific primers that target the 18S rDNA and sequencing of the PCR amplicons.

*Acanthamoeba* spp. were isolated from 100% of the samples taken from raw surface waters (river and lake) and 14% of samples from ground water (aquifers). They were not isolated from any of the samples taken after completion of water treatment, treated water storage tanks, or water from customers' taps.

All the isolates appeared to be closely related to each other and part of the T4 genotype, which has previously been found to be the most common cause of clinical AK, and also the genotype most widespread in the environment.

Future work should therefore focus on the diversity and distribution of *Acanthamoeba* spp. within household systems. Whole genome sequence of isolates might identify areas of the genome more useful for studying molecular ecology in water systems. The future development of PCR diagnostics might also improve both our understanding of prevalence and of the molecular ecology of *Acanthamoeba* spp.

Finally, a previously published protocol was adapted in order to test the susceptibility of an environmental isolate to disinfectants, in this case chlorhexidine (CHX), using a colorimetric sulforhodamine B stain. However, the consistency of the assay, previously used only with laboratory-adapted isolates, was poor, and only non-significant results were obtained.

In conclusion, *Acanthamoeba* spp. was found to be present in water entering WTW but the processes in place were effective at removing them. In contrast to previous studies, no *Acanthamoeba* spp. were isolated from tap water, and further investigation is needed to explore the plumbing, tap hygiene and water storage in domestic properties. Finally, it is not clear if the disinfectant assay protocol is suitable for environmental, low passage isolates, so further work is needed to improve the assay.

# Abbreviations and Acronyms

| Abbreviations and acronyms | Definition |
|---|---|
| AK | *Acanthamoeba* keratitis |
| AU | Absorbance units |
| cfu | Colony forming units |
| CHX | Chlorhexidine |
| CNS | Central nervous system |
| CWST | Cold water storage tank |
| DNA | Deoxyribonucleic acid |
| DPD | Diethyl-p-phenylene diamine |
| DWI | Drinking Water Inspectorate |
| EA | Environment Agency |
| EC | Electrical conductivity |
| FBC | Flat bottom clarifier |
| HPC 2D37 | Heterotrophic plate count 2 days at 37°C |
| HPC 3D22 | Heterotrophic plate count 3 days at 22°C |
| GAC | Granular Activated Carbon |
| GAE | Granulomatous Amoebic Encephalitis |
| MPS | Multipurpose contact lens solution |
| NA | Not applicable |
| NM | Not monitored |
| NNA | Non nutrient agar |
| NTU | Nephelometric turbidity units |
| Ofwat | The Water Services Regulation Authority |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PCV | Prescribed concentration or value |
| PHE | Public Health England |
| PYG | Peptone Yeast Glucose |
| SD | Standard deviation |
| SRB | Sulforhodamine B |
| TC | Total coliforms |
| TAE | Tri-acetate EDTA |
| TCA | Trichloroacetic acid |
| UV | Ultraviolet |
| WTW | Water treatment works |

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# CHAPTER 1

# General Introduction

## 1.1: Regulatory framework for water treatment in England

The safe and wholesome supply of water designed for human consumption is a vital, costly and a strongly regulated service in most developed countries. In England, the main regulatory frameworks governing the water industry include the Council Directive 98/83/EC and Water Industry Act 1991. Council Directive 98/83/EC provides guidance on water quality to the members of the European Union and came into force on 3rd November 1998. The Water Industry Act 1991 provides regulations for water companies on water supply and quality. Water companies are required to produce wholesome water for their customers under this Act. Wholesome water is defined as water that is free from any microorganisms and parasites and from any substances, which, in numbers or concentrations, constitute a potential danger to human health (DWI, 2018). This is both stated in the Water Industry Act 1991 and Council Directive 98/83/EC and is regulated in England by the Drinking Water Inspectorate (DWI). As well as the DWI, public water supply companies work closely with other government bodies including Public Health England (PHE), the Environment Agency (EA), the Water Services Regulation Authority (Ofwat) and local health authorities to ensure that both public health and economic requirements are met.

The DWI provides guidance to water companies in England and Wales on all aspects of water quality. Their role is to monitor compliance by water companies to ensure that they are meeting the requirements stated in the Water Supply (Water Quality) Regulations (2016), which are regularly updated. The guidance provides the water companies with various parameters and their limits (referred to as Prescribed Concentrations or Values (PCV) that are required to be monitored in

the water samples taken from water treatment works (WTW) and domestic properties.

The microbiological parameters within the regulations refer to *Escherichia coli (E. coli),* coliform bacteria, heterotrophic plate counts (HPC), enterococci, *Clostridium perfringens (C. perfringens)* and *Cryptosporidium* oocysts. *E. coli* is monitored as a faecal indicator and if found in water supplies indicates that there has been faecal contamination of the water (WHO, 2012). Enterococci and *C. perfringens* are also used as faecal indicators. HPC are used to monitor operational efficiency of the water treatment processes. The PCV for *E. coli,* coliforms, enterococci, and *C. perfringens* is 0 colony forming units (cfu) in 100ml of sample analysed. If these are detected in treated water, investigation by the Water Quality department is required to find the cause of failure and assess if there is an impact on public health by the water supplied.

There is currently no PCV value set in the regulations for the detection of *Cryptosporidium* oocysts in treated water. Therefore the responsibility lies with the water company to make an assessment on the risk to the public if oocysts are detected in treated water and the course of action that will need be taken. This would include informing stakeholders.

In addition to the water quality parameters, the guidance gives the numbers of samples that should to be taken at various stages of the water treatment process and the number of samples that should to be taken from customer's properties within the supply areas. Some sample results are sent to DWI ('routine

compliance sampling'), whereas others are used only to monitor the water quality performance of a WTW ('operational samples'). When companies breach the PCV limits, the water is deemed unwholesome and companies are required to inform the DWI and other stakeholders. The means of reporting events to DWI will depend on the risk to public health, which is determined by the water companies' Water Quality department. The routine compliance samples results are sent to the DWI as part of a monthly report, which also includes PCV failures that have occurred in that month. However, if there is a risk to public health from either a routine compliance sample result or operational sample result, then DWI have to be informed immediately. Following any breach, an investigation is undertaken by the water company to identify any causes that could have contributed to the breach. The DWI assesses the investigation into the incident and decides on an appropriate course of action, including making recommendations on how to prevent or reduce the risk of reoccurrence.

## 1.2: Importance of water treatment processes

An important aspect of the public water supply is to ensure that the water is microbiologically safe for human consumption. The presence of pathogenic microorganisms in drinking water was recognised in the 19$^{th}$ century when John Snow was able to trace an outbreak of cholera in London to a public water supply (Koch and Denike, 2006). This discovery was key to identifying the importance of removing bacteria from water supplies and the disinfection of drinking water was initiated. The DWI's guidelines of disinfection under section 3, Regulation 2(1) are "a process of water treatment to remove or render harmless to human health every

pathogenic microorganism and pathogenic parasite that would otherwise be present in the water and disinfection shall be construed accordingly defines disinfection" (DWI, 2012; DWI 2018).

Disinfection of drinking water is now an essential and a mandatory part of the water treatment process in most developed countries. Since its introduction, the number of deaths due to waterborne bacterial pathogens has dramatically decreased (Smith *et al.,* 2006). Despite advances in water treatments, microbial pathogens have continued to be a challenge, especially protozoan pathogens such as *Cryptosporidium* spp. and *Giardia* spp. In England and Wales in 2016, there were 5925 laboratory reported cases of cryptosporidiosis and 4492 reported cases of giardiasis (PHE, 2018).

There are many species of *Giardia,* however *Giardia duodenalis* is the only species currently known to cause a gastro-intestinal infection in humans. It can cause infection through consumption of contaminated water or food. The infection spreads by the shedding of cysts from infected individuals and can be passed on from animals to humans, so is sometimes also a zoonotic risk. Cysts can be removed by clarification and filtration, and with sufficient contact time with chemical disinfectants, cysts can be inactivated in the water treatment process (Percival *et al.,* 2004).

There have been no known outbreaks associated with the public water supply in the UK. However, *Giardia* is currently not regularly monitored by public water companies. The risk to public health from *Giardia* may lie with consumption of

water from contaminated private water supplies, which may not be as highly monitored or treated as water provided by public water companies (Horton *et al.,* 2018), or consumption of untreated water (Percival *et al.,* 2004). Further understanding of *Giardia* transmission routes, improved molecular detection techniques for environmental samples, and testing clinical samples for *Giardia* will help determine if the risk to public health is greater that currently perceived in the UK (Horton *et al.,* 2018).

However *Cryptosporidium* spp. is a regulatory parameter and is required to be monitored by public water companies in the UK. In mid-to-late 1980s *Cryptosporidium* spp. became increasingly identified as a major waterborne protozoan pathogen, causing gastro-intestinal infection.

*Cryptosporidium* spp. have been known as pathogens of animals for some time, and was first identified as a human pathogen in the 1980s (Tzipori and Widmer, 2008). Further work showed it to be a common cause of sporadic disease cases in immunocompetent individuals, as well as outbreaks associated with human-to-human transmission (Fayer and Ungar, 1986; Baxby *et al.,* 1985). *Cryptosporidium* oocysts are environmentally robust and are resistant to disinfection with chlorine, making transmission in contaminated water, whether contaminated by human or other animal faeces, difficult to control. There were several outbreaks of cryptosporidiosis reported within the UK, some of which are summarised in Table 1.1

Over time, the taxonomy and characterisation of *Cryptosporidium* spp. has developed such that while most human cases are caused by *C. parvum* (wide host range, so often zoonotic in origin), *C. hominis* (restricted mainly to humans) and *C. ubiquitum* (again wide host range, so often zoonotic in origin), there are many other *Cryptosporidium* spp. with varying degrees of host specificity (including in livestock and wildlife) and thus of zoonotic risk.

**Table 1.1: Summary of some waterborne outbreaks of cryptosporidiosis in the UK.**

| Year and species | Location | Summary of outbreak | References |
|---|---|---|---|
| **1989**<br><br>Species not identified | Swindon and Oxfordshire | 519 cases of human cryptosporidiosis. The cause of the outbreaks was *Cryptosporidium* oocysts not being effectively removed by the water treatment process as oocysts were detected in raw and final water samples. As a result of this outbreak the Government established an Expert Group to investigate *Cryptosporidium* spp. in water supplies. | Richardson, A.J. *et al.,* 1991 |
| **1997**<br><br>Species not identified) | North West London and Hertfordshire | 345 confirmed cases of cryptosporidiosis and 746,000 people advised to boil their drinking water, North West London and Hertfordshire. Source of the outbreak was inadequate filtration of the raw water from underground strata. | Boucheir, 1998 |
| **2008**<br><br>*C. cuniculus* (rabbit genotype) | Northamptonshire | 23 people exhibited cryptosporidiosis symptoms. On investigation, a rabbit carcass was found within the WTW. On investigation the oocysts isolated from the rabbit faeces and the human faeces were found to be of the same species. | Puleston *et al.,* 2014 |
| **2015**<br><br>*C. andersoni*<br><br>*C. hominis*<br><br>*C. ubiquitum* | Lancashire | Affected 712,000 people, some of whom had to boil their water for up to a month. There were no cases of cryptosporidiosis associated with this outbreak. Water supplied by Franklaw WTW in North Lancashire, which takes raw water from ground and surface sources. Oocysts initially detected by routine monitoring of water samples by the laboratory. The root cause remains unknown but thought likely to be contamination of the treated water service reservoir. The company endured severe reputational and financial repercussions, as they had to install UV treatment at the site and were fined £450,000. | DWI, 2017 |

In 1999, new regulations were introduced to protect public health from cryptosporidiosis associated with drinking water (Howe *et al.,* 2002). Water companies were required to assess the risk of the potential presence of *Cryptosporidium* oocysts in their water sources by carrying out risk assessments on WTW and the surrounding catchment areas, and to continuously monitor for *Cryptosporidium* oocysts in the final water at high-risk works and their catchment areas. In 2007, the regulations were updated such that unusual number of oocysts detected in treated water must be reported to the DWI. Due to improvements in sampling schedules, monitoring of catchment areas, and improved diagnostic and detection methods, *Cryptosporidium* oocysts can now be detected routinely by laboratories on the same day as the sample is taken, therefore potential outbreaks can be managed as soon as possible.

With increasing awareness of other protozoa as potential microbial pathogens, alongside an ageing population who are more likely to be immunocompromised, it is important that any waterborne pathogens that may pose a risk to human health are investigated so that measures can be taken to remove or mitigate the pathogens by effective water treatment. A pathogenic protozoan that has 'emerged' as a potential public health risk via public water supplies in recent years is *Acanthamoeba* spp. (Visvesvara *et al.,* 2007; Khan, 2006).

*Acanthamoeba* spp. can cause *Acanthamoeba* keratitis (AK) and granulomatous amoebic encephalitis (GAE). The risk of contracting AK by contact lens wearers due to contact with tap water is significant and the incidence of AK appears to be increasing (Seal *et al.,* 1992; Kilvington *et al.,* 2004; Radford *et al*., 2002; Carnt *et*

*al.,* 2018a) with the rise in use of prescription and cosmetic contact lens wearers. Apart from acting directly as, albeit opportunistic, pathogens, *Acanthamoeba* spp., like several other protozoa, can harbor a range of bacteria, themselves pathogenic including *Legionella* spp., *Mycobacterium* spp. and *Listeria monocytogenes* (Greub and Raoult, 2004).

## 1.3: Classification and distribution of *Acanthamoeba* spp.

*Acanthamoeba* spp. are free living opportunistic pathogens belonging to the kingdom Protozoa, and were first discovered in 1930 by Castellani (Khan, 2006). The taxonomy of members of the genus is both complex and confusing (Risler *et al.,* 2013, Fuerst, 2014). In the 1970s, *Acanthamoeba* spp. were classified according to the morphology of their cysts (see 1.4) into 18 species clustered in 3 groups (Khan, 2006). However, cyst morphology is plastic and depends on the culture conditions (Fuerst *et al.,* 2015). Whole genome sequences confirm some of these species, but many are yet to be fully sequenced or published. An extra complication is that many *Acanthamoeba* spp. genomes are themselves diverse as a result of viruses (some of which have a wide host range) inserting their own DNA and possibly transferring DNA between host *Acanthamoeba* spp. genomes (Chelkha *et al*., 2018).

Thus while laboratory studies often use type strains of particular species (although some papers call these species ‘strains’), the classification of environmental and clinical isolates tends to be based on the comparison of the 18S rRNA gene sequence (Stothard *et al.,* 1998; Maghsood *et al.*, 2005; Niyyati *et al.,* 2009; Risler

*et al.,* 2013; Fuerst, 2014). Currently, using this method around 20 *Acanthamoeba* genotypes have been described, named T1 – T21/22. A further environmental genotype (T99) appears to have been a laboratory artifact (Corsaro and Venditti, 2018). Different genotypes are defined as being at least 4% different from each other (Fuerst, 2014). Some of these genotypes are associated with a particular spp. and/or environment and others, for example T4, appear to comprise an assemblage that includes several traditionally different species (Risler *et al.,* 2013).

Not all genotypes appear to be similarly pathogenic, although cases of human infection with most genotypes have been reported. The genotypes most frequently associated with AK, for example, are T3, T4, T6 and T11 (Maghsood *et al.,* 2005; Zhao *et al.,* 2010; Ledee *et al.,* 2009). *Acanthamoeba* genotype T4 has been reported as being the most prevalent genotype in a variety of environments such as rivers, lakes, tap water and swimming pools (Maghsood *et al.,* 2005; Gavarane *et al.,* 2018), and WTW (Richard *et al.,* 2016).

*Acanthamoeba* spp. are widely distributed in diverse environments with a wide range of temperature and pH (Khan, 2006). They have been isolated from soil, dust, bottled water, chlorinated swimming pools, domestic water supplies, air conditioning units and contact lens storage cases (De Jonckheere, 1991). Other environments where they have been isolated include sewage, showerheads, ventilators and humidifiers (Schuster and Visvesvara, 2004) and from eye wash stations located within laboratories (Schuster and Visvesvara, 2004).

## 1.4: Life cycle of *Acanthamoeba* spp.

The life cycle of *Acanthamoeba* spp. consists of two stages or morphotypes: trophozoites and cysts. They are able to switch morphotype depending on their environmental conditions. This ability aids their survival in diverse and unfavourable environments, as the cysts are environmentally more resistant than the trophozoite stage (Khan, 2006). The ideal environmental conditions for growth are when the pH is in the neutral range, the temperature is approximately 30°C, and there is an abundant food source, i.e. when bacteria and organic particles are available. In this ideal environment, *Acanthamoeba* spp. exist in the trophozoite stage of their life cycle and feed through phagocytosis and pinocytosis. During this stage, the amoebae are metabolically active and exhibit spine-like structures on their surface (Khan, 2009). These are called acanthopodia and enable attachment to various surfaces. It is also during this stage that they reproduce asexually via binary fission.

In more hostile environmental conditions, the trophozoites undergo encystation and revert to the cyst stage of their life cycle (Khan, 2009). The cysts are between 5 – 20 µm, metabolically inactive and are resistant to environmental conditions, due to the cyst having a double wall, which provides greater protection from environmental conditions. In the cyst form, *Acanthamoeba* spp. can exist for several years (Khan, 2006). The cell wall of *Acanthamoeba* spp. includes ostioles through which the cysts are able to monitor the surrounding environment (Khan, 2009). The cyst stage is highly resistant to disinfectants (Dupuy *et al.,* 2014).

When the environment becomes more favourable, the cysts undergo excystation and become trophozoites again.

## 1.5: Diseases caused by *Acanthamoeba* spp.

### 1.5.1: *Acanthamoeba* keratitis (AK)

Although *Acanthamoeba* spp. is abundant in the environment and incidence of AK is low, the number of cases has been on the rise. AK is an ocular infection, which causes severe pain and significant loss of sight, or even blindness, in up to 15% of patients, (Radford *et al.,* 2002). The incidence of AK appears to be increasing in the UK, with one recent study reporting an almost three-fold increase in cases since 2010 (Carnt *et al.,* 2018a).

The main risk factor associated with AK is wearing contact lenses and contact lenses usage is said to have increased by 5-15% p.a. in the past few decades globally (Cavanagh *et al.,* 2010), although in the UK and Ireland it appears to have plateaued since around 2005 (Carnt *et al.,* 2018a). For example, a study of patients with AK in England and Wales between October 1997 and September 1999, found that 88% of patients were contact lens wearers (Radford *et al.,* 2002). In a very recent study in New Zealand, 96% of AK patients were contact lens wearers (McKelvie *et al.,* 2018). Indeed, various clinical reviews find the following risk factors for AK (Khan, 2006; Carnt and Stapleton, 2016; Szentmary *et al.,* 2018; Carnt *et al.,* 2018a):

- Contact with contaminated water whilst wearing contact lenses e.g. swimming in lakes and ponds
- Poor hygiene during contact lens use
- Not adhering to the manufacture's instructions on the usage of contact lenses
- Cleaning of contact lens with tap water
- Use of chlorine solutions for cleaning contact lenses

Several studies in the UK have suggested that the presence of *Acanthamoeba* spp. in domestic tap water was likely to be a risk factor for AK in contact lens wearers (Kilvington *et al.,* 2004; Radford *et al.,* 2002). The hardness of the water may also be associated with the risk of contracting AK from domestic water source (Seal *et al.,* 1992). It has also been suggested that the presence of limescale in tap water or water storage tanks provides a favourable environment for growth of *Acanthamoeba* spp. (Seal *et al.,* 1992).

*Acanthamoeba* infections are still mainly diagnosed by taking a corneal biopsy and culturing for *Acanthamoeba* spp. using non-nutrient agar (NNA) plates seeded with Gram-negative bacteria (Khan *et al*., 2001). The presence of trophozoites and cysts are observed using an inverted light microscope. Commercial diagnostic kits are not currently available. Specialist centres are increasingly able to use in vivo laser scanning confocal microscopy of corneal lesions, and some laboratories are using PCR or qPCR instead of (or as an adjunct to) culture, both of which appear to be much more sensitive than culture (Clarke *et al.,* 2012).

Early detection and treatment is the key to managing the disease. Various biguanides have been used to treat AK but they are not very effective at eradicating the cyst stage (Siddiqui *et al.,* 2016). Chlorhexidine (CHX) is a biguanide and has been suggested as being effective at treating AK (Heredero-Bermejo *et al.,* 2016; Ghani *et al.,* 2016; Lim *et al.,* 2008), although as an opportunistic pathogen, *Acanthamoeba* spp. are often found in association with other pathogens, including bacteria, so combination therapy is often recommended (e.g. Siddiqui *et al.,* 2016). Treatment can take up to a year, and even then, recurrence may develop in around 10% cases (Siddiqui *et al.,* 2016). A recent review of risk factors for bad outcomes of AK found delayed/mis-diagnosis, severe inflammation and use of corticosteroids to the main factors (Carnt *et al.,* 2018b).

While there are many laboratory studies of potential treatments for AK, some of which show potential, there is currently no accepted method for in vitro testing of efficacy and clinical trials tend to be limited to anecdotal reports and single case descriptions.

### 1.5.2: Granulomatous amoebic encephalitis (GAE)

This is an important clinical disease caused by *Acanthamoeba* spp., which may be less prevalent than AK, but is important because it is fatal in around 90% of cases (Siddiqui *et al.,* 2016). It is usually found in immunocompromised patients as a secondary infection (Khan, 2007), but has also been described in immunocompetent individuals. However the pathogenesis of the infection has not yet been determined and there is no effective treatment for this infection (Khan,

2007). It is believed that the amoebae enter the bloodstream through the lungs, following inhalation, or possibly the skin, and thence the bloodstream (Khan, 2009). In some studies, direct infection via the olfactory neuroepithelium can also occur. It is not known how the amoebae cross the blood brain barrier in order to enter the CNS, but it has been suggested that host inflammatory responses to the amoebae may increase the permeability of the vascular endothelium in the brain (Khan, 2009).

### 1.5.3: Immune response to *Acanthamoeba* spp. infections

*Acanthamoeba* spp. are ubiquitous in the environment and yet the number of infections caused by them is low (Khan, 2006). It appears that *Acanthamoeba* spp. requires some form of damage, e.g. corneal trauma, to infect the eye and that AGE requires immunosuppression or a pre-existing infection to develop.

When *Acanthamoeba* spp. enters the healthy eye, blinking and the tear film, plus the keratinized corneal epithelium, all serve as physical and chemical barriers to infection (Khan, 2006). This innate immunity is then supported by IgA, T-lymphocytes, natural killer cells and macrophages. Keratitis occurs when trauma, for example associated with contact lenses, enables entry of the *Acanthamoeba* spp. into the deeper layers of the cornea, and the inflammation resulting from the immune response causes further cycles of physical damage to the eye and the clinical signs seen. A competent immune system appears to be able to prevent AGE, and the exact mechanism of pathogenesis is almost completely unknown: the brain necrosis and other lesions seen may be due to the direct effects of the trophozoites or the immune response to them.

## 1.6: Symbiotic relationships with bacteria

Like many amoebae, *Acanthamoeba* spp. have a complex range of relationships with diverse bacteria, reviewed by Khan and Siddiqui, (2014) and Sun *et al.,* (2018), and summarised in Table 1.2.

**Table 1.2: Examples of interactions between *Acanthamoeba* spp. and bacteria.** Adapted from Khan and Siddiqui, (2014) and Sun *et al.,* (2018).

| Bacterial examples | Description of interaction | Relationship |
|---|---|---|
| 'Non-pathogenic strains of *E. coli* such as K12 and *Enterobacter* spp. | *Acanthamoeba* spp. graze on, phagocytose and digest bacteria as food. | Predator prey |
| Pathogenic strains of *E. coli* such as K1. *Burkholderia cepacia, Chlamydophila pneumoniae* | Evade amoebal killing; survive inside *Acanthamoeba* (even as cysts) for some time, but without multiplication. So *Acanthamoeba* aid survival of bacteria in an adverse environment or may aid transport/transmission to an alternative environment or host. | 'Sanctuary' / Commensalism<br><br>'Trojan horse' |
| *L. pneumophila, E. coli O157, Coxiella burnetii, Pseudomonas aeruginosa, Vibrio cholerae, Helicobacter pylori, Listeria monocytogenes* and *Mycobacterium avium* | Bacteria may be phagocytosed or possibly even actively invade *Acanthamoeba* in which they not only survive but replicate. In some circumstances, the bacteria may kill the amoebal host by lysis and so escape to the environment or to infect a host. | Reservoir /<br><br>Host/parasite |
| *Neochlamydia spp* | *Neochlamydia* spp act as endosymbionts, surviving within *Acanthamoeba* and causing no known harmful effects but presumably benefitting from 'sanctuary' and food availability. *Neochlamydia* infection, however, protects the amoeba from superinfection with *Legionella*. | Mutualism<br><br>(Classical symbiosis) |

These interactions are often described as symbiotic, but this encompasses a wide range of interactions (Table 1.2) including parasitism, commensalism and

mutualism. The ability of some bacteria to survive phagocytosis, and even replicate within amoeba such as *Acanthamoeba* spp. is sometimes seen (using very non-Darwinian terminology) as a 'training ground' for intracellular pathogens of animals, including humans, in that the molecular adaptations that evolved to evade destruction by protozoa are often the same as those that allow the bacteria to be intracellular pathogens and survive phagocytosis by macrophages in animal hosts (Molmeret *et al.,* 2005).

The relationship between *Acanthamoeba* spp. and *Legionella pneumophila* has been particularly well-studied. *L. pneumophila* is a Gram-negative facultative intracellular bacterium and is the causative agent of Legionnaires' disease in humans. It is commonly found in hot water storage tanks, showerheads and air conditioning systems. The infection route to humans is by inhalation of aerosols directly into the lungs. In humans, the bacteria invade the alveolar macrophages and are able to replicate inside them (Khan, 2006). Rowbotham, (1980) first reported that it is due to the similarities between macrophages and *Acanthamoeba* spp., that *L. pneumophila* is able to invade and replicate within *Acanthamoeba* spp and suggested that the infected individual might actually have inhaled amoebae containing multiple bacteria rather than free bacteria, thus increasing the infectious dose.

Further studies (Tyndall and Domingue, 1982) demonstrated that *Acanthamoeba* spp. are able to uptake *L. pneumophila,* and to support its growth (Holden *et al.,* 1984). Indeed, Cirillo *et al.,* (1999) demonstrated that *L. pneumophila* grown in *Acanthamoeba* spp. was more virulent in mice and able to replicate faster within

macrophages than that grown on agar. Thus *Acanthamoeba* spp. can play the role of a true reservoir host (Haydon *et al.,* 2002; Hallmaier-Wacker *et al.,* 2017) for *L. pneumophila* infections of humans.

With regards to water treatment, the relationship between *Acanthamoeba* spp. and Enterobacteriaceae is perhaps more significant than that with *L. pneumophila*, as these bacteria are used as indicator organisms to monitor microbiological quality of water treated by WTW. There is limited literature that discusses the relationship between *Acanthamoeba* spp. and Enterobacteriaceae, although some *E. coli* strains and *Salmonella enterica* can not only survive within *Acanthamoeba* but have increased resistance to low pH and antibiotics (Lambrecht *et al.,* 2015) when within the amoebae. Indeed, many of the virulence genes of *Salmonella* are believed have evolved as adaptations to survival in amoebae (Riquelme *et al.,* 2016).

Another important relationship is occurrence of *Acanthamoeba* spp. With communities of microorganisms associated with biofilms, as biofilms occur in the water distribution network and taps (Qin *et al.,* 2017; Taravaud *et al.,* 2018; Wang *et al.,* 2012) although little work has been done specifically on this.

In England, water companies routinely monitor for the presence of *Cryptosporidium* oocysts at WTW that are at high risk of contamination from the surrounding area. There have been reports of a relationship between *Acanthamoeba* spp. and *Cryptosporidium* oocysts (Stott *et al.,* 2003; Gomez-Couso *et al.,* 2007). *Acanthamoeba* spp. can uptake *Cryptosporidium* oocysts.

However, there are no reports on the condition of the oocysts once they have been taken up by *Acanthamoeba* spp.

## 1.7: Public health impact of *Acanthamoeba* spp.

The transmission of pathogenic organisms through public water supplies creates a potential major risk to the population, as the water is constantly being supplied in large quantities and is consumed as it is produced. Hence, most of the microbiological regulatory monitoring processes only provide results after the public has consumed the water supplied. Potential contaminants must be dealt with before the water reaches the consumer; therefore effective water treatment is an integral part of protecting the public from exposure to harmful microorganisms. Although AK is a rare infection, with an increase in the number of contact lens wearers in England since the 1990s, the incidence rate of this infection within the population may continue to rise (Cavanagh *et al.,* 2010; Carnt *et al.,* 2018a).

As contact with contaminated water and washing of lens in tap water are major risk factors (Carnt and Stapleton, 2016; Szentmary *et al.,* 2018), the study of the presence and control of *Acanthamoeba* spp. in WTW is paramount. A study on their presence in England WTW and evaluation of *Acanthamoeba* spp. at each stage of the water treatment is required to assess if there is a risk to the public from exposure to *Acanthamoeba* spp. via public water supplies.

There is some evidence of a positive relationship between the presence of *Acanthamoeba* spp. in domestic water supplies and the incidence of AK

(Kilvington *et al.,* 1990; Kilvington *et al.,* 1991; Radford *et al.,* 2002), but the source of the *Acanthamoeba* spp. remains unclear. Magnet *et al.,* (2012) sampled sewage effluents and tap water in Spain and isolated pathogenic T4 genotype of *Acanthamoeba* spp. from river and tap water. A study in Malaysia also isolated T4 genotype from samples taken from WTW (Richard *et al.,* 2016). However, there have been few studies following water from the raw source to the consumer through modern WTW (Al-Herrawy and Gad, 2017) and none published in temperate countries.

## 1.8: *Acanthamoeba* spp. in water treatment works

As discussed earlier, *Acanthamoeba* spp. have been isolated from domestic water supplies and been associated with the increasing incidence rates of AK. It is possible that the source of *Acanthamoeba* spp. in domestic supplies is from the water supply itself, i.e. the environmental *Acanthamoeba* spp. in raw water can survive the water processing plant and subsequent distribution. Few studies have investigated this, and only one pilot study has been undertaken in the UK (Shanmuganathan and Khan, 2009). Below is a summary of the processes involved in treating raw water and the likelihood of *Acanthamoeba* spp. occurrence within each stage of the treatment process.

**Raw Water source entering the water treatment**

The main source of raw water for WTW is from rivers (surface water) and aquifers (ground water) via boreholes or wells. As there is a limit set by the Environment Agency on the volume of raw water that public water companies are able to abstract from rivers (Environment Agency, 2016), water companies also store raw water in reservoirs which may be open and have other, mainly recreational, uses.

Ground water usually has much less suspended solids and so often appears clearer at source than surface water. Ground water is also much less likely to contain pathogens than surface water owing to its having been naturally filtered as it passes through the various layers of strata to reach the aquifer. Ground water from aquifers is often much older, as the percolation process can take decades.

However, there are some ground water sources with some of the characteristics of surface water. This occurs in areas where the strata contain vertical cracks and fissures, allowing surface water from rainfall to pass into the deeper levels much more quickly. Furthermore, rather than being stored in the bedrock, some water may be stored in caves. Due to the reduction of the natural filtering process that would normally occur, such water may again share some characteristics of surface water, including higher likelihood of bacteria being present. These sources of water are known as karstic or karst sources (Figure 1.1).

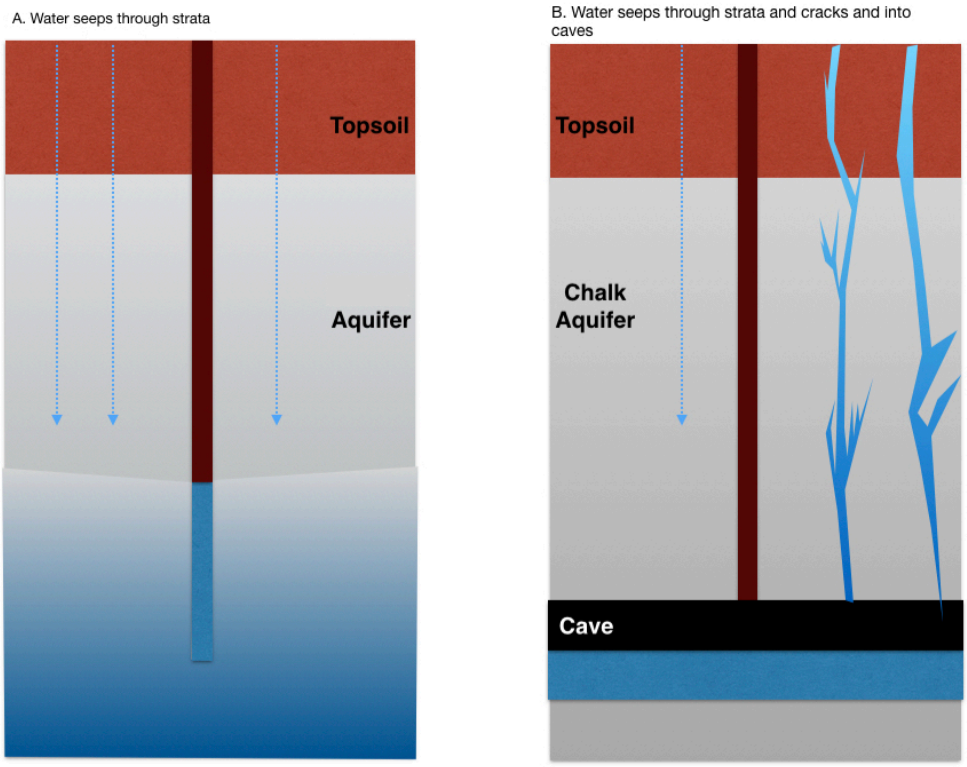


**Figure 1.1: Schematic diagram demonstrating the main differences between non-karstic groundwater sources (A) and karstic water sources (B).** Borehole water is naturally filtered by sinking through the strata (bedrock) and can take years to reach the aquifer. In certain places there are cracks in the bedrock, which allow faster flow into the aquifer, so karstic water is filtered less and can reach the aquifer within a few days or weeks. Sometimes with karstic sources the water may be extracted from a cave. Generally karstic sites exist in areas with soluble rock, or areas with fissures. Karstic sources are often in areas associated with chalk; however many areas with chalk bedrock have not had cracks and caves formed and act as a traditional borehole.

Whatever the source of raw water, it goes through a series of processes at the WTW before supply to domestic and commercial customers in the area. Depending on the source water, there are various treatment options. Non-karstic groundwater sources often require much less treatment due to the natural filtration process, whereas karstic and surface water generally have more contaminants and therefore require more treatment.

**Screening surface water**

When raw water is abstracted from a river or other surface water source, it is first passed through a series of grates and filters that remove the larger debris. At this stage there is little removal of microorganisms from the raw water and it is therefore highly likely that *Acanthamoeba* spp. will be isolated. This is supported by studies at WTW in Germany and Iran where *Acanthamoeba* spp. were isolated from river and lake water sources (Hoffmann and Michel, 2001; Mahmoudi *et al.,* 2012).

**Settling reservoirs**

Approaches to removing sediment vary. Some WTW store water in settling reservoirs. In these, water is held for locally differing times to allow heavier particles to settle by gravity (Brandt *et al.,* 2017).

**Coagulation, clarification and sedimentation**

The next stage of water treatment of surface waters involves adding chemicals promoting coagulation and the formation of a 'flocculation blanket'. The flocculation blanket traps organic matter and small particles that are still suspended in the water. This clarifies the water, making it clearer and aesthetically more acceptable by consumers. Common chemicals used for coagulation are aluminum sulphate, iron (III) sulphate and polyelectrolytes (charged synthetic polymers). In this stage, water enters the treatment process from the bottom and is pushed upwards so that water is passed through the flocculation blanket. The water at the top of the column then passes to the next stage of the process. The

coagulated material is collected in a sludge cone and discarded, which is required to maintain the efficiency of the treatment process (Brandt *et al.,* 2017).

**Filtration**

At this stage, most of the sediment should have been removed from the source water; however smaller particles may still be present. Filtration helps to remove these particles. There are various types of filtration used in WTW:

**Slow Sand Filters**: This is a biological filtration process, whereby water is passed through fine sand particles. The process is slow as it relies on the weight of water above the filter to pass water through and so require a large area; however the process is able to remove bacteria and organic matter as well as improving the turbidity of the water (Brandt *et al.,* 2017).

**Rapid Gravity Filters:** This process involves filter beds that contain anthracite that remove solid particles. The aim of this stage is to reduce the turbidity of the water to 0.3 NTU (Brandt *et al.,* 2017).

**Granulated Activated Carbon (GAC):** GAC is a porous material that offers a large surface area to improve removal of organic matter (Brandt *et al.,* 2017). The materials often used for GAC are coal, peat or coconut. As water passes through GAC, removes volatile organic compounds including many pesticides are removed, and thus the process improves the taste and odour of water.

**Membrane filtration:** These are usually synthetic semi permeable membranes which can vary in pore size and remove particles through a sieving mechanism. The pore size can be as small as 0.005 μm. This step is effective at removing *Cryptosporidium* oocysts and *Giardia* cysts (Brandt *et al.,* 2017). Filtration, flocculation, and sedimentation have been reported as being effective processes for reducing the number of *Acanthamoeba* spp. during the water treatment process (Hoffman and Michel, 2001.)

**Ozonation**

Ozone ($O_3$) is an oxidant and is used to break down large organic compounds, including many herbicides and pesticides, and improve the taste, odour and colour of raw water. It is used as a disinfectant and is more effective at killing viruses and oocysts than chlorine (Brandt *et al.,* 2017). Cursons *et al.,* (1980) found that ozone was amoebicidal in axenic conditions, but to inactivate *Acanthamoeba* spp. high ozone demand was required. The water is passed through contact tanks with ozone. At the WTW, a liquid oxygen tank is present which is connected to an ozone generator. When the role of ozone is completed, an ozone destructor is used to convert the ozone to oxygen.

**Disinfection**

Chlorine is a well-known disinfectant, commonly used in a solid or liquid form as sodium hyperchlorite. It is a major disinfection agent in water treatment processes in England and Wales (Brandt *et al.,* 2017). The amount of chlorine in drinking water is carefully monitored, and reported as free chlorine and total chlorine. Free chlorine, or residual chlorine, is unreacted chlorine, i.e. that available for

disinfection, whereas combined chlorine is not – thus total chlorine is the sum of the two. The range of residual chlorine recommended by World Health Organization (WHO, 2011) is 0.2mg/l – 0.5mg/l.

When chlorine is dissolved in water, it mostly forms hypochlorous acid (HOCl) . However, at higher pH the hypochlorite ion OCl- will form (Brandt *et al.,* 2017). "Free chorine" refers to all chlorine in the water as hypochlorous acid, hypochlorite, and the gaseous form ($Cl_2$). As soon as chlorine is added to the water, some will react with organic material or other chemicals, meaning there is less available for disinfection. This is a particular issue for water sources that contain ammonia ($NH_3$) as the chlorine reacts with ammonia to form chloramines (Brandt *et al.,* 2017), a common component of 'combined chlorine'.

*Acanthamoeba* spp. can be resistant to chlorine, (De Jonckheere and Van De Voorde, 1976) at a chlorine concentration of 4mg/ml and contact time of 3 hours, and some pathogenic strains have been reported as resistant to up to 40mg/ml chlorine. Despite this *Acanthamoeba* spp. were not isolated in treated water at German treatment works after disinfection with chlorine (Hoffman and Michel, 2001). Dupuy *et al.,* (2014) studied the effectiveness of several disinfectants, chlorine, monochloramine and chlorine dioxide on cysts and trophozoites and found that chlorine dioxide was the most effective. However, chlorine dioxide is not a routinely used disinfectant in WTW in England. In a more recent study (Moon *et al.,* 2018) chloroquine was shown to have potential disinfectant properties against *Acanthamoeba* spp.

Another method of disinfection is to pass the water through ultra violet (UV) light, which has been introduced to WTW particularly to control cryptosporidiosis (Hijnen *et al.*, 2006). There appears to be a consensus that while UV can destroy *Acanthamoeba* infectivity, it requires higher doses to do so than are routinely used for bacterial or cyptosporidial disinfection (Chang *et al.,* 1985; Hijnen *et al.,* 2006)

Some work has been carried out on alternative approaches to removing protozoan cysts from water. Solar and photocatalytic disinfection of water was not effective against the cysts of *Acanthamoeba polyphaga* (Lonnen *et al.,* 2005). Pulsed Electric Field (PEF) (Vernhes *et al.,* 2002), which involves applying electrical fields to the water to disrupt the membrane potential of living cells, was found to be effective at eliminating trophozoites of *Naegleria lovaniensis* directly from the river water but has not been tested against *Acanthamoeba* spp.

**Post-processing: storage and distribution**

After leaving the WTW, water is taken either to reservoirs, more commonly referred to as storage or service reservoirs, or to water towers. These reservoirs are designed to prevent contamination with organic material. Water is transferred to the reservoir under high pressure via trunk mains.

Storage reservoirs are designed to store water, so that the treatment works can process water at a constant rate. The reservoirs therefore act as a buffer, releasing more water during peak demand and replenishing during periods of low demand. Storage reservoirs need to be monitored carefully to ensure good

turnover of water and internal inspections are carried out on a regular basis to ensure the integrity of the reservoir is good.

Upon leaving the reservoir, water is fed to the distribution network. Reservoirs used to be placed on high ground, using gravity to maintain positive pressure in the network; however, this pressure is nowadays more commonly achieved by the use of booster pumps. Positive pressure ensures that if there is a burst or leak, water will leak out and prevent ingress to the network. Distribution mains then distribute water under pressure to domestic and commercial properties.

Traditionally, buildings in the UK had cold water storage tanks (CWST) to maintain a back up of water should there be an issue in the network. The cold kitchen tap, assumed to be the main source for drinking, would still have been on the rising main, however, as the quality of water that has passed through a CWST may become compromised. More recently, new build houses are less likely to have CWST, although large industrial buildings or high-rise flats may still have these. The Water Supply (Water Fittings) Regulations (1999) (DWI, 1999) are in place for the maintenance of potable water CWSTs so as to maintain the quality of water. They describe the requirements for the 'design, installation and maintenance of plumbing systems, water fittings and water-using appliances', and water companies are responsible for their enforcement.

*Acanthamoeba* spp. have been isolated from water samples taken from taps that were both tank-fed and mains-fed (Seal *et al.,* 1992; Shoff *et al.,* 2008).

## 1.9: Rationale, aims and objectives of the study

AK is increasing in incidence in the UK and appears to be associated with water and wearing contact lenses. The sources of *Acanthamoeba* are not known: *Acanthamoeba* spp. are known to exist in the environment, and have been found in tap water in homes, but whether this results from *Acanthamoeba* surviving processing at the WTW and subsequent distribution or if the water becomes contaminated at a later stage is unknown. There are few studies on the occurrence of *Acanthamoeba* spp. in WTW, and only one pilot study in England (Shanmuganathan and Khan, 2009).

There is evidence to suggest that contact lens wearers occasionally wash their lenses with tap water, and of course they may simply splash tap water in their eyes when washing etc. With the rise in contact lens wearing, it is important to consider the effectiveness of water treatment in eliminating *Acanthamoeba* spp. The presence of *Acanthamoeba* spp. in water has a significant impact on public health because not only they can cause AK via contaminated water, but because they can also protect bacteria from the disinfection process and act as reservoirs for important pathogens such as *Legionella*.

This study aimed to investigate the prevalence of *Acanthamoeba* species at various stages of the water processing and distribution system, and investigate any factors that might be associated with the presence or absence of, and therefore the risk posed by, waterborne *Acanthamoeba* spp.

To achieve this overarching aim, four objectives and hypothesis were set:

1. To study the prevalence of *Acanthamoeba* spp. in ground and surface raw water sources supplying four WTW. Hypothesis: *Acanthamoeba* spp. will be more prevalent in surface water than ground water.
2. To investigate the prevalence of *Acanthamoeba* spp. at various stages of the water treatment and distribution process, including in domestic properties served by the WTW. Hypothesis: Current water treatment processes will be effective at removing *Acanthamoeba* spp. from raw water sources.
3. To compare the genotypes of any *Acanthamoeba* spp. detected in raw water and at various stages of the water treatment process. Hypothesis: The *Acanthamoeba* genotype isolated from raw water sources will be T4.
4. To assess a published assay for testing the effectiveness of disinfectants on environmental *Acanthamoeba* spp. Hypothesis: The assay will be suitable to use with environmental *Acanthamoeba* spp. isolates.

# CHAPTER 2

# General Materials and Methods

## 2.1: Ethics Statement

This research was reviewed and approved by the SVMS Ethics Committee (approval number 2637181114). All experiments were carried out in accordance with the code of Research Conduct and Research Ethics guidelines set forth by SVMS, University of Nottingham.

## 2.2: Details of Water Treatment Works

Four Water Treatment Works (WTW A, B, C and D) located in the South East of England (Figure 2.1) were chosen to represent the main types of raw water and water treatments undertaken in England. Sampling was carried out at each location over a period of nine months in 2009, and additional sampling was carried out in 2017 at WTW D. The total output of treated water of all of the WTW was approximately 322 megalitres per day and combined, they supplied approximately 828,500 people.

**WTW A** was located near Bushey, Hertfordshire. The works received karstic water from chalk aquifers, pumped through approximately 18 boreholes located over a wide area surrounding the site. These chalk aquifers take a few days to weeks to receive water. The number of boreholes in use at any time was determined by the water demand and often boreholes nearer to the site were used, as it required less energy and cost to pump water to the works. During high demand periods the boreholes further away are used. The output of WTW A was approximately 160 megalitres per day and supplied around 440,000 people.

**WTW B** was located near Codicote, Hertfordshire, and had a single borehole, which pumped ground water from a chalk aquifer. This was a non-karstic source, which takes months to filter water. The maximum capacity was approximately 0.655 megalitres per day and the population served was around 2,500.

**WTW C** was located near Watford, Hertfordshire and had three boreholes. It abstracted water from chalk aquifers, which, depending on demand ran in rotation. This was another non-karstic groundwater site, where the water would take months to reach the aquifer. The maximum output capacity was approximately 21 megalitres per day and the population served was around 86,000.

**WTW D** was located in Egham, Surrey, and its intake was surface water from the River Thames that lies adjacent to the treatment works, although in exceptional circumstances, such as a pollution event on the river, water could be extracted from a nearby lake, also used for recreational/leisure activities. The maximum capacity was approximately 140 megalitres per day and the population served was around 300,000.

**The recreational lake** was also located in Surrey. It was used as an intake for WTW D when required and used by members of the public for recreational activities such as water sports.

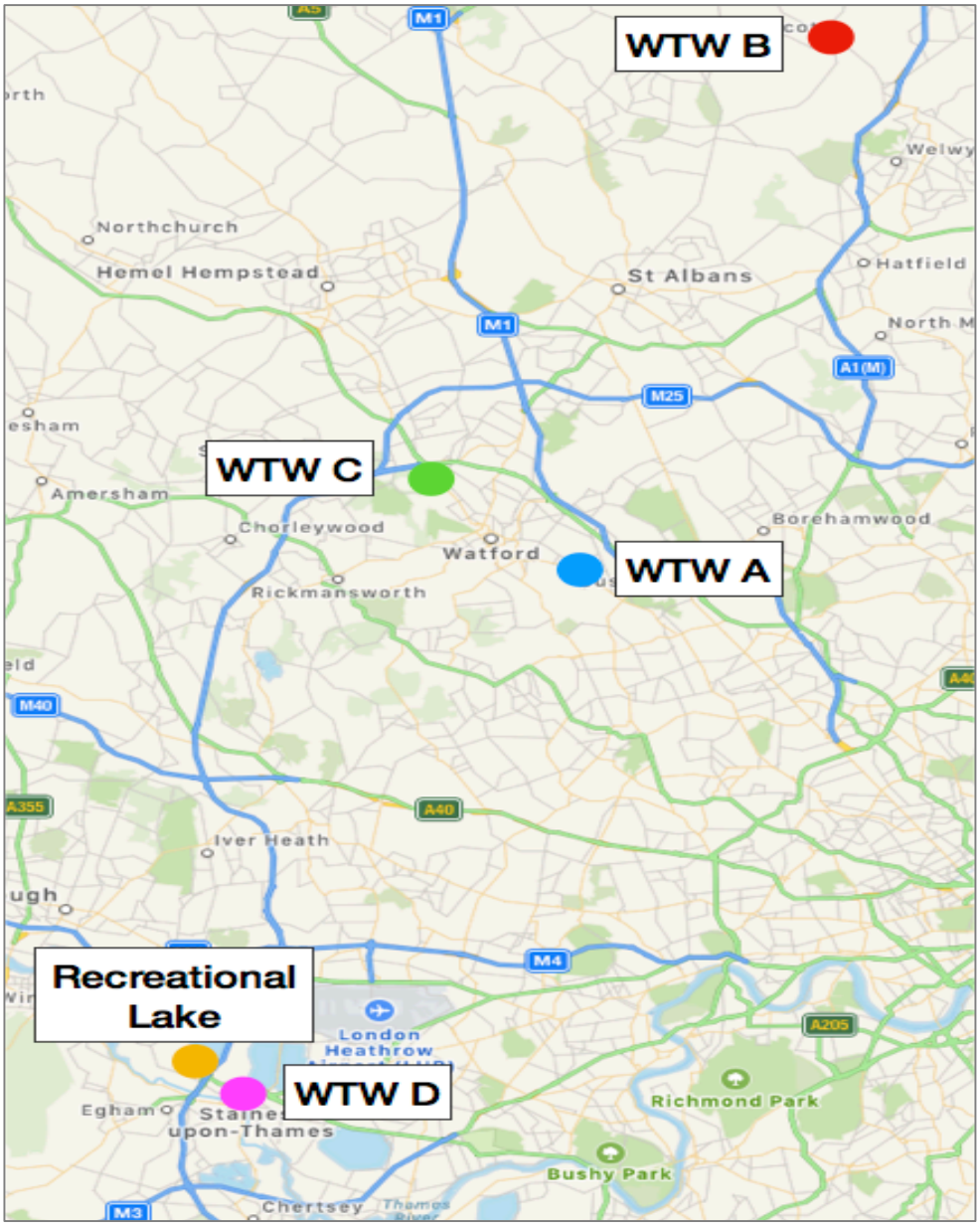


**Figure 2.1: Location of Water Treatment Works (WTW) and the recreational lake examined in this study.** WTW A, WTW B, WTW C, WTW D and the recreational lake are located in the South East of England.

## 2.3: Sampling points at each Water Treatment Works

### 2.3.1: Water treatment works A (karstic ground water)

**Table 2.1: Sampling points at WTW A.** The numbered points correspond to those in Figure 2.2.

| Treatment stage | Sampling point | Type of treatment |
|---|---|---|
| **Source raw water -aquifer (karstic water)** | 1. Inlet 1<br>2. Inlet 2 | Raw water from the ground was taken in via two inlets to be treated. The raw water from both of the inlets was combined for the treatment process. |
| **Partially treated** | 3. GAC inlet 1<br>4. GAC inlet 2 | Water sampled prior to passing through GAC filters. |
| | 5. GAC filters | Samples passed through one of 12 GAC filter beds. Samples were taken from each of these, to monitor if one of the GAC filters was not treating water effectively (and required the carbon regenerating). For this study samples were collected from a predetermined filter on a rotational basis. |
| | 6. GAC basin 1<br>7. GAC basin 2 | The GAC basin was the combined outlet of the active GACs and allowed sampling of each of the two steams prior to chlorination. |
| | 8. Pre membrane 36”<br>9. Pre membrane 27” | The pre-membrane sample point was located after the water had been disinfected (chlorination) but prior to the water entering the membrane filtration plant. 36” and 27” refers to the size of the pipe which water was passed through. |
| **Treated** | 10. Final treated water 27”<br>11. Final treated water 36” | Post filtration by membrane filtration and water had been disinfected. Final treated water distributed to service reservoir by two main pipes. The quality of water supplied by 27” and 36” was identical. |
| | 12 – 17 service reservoirs | The two streams both fed into multiple closed reservoirs near the plant. These reservoirs could be fed via either final water stream. From here they supplied the customers. |
| | 18 – 22 domestic properties | Water from customers’ kitchen taps. |

WTW A received karstic water from 18 boreholes and water entered the WTW and was processed through two parallel streams (Figure 2.2). Samples were taken at various stages in the treatment process, described and shown in Table 2.1 and Figure 2.2. Water was taken from boreholes followed by GAC filtration, then disinfected with hypochlorite before entering the membrane filtration plant. The membrane filtration plant was installed in the late 1990s following an outbreak of cryptosporidiosis in the area fed by the works. The works has online monitors for chlorine, turbidity and electrical conductivity. The contact time for disinfection was 15 minutes at 10mg.min/l. The free chlorine target of final treated water was 0.3mg/l. Treated water was then fed into service reservoirs, located adjacent to the site, before distribution to an area in North London.

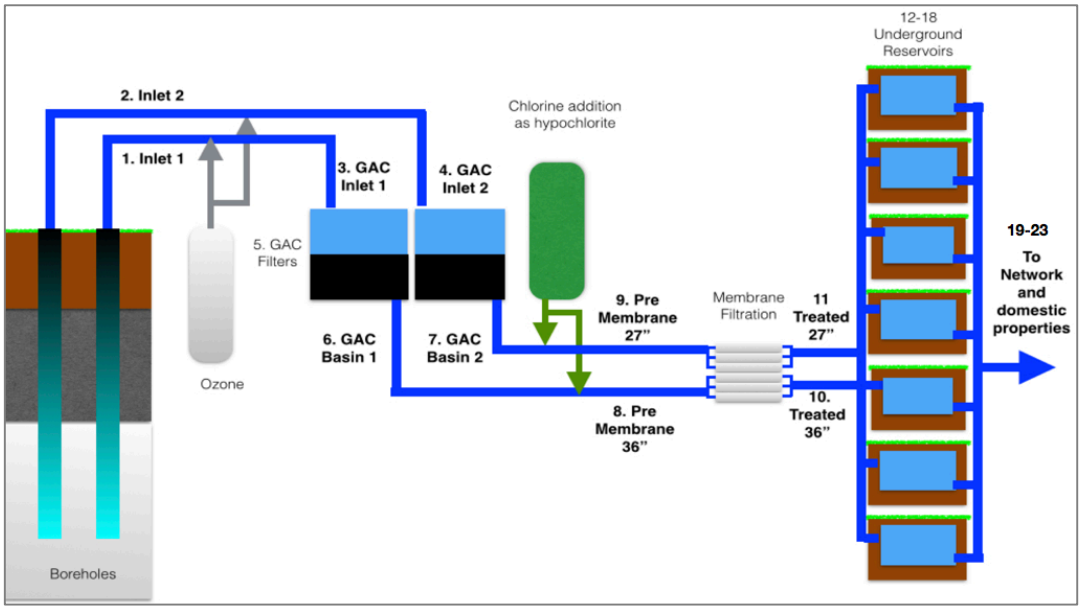


**Figure 2.2: Schematic diagram of WTW A.** This works intakes karstic ground water. The main filtration step was ultra-membrane filtration. The numbers on the sampling points correlate to the numbers in table 2.1, which explains each of the sampling points in more detail.

### 2.3.2: Water treatment works B (non-karstic groundwater)

**Table 2.2: Sampling points at WTW B.** The numbered points correspond to those in Figure 2.3.

| Treatment Stage | Sampling point | Type of treatment |
|---|---|---|
| **Raw** | 1. Raw | Raw water from borehole was fed into the plant. |
| **Treated** | 2. Final treated water | Treated water after the raw water had passed through both UV and chlorination. |
| | 3. Service reservoir | Treated water was stored here before distributed to customers. |
| | 4. Domestic property | Water from customers' taps. |

WTW B had a single borehole taking water from a non-karstic groundwater aquifer, and the treatment process occurred on site (Table 2.2 and Figure 2.3). Water was initially disinfected by UV followed by further disinfection with chlorine. The contact time of chlorine was 15 minutes with 10mg.min/l. The target for free chlorine of final treated water was 0.3mg/l. Samples were taken at various stages in the treatment process, described and shown in Table 2.2 and Figure 2.3. Water was pumped to a water tower that acted as a service reservoir. From here water was distributed to the nearby area.

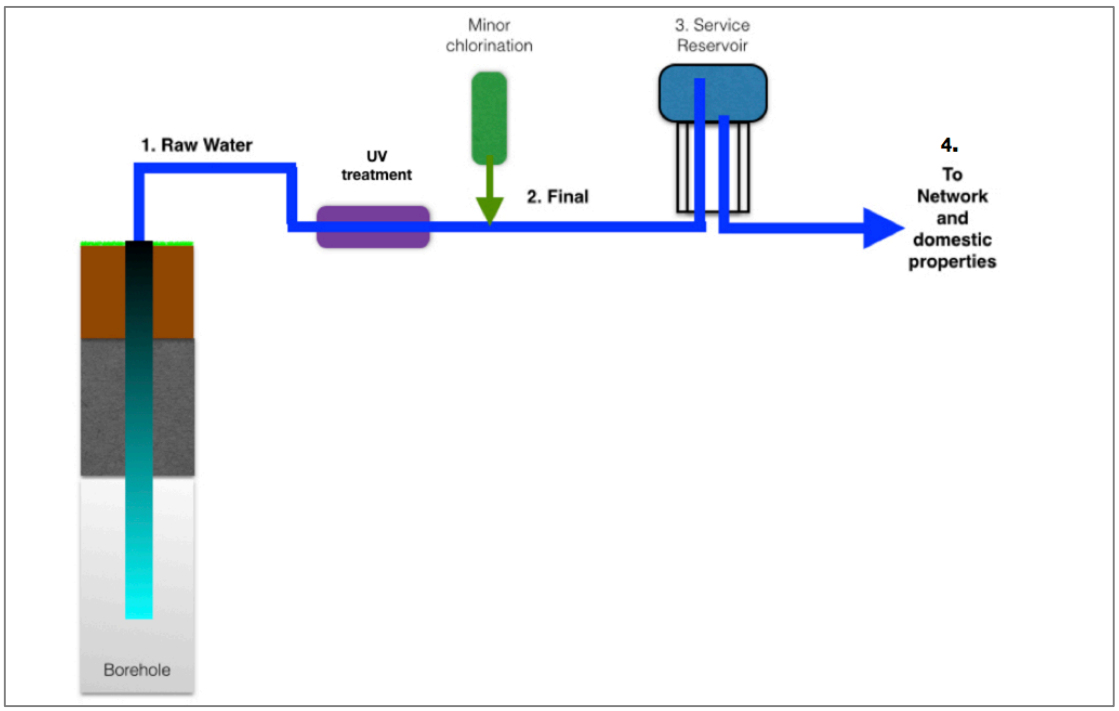


**Figure 2.3: Schematic diagram of WTW B**. Due to the natural filtration that occurred as water percolated to the aquifer, the extracted water only needed to be disinfected using UV treatment and chlorine dosing.

### 2.3.3: Water treatment works C (non-karstic groundwater)

**Table 2.3: Sampling points at WTW C.** The numbered points correspond to those in Figure 2.4.

| Treatment Stage | Sampling point | Type of treatment |
|---|---|---|
| **Raw** | 1. Raw 1<br>2. Raw 2<br>3. Raw 3 | Raw water from boreholes was fed into the plant. |
| **Treated** | 4. Final treated water | Treated water after water had been subject to chlorination. |
| | 5. Service reservoir | Treated water was stored here before distributed to customers. |
| | 6. Domestic property | Water from customer's taps. |

WTW C had three boreholes, and depending on demand, either one or two of these were running at any time on rotation. Unlike WTW B, there was no UV

treatment stage so the water was superchlorinated (to around 1mg/L) before entering a contact tank that allowed around two hours for disinfection. Upon leaving the contact tank, sodium bisulphite was added to reduce the concentration of chlorine ($NaHSO_3 + Cl_2 + H_2O \rightarrow NaHSO_4 + HCl$).

The contact time was 15 minutes with 15mg/min/l. The target concentration of free chlorine of final treated water was 0.3mg/l. Water was then fed to one of two service reservoirs before being distributed to the network. Samples were taken at various stages in the treatment process, described and shown in Figure 2.4 and Table 2.3.

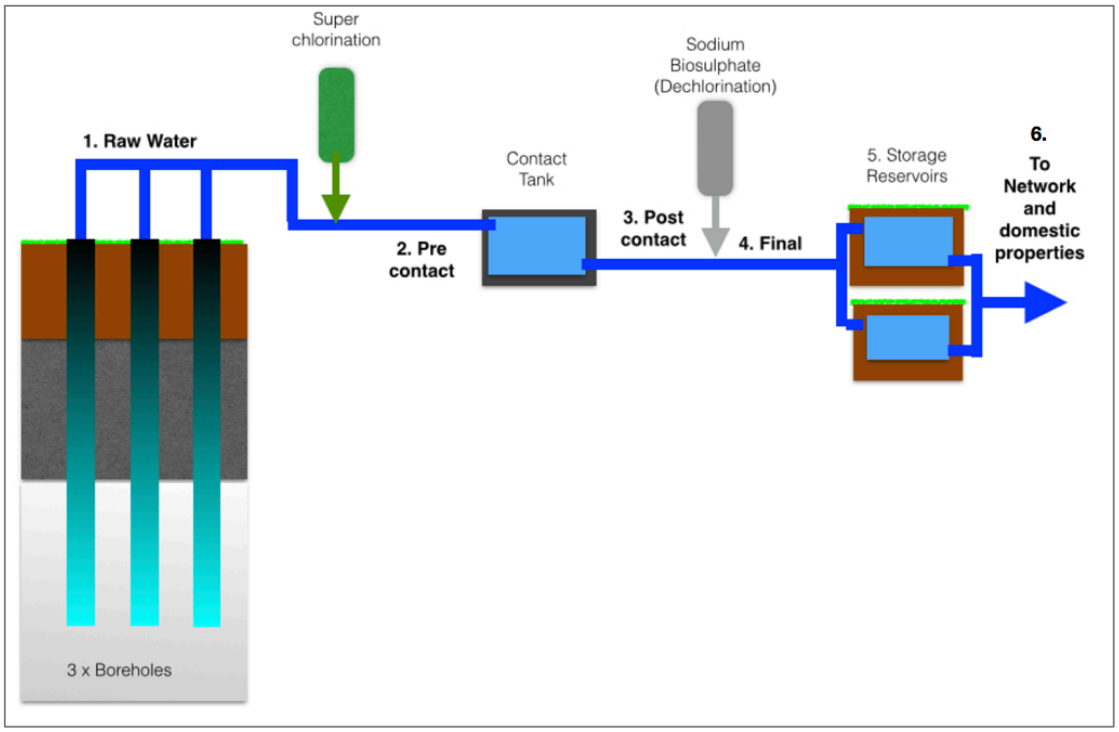


**Figure 2.4: Schematic diagram of WTW C.** There was no UV treatment, and water was 'superchlorinated'. It was then treated with sodium biosulphite to reduce residual chlorine.

### 2.3.4: Water treatment works D (surface water)

**Table 2.4: Sampling points at WTW D.** The numbered points correspond to those in Figure 2.5.

| Treatment Stage | Sampling point | Type of treatment |
|---|---|---|
| **Source raw water** | 1. River | Water was abstracted from the river (surface water), and large debris was removed. |
| | 2. Raw | Water from a local lake was pumped into the works and mixed with the river water. |
| **Partially treated** | 3. Pre – ozone | Due to the water source, ozone was added at various stages to aid with breaking down compounds and biological material. Stage 3 was immediately prior to the first ozone treatment. |
| | 4. Sedimentation plant | The sedimentation plant removed sediment from the water to improve its appearance and clarity. Flocculants were added that caused sediment to bind together, making it heavier and therefore sink. |
| | 5. FBC 1<br>6. FBC 2 | There were two flat bottom clarifiers (FBC). These allowed for further sedimentation of the water, improving its clarity further |
| | 7. Intermediate ozone inlet | Water was passed through rapid gravity filters (RGF) prior to the second stage of ozonation of the water. |
| | 8. GAC inlet | This sample was taken after intermediate ozonation but prior to the GAC filters. There were several filters at the treatment plant with one common inlet |
| | 9. GAC outlet | Post GAC (filtration step) This was the combined outlet after the water had passed through the GACs |
| | 10. Dechlorination | Water was super-chlorinated before entering the contact tank. After the contact tank, sodium bisulphite was added to reduce the residual chlorine to more tolerable concentrations. |
| **Treated** | 11. Treated water | Treated water left the treatment stage of the works after dechlorination |
| | 12. Link main | The link main could either send excess water to another treatment plant, or receive treated water from another plant depending on demand via the link main. |
| | 13 – 20: service reservoirs | Treated water was stored in multiple closed reservoirs near the works; from here they supplied customers. |
| | 21 – 23: Domestic properties | Water from customer's kitchen taps. |

WTW D mainly took water from the River Thames adjacent to the works, although if required water was also taken from a nearby recreational lake. The sampling points at the WTW are described in Table 2.4 and Figure 2.5. In addition to samples taken at the WTW, 2L of water were taken directly from the lake. It was not subjected to any water treatment as the lake is used as an emergency back-up supply should water not be available for abstraction from the River Thames (e.g. pollution incidents such as a fuel spill). This sample was taken using a sterile dipper. On entering the works, water passed through several screens to remove larger debris. After an initial ozone treatment, water then entered the sedimentation plant followed by flat bottom clarifiers and rapid gravity filters. These were designed to remove further sediment from the water.

After the water had been further treated with ozone, it was filtered using GAC. As at WTW C, it was then disinfected with high concentrations of chlorine ('superchlorinated') before entering the contact tank, and then dechlorinated and fed to service reservoirs. The contact time was 15 minutes with 15mg.min/l. The free chlorine of final treated water aimed to be 0.3mg/l. There was also a 'link main' prior to the service reservoirs, connecting to two other treatment works. Thus the water distributed from WTW D eventually had multiple sources. Indeed, the flow could be either way in this link main, depending on different site requirements.

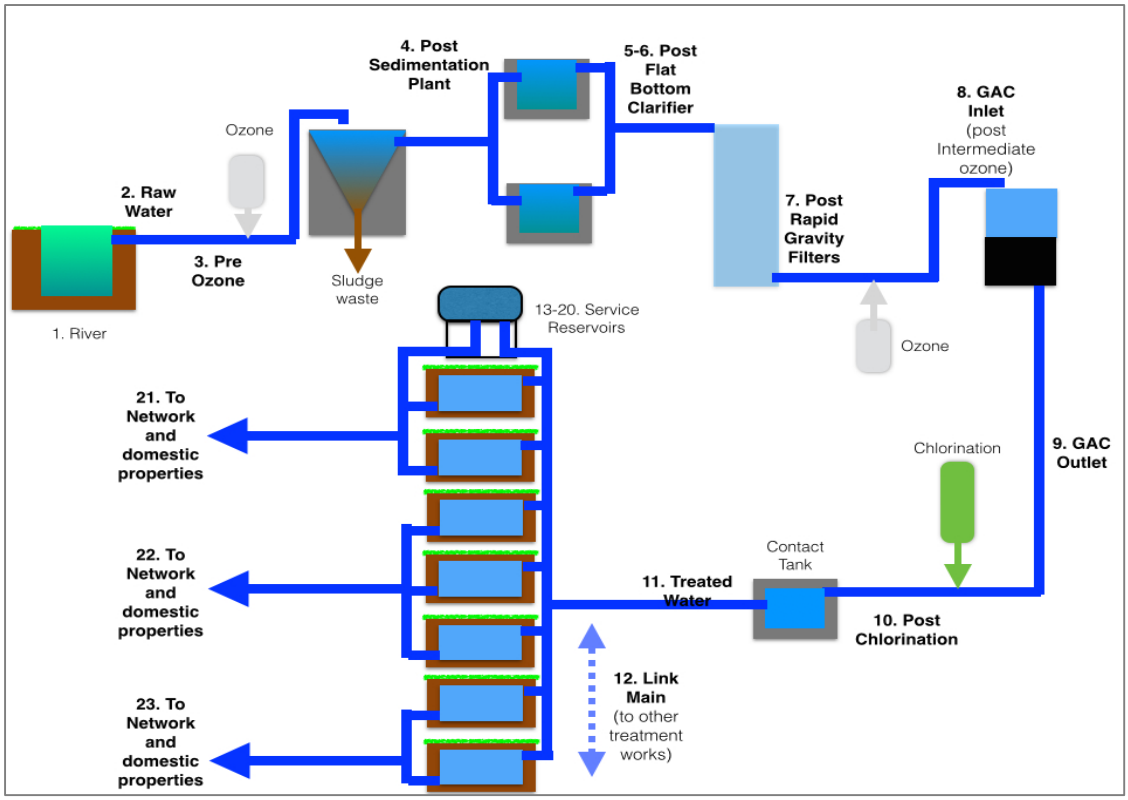


**Figure 2.5: Schematic diagram of WTW D.** Due to high turbidity of the raw water, the water was subject to treatment to remove this via the sedimentation plant, flat bottom clarifiers and rapid gravity filters.

### 2.3.5: Summary of samples collected for the study

**Table 2.5: A summary of the number of samples collected for the study.** The table summarises the number of samples taken from each site for the study, from each water treatment works and downstream domestic properties.

| WTW | No of raw water samples | No of part treatment water samples | No of treated water samples | No of samples from domestic properties |
|---|---|---|---|---|
| **A** | 18 | 63 | 81 | 45 |
| **B** | 9 | 0 | 18 | 9 |
| **C** | 27 | 0 | 18 | 9 |
| **D** | 18 | 81 | 90 | 27 |
| **Recreational lake** | 9 | N/A | N/A | N/A |

## 2.4: Distribution of water from WTW to customers

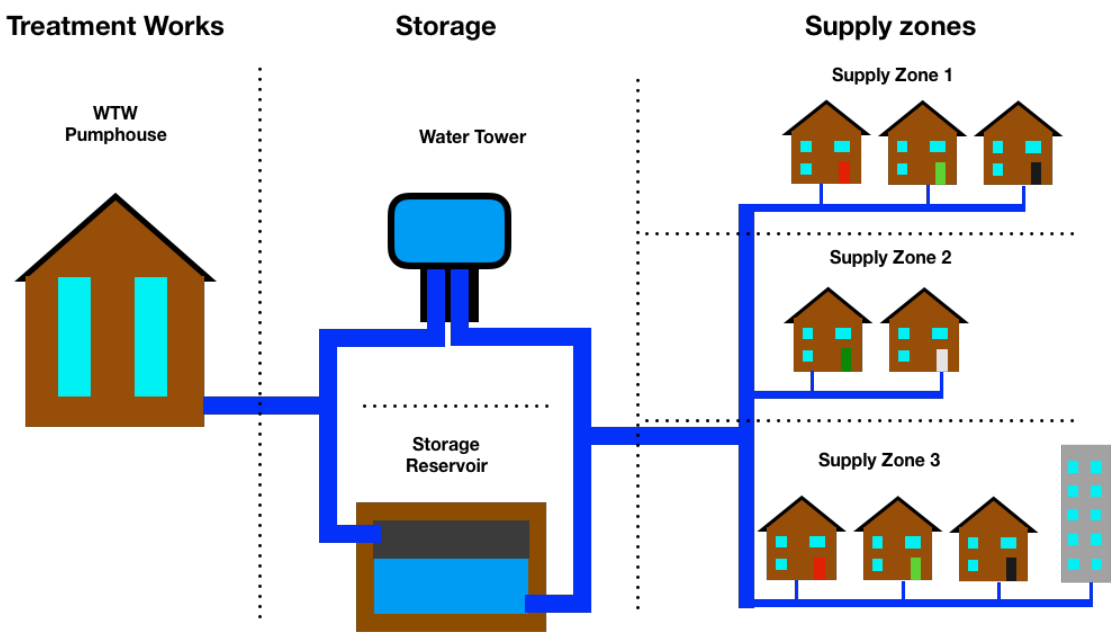


**Figure 2.6: A schematic diagram of water distribution from WTW to customers.**

After the water had been treated at the treatment works, it was pumped to and stored in either a service reservoir or a water tower (Figure 2.6). Then, depending on demand, water was pumped using boosters to customers' properties. This was to allow the treatment process to run at a constant pace, improving the effectiveness of treatment. Such storage of treated water also generally allows for an adequate supply of water should the treatment process fail or stop temporarily. The arrangements for water entering and leaving storage reservoirs had to be considered to ensure that there was a good turnover of water, and ensuring that the risk of stagnating water was reduced, as this have led to contamination of the supply. Service reservoirs were checked and cleaned every 10 years, or sooner if a potential issue was detected.

## 2.5: Sampling process of water samples

### 2.5.1: Collection of water samples

Each of the WTW had taps at most treatment stages to enable collection of water samples for routine water quality monitoring. Samples were available for this study from raw water, part treated water, treated water and some domestic properties supplied by the works (described in more detail in section 2.2). The water samples were collected aseptically in sterile screw cap bottles (Aurora Scientific, Bristol, UK) containing sodium thiosulfate to neutralise excess chlorine up to a concentration of 5mg/l. by trained sampler technicians as part of routine water quality monitoring. Extra samples were taken for this study. Samples were collected using the following procedures, as described in Microbiology of Drinking Water - Part 2 - Practices and Procedures for Sampling (2010). A different method was used in customer properties as the use of heat has the potential to damage certain fixture and fittings.

Temperature, time of sampling, pH, chlorine concentration (free and total chlorine), turbidity and electrical conductivity at $20^0$C were recorded for each sample by the laboratory and sampling teams. HPC and total coliforms were also analysed and recorded by the microbiology team. These were analysed as part of routine testing to continuously monitor water quality using methods stated by the Microbiology of Drinking Water (http://www.standingcommitteeofanalysts.co.uk).

### 2.5.2: Sample collection at WTW by a trained sampler technician

1. The tap was run for the time indicated by the sample point label provided at

each sampling point using a calibrated timer. The runtime was determined by calculating the volume required to turnover water in the pipework three times. Water was flushed at the outlet for a time calculated to allow three turnovers of water in the pipework to the outlet. The Water Supply (Water Quality) Regulations 2016 state that the sample should be representative of the distribution system rather than the tap itself.

2. After the run time, samples relating to chemical and physical properties were collected using designated bottles. For sample points after chlorine disinfection, this included an on-site chlorine check (described below).
3. The tap was turned off. Using a flamer, the end of the tap to the isolating handle was heat sterilized, until steam was seen at the nozzle.
4. The tap was turned back on and allowed to run for approximately 10-30 seconds.
5. Using an aseptic technique, water samples for routine microbiological examination were then taken.
6. An extra 2L of water (in 2 x 1L sterile bottles) were collected for *Acanthamoeba* spp. detection in this project.

### 2.5.3: Sample collection at domestic properties by trained sampler technician

1. First it was established that the tap came directly from the mains supply (i.e. water was not stored in a domestic cistern beforehand and there was no further treatment Figure 2.7).
2. The tap was run for two minutes using a calibrated timer.
3. Samples relating to chemical and physical properties were collected using

designated bottles. This included an onsite chlorine check (outlined below)

4. The tap was turned off and the end of the tap was disinfected using a 1000ppm chlorine solution.
5. The solution was given three minutes contact time using the calibrated timer.
6. The tap was then run. A free chlorine check was undertaken to ensure the concentration was within 0.10mg/l of the original reading.
7. Using an aseptic technique, samples for routine microbiological examination were then taken.
8. An extra 2L (2 x 1L sterile bottles) were collected for this project and the analysis of *Acanthamoeba* spp.

If a sample taken from a domestic property fails, water companies are required to undertake an investigation to ensure that there is no risk to public health by the water supplied. With microbiological failures, this involves understanding if the issue was due to the condition of the tap, or if there is a wider issue. A swab of the tap is taken, followed by a microbiological sample after the tap is turned on. After this, the process is the same as for a routine sample, where the tap is run and disinfected prior to another sample being taken. To ensure there are no issues in the network, a sample is taken from neighboring properties either side, using the standard disinfection procedure. If required, customers will be informed and put on boil notice till the investigation is complete and the water is deemed fit for consumption.

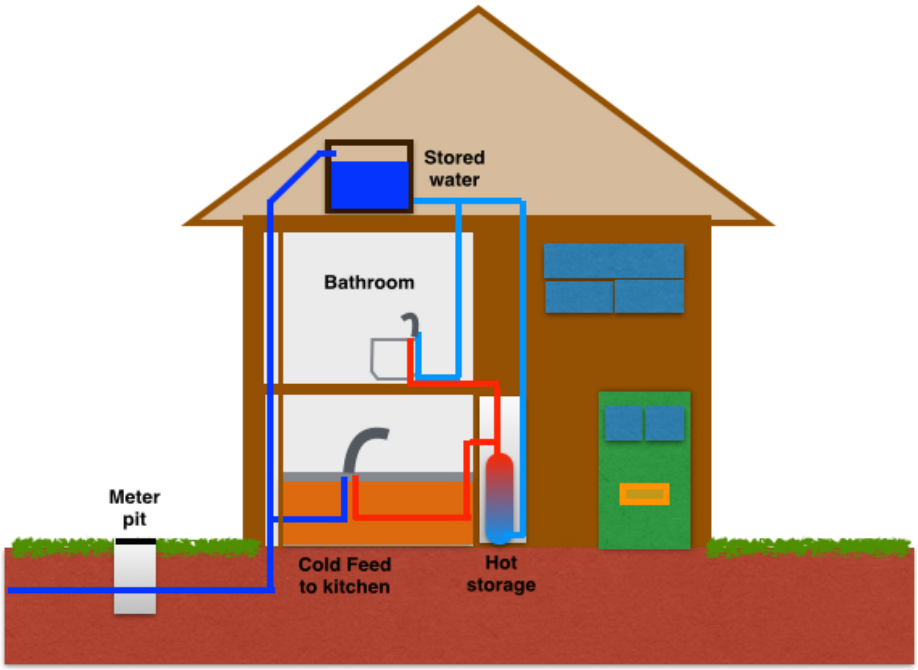


**Figure 2.7: An example of a water system in a domestic property.** Water companies in England and Wales are required to analyse water from a suitable tap to check the quality that should be representative of the mains supply. Provided there is no domestic treatment installed, this is usually the cold kitchen tap. Other outlets may have been fed through other systems including tanks and so may not be representative of the mains incoming, hence were not sampled for this study.

Domestic properties in England used to be designed with a cold-water storage tank, usually located in the roof space. Although more recent new builds generally do not have cold water storage tanks, and many older buildings have had these removed, there are many properties that still have a tank. However, the cold kitchen tap should come directly from the mains, although some properties have installed treatment, including softeners and filters, prior to this. It used to be common in the UK to have separate hot and cold taps, however it is now more common to find mixer taps. Most mixer taps are designed to prevent blending of hot and cold water until the outlet. This is usually achieved by running separate pipes through the nozzle of the tap, which is considered more hygienic, as the hot water may be subject to a deterioration of the water. However some may blend at

the base of the nozzle, allowing mains cold water and hot water to mix earlier.

Sampling frequency was determined by the population size in each ‘zone’. Zones are decided by a number of factors. The first was the WTW that supplied the area. The second was the population. DWI require that zones in England and Wales have a population of no greater than 100,000. During the sampling period, properties were randomly selected and sampled from each zone that was supplied from the WTW under investigation every month.

### 2.5.4: Onsite tests for chlorine concentration (Palintest, 2016)

1. Chlorine concentration was measured onsite by the sampler technician collecting the water samples, using a calibrated Palin 1000 chlorometer and following the manufacturer’s instructions. This device measured both free and total (i.e. free and ‘combined’ chlorine)
2. A 10ml of sample of the water was placed in the chlorometer, and the unit calibrated to zero.
3. DPD1 (N,N-diethyl-p-phenylenediamine) buffered solution was added to the solution and shaken after the cap was replaced. This reacts with free chlorine to produce a pink product (‘Würster dye’) the intensity of which was then measured and recorded. This gave the free chlorine result.
4. DPD3 was then added. This acidifies the solution and contains potassium iodide, which reacts with chloramines (chlorine combined with organic material) to release iodine, which in turn reacts with the DPD to produce the pink product. The solution was left to react for 2 minutes using a calibrated timer, after which the colour intensity was measured and recorded to give

the total chlorine concentration.

## 2.6: Isolation of *Acanthamoeba* spp. by membrane filtration

The water samples were handled aseptically and passed through sterile funnels (Pall) with 47mm diameter gridded cellulose nitrate membrane filters of 0.45 μm pore size (Sartorius Biotech GmbH, Goettingen, Germany). The membrane was placed on the base of the funnel with the gridded side facing upwards. Each sample was passed through a different funnel to prevent cross contamination of samples. NNA plates (purified agar, Oxoid Ltd, Basingstoke, UK, Appendix B) with Gram negative bacterial lawn (as a nutrient source for *Acanthamoeba* spp.) were prepared by pouring 10 ml of *Escherichia coli* broth (Appendix C) onto NNA plates. NNA was used to minimise bacterial growth. The excess bacterial broth was removed after two minutes with a sterile pipette and the plates were left to dry for 2 hours.

Once dry, the plates were inoculated with the environmental specimen, the membrane filters from the filtration of water samples. The filter membranes were cut into quarters and placed on the NNA plate with *E. coli*, with the gridded side touching the surface of the agar (Khan, 2006). The plates were incubated at $30^0$C in a humidified incubator, and the presence of *Acanthamoeba* spp. was confirmed using morphology and morphometry of trophozoites and cysts daily for up to fourteen days using a light microscope (Figures 2.8 and 2.9). The *Acanthamoeba* spp. positive plates were then stored at $4^0$C for further analysis.

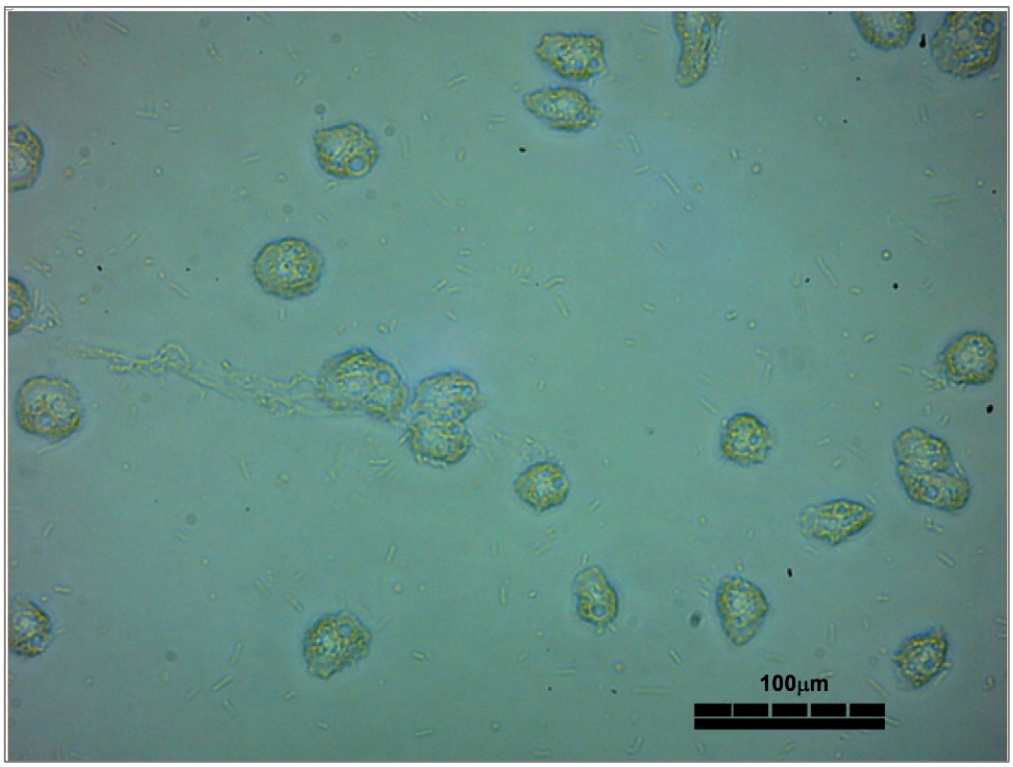


**Figure 2.8: *Acanthamoeba* trophozoites grown in PGY medium**

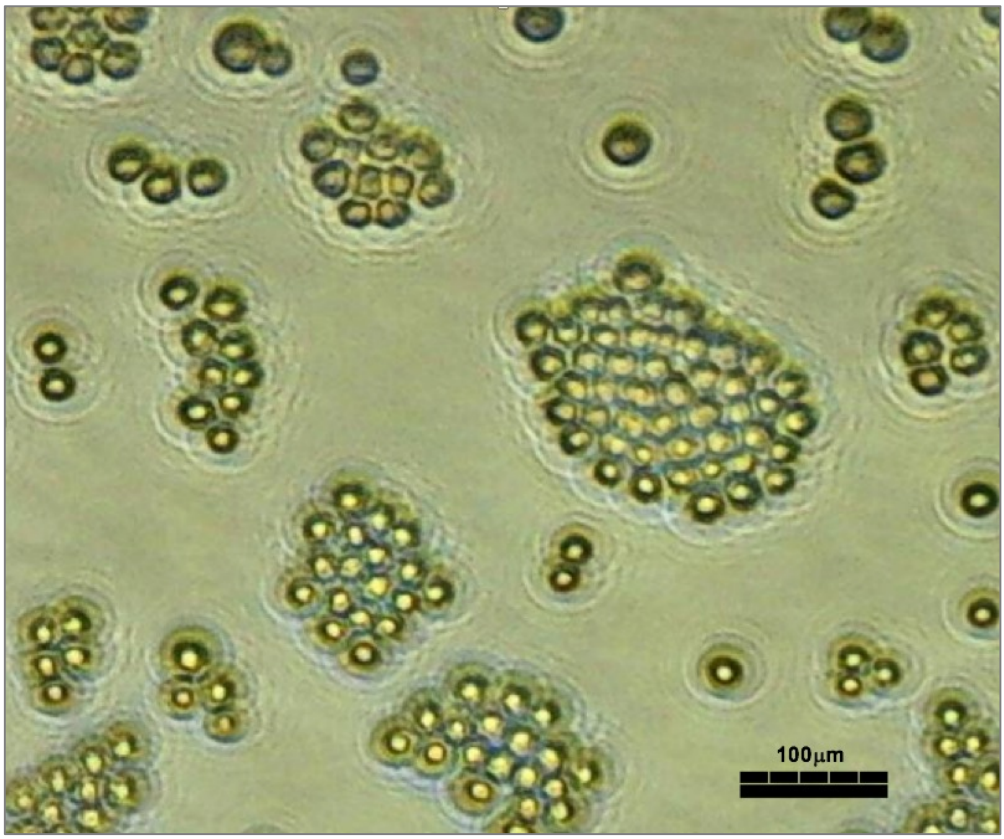


**Figure 2.9: *Acanthamoeba* cysts developed on the surface of NNA.**

## 2.7: Axenic culture of *Acanthamoeba* spp.

Approximately 1mm pieces of agar containing *Acanthamoeba* trophozoites or cysts were cut and placed on to another plate of non-nutrient agar covered with a heat-killed bacterial lawn. This was undertaken by heating the culture of *E. coli* at $75^0$C in a water bath for 10 minutes. After 24 – 48 hours, the plates were observed under the microscope and once the trophozoites had developed, 1mm of agar was cut. This agar piece was placed in a T-75 culture flask with 20ml PYG (Appendix A) and 1ml of antibiotic solution. The flask was incubated at $30^0$C and observed daily for the presence of *Acanthamoeba* spp. using a light microscope. Once the trophozoites had grown in the flask, the PYG and the agar from the flask were discarded and replaced with PYG and 0.5ml of antibiotic solution. The flask was incubated $30^0$C and observed daily for the presence of trophozoites. Once the trophozoites were present in the flask, the contents of the flask were transferred into a sterile 50ml centrifuge tube and centrifuged at 3,000 rpm for 5 minutes. The supernatant was discarded and 20ml of PYG was added to the centrifuge tube to suspend the pellet. Then the content of the centrifuge tube was transferred into T-75 flask. The flask was incubated at $30^0$C and once the trophozoites were present, 10ml were transferred from the flask to a 50ml centrifuge tube and stored at $-20^0$C.

## 2.8: Statistical analysis

Simple descriptive statistics (mean, standard deviation, median, quartiles etc.) were undertaken in Microsoft Excel. One way analysis of variance (ANOVA) and Tukey's HSD ('honestly significant difference') test for pairwise comparison of means were used to compare the results from different water sources, using the online VassarStats webpage, http://vassarstats.net/anova1u.html.

# Chapter 3

# *Acanthamoeba* spp. in ground and surface water in South East England

## 3.1: Introduction

This chapter describes studies on the presence of *Acanthamoeba* spp. in ground and surface water entering WTW in South East of England. Most WTW abstract water from either surface sources or deep aquifers and, depending on the likely chemical and microbiological 'contaminants' of the water, subsequent treatment processes are bespoke designed to ensure the provision of wholesome water to consumers. Effective treatment of the water therefore depends on understanding what contaminants might be present in the raw water entering the WTW.

As discussed in chapter 1, *Acanthamoeba* spp. are found in diverse environments, especially in aquatic environments such as surface and ground water. Several studies have demonstrated the presence of *Acanthamoeba* spp. in river water abstracted to provide drinking water, for example in Japan (Edagawa *et al.,* 2009) and Iran (Mahmoudi *et al.,* 2012), and similar studies have been undertaken on water from aquifers, for example in Mexico (Ramirez *et al.,* 2006). Although the incidence of *Acanthamoeba* spp. keratitis among contact lens wearers is increasing in the UK (Radford *et al.,* 2002 and Carnt *et al.,* 2018a), and believed to be strongly associated with tap water (Kilvington *et al.,* 2004), little is known about *Acanthamoeba* spp. in the wider UK environment and the potential sources of domestic tap water contamination.

To address this gap in knowledge, this study investigated the prevalence of *Acanthamoeba* spp. in raw water entering four WTW in South East of England.

The sites were chosen in order to represent the main types of water source used in the UK: ground (aquifers, both karstic and non-karstic) and surface water (river and lake). Karstic water (Figure 1.1) is classified as a ground water source, however it exhibits characteristics of both ground and surface water owing to the way the water reaches the aquifer.

Given its life cycle and natural history, the hypothesis tested was that *Acanthamoeba* spp. would be detected more frequently in surface water sources due to the availability of nutrient sources (i.e. bacteria), and less frequently, if at all, in ground water sources due to an expected lower bacterial/nutrient presence.

## 3.2: Materials and Methods

### 3.2.1 Sources of water samples

Raw water samples were collected over nine months, from November 2009 to August 2010 (excluding January 2010), from five locations in South East of England, as described in Chapter 2 (Figure 2.1 is a map of the locations of the WTW). These sites were all sources of raw water to a public water company that provides domestic drinking water. Raw water to one of the WTW, site D, was sampled again from February 2017 to January 2018.

A total of 74, two litres samples were collected; the number of samples ranged between 8 and 21 at each site (Figure 3.1). With the exception of a recreational lake, where two samples were taken early in the afternoon, all samples were taken between 08.00 and 13.00.

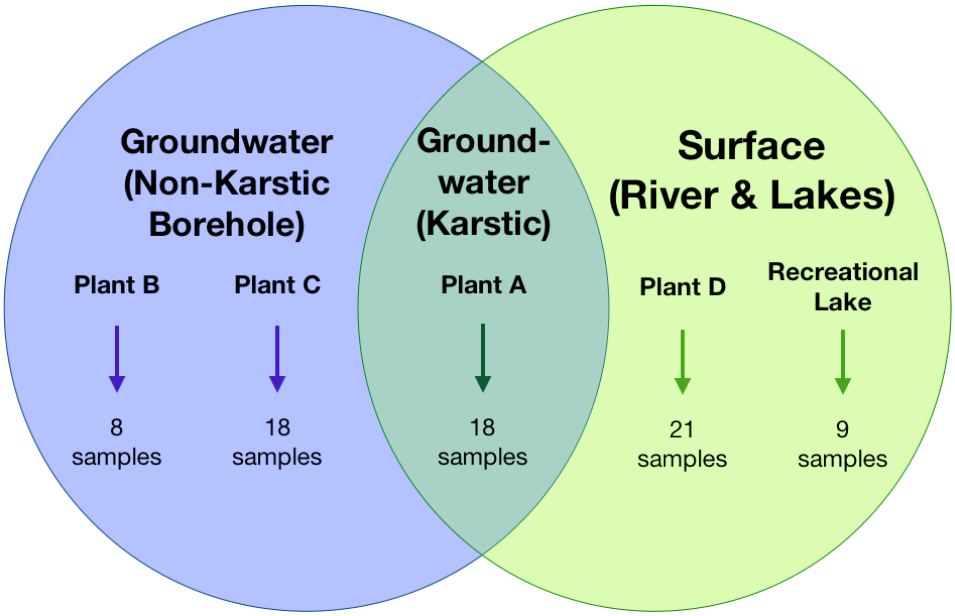


**Figure 3.1*:* Number of samples taken from ground and surface water sources.** WTW A used karstic water pumped via boreholes (18 samples in total), WTW B and C used non-karstic groundwater from boreholes (26 samples in total), and WTW D took surface water from a river and a lake (30 samples in total). See Figure 2.1 for locations of WTW. Detailed descriptions of each WTW are set out in Chapter 2, and summarised in Table 3.1.

**Table 3.1: Summary of sampling by source.** Sampling at all locations was carried out between November 2009 and August 2010, but for WTW D, sampling was repeated from February 2017 to January 2018. One 2L water sample was taken on each occasion.

| WTW | Water source | Sources sampled | Number of samples | Comments |
|---|---|---|---|---|
| A | Ground (Karstic) | Water was pumped from aquifers via boreholes where raw water entered the works through inlets 1 and 2 | 18 | Both supply water simultaneously. The water entering via both of the inlets were the same. |
| B | Groundwater (non-karstic) | Single borehole | 8 | |
| C | Groundwater (non-karstic) | Three boreholes<br>A<br>B<br>C | <br>9<br>1<br>8 | Abstraction mainly from A and C, with B used as standby. |
| D | Surface water | River<br>Lake | 21<br>9 | |

### 3.2.2: Water sample analytical assays

The detailed protocol for the isolation of *Acanthamoeba* spp. is given in Chapter 2. Briefly, each 2L water sample was concentrated by membrane filtration. The filters were then placed on non-nutrient agar plates seeded with *E.coli* as a food source, and incubated at 30°C for up to 14 days. After incubation, the plates were viewed under a light microscope for the detection of characteristic *Acanthamoeba* spp. trophozoites and cysts.

The water company laboratory monitored the pH, turbidity, electrical conductivity and bacterial counts for each month, routinely, and these data were also collected for comparison with the *Acanthamoeba* spp. results.

## 3.3: Results

**Water treatment works A: karstic ground water source**

The temperature, pH, turbidity, electrical conductivity, bacterial counts and the presence of *Acanthamoeba* spp. are all summarised in Tables 3.2 and 3.3. The water temperature at WTW A varied by 1°C during the study period, and the pH of the water was consistently neutral (mean 7.1: SD 0.1). The conductivity (a measure of ionic concentrations) across both inlets was consistent (mean 714.4; SD 13.5) and turbidity ranged from 0.10 – 0.46 NTU (mean 0.2; SD 0.1). Bacteria were consistently found in both inlets, including both coliforms (mean 8.9 cfu/100ml; SD 10.5; range 0-41) and *E. coli* (mean 1.6 cfu/100ml; SD 2.8; range 0-11). *Acanthamoeba* spp. was detected in both inlet 1 and inlet 2 in April 2010.

**Table.3.2: *Acanthamoeba* spp. isolation and summary of bacteriological and physical data for WTW A inlet 1**. (*For *Acanthamoeba* spp., - indicates not detected in the sample volume analysed, and + detected in a 2L sample of water). Raw data shown in Appendix E.

| Month | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (cfu/ml) | HPC 3D22 (cfu/ml) | Total coliforms (cfu/100 ml) | *E.coli* (cfu/100 ml) | Detection of *Acanthamoeba* spp by culture and microscopy* |
|---|---|---|---|---|---|---|---|---|---|
| Nov-09 | 11.9 | 7.1 | 712 | 0.20 | 3 | 20 | 9 | 1 | - |
| Dec-09 | 11.0 | 7.1 | 687 | 0.15 | 8 | 43 | 3 | 1 | - |
| Feb-10 | 11.8 | 7.0 | 724 | 0.27 | 2 | 30 | 0 | 0 | - |
| Mar-10 | 10.9 | 7.0 | 702 | 0.16 | 8 | 34 | 6 | 0 | - |
| Apr-10 | 10.9 | 7.0 | 712 | 0.16 | 0 | 9 | 3 | 2 | + |
| May-10 | 12.1 | 7.0 | 710 | 0.15 | 0 | 10 | 5 | 1 | - |
| Jun-10 | 11.0 | 7.0 | 712 | 0.2 | 3 | 2 | 1 | 0 | - |
| Jul-10 | 12.1 | 7.0 | 702 | 0.14 | 1 | 3 | 22 | 1 | - |
| Aug-10 | 12.1 | 7.0 | 719 | 0.14 | 5 | 12 | 11 | 0 | - |
| Mean (1d.p) | 11.5 | 7.0 | 708.9 | 0.2 | 3.3 | 18.1 | 6.7 | 0.7 | |
| SD (1 d.p) | 0.6 | 0.0 | 10.8 | 0.0 | 3.1 | 14.5 | 6.8 | 0.7 | |

**Table 3.3: *Acanthamoeba* spp. isolation and summary of bacteriological and physical data for WTW A inlet 2.** (*For *Acanthamoeba* spp., - indicates not detected in the sample volume analysed, and + detected in a 2L sample of water). Raw data shown in Appendix E.

| Month | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (cfu/ml) | HPC 3D22 (cfu/ml) | Total Coliforms (cfu/100ml) | *E.coli* (cfu/100 ml) | Detection of *Acanthamoeba* spp by microscopy* |
|---|---|---|---|---|---|---|---|---|---|
| Nov-09 | 11.8 | 7.1 | 715 | 0.14 | 3 | 38 | 6 | 1 | - |
| Dec-09 | 11.3 | 7.2 | 725 | 0.11 | 2 | 46 | 21 | 11 | - |
| Feb-10 | 12.0 | 7.1 | 723 | 0.46 | 1 | 30 | 0 | 0 | - |
| Mar-10 | 10.9 | 7.1 | 718 | 0.16 | 7 | 45 | 41 | 6 | - |
| **Apr-10** | 10.9 | 7.1 | 714 | 0.14 | 0 | 10 | 1 | 0 | **+** |
| May-10 | 11.9 | 7.1 | 688 | 0.13 | 1 | 13 | 4 | 1 | - |
| Jun-10 | 11.1 | 7.1 | 732 | 0.1 | 1 | 12 | 3 | 0 | - |
| Jul-10 | 12.2 | 7.1 | 735 | 0.13 | 0 | 7 | 6 | 0 | - |
| Aug-10 | 12.1 | 7.1 | 730 | 0.12 | 1 | 18 | 18 | 3 | - |
| Mean (1d.p) | 11.6 | 7.1 | 720.0 | 0.2 | 1.8 | 24.3 | 11.1 | 2.4 | |
| SD (1d.p) | 0.6 | 0.0 | 14.1 | 0.10 | 2.2 | 15.6 | 13.4 | 3.8 | |

**Water treatment works B: non-karstic ground water source**

The temperature, pH, turbidity, electrical conductivity, bacterial counts and the presence of *Acanthamoeba* spp. are all summarised in Table 3.4. The borehole was not available for sampling in May 2010. Water temperatures varied by 2.2°C during the study period, and the pH of the water was consistently neutral (mean 7.1; SD 0.1). The conductivity was consistent (mean 499.4; SD 12.9) and turbidity ranged from 0.09 – 0.34 NTU (mean 0.2, SD 0.1). Bacteria were rarely found and no coliforms or *E. coli* were detected. *Acanthamoeba* spp. was detected only in the November 2010 sample.

**Table 3.4: *Acanthamoeba* spp. isolation and summary of bacteriological and physical data for WTW B**. (*For *Acanthamoeba* spp., - indicates not detected in the sample volume analysed, and + detected in a 2L sample of water). Raw data shown in Appendix F.

| Month | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (cfu/ml) | HPC 3D22 (cfu/ml) | Total Coliforms (cfu/100ml) | *E.coli* (cfu/100ml) | Detection of *Acanthamoeba* spp. by microscopy* |
|---|---|---|---|---|---|---|---|---|---|
| **Nov-09** | NM | 7.0 | 484 | 0.14 | 0 | 0 | 0 | 0 | **+** |
| Dec-09 | 10.8 | 7.2 | 520 | 0.09 | 0 | 0 | 0 | 0 | - |
| Feb-10 | 11.5 | 7.1 | 509 | 0.15 | 1 | 0 | 0 | 0 | - |
| Mar-10 | 9.8 | 7.1 | 503 | 0.1 | 0 | 0 | 0 | 0 | - |
| Apr-10 | 11.9 | 7.1 | 511 | 0.15 | 0 | 1 | 0 | 0 | - |
| Jun-10 | 11.8 | 7.1 | 482 | 0.34 | 0 | 2 | 0 | 0 | - |
| Jul-10 | 12 | 7.2 | 498 | 0.14 | 0 | 0 | 0 | 0 | - |
| Aug-10 | 11.9 | 7.3 | 488 | 0.23 | 1 | 0 | 0 | 0 | - |
| Mean (1d.p) | 11.4 | 7.1 | 499.4 | 0.2 | 0.3 | 0.4 | 0.0 | 0.0 | |
| SD (1d.p) | 3.9 | 0.1 | 12.9 | 0.1 | 0.4 | 0.7 | 0.0 | 0.0 | |

**Water treatment works C: non-karstic ground water source**

WTW C received water from three different boreholes, although generally only one or two of the boreholes was run at any one time, depending on the demand. Borehole 1 was operational throughout the sampling period. Borehole 2 was operating in standby mode and was only monitored for one month in August 2010. Borehole 3 was operational throughout the study period with the exception of August. The temperature, pH, turbidity, electrical conductivity, bacterial counts and the presence of *Acanthamoeba* spp. are all summarised in table 3.5. The water temperature varied by 2.0°C during the study period (mean 11.6°C; SD 0.7) and the pH of the water was consistently neutral (mean 7.0; SD 0.0). The conductivity was consistent (mean 589.4; SD 15.9) and turbidity ranged from 0.08 – 0.26 NTU (mean 0.2; SD 0.1). Bacteria were rarely found and coliforms and *E. coli* were found only from borehole 2 in August. *Acanthamoeba* spp. were detected only in April 2010 and May 2010 samples (Table 3.5).

**Table 3.5: *Acanthamoeba* spp. isolation and summary of bacteriological and physical data for WTW C.** (For A*canthamoeba* spp., - indicates not detected, and + detected in a 2L water sample). Raw data shown in Appendix G.

| Month | Borehole Number | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (cfu/ml) | HPD2D22 (cfu/ml) | Total Coliforms (cfu/100ml) | E.coli (cfu/100ml) | Detection of *Acanthamoeba* spp. by microscopy* |
|---|---|---|---|---|---|---|---|---|---|---|
| Nov-09 | 1 | 12.9 | 7.0 | 587 | 0.09 | 1 | 0 | 0 | 0 | - |
| Dec-09 | 1 | 10.9 | 7.1 | 585 | 0.2 | 0 | 0 | 0 | 0 | - |
| Feb-10 | 1 | 11.4 | 7.0 | 588 | 0.14 | 0 | 0 | 0 | 0 | - |
| Mar-10 | 1 | 11.5 | 7.0 | 576 | 0.15 | 0 | 1 | 0 | 0 | - |
| **Apr-10** | 1 | 11.8 | 7.0 | 613 | 0.14 | 0 | 0 | 0 | 0 | **+** |
| **May-10** | 1 | 11.1 | 7.0 | 593 | 0.17 | 0 | 0 | 0 | 0 | **+** |
| Jun-10 | 1 | 11.6 | 7.1 | 573 | 0.26 | 1 | 0 | 0 | 0 | - |
| Jul-10 | 1 | 12.0 | 7.0 | 593 | 0.14 | 0 | 0 | 0 | 0 | - |
| Aug-10 | 1 | 11.7 | 7.1 | 577 | 0.21 | 1 | 0 | 0 | 0 | - |
| Aug-10 | 2 | 11.6 | 7.1 | 566 | 0.17 | 1 | 3 | 3 | 1 | - |
| Nov-09 | 3 | 12.9 | 7.0 | 598 | 0.08 | 1 | 0 | 0 | 0 | - |
| Dec-09 | 3 | 10.9 | 7.0 | 590 | 0.18 | 0 | 0 | 0 | 0 | - |
| Feb-10 | 3 | 11.6 | 7.0 | 598 | 0.15 | 0 | 0 | 0 | 0 | - |
| Mar-10 | 3 | 10.1 | 7.0 | 576 | 0.09 | 0 | 0 | 0 | 0 | - |
| Apr-10 | 3 | 11.8 | 7.0 | 624 | 0.12 | 0 | 0 | 0 | 0 | - |
| **May-10** | 3 | 11.2 | 7.0 | 593 | 0.13 | 1 | 0 | 0 | 0 | **+** |
| Jun-10 | 3 | 11.6 | 7.0 | 567 | 0.18 | 0 | 0 | 0 | 0 | - |
| Jul-10 | 3 | 12.0 | 7.0 | 612 | 0.18 | 0 | 0 | 0 | 0 | - |
| Mean (1d.p) | N/A | 11.6 | 7.0 | 589.4 | 0.2 | 0.3 | 0.2 | 0.2 | 0.1 | |
| SD (1d.p) | N/A | 0.7 | 0.0 | 15.9 | 0.1 | 0.5 | 0.7 | 0.7 | 0.2 | |

**Water treatment works D: surface water sources**

The source of raw for WTW D was surface water; it was sampled between November 2009 and August 2010, then again from February 2017 to January 2018. Raw water was abstracted from the River Thames and a local recreational lake, if required.

**Surface water (River Thames)**

The temperature, pH, turbidity, electrical conductivity, bacterial counts and the presence of *Acanthamoeba* spp. from the River Thames are all summarised in Table 3.6. The water temperature varied by 16°C during the study periods (range 6.2-22.1°C, mean 13.6°C; SD 6.7) and the pH of the water was consistently slightly alkaline (mean pH 8.0; SD 0.1). The conductivity was also fairly consistent (mean 609.2; SD 30.6), and turbidity relatively high and variable (range from 1.1 – 38 NTU, mean 6.2; SD 8.8). Few bacterial tests were done on the raw river water, but when they were analysed, the coliform and *E. coli* counts were high (860-24200 and 170 to 4610 cfu/100ml, respectively). *Acanthamoeba* spp. were detected in every sample analysed.

**Table 3.6: *Acanthamoeba* spp. isolation and summary of bacteriological and physical data for River Thames water fed to WTW D**. (For *Acanthamoeba* spp., - indicates not detected and + detected in a 2L sample of water. Elsewhere in the table, NM indicates not monitored). Raw data available in Appendix H and I.

| Month | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (cfu/ml) | HPC 3D22 (cfu/ml) | Total Coliforms (cfu/100ml) | E.coli (cfu/100ml) | Detection of *Acanthamoeba* spp. by microscopy* |
|---|---|---|---|---|---|---|---|---|---|
| **Nov-09** | 12.0 | 7.9 | 593 | 5.2 | NM | NM | NM | NM | + |
| **Dec-09** | 12.0 | 7.9 | 593 | 5.2 | NM | NM | NM | NM | + |
| **Feb-10** | 6.4 | 7.9 | 563 | 8.0 | NM | NM | NM | NM | + |
| **Mar-10** | 8.2 | 8.0 | 591 | 5.1 | NM | NM | NM | NM | + |
| **Apr-10** | 10.1 | 8.2 | 606 | 4.1 | NM | NM | NM | NM | + |
| **May-10** | 13.6 | 8.1 | 572 | 2.2 | NM | NM | NM | NM | + |
| **Jun-10** | 19.2 | 7.9 | 624 | 3.0 | NM | NM | NM | NM | + |
| **Jul-10** | 19.2 | 7.9 | 624 | 3.0 | NM | NM | NM | NM | + |
| **Aug-10** | 19.2 | 8.1 | 637 | 1.2 | NM | NM | NM | NM | + |
| **Feb-17** | NM | 8.0 | 603 | 5.6 | NM | NM | 1300 | 308 | + |
| **Mar-17** | 9.9 | 8.0 | 600 | 19.0 | NM | NM | NM | NM | + |
| **Apr-17** | 13.8 | 7.8 | 622 | 2.0 | NM | NM | NM | NM | + |
| **May-17** | 18.0 | 7.9 | 569 | 2.6 | NM | NM | NM | NM | + |
| **Jun-17** | 17.8 | 8.0 | 632 | 1.8 | NM | NM | NM | NM | + |
| **Jul-17** | 22.1 | 8.0 | 625 | 1.8 | NM | NM | NM | NM | + |
| **Aug-17** | NM | 7.9 | 615 | 1.9 | NM | NM | NM | NM | + |
| **Sep-17** | 15.3 | 8.0 | 624 | 1.1 | NM | NM | NM | NM | + |
| **Oct-17** | 14.1 | 8.0 | 655 | 1.2 | NM | NM | 860 | 170 | + |
| **Nov-17** | NM | 7.9 | 674 | 1.1 | NM | NM | NM | NM | + |
| **Dec-17** | 6.2 | 7.8 | 623 | 18.0 | NM | NM | NM | NM | + |
| **Jan-18** | 7.5 | 7.9 | 548 | 38.0 | NM | NM | 24200 | 4610 | + |
| **Mean (1.d.p)** | 13.4 | 8.0 | 609.2 | 6.2 | | | 8786.7 | 1696.0 | |
| **SD (1 d.p)** | 6.7 | 0.1 | 30.6 | 8.8 | | | 13350.2 | 2524.5 | |

**Recreational lake surface water**

The recreational lake was used as a reserve source for WTW D. The temperature, pH, turbidity, electrical conductivity, bacterial counts and the presence of *Acanthamoeba* spp. from the lake are all summarised in Table 3.7. The water temperature varied by 17.6°C during the study period (range 5-22.6°C, mean 13.5°C SD 6.1), and the pH of the water was again consistently alkaline (mean pH 8.1; SD 0.2). The conductivity was fairly consistent (mean 639.8, SD 26.1), but the turbidity was lower and less variable than water from the River Thames (range from 0.59 – 2.8 NTU, mean 1.3; SD 0.9). Bacteria, including *E. coli*, were detected in every sample apart from those taken in May. *Acanthamoeba* spp. were detected in every sample taken, and cryptosporidial oocysts were detected in the one sample tested, in February, 2010.

**Comparison of raw water sources**

Three main types of water source were sampled and the results from each source are summarised in Table 3.8. The water temperatures were on average similar (mean 12.3°C) from all water sources, however surface water temperatures (site D) were much more variable than those from aquifers (sites A-C), whether karstic (site A) or ground water (sites B and C) (Figure 3.2).

**Table 3.7: *Acanthamoeba* spp. isolation and summary of bacteriological and physical data for recreational lake**. (For *Acanthamoeba* spp., - indicates not detected and + detected in a 2L water sample. NM- Not monitored). Raw data available in Appendix H.

| Month | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37C (cfu/ml) | HPC 3D22C (cfu/ml) | Total Coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy* | Presence of Cryptosporidium oocysts |
|---|---|---|---|---|---|---|---|---|---|---|
| **Nov-09** | 11.2 | 7.8 | 659 | 0.59 | 30 | 1000 | 29 | 0 | + | NM |
| **Dec-09** | 5.0 | 7.9 | 652 | 0.87 | <10 | 170 | 14 | 9 | + | NM |
| **Feb-10** | 5.6 | 7.8 | 665 | 1.10 | 30 | 110 | 3 | 2 | + | + |
| **Mar-10** | 10.6 | 8.3 | 650 | 2.80 | 30 | 20 | 0 | 0 | + | NM |
| **Apr-10** | 14.1 | 8.2 | 660 | 0.52 | 10 | 70 | 2 | 2 | + | NM |
| **May-10** | 14.0 | 8.1 | 629 | 0.60 | 570 | 880 | 0 | 0 | + | NM |
| **Jun-10** | 19.8 | 8.2 | 647 | 1.70 | 60 | 2180 | 21 | 8 | + | NM |
| **Jul-10** | 22.6 | 8.3 | 590 | 2.60 | 100 | 710 | 130 | 0 | + | NM |
| **Aug-10** | 18.5 | 8.1 | 606 | 1.10 | 360 | 1750 | 145 | 14 | + | NM |
| Mean (1d.p) | 13.5 | 8.1 | 639.8 | 1.3 | 148.8 | 765.6 | 38.2 | 3.9 | | |
| SD (1d.p) | 6.1 | 0.2 | 26.1 | 0.9 | 198.0 | 779.8 | 57.3 | 5.2 | | |

**Table 3.8: Summary and comparison of raw water sources at each WTW and recreational lake.** The mean, SD and range of temperature, pH, electrical conductivity (EC), turbidity, heterotrophic plate count (HPC) 2D37, HPC 3D22, total coliforms, *E.coli* and *Acanthamoeba* spp. detection of the raw water source of all WTW in this study. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, NM denotes data not monitored as part of routine sample analysis, raw data is in appendix E-I).

| Source | Statistics (1d.p) | Temperature (°C) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (cfu/ml) | HPC 3D22 (cfu/ml) | Total Coliforms (cfu/100ml) | E.coli (cfu/100ml) | % of samples positive for the detection of *Acanthamoeba* spp. :% of months *Acanthamoeba* spp detected |
|---|---|---|---|---|---|---|---|---|---|---|
| **WTW A Ground (Karstic)** | Mean | 11.6 | 7.07 | 714 | 0.17 | 2.56 | 21.22 | 8.89 | 1.56 | **11** (2 of 18 samples positive) : **11** (1 of 9 months) |
| | ±SD | 0.5 | 0.06 | 13 | 0.08 | 2.71 | 14.96 | 10.54 | 2.79 | |
| | Range | 10.9-12.2 | 7.0-7.2 | 687-735 | 0.10-0.46 | 0-8 | 2-46 | 0-41 | 0-11 | |
| | n | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | |
| **WTW B ground** | Mean | 11.4 | 7.14 | 499 | 0.17 | 0.25 | 0.38 | 0.00 | 0.00 | **13** (1 of 8 samples positive) : **13** (1 of 8 months) |
| | ±SD | 3.9 | 0.09 | 13 | 0.08 | 0.43 | 0.70 | 0.00 | 0.00 | |
| | Range | 9.8-12.0 | 7.0-7.3 | 482-520 | 0.09-0.34 | 0-1 | 0-2 | N/A | N/A | |
| | n | 7 | 8 | 8 | 8 | 8 | 8 | 8 | 0 | |
| **WTWC ground** | Mean | 11.6 | 7.02 | 589 | 0.15 | 0.33 | 0.22 | 0.17 | 0.06 | **17** (3 of 18 samples positive) : **22** (2 of 9 months) |
| | ±SD | 0.7 | 0.04 | 16 | 0.05 | 0.49 | 0.73 | 0.71 | 0.24 | |
| | Range | 10.10-12.90 | 7.0-7.1 | 566-624 | 0.08-0.026 | 0-1 | 0-3 | 0-3 | 0-1 | |
| | n | 18 | 18 | 18 | 18 | 18 | 18 | 18 | 18 | |
| **WTW D river** | Mean | 13.4 | 7.96 | 609 | 6.24 | NM | NM | 8786.7 | 1696.0 | **100** (21 of 21 samples positive) :**100** (21 of 21 months) |
| | ±SD | 6.7 | 0.10 | 31 | 8.81 | NM | NM | 13350.2 | 2524.5 | |
| | Range | 6.2-22.1 | 7.80-8.20 | 548-674 | 1.10-38.0 | N/A | N/A | 860-24200 | 170-4610 | |
| | n | 18 | 21 | 21 | 21 | N/A | N/A | 3 | 3 | |
| **Lake** | Mean | 13.5 | 8.08 | 640 | 1.32 | 77.50 | 667.78 | 38.22 | 3.89 | **100** (9 of 9 samples positive) : **100** (9 of 9 months) |
| | ±SD | 6.1 | 0.20 | 26 | 0.86 | 113.63 | 817.92 | 57.30 | 5.16 | |
| | Range | 5.0-22.6 | 7.8-8.3 | 590-665 | 0.52-2.80 | 10-570 | 20-2180 | 0-145 | 0-14 | |
| | n | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | |

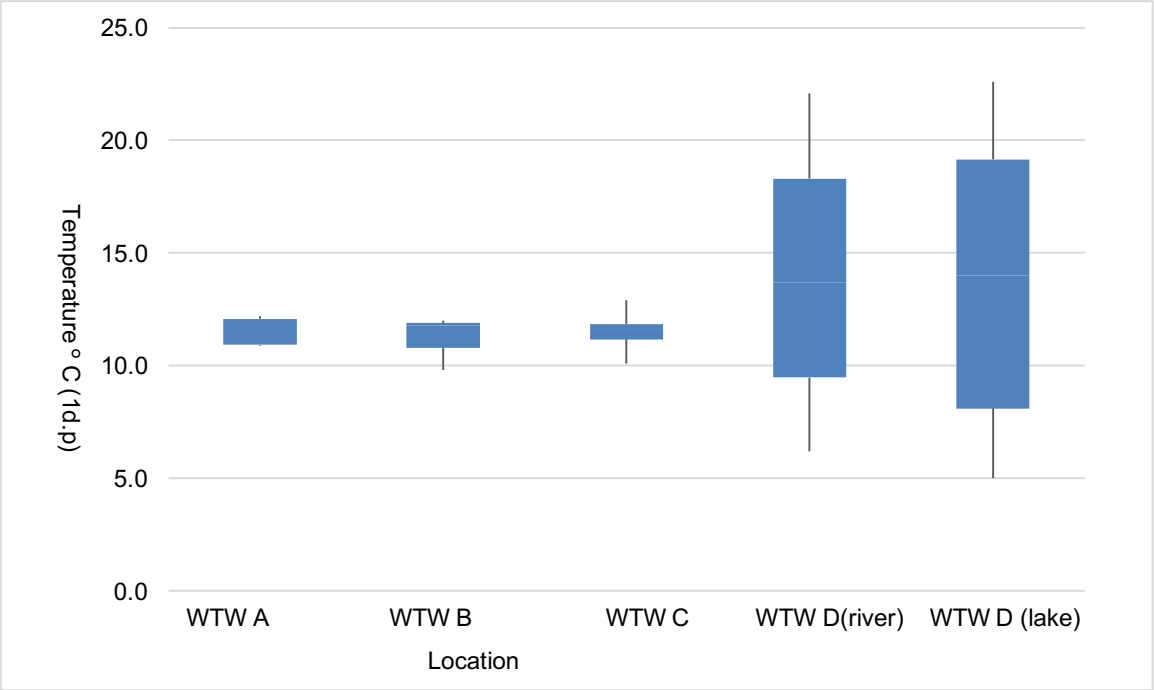


**Figure 3.2: Box plots of temperatures of different water sources** (median, Q1-3 and range). Sites A-C were from aquifers. Site A was a karstic source, sites B and C non-karstic ground water sources. WTW D took water from surface water of a river and lake, which are labelled separately.

The pH was neutral to slightly alkaline for all water sources: both surface water sources were slightly more alkaline than aquifer sources (one way ANOVA, *p*=<0.01). The conductivity, which was fairly constant within each water source, differed significantly between all sites (ANOVA, p=<0.01) apart from between ground water at WTW C and river water at WTW D. Turbidity was similar at all sites except for river water (WTW D), which was significantly more turbid (ANOVA, *p*=<0.01).

HPC, coliforms and *E. coli* counts varied significantly between water sources. Heterotrophic plate counts at 37°C (not available for the River Thames) were very similar at all sites apart from the lake, where they were significantly higher

($p$=<0.01). Heterotrophic plate counts at 22°C (again not available for the River Thames) were higher than at 37°C and again significantly higher for lake water (site D) ($p$=<0.01) than for aquifer sources (WTW A to C). The difference between bacterial counts at the karstic aquifer (WTW A) and non-karstic groundwater aquifer sites (B and C) was significant ($p$=<0.01) when the WTW D data were not included in the analysis.

In an ANOVA analysis of all five waters sources (i.e. including the three counts for the River Thames), only the River Thames's extremely high counts, were significantly different from counts at the other sites. However, if the river results were not included, then the lake counts were also significantly higher ($p$=<0.01) than the borehole coliform counts, and if only aquifer counts were compared then the karstic water had significantly higher coliform counts than the non-karstic groundwater samples ($p$=<0.01). Similarly, the river water had significantly higher *E. coli* counts than the other water sources. If this was excluded from the analysis, the ANOVA was still significant ($p$=<0.01) but the only significant difference in the Tukey HSD pairwise comparison of means was between site B (ground water with no positives) and the lake samples. If only aquifer samples were compared, then neither the ANOVA ($p$=0.03) nor Tukey analyses revealed any significant differences in *E. coli* counts between water sources, *Acanthamoeba* spp. were detected in 11-17% of aquifer samples, but consistently (100%) of surface water samples.

## 3.4: Discussion

*Acanthamoeba* spp. were detected by culture in raw water sources in WTW in the South East of England that provide drinking water to domestic and commercial consumers. They were isolated at least once during the sampling period from all raw water sources, but much more frequently from surface water than aquifers (ground water). This confirms previous reports (Ramirez *et al.,* 2006; Hoffmann and Michel, 2001), which demonstrated *Acanthamoeba* spp. in both borehole and surface water respectively. The findings also confirm our hypothesis set out at the beginning of this study that surface water would be more likely to contain *Acanthamoeba* spp. than ground water. Detection by culture, or at least by the culture method used in this study, is qualitative rather than quantitative, and detects only living, culturable protozoa and is of unknown sensitivity. Alternative approaches, such as PCR, are increasingly used for the diagnosis of clinical infection, but not for environmental sampling (Clarke *et al.,* 2012). PCR might be more sensitive and also detect a greater diversity of *Acanthamoeba* spp., and might also yield more quantitative results (see chapter 5).

No seasonality in prevalence by culture was observed. Rather *Acanthamoeba* spp. were detected only sporadically in aquifer water but constantly in surface water samples. This suggests that surface water might provide a greater public health risk via the drinking water system than aquifer water.

Water parameters, apart from the temperature of surface water, were largely consistent over time within each source but varied between sources. Average temperatures were similar over time and between water sources, but fluctuated widely with season for surface water, doubtless reflecting seasonal changes in weather and therefore temperature above ground. However, the lack of seasonality of *Acanthamoeba* spp. detection in water suggests temperature was not a significant factor in its prevalence during this study (although, as already indicated, it might still drive the diversity and/or abundance of protozoa).

The differences in pH between surface and aquifer samples probably reflect local geology and the exposure of surface water to run off. The total pH range observed across all water sources and time (pH 7.0-8.3) is suitable for the growth of *Acanthamoeba* spp. (Khan, 2006).

The variation in conductivity between water sources (but not over time) reflects soluble ions. The electrical conductivity of ground water sources was lower than surface water sources, indicating that there were less dissolved ions in ground water than surface water, presumably reflecting exposure to surface run off from various inorganic and organic sources. This might affect the ability of both bacteria and *Acanthamoeba* spp. to grow and survive in aquifers. Similarly, the turbidity of river water was significantly higher in river water than aquifer water, but with considerable variation from month to month, probably driven by weather, erosion and run off from soil.

It was hypothesized that *Acanthamoeba* spp. would be detected more frequently in surface water sources due to the availability of food sources (i.e. bacteria), and less frequently, if at all, in ground water sources due to a presumed lower bacterial presence. Bacterial loads did, indeed, vary between water sources. HPC, especially of bacteria growing at lower temperatures, were much higher in surface water than in aquifer water, but also slightly higher in karstic than ground water samples. This is readily explainable in terms of nutrient, particularly carbon, availability. Surface waters will contain high nutrient loads from run off and the diverse microbial and other communities sustained. The strata above the aquifers in this study involved chalk beds, which is a good natural source of filtration. However, it would also be expected that the karstic water, having reached the aquifer through cracks rather than solely filtered through solid rock, would have higher nutrient concentrations and thus support a higher bacterial load. This seems to be the case. The low coliform and *E. coli* counts from the karstic water source might also reflect surface contamination reaching the aquifer.

Thus, *Acanthamoeba* spp. are detected more frequently in surface waters, possibly due to their higher loads of bacteria than aquifers, and particularly than ground water aquifers. Furthermore, no other parameters measured correlated with the presence or absence of *Acanthamoeba* spp. However, whether or not these observations are related or simply coincidence cannot be determined from the data collected, and changes in other water parameters over time (e.g. turbidity) lend no support to any causal link.

In addition to posing a potential risk to drinking water, the isolation of *Acanthamoeba* spp. from the river and lake supports a further risk factor for *Acanthamoeba* keratitis, that of direct contact with contaminated water while wearing contact lenses, e.g. swimming in lakes and rivers (Khan, 2006).

# Chapter 4

# The effectiveness of water treatment works in eliminating *Acanthamoeba* species.

## 4.1: Introduction

Chapter 3 described the results of a prevalence study of *Acanthamoeba* spp. in raw water entering four WTW in South East of England. *Acanthamoeba* spp. can cause a range of clinical diseases in humans, but as discussed in chapter 1, of particular importance in the UK is keratitis (AK), particularly among contact lens wearers. Infection is strongly associated with tap water (Kilvington *et al.,* 2004 and Seal *et al.,* 1992) although direct contact with contaminated water, for example through water sport activities, is another well-documented transmission route (Khan, 2006). The results in Chapter 3, demonstrated *Acanthamoeba* spp. in the raw water sources supplying all the studied WTW, but particularly in surface water sourced from the River Thames and a recreational lake, i.e. in water used both for domestic potable water supply and for recreation.

In this chapter, the results of the investigation of the presence of *Acanthamoeba* spp. at different stages of processing through WTW are described. Each WTW (described in detail in Chapter 2) had different treatment processes for ensuring that the water supplied to homes is wholesome. The source water determined the treatment processes for the WTW, whether surface or ground. However, these processes were developed with pathogens other than *Acanthamoeba* spp. in mind. Due to this, little is known about their effectiveness in removing *Acanthamoeba* spp. from raw water, or the sources of the *Acanthamoeba* spp found, for example, in domestic tap water (Kilvington *et al.,* 2004).

## 4.2: Materials and Methods

### 4.2.1: Sample collection

Water samples were collected over nine months, from November 2009 to August 2010 (excluding January 2010), from four WTW, as described in Chapter 2 (Figure 2.1 for a map of locations of WTW), and from a random selection of properties supplied by those WTW. The WTW were chosen in order to represent the main types of water source and WTW used in England. Detailed descriptions of each WTW are set out in Chapter 2, and summarised in Table 4.1.

Water samples collected included raw water (described in Chapter 3), part treated water, treated water and water from some domestic properties supplied by the WTW. Samples were collected aseptically in sterile screw cap bottles as part of routine water quality monitoring, as determined by The Microbiology of Drinking Water (SCA, 2010). All protocols are described in more detail in Chapter 2 (section 2.2).

**Table 4.1: Summary of number of samples taken from WTW.** Sampling were taken from raw source, part treated water, treated water, treated water storage and from domestic properties. Sampling was carried out between November 2009 and August 2010, and again from WTW D in February 2017 to January 2018 (D* in the table). A 2L water sample was taken from each sampling point. There were no samples taken from part treated stages at WTW B and WTW C as there is no sampling points at this this stage.

| WTW | Raw samples | Partially treated samples | Treated samples from WTW | Treated sample storage | Domestic property samples | Total number of samples |
|---|---|---|---|---|---|---|
| **A Ground (karstic) water** | 18 | 63 | 18 | 63 | 45 | 207 |
| **B Ground water** | 8 | 0 | 8 | 9 | 9 | 34 |
| **C Ground water** | 18 | 0 | 9 | 18 | 9 | 54 |
| **D Surface water** | 9 | 81 | 18 | 72 | 27 | 207 |
| **D* Surface water** | 12 | 108 | 12 | 0 | 0 | 132 |

The temperature, time of sampling, pH, chlorine concentration (free and combined chlorine), turbidity, electrical conductivity at 20°C and bacterial counts were recorded for each sample, and 2L water samples were collected for *Acanthamoeba* spp. detection. Diagrams explaining the processes and sampling points for each WTW are given in Chapter 2 (section 2.3). Water samples were analysed by membrane filtration, and the filters placed on non-nutrient agar plates with *E. coli* as a food source, and incubated at 30°C for up to 14 days, then

examined microscopically for the presence of trophozoites and/or cysts as described in chapter 2 (sections 2.4 onwards).

## 4.3: Results

**Water treatment works A**

During the sampling period, 207 samples, including raw water samples, were collected and tested for the presence of *Acanthamoeba* spp. from WTW A (Table 4.1). Over the sampling period, *Acanthamoeba* spp. were detected in April in the raw water sources, as already described in chapter 3, but nowhere else in the WTW (Table 4.2). The average temperature of the samples taken from raw water to domestic properties was in the range11.6°C to 12.9°C (Table 4.3), and while most water chemical parameters did not change through the treatment and distribution process, treatment at the WTW approximately halved the turbidity of the raw water.

Low numbers of bacteria were detected in the raw water samples, as described in Chapter 3, but fewer and no coliforms, were detected after water treatment. However, while the mean bacterial counts increased in samples collected from domestic properties, the SD (Table 4.4) and raw data (Appendix E) indicates this resulted from a very small number of individual properties with high bacterial counts.

**Table 4.2: The detection of *Acanthamoeba* spp. at each step of water treatment at WTW A.** Samples were taken between November 2009 and August 2010 (Appendix E). Sampling points are as described in Table 2.1. A schematic diagram of WTW is in chapter 2 (figure 2.2). (+ *Acanthamoeba* spp. Isolated, - *Acanthamoeba* spp. not isolated).

| Sampling points | Nov 09 | Dec 09 | Feb 10 | Mar 10 | Apr 10 | May 10 | Jun 10 | Jul 10 | Aug 10 |
|---|---|---|---|---|---|---|---|---|---|
| 1. Inlet 1 | - | - | - | - | + | - | - | - | - |
| 2. Inlet 2 | - | - | - | - | + | - | - | - | - |
| 3. (GAC) inlet 1 | - | - | - | - | - | - | - | - | - |
| 4. (GAC) inlet 2 | - | - | - | - | - | - | - | - | - |
| 5. GAC filters | - | - | - | - | - | - | - | - | - |
| 6. GAC basin 1 | - | - | - | - | - | - | - | - | - |
| 7. GAC basin 2 | - | - | - | - | - | - | - | - | - |
| 8. Pre membrane 36 | - | - | - | - | - | - | - | - | - |
| 9. Pre membrane 27 | - | - | - | - | - | - | - | - | - |
| 10. Treated water 36 | - | - | - | - | - | - | - | - | - |
| 11. Treated water 27 | - | - | - | - | - | - | - | - | - |
| 12. Service reservoir 1 | - | - | - | - | - | - | - | - | - |
| 13. Service reservoir 2 | - | - | - | - | - | - | - | - | - |
| 14. Service reservoir 3 | - | - | - | - | - | - | - | - | - |
| 15. Service reservoir 4 | - | - | - | - | - | - | - | - | - |
| 16. Service reservoir 5 | - | - | - | - | - | - | - | - | - |
| 17. Service reservoir 6 | - | - | - | - | - | - | - | - | - |
| 18. Service reservoir 7 | - | - | - | - | - | - | - | - | - |

**Table 4.3: Physical and chemical properties of water at WTW A.** The mean, SD and range of temperature, pH, electrical conductivity (EC) and turbidity of the untreated water, part treated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, NM denotes data not monitored as part of routine sample analysis, raw data is in appendix E).

| Water type | Temperature (°C) (1d.p) | | pH (1d.p) | | EC@ 20°C (uS/cm) | | Turbidity (NTU) (2d.p) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=18) | 11.6 | 0.5 | 7.1 | 0.1 | 714 | 13 | 0.20 | 0.08 |
| | R (10.9 - 12.2) | | R (7.0 – 7.2) | | R (687-735) | | R (0.1-0.5) | |
| Part treated (n=16) | NM | | 7.1 | 0.1 | 723 | 18 | 0.20 | 0.10 |
| | | | R (7.0 – 7.3) | | R (697 – 765) | | R (0.1 – 0.4) | |
| Treated (n=63) | 11.6 | 0.4 | 7.0 | 0.1 | 717 | 13 | 0.11 | 0.04 |
| | R (10.9 – 12.0) | | R (6.9 – 7.1) | | R (691 – 744) | | R (0.05 – 0.20) | |
| Treated water at service reservoir (n=44) | 12.0 | 1.7 | 7.1 | 0.1 | 711 | 18 | 0.10 | 0.06 |
| | R (9.8-19.3) | | R (7.0 – 7.3) | | R (691 – 744) | | R (0.05 – 0.20) | |
| Domestic properties (n=45) | 12.9 | 4.2 | 7.1 | 0.1 | 687 | 45 | 0.10 | 0.08 |
| | R (13.0-21.4) | | R (7.1 – 7.4) | | R (687 – 744) | | R (0.15 – 0.4) | |

**Table 4.4: Summary of the bacteriological results from WTW A**. The mean heterotrophic plate count (HPC) 2D37, HPC 3D22, total coliforms and *E.coli* of the untreated water, part treated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, NM denotes data not monitored as part of routine sample analysis, raw data is in appendix E).

| Water type | HPC 2D37 (cfu/ml) (1d.p) | | HPC 3D22 (cfu/ml) (1d.p) | | Total coliforms (cfu/100ml)* (1d.p) | | *E.coli* (cfu/100ml) (1d.p)* | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **Untreated (n=18)** | 2.6 | 2.7 | 21.2 | 14.9 | 8.9 | 10.5 | 1.6 | 2.8 |
| | R (0-8) | | R (2-46) | | R (0-41) | | R (0 – 11) | |
| **Part treated (n=34)** | 1.5 | 2.4 | 31.0 | 98.0 | 0.2 | 1.1 | 0 | 0 |
| | R (0-9) | | R (0 – 560) | | R (0-6) | | | |
| **Treated (n=18)** | 0.1 | 0.2 | 0.1 | 0.3 | 0 | 0 | 0 | 0 |
| | R (0 – 1) | | R (0 – 1) | | | | | |
| **Treated water at service reservoir (n=63)** | 2.2 | 16.8 | 1.6 | 4.1 | 0 | 0 | 0 | 0 |
| | R (0 – 133) | | R (0-21) | | | | | |
| **Domestic properties (n=45)** | 14.0 | 47.6 | 11.3 | 45.5 | 0 | 0 | 0 | 0 |
| | R (14 – 248) | | R (11 – 291) | | | | | |

**Water treatment works B**

Over nine months, 34 samples were collected and tested for *Acanthamoeba* spp. from WTW B. *Acanthamoeba* spp. was isolated only from the raw source and only in November as described in Chapter 3. *Acanthamoeba* spp. was not isolated in the final treated water or the water tower (Table 4.5), where water was stored before it was supplied to customers.

The mean temperature of samples taken from untreated raw water to domestic water was between 11.4°C to 13.2 °C (Table 4.6). There was no significant

difference in turbidity, pH and electrical conductivity between untreated water and the water at domestic properties. At this site coliforms were not detected at any stage. The HPC count was higher at domestic properties than at the WTW (Table 4.7).

**Table 4.5 The detection of *Acanthamoeba* spp. in at each step during treatment at WTW B**. A schematic diagram of WTW is in chapter 2 (figure 2.3). (+ *Acanthamoeba* spp. Isolated, - *Acanthamoeba* spp. not isolated). In May 2010 the raw and final treated sampling point was not monitored (NM), as it was not available to take sample from.

| Sampling points | Nov 09 | Dec 09 | Feb 10 | Mar 10 | Apr 10 | May 10 | Jun 10 | Jul 10 | Aug 10 |
|---|---|---|---|---|---|---|---|---|---|
| 1. Raw | + | - | - | - | - | NM | - | - | - |
| 2. Treated water | - | - | - | - | - | NM | - | - | - |
| 3. Service reservoir | - | - | - | - | - | - | - | - | - |

**Table 4.6: Physical and chemical properties of water at WTW B**. The mean, SD and range of temperature, pH, electrical conductivity (EC) and turbidity of the untreated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, raw data is in appendix F, *n=8 was used to calculated the mean and SD of untreated sample temperature).

| Water type | Temperature (°C) (1d.p) | | pH (1d.p) | | EC@ 20°C (uS/cm) | | Turbidity (NTU) \|(2d.p) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=9*) | 11.4 | 0.8 | 7.1 | 0.1 | 499 | 14 | 0.17 | 0.08 |
| | R (9.8-12.0) | | R (7.0-7.3) | | R (484 – 520) | | R (0.09-0.34) | |
| Treated (n=9) | 11.3 | 0.8 | 7.1 | 0.1 | 507 | 12 | 0.14 | 0.06 |
| | R (10.0 – 12.0) | | R (7.0 – 7.3) | | R (490 – 520) | | R (0.09 –0.26) | |
| Treated water at storage tower (n=9) | 11.9 | 3.2 | 7.2 | 0.1 | 550 | 60 | 0.13 | 0.71 |
| | R (7.0 – 16.6) | | R (7.1-7.4) | | R (488 – 684) | | R (0.07 -0.24) | |
| Domestic properties (n=9) | 13.2 | 3.4 | 7.3 | 0.2 | 569 | 70 | 0.12 | 0.05 |
| | R (9.2 – 20.0) | | R (7.1 – 7.6) | | R (514 – 684) | | R (0.06-0.23) | |

**Table 4.7: Summary of the bacteriological results from WTW B.** The mean heterotrophic plate count (HPC) 2D37, HPC 3D22, total coliforms and *E.coli* of the untreated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, raw data is in appendix E).

| Water type | HPC 2D37 (cfu/ml) | | HPC 3D22 (cfu/ml) | | Total coliforms (cfu/100ml) | | *E.coli* | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| **Untreated (n=9)** | 0.3 | 0.5 | 0.4 | 0.7 | 0 | 0 | 0 | 0 |
| | R (0-1) | | R (0 – 2) | | | | | |
| **Treated (n=9)** | 0.4 | 0.7 | 0 | 0 | 0 | 0 | 0 | 0 |
| | R (0 – 2) | | | | | | | |
| **Treated water at storage tower (n=9)** | 0.1 | 0.1 | 0.4 | 0.7 | 0 | 0 | 0 | 0 |
| | R (0 -2) | | R (0 -2) | | | | | |
| **Domestic properties (n=9)** | 10.3 | 30.1 | 4.7 | 13.3 | 0 | 0 | 0 | 0 |
| | R (0-91) | | R (0-40) | | | | | |

**Water treatment works C**

Over nine months, 54 samples were collected and tested for the presence of *Acanthamoeba* spp. from WTW C. *Acanthamoeba* spp. was isolated only from raw water and only in April, as described in Chapter 3 and was not detected in the service reservoir or final treated water (Table 4.8).

The mean temperature of the samples taken from raw water to domestic properties was in the range11.6°C to 12.2°C (Table 4.9), and while most water chemical parameters did not change through the treatment and distribution

process, treatment at the WTW approximately halved the turbidity of the raw water.

Low numbers of bacteria were detected in the raw water samples, as described in Chapter 3, but fewer and no coliforms, were detected after water treatment or in domestic properties (Table 4.10).

**Table 4.8: The detection of *Acanthamoeba* spp. at each step during treatment at WTW C. between November 2009 – August 2010**. A schematic diagram of WTW is in chapter 2 (figure 2.4). (+ *Acanthamoeba* spp. Isolated, - *Acanthamoeba* spp. not isolated). Sampling point for raw 2 was not monitored (NM), as it was unavailable in November 2009 – July 2010. Sampling point for raw 3 was not monitored, as it was unavailable in August 2010.

| Sampling points | Nov 09 | Dec 09 | Feb 10 | Mar 10 | Apr 10 | May 10 | Jun 10 | Jul 10 | Aug 10 |
|---|---|---|---|---|---|---|---|---|---|
| 1. Raw 1 | - | - | - | - | + | + | - | - | - |
| 2. Raw 2 | NM | NM | NM | NM | NM | NM | NM | NM | - |
| 3. Raw 3 | - | - | - | - | - | + | - | - | NM |
| 4. Treated water | - | - | - | - | - | - | - | - | - |
| 5. Service reservoir 1 | - | - | - | - | - | - | - | - | - |
| 6. Service reservoir 2 | - | - | - | - | - | - | - | - | - |

**Table 4.9: Physical and chemical properties of water at WTW C.** The mean, SD and range of temperature, pH, electrical conductivity (EC) and turbidity of the untreated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, raw data is in appendix G, *n=8 was used to calculated the mean and SD of domestic properties sample temperature).

| Water type | Temperature (°C) | | pH | | EC@ 20°C (uS/cm) | | Turbidity (NTU) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=17) | 11.6 | 0.7 | 7.0 | 0.0 | 589 | 16 | 0.15 | 0.1 |
| | R (10.1 – 12.9) | | R (7.0 – 7.1) | | R (566 – 624) | | R (0.08 –0.26) | |
| Treated (n=9) | 11.8 | 0.6 | 7.1 | 0.1 | 597 | 12 | 0.10 | 0.03 |
| | R (11.1 – 12.9) | | R (7.0 – 7.2) | | R (578 – 609) | | R (0.05 –0.17) | |
| Treated water at service reservoir (n=18) | 11.8 | 1.1 | 7.1 | 0.1 | 592 | 13 | 0.10 | 0.03 |
| | R (9.4 – 13.6) | | R (7.0 – 7.2) | | R (560-610) | | R (0.06-0.18) | |
| Domestic properties (n=9) | 12.2 | 2.8 | 7.0 | 0.1 | 613 | 24 | 0.14 | 0.1 |
| | R (8.2-16.4) | | R (6.9-7.1) | | R (579-666) | | R (0.05-0.43) | |

**Table 4.10: Summary of the bacteriological results from WTW C.** The mean heterotrophic plate count (HPC) 2D37, HPC 3D22, total coliforms and *E.coli* of the untreated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, raw data is in appendix G).

| Water type | HPC 2D37 (cfu/ml) | | HPC 3D22 (cfu/ml) | | Total coliforms (cfu/100ml) | | *E.coli* (cfu/100ml) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=17) | 0.3 | 0.5 | 0.2 | 0.7 | 0.2 | 0.7 | 0.1 | 0.2 |
| | R (0-1) | | R (0-3) | | R (0-3) | | R (0-1) | |
| Treated (n=9) | 0.3 | 1.0 | 0.1 | 0.3 | 0 | 0 | 0 | 0 |
| | R (0-3) | | R (0-1) | | | | | |
| Treated water at service reservoir (n=18) | 0.2 | 0.7 | 3.6 | 5.0 | 0 | 0 | 0 | 0 |
| | R (0-3) | | R (0-20) | | | | | |
| Domestic properties (n=9) | 0.3 | 0.7 | 0 | 0 | 0 | 0 | 0 | 0 |
| | R (0-2) | | | | | | | |

**Water treatment works D**

Over nine months, 207 samples were collected and tested for the presence of *Acanthamoeba* spp. in WTW D, including the raw water samples. This site was sampled again from raw water stage to final treated water stage from February 2017 to January 2018, when 99 samples were analysed. The raw water source for this works was surface water, largely from the River Thames but also, if required, water was taken from the lake, and both sources were consistently positive for *Acanthamoeba* spp. as described in Chapter 3. The results of sampling throughout WTW D during the two sets of sampling period are shown in Table 4.11 and Table 4.12. *Acanthamoeba* spp. was isolated in every month of the study from the pre-ozone sampling points, and with the exception of March, from the

sedimentation plant. It was also isolated from flat bottom clarifiers over several months, and, in April-May from post rapid gravity filters and a GAC inlet. However, *Acanthamoeba* spp. was not isolated any further into the WTW process, including in the final treated water or the service reservoirs.

The mean temperature of the samples taken from raw water to domestic properties was in the range11.6°C to 12.2°C (Table 4.13), and while most water chemical parameters did not change through the treatment and distribution process, treatment at the WTW reduced greatly the turbidity of the raw water.

Large numbers of bacteria were detected in the raw water samples, as described in Chapter 3, but fewer and no coliforms, were detected after water treatment or in domestic properties (Table 4.14).

**Table 4.11:Detection of *Acanthamoeba* spp. at each step during treatment at WTW D (samples taken November 09 – August 10).** A schematic diagram of WTW is in chapter 2 (figure 2.5). (+ *Acanthamoeba* spp. Isolated, - *Acanthamoeba* spp. not isolated). Raw data is available in Appendix H.

| Sampling points | Nov 09 | Dec 09 | Feb 10 | Mar 10 | Apr 10 | May 10 | Jun 10 | Jul 10 | Aug 10 |
|---|---|---|---|---|---|---|---|---|---|
| 1. River and lake | + | + | + | + | + | + | + | + | + |
| 2. Raw | + | + | + | + | + | + | + | + | + |
| 3. Pre-ozone | + | + | + | + | + | + | + | + | + |
| 4. Post sedimentation plant | + | + | + | - | + | + | + | + | + |
| 5. Post flat bottom clarifier 1 | - | - | - | - | + | + | - | - | - |
| 6. Post flat bottom clarifier 2 | + | + | _ | - | - | - | - | - | - |
| 7. Post rapid gravity filters | - | - | - | - | + | + | - | - | - |
| 8. GAC inlet | - | - | - | - | + | - | - | - | - |
| 9. GAC outlet | - | - | - | - | - | - | - | - | - |
| 10. Post chlorination | - | - | - | - | - | - | - | - | - |
| 11. Treated water | - | - | - | - | - | - | - | - | - |
| 12. Link main | - | - | - | - | - | - | - | - | - |
| 13. Service reservoir 1 | - | - | - | - | - | - | - | - | - |
| 14. Service reservoir 2 | - | - | - | - | - | - | - | - | - |
| 15. Service reservoir 3 | - | - | - | - | - | - | - | - | - |
| 16. Service reservoir 4 | - | - | - | - | - | - | - | - | - |
| 17. Service reservoir 5 | - | - | - | - | - | - | - | - | - |
| 18. Service reservoir 6 | - | - | - | - | - | - | - | - | - |
| 19. Service reservoir 7 | - | - | - | - | - | - | - | - | - |
| 20. Service reservoir 8 | - | - | - | - | - | - | - | - | - |

**Table 4.12:Detection of *Acanthamoeba* spp. at each step during treatment at WTW D (samples taken February 17 – January 18).** A schematic diagram of WTW is in chapter 2 (figure 2.5). (+ *Acanthamoeba* spp. Isolated, - *Acanthamoeba* spp. not isolated). Raw data is available in Appendix I.

| Sampling points | Feb 17 | Mar 17 | Apr 17 | May 17 | Jun 17 | Jul 17 | Aug 17 | Sept 17 | Oct 17 | Nov 17 | Dec 17 | Jan 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. River | + | + | + | + | + | + | + | + | + | + | + | + |
| 2. Raw | + | + | + | + | + | + | + | + | + | + | + | + |
| 3. Pre-ozone | - | + | + | - | - | - | + | + | - | - | - | - |
| 4. Post sedimentation | - | - | - | - | - | - | - | - | - | - | - | - |
| 5. Post FBC 1 | - | - | - | - | - | - | - | - | - | - | - | - |
| 6. Post FBC 2 | - | - | _ | - | - | - | - | - | - | - | - | - |
| 7. Post rapid gravity filters | - | - | - | - | - | - | - | - | - | - | - | - |
| 8. GAC inlet | - | - | - | - | - | - | - | - | - | - | - | - |
| 9. GAC outlet | - | - | - | - | - | - | - | - | - | - | - | - |
| 10. Post chlorination | - | - | - | - | - | - | - | - | - | - | - | - |
| 11. Treated water | - | - | - | - | - | - | - | - | - | - | - | - |

**Table 4.13: Physical and chemical properties of water at WTW D (samples taken November 09 – August 10).** The mean, SD and range of temperature, pH, electrical conductivity (EC) and turbidity of the untreated water, part treated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, NM denotes data not monitored as part of routine sample analysis, *n=9 was used to calculated the mean and SD of treated water sample temperature, raw data is in appendix H)

| Water type | Temperature (°C) | | pH | | EC@ 20°C (uS/cm) | | Turbidity (NTU | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=9) | 13.2 | 4.9 | 8.0 | 0.1 | 600 | 25 | 4.11 | 2.03 |
| | R (6.4-19.2) | | R (7.9-8.2) | | R (563-637) | | R (1.2-8.0) | |
| Part treated (n=30) | NM | | 7.4(0.4) | 0.4 | 607 | 24 | 1.17 | 1.30 |
| | | | R (7.0-8.2) | | R (564-643) | | R (0.24- 6.5) | |
| Treated (n=18)* | 12.8 | 5.8 | 7.4 | 0.1 | 601 | 27 | 0.16 | 0.06 |
| | R (5.3 – 21.8) | | R (7.2-7.6) | | R (553 – 667) | | R (0.09-0.30) | |
| Treated water at service reservoir (n=72) | 12.7 | 5.2 | 7.4 | 0.1 | 617 | 34 | 0.18 | 0.07 |
| | R (5.2 – 21.8) | | R (7.2-7.8) | | R (548-693) | | R (0.09-0.47) | |
| Domestic properties (n=27) | 13.4 | 4.8 | 7.3 | 0.1 | 622 | 28 | 0.16 | 0.04 |
| | R (6.4-21.1) | | R (7.2 – 7.5) | | R (576-693) | | R (0.10-0.24) | |

**Table 4.14: Summary of the bacteriological results from WTW D (samples taken November 09 – August 10).** The mean heterotrophic plate count (HPC) 2D37, HPC 3D22, total coliforms and *E.coli* of the untreated water, part treated, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, raw data is in appendix H).

| Water type | HPC 2D37 (cfu/ml) | | HPC 3D22 (cfu/ml) | | Total coliforms (cfu/100ml) | | *E.coli* (cfu/100ml) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated | NM | | NM | | NM | | NM | |
| Part treated (n=46) | 2141.6 | 13248.1 | 1991.2 | 3916.3 | 725.1 | 2990.0 | 60.8 | 178.4 |
| | R (0-90000) | | R (0-20000) | | R (0-20100) | | R (0-1000) | |
| Treated (n=18) | 14.8 | 32.3 | 0.9 | 1.6 | 0 | 0 | 0 | 0 |
| | R (0-119) | | R (0-6) | | | | | |
| Treated water at service reservoir (n=72) | 0.1 | 0.4 | 1.3 | 3.3 | 0 | 0 | 0 | 0 |
| | R (0-12) | | R (0-18) | | | | | |
| Domestic properties (n=27) | 9.4 | 38.3 | 5.4 | 23.78 | 0.04 | 0.2 | 0 | 0 |
| | R (0-199) | | R (0-124) | | R (0-1) | | | |

**Table 4.15: Physical and chemical properties of water at WTW D (samples taken February 17 – January 18).** The mean, SD and range of temperature, pH, electrical conductivity (EC) and turbidity of the untreated water, part treated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, NM denotes data not monitored as part of routine sample analysis, *n=12 was used to calculated the mean and SD of untreated water sample pH raw data is in appendix I).

| Water type | Temperature (°C) | | pH | | EC@ 20°C (uS/cm) | | Turbidity (NTU) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=9) | 13.9 | 5.2 | 7.9 | 0.1 | 616 | 34 | 7.8 | 11.5 |
| | R (6.2-22.1) | | R (7.8-8.0) | | R (548-674) | | R (1.1-38.0) | |
| Part treated (n=35) | NM | | 7.4 | 0.3 | 593 | 149 | 1.3 | 1.8 |
| | | | R (7.0-8.1) | | R (570-686) | | R (0.13-10.0) | |
| Treated (n=12) | 13.8 | 5.2 | 7.3 | 0.1 | 653 | 27 | 0.16 | 0.09 |
| | R (5.1-22.1) | | R (7.2-7.4) | | R (607-699) | | R (0.11-0.38) | |

**Table 4.16: Summary of the bacteriological results from WTW D (samples taken February 17 – January 18).** The mean heterotrophic plate count (HPC) 2D37, HPC 3D22, total coliforms and *E.coli* of the untreated water, part treated water, treated water from the WTW, service reservoir where treated water is stored prior to being supplied and domestic properties. (n denotes the sample size used to calculate the mean and SD, R denotes the data range, NM denotes data not monitored as part of routine sample analysis, *n=9 was used to calculated the mean and SD of treated water sample HPC 2D37 raw data is in appendix I).

| Water type | HPC 2D37 (cfu/ml) | | HPC 3D22 (cfu/ml) | | Total coliforms (cfu/100ml) | | *E.coli* (cfu/100ml) | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Untreated (n=3) | NM | | NM | | 8786.7 | 13350 | 1696 | 2524.5 |
| | | | | | R (860-24200) | | R (170-4610) | |
| Part treated (n=78) | 60.68 | 295.5 | 227.8 | 605.4 | 218.2 | 851.5 | 46.8 | 199.4 |
| | R (0-2600) | | R (0-3500) | | R (0-4350) | | R (0-1200) | |
| Treated (n=12)* | 0.5 | 0.76 | 0.08 | 0.29 | 0 | 0 | 0 | 0 |
| | R (0-2) | | R (0-1) | | | | | |

**Samples from domestic properties**

In total, 90 domestic properties served by WTW D were sampled and tested for the presence of *Acanthamoeba* spp. and chlorine concentration. The results are shown in Table 4.17. No domestic samples were positive for *Acanthamoeba* spp.

**Table 4.17: The distribution of *Acanthamoeba* spp. and free chlorine concentrations at domestic properties served by WTW.** n denotes the sample numbers used to calculate the mean and SD of free chlorine concentration.

| WTW | Number of properties sampled | Total no. samples (n) | No of samples positive for *Acanthamoeba* spp. | Free chlorine concentration (mg/L) | | |
|---|---|---|---|---|---|---|
| | | | | Mean | SD | Range |
| **A** | 5 | 45 | 0 | 0.21 | 0.10 | 0.21-0.53 |
| **B** | 1 | 9 | 0 | 0.16 | 0.06 | 0.10-0.30 |
| **C** | 1 | 9 | 0 | 0.20 | 0.08 | 0.12-0.40 |
| **D** | 3 | 27 | 0 | 0.35 | 0.13 | 0.08-0.60 |

WTW A, which used karstic water, supplied water to the north and west of London. One sample was taken from each of the supply zones for the months of the study period, except January 2010. *Acanthamoeba* spp. was not isolated in any of these samples. The average free chlorine was 0.21mg/l (SD 0.10mg/l).

WTW B and C, which used source water from boreholes, produced much less water than the WTW A and D and so only supplied one zone each. One property was analysed from each of these zones each month for the presence of *Acanthamoeba* spp. but *Acanthamoeba* spp. was not isolated. The average free

chlorine for properties supplied by WTW B and WTW C over the study period was 0.16mg/l (SD 0.06 mg/l) and 0.20mg/l (SD 0.08 mg/l) respectively.

WTW D, which took surface source water, supplied three water zones to the west of London and a population of 290,000. One sample was taken from each of these zones during the study period months. As with all other domestic properties sampled, *Acanthamoeba* spp. was not isolated from any of the three households supplied by this WTW. The average free chlorine for zones supplied by WTW D was 0.34mg/l (SD 0.13 mg/l).

## 4.4: Discussion

Chapter 3 described how *Acanthamoeba* spp. were consistently detected in raw water at WTW D, where water was sourced from either the River Thames or a lake. However, *Acanthamoeba* spp. were only occasionally found in ground and karstic water, at WTW A, B and C. Given the distribution of *Acanthamoeba* spp. in raw water sources, it was perhaps not surprising that none were detected in treated or part treated water at WTW A, WTW B, and WTW C.

At WTW D, however, where the raw water consistently contained *Acanthamoeba* spp., it was also detected at early stages of the treatment processes. It was detected in the river and raw water for all samples taken in both sampling periods, although no samples after the sedimentation and filtration stages. There was some variation in at which stage the amoebae were removed. As this WTW treated raw surface water, there was also a higher presence of bacteria in the initial water, and

this may have provided a food source for *Acanthamoeba* spp. (Table 4.14 and 4.16).

As one of the aims of WTWs is to remove bacteria, it might be expected that *Acanthamoeba* spp. would not be detected in the final water, as the amoebae would have lost their food source. The low numbers of bacteria present in raw water supports this argument for WTW A, WTW B, and WTW C (Table 4.4, 4.7 and 4.10). However, *Acanthamoeba* spp. can survive adverse conditions by encystation, and should have been recovered by culture. It may be that other aspects of the water processing also help remove *Acanthamoeba* spp. or it could be that the culture method used did not provide the right conditions for encysted amoebae to change to detectable trophozoites. If the last assumption is true, then future studies might benefit from the use of PCR as well as culture.

As WTW D was designed to treat surface raw water to produce drinking water, and surface water is known to have a higher likelihood of microbiological, chemical, and physical contaminants (Tallon *et al.,* 2005), the treatment process at WTW D was far more complex than at sites A, B and C.

During the November 2009 to August 2010 sampling period *Acanthamoeba* spp. were isolated from 100% of the samples taken from pre-ozonation, 89% of post sedimentation stage samples, 22% from flat bottom clarifiers, 22% from post rapid gravity filters and 11% GAC inlet. During February 2017 to January 2018, 33% of samples taken from the pre-ozonation had *Acanthamoeba* spp. For the part treatment stages of water treatment at this site there were still large numbers of

bacteria present when compared to the part treatment stage of WTW A. Thus, in addition to the removal of bacteria as a food supply leading to amoebal death, it may be that sedimentation stages are particularly important for the removal of *Acanthamoeba* spp.

There was no isolation of *Acanthamoeba* spp*.* in the final treated water at any of the WTW in this study, nor the storage reservoirs and the water towers prior to distribution associated with the WTW. As this is the case for all of the WTW examined in this study, it is highly likely that the WTW and associated reservoirs and distribution supplies are not sources of *Acanthamoeba* spp. that have previously been reported to be isolated from taps in UK by Kilvington *et al.,* (2004).

This study in England, agrees with the findings of others who have found modern WTW to be quite efficient at removing amoebae in general, and sometimes *Acanthamoeba* more specifically (e.g. Hoffmann and Michel, 2001 and Amer, 2012). In this study also, however, no *Acanthamoeba* were found in tap water at homes supplied by these WTW, and this is at odds with previous studies in the UK that have found high rates of contamination at such sites (Seal, 1992; Kilvington *et al.,* 2004) and in other countries such as Iran (Manesh *et al.,* 2016). This may suggest that it is unlikely for *Acanthamoeba* spp. to grow within the water supply once it has been removed unless there is some form of reintroduction, such as potential contamination during burst pipe repair. One recent study (Taravaud *et al.,* 2018) found seasonal growth of *Acanthamoeba* spp. in complex biofilms in water storage towers, but this was by PCR not culture.

Another possible source of reintroduction of *Acanthamoeba* spp. to the network could be a burst on a distribution pipe or the presence of biofilms within the pipes. Mains water is generally under pressure so that if a burst occurs, the risk of ingress to the network is low. However if the repair is carried out using the wrong equipment, or contaminated parts caused by poor storage and handling, this could lead to the contamination of the water. Also any biofilms within the distribution system will act as a barrier and protect any amoeba behind the biofilm from chlorination. The biofilm might also provide a suitable food source for their growth, which may lead to growth of *Acanthamoeba* spp. within the pipes (Stockman *et al.,* 2011).

*Acanthamoeba spp.* was not isolated from any of the households supplied by any of the WTW in this study. When water is distributed around the supply network, residual chlorine should be present to ensure that bacteria are unable to grow and the water remains microbiologically safe until consumption. Although all of the samples taken from the domestic properties had residual chlorine, the concentration of this was not sufficient to kill *Acanthamoeba* spp. cysts (Khan, 2009). For this study the samples in domestic properties were taken from the kitchen, for where water should be supplied by the main cold feed.

No samples were taken from the internal storage systems, where chlorine concentrations would be expected to be negligible, temperatures might be higher and there is the possibility of bacterial contamination and growth.

The study of domestic tap water in England reported by Kilvington *et al*., (2004), in which *Acanthamoeba* spp. were isolated, sampled water from taps in various

locations within each property including cold taps from bathrooms, cloakrooms, and mixer taps from bathrooms and bedrooms. It is very likely that the immediate sources of water for these were internal storage tanks, which if not maintained, may well have biofilms.

An 'average' domestic plumbing system is described is chapter 2 (Figure 2.7). Water to the cold kitchen tap is generally straight off the rising main as the quality and safety of water after storage cannot be guaranteed. The rising main provides water direct from the distribution network. The majority of properties in the UK still have a cold water storage tank in the roof space, which feeds the down services to other cold water outlets and the hot water services. These systems are not designed to be used as potable water as there is a risk that the water may not turnover, leading to stagnating water which could encourage microbial growth. Also, there is the risk of ingress as the systems may not be suitably sealed, leading to a potential reintroduction of *Acanthamoeba* spp. A study in Florida (Shoff *et al.,* 2008), took swabs from water storage tanks, as this was the way they determined presence of *Acanthamoeba* spp. in tap water, and *Acanthamoeba* spp. were isolated from 2.8% of the samples taken.

The study described in this chapter used an aseptic technique that involved disinfecting the end of the tap. The sampling technique will have an impact on the results as the swabbing process may disturb biofilms, which may be present in the ends of the taps. Kilvington *et al.,* (2004) reported that biofilms were observed on the swabs taken. The microorganisms within the biofilm can provide a food source

for *Acanthamoeba* spp. and thus it is more likely to record a positive reading (Stockman *et al.,* 2011).

Future studies could involve taking swabs from the tap before the disinfecting process as well as the water sample to see if there will be any difference in results. In addition a survey of the property could be undertaken to check for the hygiene of domestic taps, type of taps (e.g. single or mixer taps) and swabs of the surrounding area around the sink to check if *Acanthamoeba* spp. can be isolated. It would also be interesting to compare culture and PCR, of both membranes and swabs. A brief comparison of culture and PCR is described in Chapter 5. As chlorine levels used by WTW is not very effective against *Acanthamoeba* spp., the effectiveness of other disinfectants on the isolates from this study should also be carried out to determine if they could kill or deactivate *Acanthamoeba* spp. An attempt to employ a colorimetric for testing disinfectants in described in Chapter 6.

Finally, it was interesting that a very small number of domestic taps had very high bacterial and coliform counts. It is not possible to determine the reason for this, but it seems likely that a very local contamination event had taken place.

# Chapter 5

# Molecular characterisation of *Acanthamoeba* species isolated from surface water.

## 5.1: Introduction

This chapter focuses on the molecular characteristics of *Acanthamoeba* isolates from WTW D collected between February 2017 and January 2018. This site was chosen for resampling as its main water source is the River Thames (surface water), which had previously yielded many positive *Acanthamoeba* cultures. The sampling points are described in chapter 2, section 2.3, and the results of culture have been described in Chapters 3 and 4. Two hypotheses were tested in the study described in this Chapter.

1) The environmental *Acanthamoeba* spp. isolated from raw water represent a diverse community of species and genotypes, as has been found in some previous environmental studies (see Chapter 1 for a review).
2) The use of PCR directly on the membrane filters rather than just of cultured *Acanthamoeba* spp. isolates would reveal a wider diversity of *Acanthamoeba* spp. than culture, and possibly novel genotypes.

In addition, molecular characterisation was seen as a method to confirm that the isolates obtained from culture were, indeed, *Acanthamoeba* spp.

The taxonomy and characterisation of *Acanthamoeba* spp. are discussed in Chapter 1. Currently, around 20 *Acanthamoeba* genotypes, T1 – T21/22, have been described, based on 18S rRNA sequencing (Stothard *et al.,* 1998; Maghsood *et al.*, 2005, Niyyati *et al.,* 2009) of environmental and clinical isolates. A further environmental genotype (T99) has recently been shown not to be a novel

genotype but rather to have result from chimeric sequences (Corsaro and Venditti, 2018). Some of these genotypes are associated with a particular spp. and/or environment (ecotype) and others, for example T4, appear to comprise an assemblage of related species (sometimes referred to as strains) compared to whole genome sequencing (Risler *et al.,* 2013) and are found in a variety of environments globally. Figure 5.1 shows a phylogenetic tree of published rRNA sequences that demonstrates not only the lack of species-genotype identity, but how certain combinations of genotypes form higher level clusters.

Not all genotypes appear to be similarly pathogenic, although cases of human infection with most genotypes have been reported. The genotypes most frequently associated with keratitis are T3, T4, T6 and T11, particularly genotype T4 (Maghsood *et al.,* 2005; Zhao *et al.,* 2010; Ledee *et al.,* 2009). *Acanthamoeba* genotype T4 has also been reported as the genotype most prevalent in the environment, having been isolated from various environments, including rivers, lakes, tap water and swimming pools (Maghsood *et al.,* 2005; Gavarane *et al.,* 2018). As this genotype is both the most prevalent in the environment and the most common pathogenic genotype detected, it poses the most significant risk to public health.

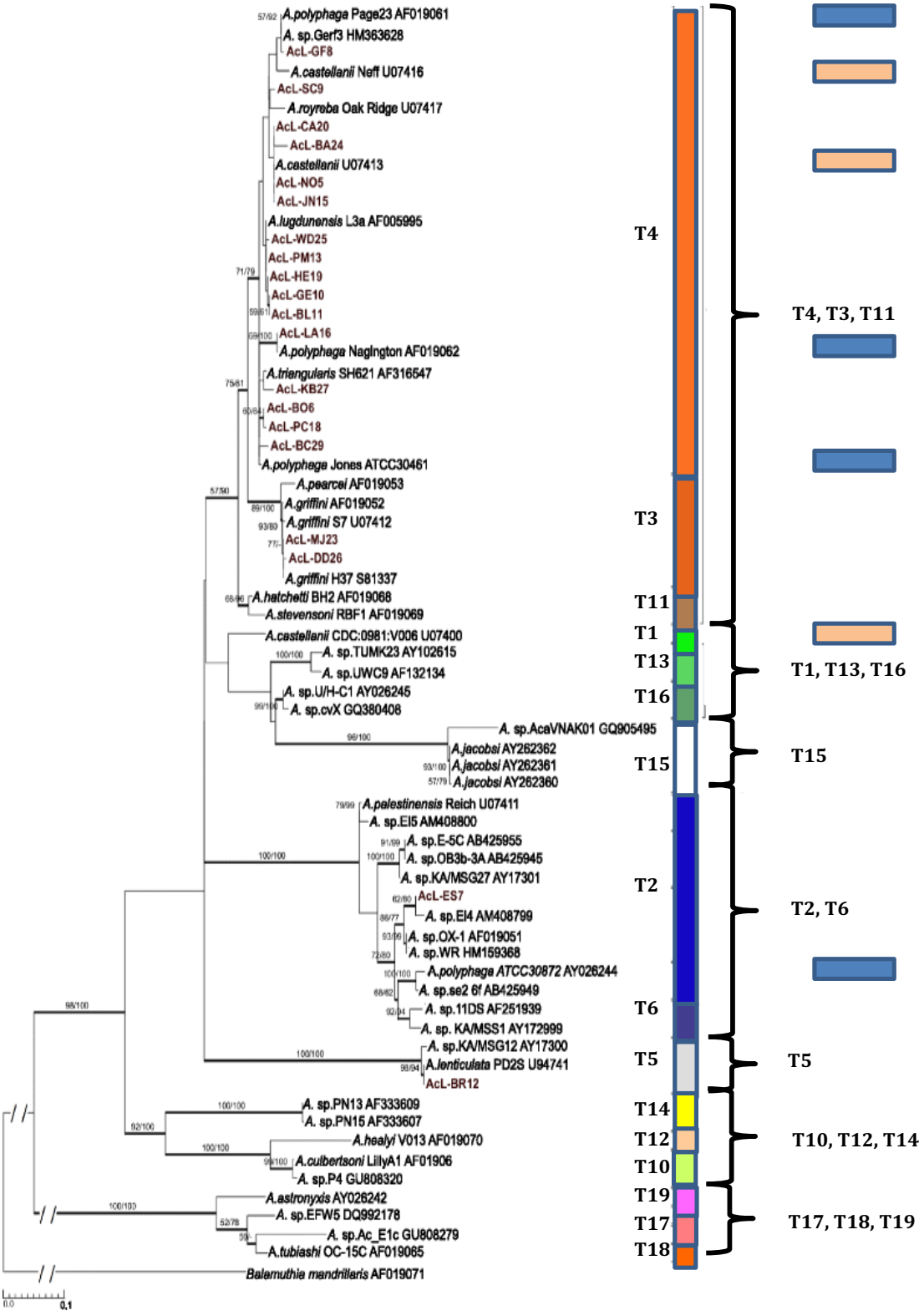


**Figure 5.1: Phylogenetic tree of partial small sub-unit rDNA sequences of *Acanthamoeba* spp.** The tree is inferred from the maximum likelihood method, showing how isolates fall within clades associated with genotypes and/or genotypic combinations but not, by this method, species. Genotypes (T1-19) are indicated, and, in the far right hand column, blue boxes indicate *A. polyphaga* and orange boxes *A. castallenii.* Based on Risler *et al* (2013). The scale (bottom left)

indicates % nucleotide difference, for 737 - 1019bp sequences, and bootstrap differences are indicated for branching nodes.

The usual approach to genotyping *Acanthamoeba* is PCR of the small unit rRNA gene; either of environmental/ clinical samples directly, or of cultured isolates, followed by sequencing of the resultant amplicons. Several approaches and primers for the PCR assay have been published, but those chosen for this study were the genus specific primers JDP1 and JDP2 (Schroeder *et al.,* 2001; Ledee *et al.,* 2009; Zhao *et al.,* 2010). The variety of approaches used, combined with only short sequences often being obtained, can make accurate characterisation difficult. However, it enables identification to the genus level and some idea of which genotype, or genotype cluster, the detected amoebae might belong to.

## 5.2: Materials and methods

### 5.2.1: Sample collection and DNA extraction

Water samples (2L) from WTW D were passed through membrane filters, and cultured as described in Chapter 2 (section 2.6). Then the frozen axenic culture of the isolates (chapter 2, section 2.8) was used for DNA extraction. Culture results are described in Chapters 3 and 4. In addition, in June 2018, four 2L raw water samples and four treated water samples were filtered, and the solid, trapped debris was scraped off into 2 ml of PBS for DNA extraction. The suspension was transferred to a 50ml centrifuge tube and a pellet produced by centrifugation at 3000rpm for 5 minutes. The supernatant was discarded and the pellet was stored at 4°C for 24 hours. The pellets were re-suspended in 200 µl of PBS, and DNA was extracted using a Qiagen DNAEasy extraction kit, following the

manufacturer's instructions. The quality and quantity of DNA was determined by Thermo NanoDrop following the manufacturer's instructions, and DNA was stored at -20°C.

### 5.2.2: Polymerase chain reaction (PCR)

PCR was carried out using the Qiagen PCR multiplex kit. The total volume of the mix was 20µl. Genus specific primers JDP1 and JDP2 were used (Schroeder *et al.,* 2001, Zhao *et al.,* 2010 and Booton *et al.,* 2002). Table 5.1 gives details of the PCR reagent mix. The mix was centrifuged for 20 seconds then placed in the PCR machine. The PCR cycle was 7 minutes at 95°C, 1 minute at 95°C, 1 minute at 60°C and 2 minutes at 72°C. The cycle was repeated 35 times.

**Table 5.1: Reagents used for PCR of putative *Acanthamoeba* DNA.**

| Reagent | Quantity (µl) |
|---|---|
| Master mix | 10 |
| Loading dye | 2 |
| Water | 5 |
| Forward primer (JDP1) 5'-GGCCCAGATCGTTTACC-3' | 1 |
| Reverse Primer (JDP2) 3'-TCTCACAAGCTGCTAGG-5' | 1 |
| DNA solution (from sample) | 1 |

Amplicons were visualised by electrophoresis through a 1.5% agarose gel in TAE buffer with ethidium bromide and viewed under a UV source, and a 100bp ladder was used as an aid to estimate the size of the DNA fragments. A negative control

was also used, which was DNA free water. The expected amplicon size was 423bp to 551bp (Schroeder *et al.,* 2001).

### 5.2.3: Sequencing

For sequencing, amplicons were purified using a Monarch PCR and DNA clean-up kit following manufacturer's instructions. They were then Sanger sequenced through a commercial supplier (Source BioScience, https://www.sourcebioscience.com/services/genomics/sanger-sequencing-services/) using conserved internal primers Acanth892 (3'CCAAGAATTTCACCTCTGAC5') and Acanth892C (5'-GTCAGAGGTGAAATTCTTGG-3'). Raw sequences were trimmed of obvious nonsense reads, and were compared with a published sequences using the online Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences obtained from the forward primer were made complement, and they and the reverse primer sequences were aligned with each other and a published sequence of a T4 isolate (KU936119), then trimmed using Seaview (http://doua.prabi.fr/software/seaview ), which was also used to construct simple trees.

### 5.2.4: Phylogenetic tree

More formal phylogenetic analysis (done with Professor Richard Emes) involved downloading a range of published and genotyped sequences through NCBI, and careful trimming of sequences, including removal of large strings of poor sequence, to maximise meaningful alignment. Sequences were then aligned and a tree produced, again through Seaview.

## 5.3: Results

DNA was extracted from 36 isolates obtained between February 2017 and January 2018, and all 8 filter membrane samples collected in June 2018 from four river and four treated water samples. PCR produced amplicons of the expected size from 35 out of 36 isolates (Figure 5.2) and from two of the membrane filter samples analysed in June 2018 (one river and one treated water sample) (Tables 5.2 and 5.3).

**Table 5.2: PCR and sequencing of cultured *Acanthamoeba* spp.** Sampling period was February 2017 to January 2018. Details of sampling points are described in chapter 2. Isolate genotype was determined using the NCIB BLAST.

| Sample number | Month of sampling | Sampling point | PCR band between 423 - 551 bp | Isolate genotype (from BLAST) | Closest sequence in Blast |
|---|---|---|---|---|---|
| A9 | Feb-17 | River | Yes | T4 | MF139794.1 |
| A8 | Feb-17 | Raw | Yes | T4 | MF139794.1 |
| JB1 | Mar-17 | River | Yes | T4 | KX682341.1 |
| JB2 | Mar-17 | Raw | **No** | N/A | N/A |
| JB3 | Mar-17 | Pre-ozone | Yes | T4 | KX682341.1 |
| JR3 | Apr-17 | River | Yes | T4 | KX682341.1 |
| JB4 | Apr-17 | Raw | Yes | T4 | KP233867.1 |
| JR1 | Apr-17 | Pre-ozone | Yes | T4 | KX682341.1 |
| JR2 | Apr-17 | Pre-ozone | Yes | T4 | MF139794.1 |
| JB5 | May-17 | River | Yes | T4 | MF139794.1 |
| JB6 | May-17 | Raw | Yes | T4 | KX682341.1 |
| JB7 | Jun-17 | River | Yes | T4 | KX682341.1 |
| JB8 | Jun-17 | Raw | Yes | T4 | KX682341.1 |
| JB9 | Jul-17 | River | Yes | T4 | MF139794.1 |
| JB10 | Jul-17 | Raw | Yes | T4 | KP233865.1 |
| JB11 | Aug-17 | River | Yes | T4 | KX682341.1 |
| JB12 | Aug-17 | Raw | Yes | T4 | KX682341.1 |
| A2 | Aug-17 | Pre-ozone | Yes | T4 | KX682341.1 |
| JR4 | Sep-17 | River | Yes | T4 | KX682341.1 |
| JB13 | Sep-17 | Raw | Yes | T4 | MF139794.1 |
| A1 | Sep-17 | Pre-ozone | Yes | T4 | MH024485.1 |
| JR5 | Oct-17 | River | Yes | T4 | MF139794.1 |
| JB14 | Oct-17 | Raw | Yes | T4 | KX682341.1 |
| JR6 | Nov-17 | River | Yes | T4 | MF139794.1 |
| JR7 | Nov-17 | River | Yes | T4 | KX682341.1 |
| JB15 | Nov-17 | Raw | Yes | T4 | MF139794.1 |
| JR8 | Dec-17 | River | Yes | T4 | KX682341.1 |
| JR9 | Dec-17 | River | Yes | T4 | KP233867.1 |
| JR10 | Dec-17 | River | Yes | T4 | KP233867.1 |
| A3 | Dec-17 | River | Yes | T4 | KX682341.1 |
| A4 | Dec-17 | River | Yes | T4 | KX682341.1 |
| A5 | Dec-17 | River | Yes | T4 | KX682341.1 |
| JB16 | Dec-17 | Raw | Yes | T4 | KX682341.1 |
| JR11 | Jan-18 | River | Yes | T4 | KX682341.1 |
| A6 | Jan-18 | River | Yes | T4 | MF139794.1 |
| A7 | Jan-18 | Raw | Yes | T4 | KX682341.1 |

**Table 5.3: PCR and sequencing of DNA extracted directly from membrane filters.** Samples were taken in June 2018. Details of sampling points are described in chapter 2. Isolate genotype was determined using NCIB BLAST.

| Sample number | Month of sampling | Sampling point | PCR band between 423 - 551 bp | Isolate genotype (from BLAST) | Closest sequence in Blast |
|---|---|---|---|---|---|
| JB17 | Jun-18 | River | No | N/A | N/A |
| JB18 | Jun-18 | Raw | No | N/A | N/A |
| JB19 | Jun-18 | River | No | N/A | N/A |
| **JB20** | **Jun-18** | **River** | **Yes** | **T4** | **MH024483.1** |
| **JB21** | **Jun-18** | **Treated** | **Yes** | **T4** | **MH024485.1** |
| JB22 | Jun-18 | Treated | No | N/A | N/A |
| JB23 | Jun-18 | Treated | No | N/A | N/A |
| JB24 | Jun-18 | Treated | No | N/A | N/A |

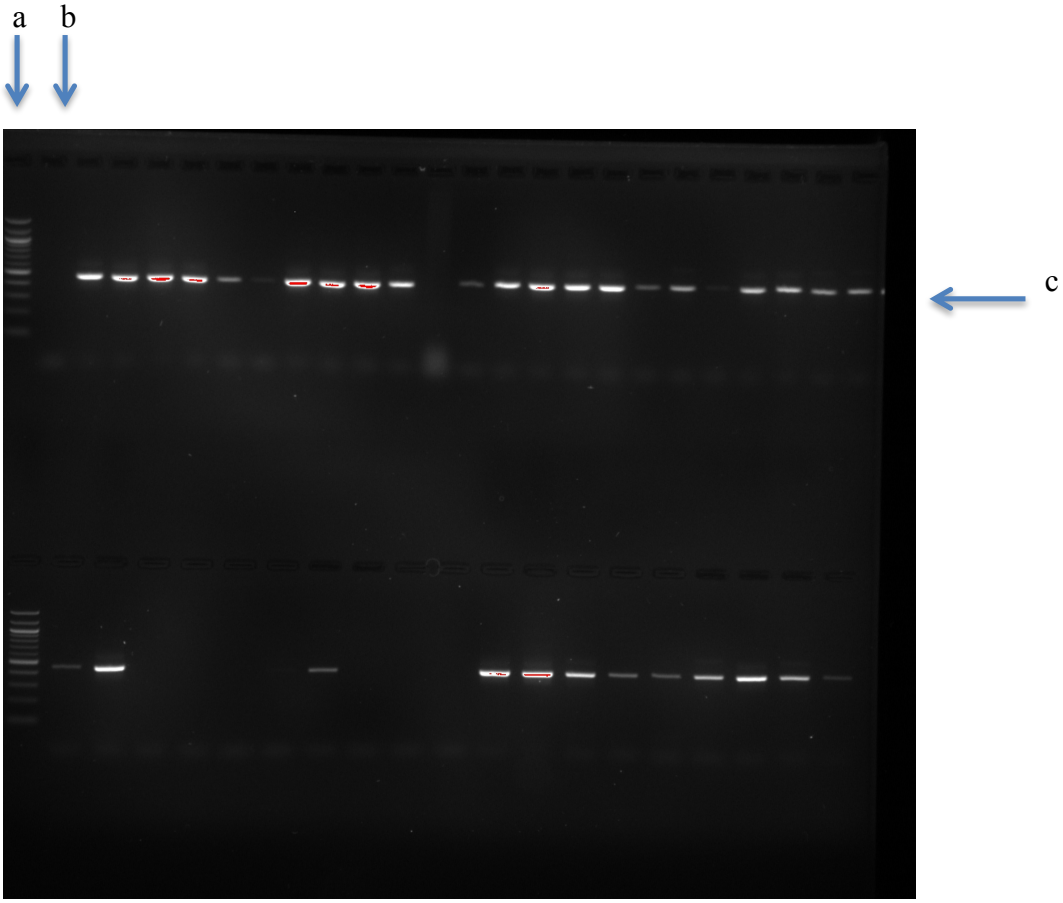


**Figure 5.2: PCR amplicons of *Acanthamoeba* rDNA.** The PCR was carried out using genus-specific primers JDP1 and JDP2. A 100bp ladder marker was run alongside the samples (left lane one, a) as well as a negative control (left lane 2, b). The bands at 500bp (c) are *Acanthamoeba* spp. Sample A1 – A9 and JB 1 – 14 were run on the top horizontal part of the gel and samples JB15 – JB24 and JR1-JR11 was run on the bottom horizontal part of the gel.

Sanger sequencing and BLAST confirmed that all the amplicons were, indeed, of *Acanthamoeba* spp. The most similar sequences to those obtained in this study were of genotypes T4 (Figure 5.3, Tables 5.2 and 5.3).

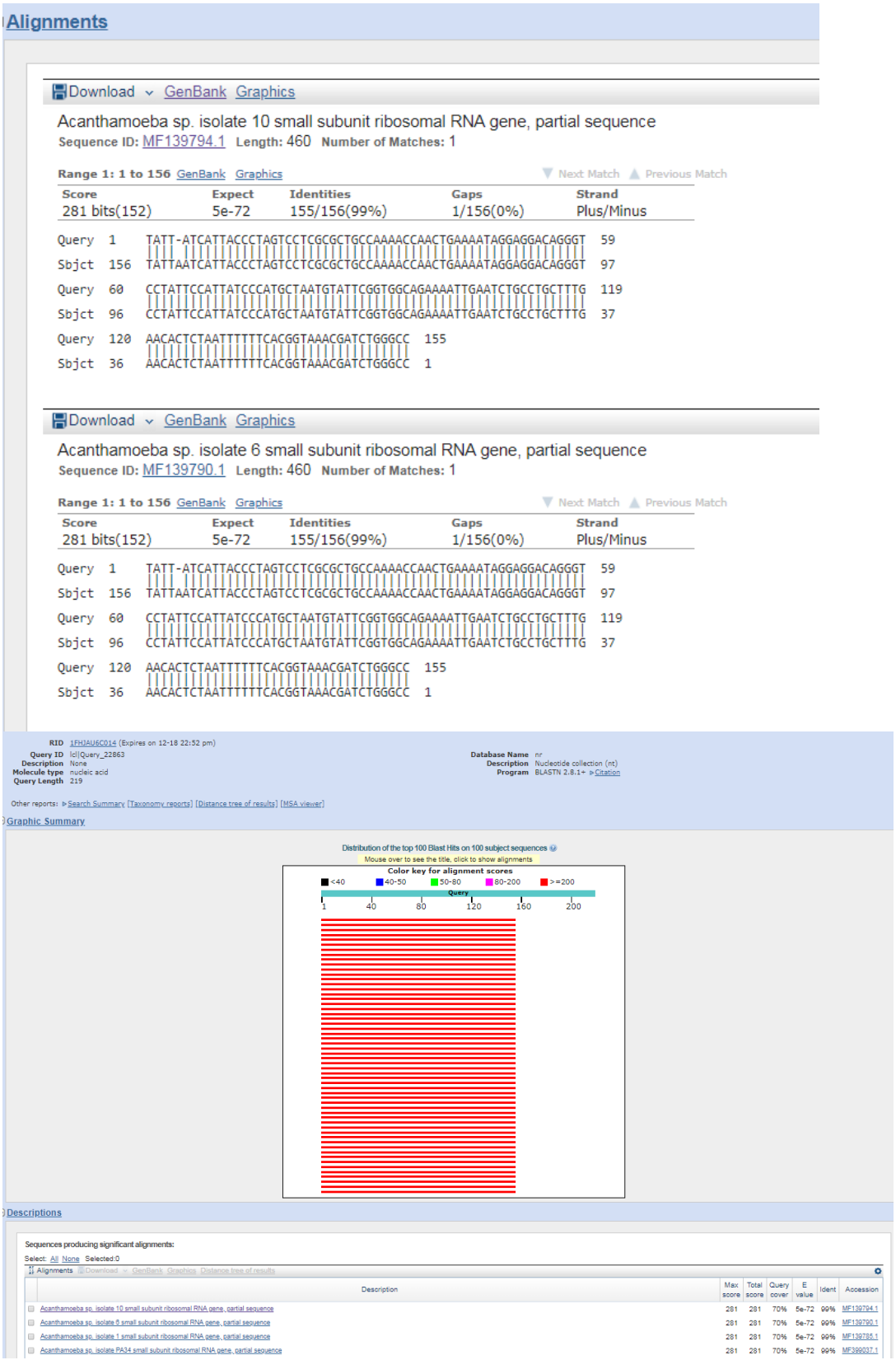


**Figure 5.3: Example images for BLAST search.** In this case, the Sanger sequence of the JB10 PCR product, using the forward sequencing primer. This is an example of a BLAST search, which was used to find the closest match to the genotypes isolated in this study (Table 5.2). Aligned sequences were also similar

to published sequences, although sequencing reactions had not spanned the entire amplicon (Figure 5.4).

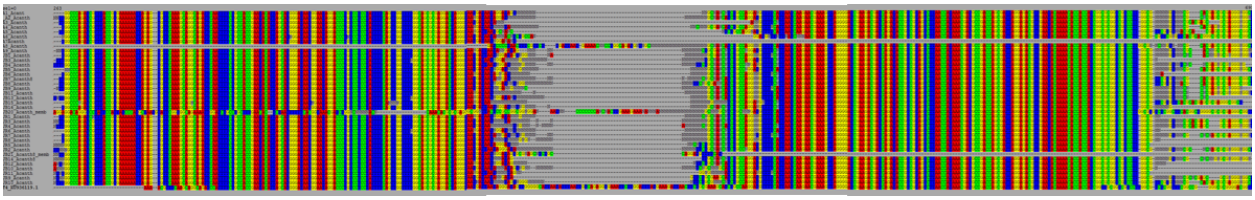

**Figure 5.4 Alignment of sequences of PCR amplicons from both cultures and membranes.** The 5' ends of A8 and JB20, and the 3' ends of A7 and JB21 consistently produced either no reaction or nonsense sequence, this is the grey area in the figure, and so were dropped from further analyses. The bottom line is the sequence of a T4 genotyped *Acanthamoeba* for comparison with the sequence from this study (KU936119 in the NCBI database).

In order to construct a phylogenetic tree, relevant sequences of genotyped *Acanthamoeba* spp. representing a range of genotypes were sourced from the NCBI database and compared with the sequences generated in this study. Type sequences were: T1 KM015457.1, MF176163.1, GQ924682.2; T2/6 EU934071.1; T2 LC184519.1, HF930510.1; T3 LC086296.1, T3 KJ094666.1, KJ094666.1; T4 KU936119.1, KU936118.1, KU936102.1, KU936110.1; T5 MF076665.1, MF076655.1; T11 KU936113.1, KU936108.1, KX688040.1. Ends were trimmed and the centre region missing from the field sequences removed to maximise meaningful alignment (Figure 5.5). Based on draft trees, obviously incorrectly labelled and partial gene sequences were removed. The BIONJ algorithm (Gascuel,1997) for neighbour-joining distance-matrix generation was used in SeaView, in order to produce the final phylogenetic tree generated in FigTree (http://tree.bio.ed.ac.uk/software/figtree/ ) (Figure 5.6).

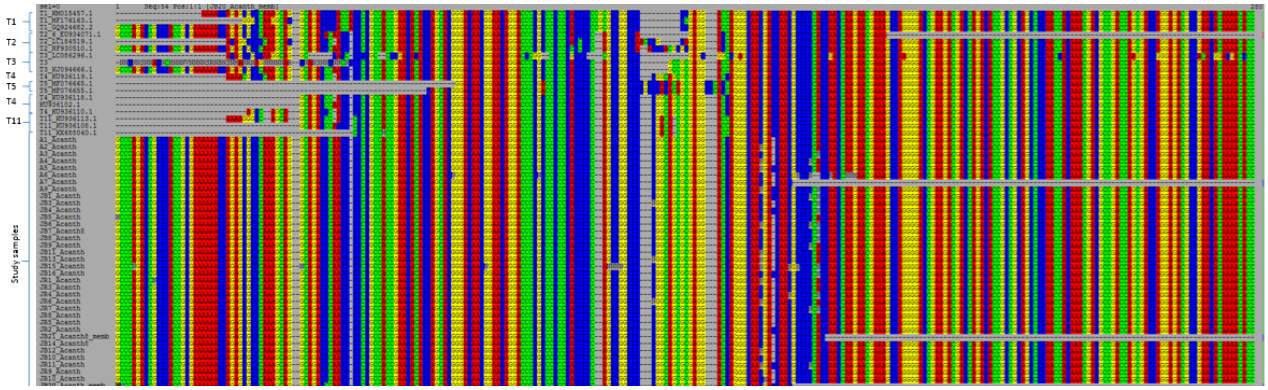

**Figure 5.5: Alignment of sequences of PCR amplicons from both cultures and membranes in this study with representative sequences of published genotypes from NCBI database. These sequences were used to construct the phylogenetic tree (Figure 5.6).**

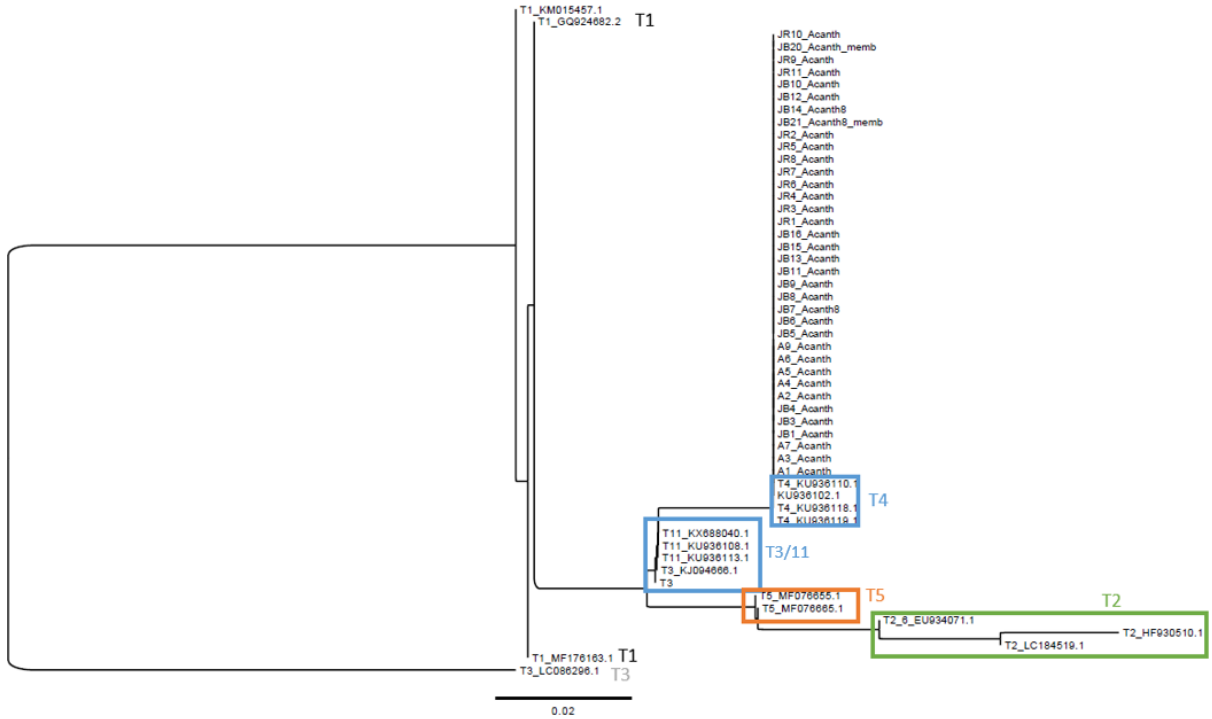


**Figure 5.6: BIONJ distance tree (JC), of sequences of the rRNA gene of *Acanthamoeba* spp.** Image generated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/) of sequences of PCR amplicons from both cultures and membranes in this study with representative sequences of published genotypes (see Figure 5.5), which are indicated by boxes and labels. The tree shows that the sequences from this study are closely related to T4 genotype.

## 5.4: Discussion

PCR and sequencing confirmed *Acanthamoeba* in 35 out of 36 cultures. There was no obvious reason for the negative PCR result from one sample, as microscopy revealed typical trophozoites, and the DNA extracted was of good quantity and quality (725.9ng/µl and 260/280 ratio was 1.95). This may be due to contamination with PCR inhibitors, or possibly the presence of a similar-looking amoeboid. PCR found only 2 out of 8 membrane filter samples positive. Unfortunately culture was not attempted from the same samples so it is not clear if this reflects a need to optimise the PCR, PCR inhibiting material, or if those 6/8 samples were truly negative.

Sanger sequencing and BLAST analysis suggested that all the *Acanthamoeba* detected were of genotype T4. This is perhaps not surprising as T4 has been found to be the most environmentally widespread genotype in previous studies (Niyyati *et al.,* 2009: Niyyati *et al.,* 2015; Magliano and Alferi*,* 2009; Kao *et al.,* 2014). It was hoped that any biases in determining the diversity of environmental *Acanthamoeba* spp. might have been overcome by direct PCR and sequencing of the membrane filters, but only two samples were positive at PCR, so it is perhaps again not surprising that both appeared to be most closely related to T4.

Further analysis, including alignment with published and genotyped sequences confirmed that all the sequences generated within this study formed part of the same clade as T4 genotypes, and part of a larger clade with T3 and T11 as described by Risler *et al.,* (2013). Indeed, the new sequences were all identical to

each other and to several published T4 sequences. Larger sequences would doubtless provide more discriminatory power and perhaps reveal subtle differences between these new isolates and other T4 sequences.

The results from this study thus agree with the findings of previous studies (Maghsood *et al.,* 2005; Gavarane *et al.,* 2018; Richard *et al.,* 2016), in which T4 was the most prevalent genotype in the environment. As discussed already, a greater diversity of genotypes might have been expected from the environment, but it may be that T4 are easier to culture than other genotypes. Alternatively, there might be a relatively narrow diversity of *Acanthamoeba* genotypes in temperate UK (most diverse genotypes have come from warmer climates, (Rahdar *et al.,* 2012). Or it may be that the Thames and the lake sampled are particularly contaminated with T4 genotype from human and animal excretion (Niyyati *et al*., 2009).

The isolation of the T4 genotype, which is that most commonly associated with AK reinforces the risk to public health of swimming in such waters. And reinforces the important finding that WTW seemed effective at removing *Acanthamoeba* spp. from raw water.

# Chapter 6

# Adaption of a sulforhodamine B (SRB) assay to determine the effectiveness of disinfectants on environmental *Acanthamoeba* isolate.

## 6.1: Introduction

This chapter focuses on attempts to optimise a sulforhodamine B (SRB) assay that could be used to determine the effectiveness of disinfectants on environmental *Acanthamoeba* spp. isolates. The approach taken was based on that described by Ortega-Rivas *et al.,* 2016 and Orellana and Kasinski, 2016. The model system was chlorhexidine (CHX) and environmental *Acanthamoeba* spp. isolated from river sample taken from WTW D in 2017 (Chapter 2).

*Acanthamoeba* keratitis (AK) is a sight-threating infection, which, if misdiagnosed or if treatment is delayed, can lead to blindness (Chapter 1). Currently there is no specific treatment for AK (Lorenzo-Morales *et al.,* 2015), not least because *Acanthamoeba* spp. has two stages of life cycle, metabolically active trophozoites and metabolically largely inactive, and therefore resistant, cysts. If cysts are not completely eradicated, there is a possibility of the infection re-occurring as the cysts revert, in less harsh conditions, to trophozoites.

As already discussed in Chapter 1, AK is particularly associated with wearing contact lenses, and although no *Acanthamoeba* were found in domestic taps in this study, previous research has suggested that domestic, as well as environmental, water is an important source of contamination (discussed in Chapter 4). Therefore control of infection might need to rely more on decontamination of potentially contaminated lenses than removal of *Acanthamoeba* spp. during water processing. Table 6 summarises the effectiveness of a range of disinfectants against *Acanthamoeba* spp.

**Table 6.1: Some of the disinfectants that have been tested on *Acanthamoeba* spp.**

| Disinfectant | Method | Effectiveness of disinfectant | References |
|---|---|---|---|
| Hydrogen peroxide and chlorhexidine gluconate | Microtiter plates were seeded with cysts then disinfectants were added to the plate. Then *E.coli* was added to the plate to turn the cysts to trophozoites, this is to investigate the number of cysts left after exposure to the disinfectants. | Hydrogen peroxide (3%) and chlorhexidine gluconate (0.004%) given enough contact time were effective at killing cysts. | Kilvington *et al.,* 1991 |
| Propamidine isethionate, chlorohexidine, gentamicine, povidone iodine | Microtiter plate method using cysts. Then NNA with bacteria were used to check for the presence of trophozoites after the cysts have been exposed to the disinfectants. | Chloroxidine was found to be the most effective against cysts. | Ghani *et al.,* 2016 |
| Chloroquine | Study was carried out in Korea where multipurpose disinfecting solutions (MPS) were tested with chloroquine. The method used was incubating axenic amoeba culture with test solution then testing for the presence of trophozoites by growing the amoeba on agar with heat-killed bacteria. | Chloroquine has the potential to be effective against *Acanthamoeba* spp. | Moon *et al.,* 2018 |

| Disinfectant | Method | Effectiveness of disinfectant | References |
|---|---|---|---|
| Photocatalytic reactors | Two types of photocatalytic reactors were tested to determine their effect on *Acanthamoeba* trophozoites in the presence of *E.coli.* The reactors used were suspended and immobilised titanium dioxide. | Suspended and immobilised titanium dioxide were found to effective against *Acanthamoeba* spp. | Adan *et al.,* 2018 |
| Novel MPS (ASP-57) | Two commercial MPS and a novel MPS with ASP-57 compound was tested against *Acanthamoeba* trophozoites and cyst. To test the effectiveness of trophozoites alamar blue stain was used and for cysts Trypan blue stain was used. | The commercial MPS showed minimal effectiveness against *Acanthamoeba* when compared to the novel MPS. | Fears *et al.*, 2018 |
| Hydrogen peroxide solution with polyacrylonitrile catalyst impregnated with ferric chloride or ferric sulphate | A catalyst was added to one step commercial hydrogen peroxide contact lens solutions. The effectiveness of this was tested using the most probable number approach. | The addition of a catalyst to the hydrogen peroxide solution was found to be more efficient against cysts. | Kilvington and Winterton, 2017 |

CHX is biguanide, which has been used to treat AK either alone or combined with other drugs e.g. propamidine (Seal *et al.,* 1996; Ghani *et al.,* 2016). It has been reported to be effective against *Acanthamoeba* cysts and trophozoites (Heredero-Bermejo *et al.,* 2016, Hoon *et al.,* 2011, Ghani *et al.,* 2016; Lim *et al.,* 2008). CHX binds to the cell membrane of the amoeba causing lysis of the cell. It therefore seemed to be the best disinfectant with which to test the assay system.

There are currently no standard protocols for investigating the effectiveness of disinfectants against *Acanthamoeba* spp. although standards for bacteriocides do exist (Fears *et al.,* 2018). Some assays (Table 6.1) have been aimed at trophozoites and other cysts. The treatment times vary between assays, as do the mechanisms for counting cells. The assay chosen for this study was based on that described by Ortega-Rivas *et al.,* (2016), which was used to find the effectiveness of CHX on clinical strains of *Acanthamoeba* spp. They used 96 well plates to facilitate high throughput screening of the effectiveness of compounds against *Acanthamoeba* spp, as did McBride *et al.,* (2005). However, Ortega-Rivas *et al.,* (2016) used sulforhodamine B (SRB), a bright pink vital stain (SRB) that binds to proteins of viable cells and is widely used to test cytotoxicity of drugs on cells (Orellana and Kasinski, 2016), whereas McBride *et al.,* (2005) used alamarBlue stain. A further problem with previously published studies of disinfectants is that they often use laboratory-adapted *Acanthamoeba* strains, whereas as low passage environmental isolates may exhibit different characteristics.

The aim of this chapter was to assess and if possible optimise an assay that could then be used to determine the effectiveness of other disinfectants on environmental *Acanthamoeba* spp.

## 6.2: Method

### 6.2.1: Basic protocol for assay

All of the steps were carried out in a class 2 biosafety cabinet under sterile conditions. Stock low passage culture of *Acanthamoeba* spp. isolated from the river sample from WTW D in April 2017 (Chapter 3) were grown in PYG as described in Chapter 2. All five 96 well plates were set-up using the isolate from this sample which was filtered on a single NNA plate. For use with this assay, 5ml of *Acanthamoeba* spp, culture was added to 75ml flask with 30 ml PYG, with 0.1% of antibiotic solution (Sigma penicillin-Streptomycin P4333) added to prevent bacterial growth, and incubated at 30°C for 18 – 24 hours so that the culture was at the trophozoite stage. The flask was then removed from the incubator and placed on an ice pack to detach the trophozoites from the base of the culture flask. The flask was viewed under the microscope to ensure that there were trophozoites in the culture. At this stage the flask was also checked to see that it was not contaminated with bacteria. If there was excessive bacterial presence the flask was discarded and another flask was set-up.

A Sedgewick Rafter was used to calculate the dilution of stock required to seed each well of the 96 well plate (Greiner Cellstar Sigma M0812) with 3000 trophozoites. The final volume of culture was made up to 100µl.

The plate was left at room temperature for 60 minutes for trophozoites to adhere to the wall of the plate, and then checked under the microscope to ensure there were trophozoites in each well.

The CHX solution (Sigma 282227) (0.6 µM, 1.25 µM 2.5 µM, 5 µM, 10 µM, 20 µM) was added to the wells of the plate, as shown in Figure 6.1.

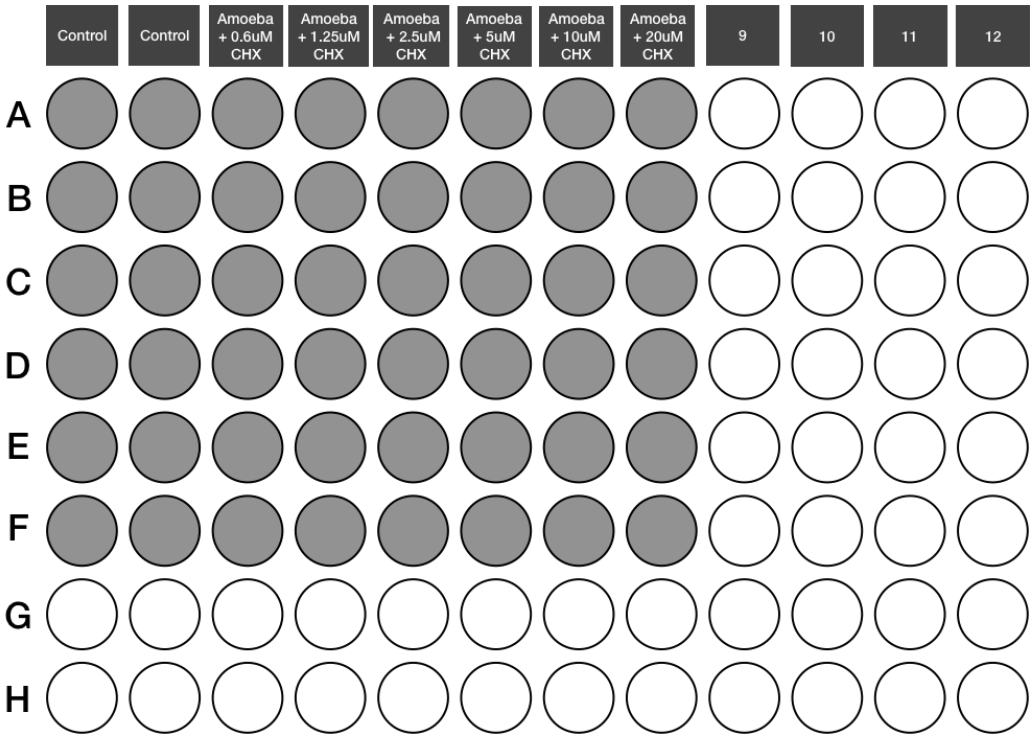


**Figure 6.1: The standard method for testing Chlorhexidine (CHX) against Acanthamoeba in a 96 well plate.** Wells 1A to 1F and 2A to 2F were controls, containing only *Acanthamoeba* trophozoites in PYG. Wells 3A-3F, 4A-4F, 5A-5F, 6A-6F, 7A-7F and 8A-8F contained *Acanthamoeba* trophozoites in PYG and serial dilutions of CHX.

The plate was incubated for 72 hours at 30°C in a humidified incubator. After this, 25µl of cold TCA (Sigma T6399) was added to fix the trophozoites to the well. The plate was then incubated at 4°C for 1 hour. The supernatant was discarded from

the well by inverting the plate. The well was washed three times with distilled water by submerging them gently in a tub of water. It was then tapped onto a paper towel to remove excess water and left to air dry until there was no visible sign of water in the wells. Any trophozoites present in the wells were stained by adding 25µl of SRB solution to each well and plate was incubated at room temperature for 20 minutes. After this time, the wells were washed three times with 1% acetic acid to remove the unbound SRB and the plate was air dried. Finally, 100µl of Tris base was added to each well to solubilise the dye, the plate was placed on a shaker for 10 minutes and then in the microplate spectrophotometer (Labtech microplate absorbance reader) and the absorbance was measured at 492nm and 630nm.

### 6.2.2 Adaptation to the basic protocol assay

In early trials, using the above assay, the wells were turbid with bacterial growth (presumably carried over from the original environmental sample) after 72 hours incubation at 30°C. Therefore, the concentration of antibiotics was increased to 0.3% to prevent bacterial contamination of *Acanthamoeba* spp. culture during the incubation period.

## 6.3: Results

In total, five plate assays were undertaken in which bacterial overgrowth was not seen. The raw data are given in appendix K. The outliers were removed and results for each concentration of CHX from each plate were collated and the mean and SD was calculated at both 492nm and 630nm wavelength. The data are

summarised in the Table 6.1 and Figure 6.2. The control wells were *Acanthamoeba* spp. in PYG.

**Table 6.2: Results of testing dilutions of CHX against environmental *Acanthamoeba* isolate.** Raw data is available in appendix K. The mean and SD was calculated using the data from the five 96 well plates set-up. R denotes the data range.

| CHX concentration (µM) | Mean absorbance at 492nm (4d.p) | SD at 492nm (4d.p) | Mean absorbance at 630nm (4d.p) | SD at 630nm (4d.p) |
|---|---|---|---|---|
| Control | 0.3040 | 0.0429 | 0.2675 | 0.0248 |
| | R (0.2627-0.3531) | | R (0.2315 – 0.3874) | |
| Control | 0.2969 | 0.0531 | 0.2698 | 0.0186 |
| | R (0.2294 – 0.3713) | | R (0.2316 – 0.3048) | |
| 0.6 | 0.2950 | 0.0465 | 0.2528 | 0.0180 |
| | R (0.2371 – 0.3636) | | R (0.2218 – 0.2834) | |
| 1.25 | 0.2871 | 0.0534 | 0.2473 | 0.2382 |
| | R (0.2214 - 0.4500) | | R (0.2101 – 0.3116) | |
| 2.5 | 0.2748 | 0.0309 | 0.2409 | 0.0193 |
| | R (0.2373 – 0.3587) | | R (0.2902 – 0.2790) | |
| 5 | 0.2773 | 0.0315 | 0.2436 | 0.0232 |
| | R (0.2165 – 0.3240) | | R (0.1967 – 0.2757) | |
| 10 | 0.2690 | 0.0414 | 0.2316 | 0.0220 |
| | R (0.2151 – 0.3669) | | R (0.1910 – 0.2808) | |
| 20 | 0.2754 | 0.0468 | 0.2347 | 0.0236 |
| | R (0.2078 – 0.4302) | | R (0.1937 – 0.2811) | |

Overall, whichever absorbance was used, there was a possible trend towards reduced absorbance at higher concentrations of CHX, but the range of values within and between tests was so great this was clearly not significant.

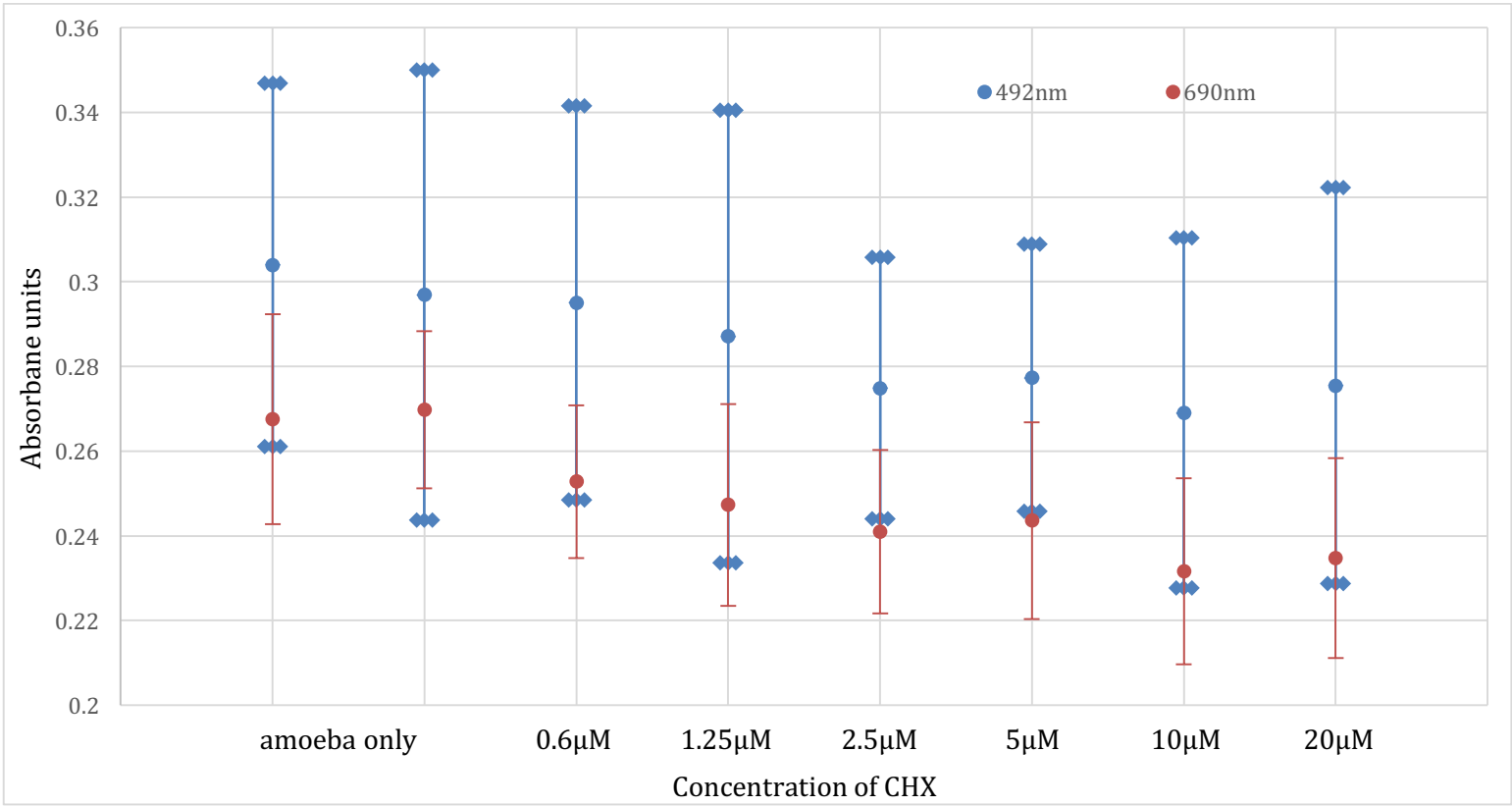


**Figure 6.2: Results of testing CHX against environmental *Acanthamoeba* spp.** Red dots indicate mean absorbance at 630nm and blue dots mean absorbance at 492nm. Vertical lines are error bars (±1 SD of the mean value). The values are in Table 6.2.

## 6.4: Discussion

Overall the SRB assay (Table 6.1) did not show any clear effect of CHX on the growth or survival of environmental *Acanthamoeba* isolate. A slight trend was seen in the results, but this was clearly not significant owing to the wide range of absorbances. Whether this was due to a combination of resistance and diversity within low passage environmental isolates or the assay itself is not clear.

There were many challenges with setting up the assay, in which many small errors can accumulate, and which in turn could lead to a large variation in the results. The two main areas of potential errors include:

1. Consistent number of trophozoites in each well. The aim was to seed each well with 3000 trophozoites; however the method does not take into account the variability of cell count, or cell 'health', actually placed in each well. Counting the number of trophozoites using a Sedgwick rafter only provides an estimate. Furthermore, the amoebae can clump together when being cultured; this might mean the number of trophozoites contained in some wells will be much greater than in others, which could lead to variations in results.
2. Bacterial contamination. Although antibiotics were used to minimize bacterial presence in the culture, after the 72 hours incubation the cultures started turning turbid using the published protocol. This was

reduced by increasing the volume of antibiotics used when setting up the culture for the assay. In addition to simple bacterial contamination, some *Acanthamoeba* may have been carriers of bacteria, protecting them from the antibiotics.

At the same time as this assay was being trialled, other approaches were being trialled in the same laboratory by other students, using laboratory-adapted strains and different stains, and also assaying cysts (Arthur, 2018; Barnish, 2018). Similar inconsistency issues were found with some of these assays. Future development of this assay should incorporate some of the findings from that work, for example trialling calcofluor stain, and developing a cyst assay.

In order to make this assay effective for environmental *Acanthamoeba* isolates, the following could be tested:

1. The Sedgewick Rafter has 100 squares in a 10 by 10 grid; however only 10 random squares were counted to estimate the volume required to seed each well with 3000 trophozoites. Although time consuming, all 100 squares could be counted to get more accurate count of *Acanthamoeba spp*. in each well.
2. The process required washing the plates. During this washing process, it is possible that some of the trophozoites may have been lost causing some variation in the results. A possible solution to this would be to use an automated pipette with a small capillary tube. Alternatively, different plates could be tested (different plastics can cause differing degrees of

adhesion) or better ways of getting the trophozoites to adhere, such as pre-coating the plates..

3. Optimisation of axenic culture by increasing the number of times a piece of agar containing *Acanthamoeba* spp. was re-grown on NNA to remove bacterial contamination (Niyyati *et al.,* 2013). The downside of this would be that the amoebae might become laboratory-adapted and their resistance/susceptibility change. Another option might be to acid treat the cultures to eliminate bacteria (Lorenzo-Morales *et al.,* 2015). To check if there were any bacteria present, the supernatant from the assay could be cultured on a nutrient agar to assess whether bacteria were present in the wells.

# Chapter 7

# General discussion and future studies

## 7.1: General discussion

*Acanthamoeba* spp. are environmentally widespread and pose an important, and increasing, public health risk in the UK (Carnt *et al.,* 2018a). The sources of infection, however, are not always clear. Domestic water (e.g. it has been found associated with taps in homes) or raw water (e.g. through water sports) appears to be the most common risk factor (Khan, 2006; Carnt and Stapleton, 2016; Szentmary *et al.,* 2018; Carnt *et al*., 2018a). This thesis describes a series of studies to investigate *Acanthamoeba* spp. in raw water entering drinking WTW in South East of England, the ability of WTW to remove these protozoa, and the presence of *Acanthamoeba* spp. in drinking tap water. In addition, the *Acanthamoeba* spp. detected were genotyped – the first time this has been done for environmental rather than clinical isolates in the UK. Finally, as controls within the domestic setting may require local treatment, an attempt was made to develop an assay for testing the susceptibility of trophozoites against disinfectants.

The study described in Chapter 3 demonstrated that *Acanthamoeba* spp. can be found consistently in surface water sources, in this case the River Thames and a recreational lake. Both water sources will have contained run-off from soil, and both had high bacterial content, and they may have been sources of nutrition for the protozoa. The sampling and testing techniques were not quantitative. They were not titrated and thus were either isolation positive or negative (all surface water sources were isolation positive). It is not possible to determine whether or not there were seasonal differences in the number of protozoa present. *Acanthamoeba* spp. (and their bacterial food source) grows at different rates at

different temperatures (Duarte *el al.,* 2013). The temperature of surface water changed markedly with different seasons. Thus it may be that the public health risk is not uniform throughout the year. It would be interesting in future studies to attempt more quantitative approaches to detection – perhaps through serial dilutions of water and/or an optimized qPCR.

It was hypothesized that a wider diversity of *Acanthamoeba* spp. might be detected in raw water than the T4 clade usually detected in UK clinical samples (Maghsood *et al.,* 2005), as reported from environmental studies elsewhere in the world. Studies in Latvia (Gavarane *et al.,* 2018), Taiwan (Kao *et al.,* 2012) and Iran (Rahdar *et al.,* 2012) have found genotypes T4, T2, T5, T6, T7, T8, T11 and T15. However, genotyping of the raw water isolates from cultures and of a small number of DNA samples taken straight from water filters in this study found only T4 genotypes in this study. As only a few direct PCR samples were sequenced, this may reflect ease of culture rather than a lack of diversity. It would be interesting to test larger numbers of environmental samples by direct PCR. This, however, will require further optimization of the PCR approach. Indeed, PCR rather than culture might be a more efficient means of detecting *Acanthamoeba* spp. in water samples should surveillance be needed. Recent studies of clinical cases found PCR to be much more sensitive than culture (Gatti *et el.,* 2010).

So many of the *Acanthamoeba* spp. isolated in this study were T4, i.e. of a genotype known to be associated with human disease. This reinforces the risk of infection associated with water sports, and possibly water supplies.

It would also be interesting in future studies to use a wider range of PCR primers in order to amplify, and sequence, a larger portion of the rRNA gene. The approach used in this study allowed confirmation that all (except one) isolates were indeed *Acanthamoeba* spp., and furthermore all were part of the T4 genotype. However, apart from artefactual differences, no significant differences between the sequences of isolates were found in this study. That may reflect the relatively short sequences tested. The approach taken in this study was that commonly used for genotyping but most phylogenetic studies compare the full length of the rRNA gene, or at least sequences >2kb (Fuerst *et al.,* 2015; Qvarnstrom *et al.,* 2013).

Fewer samples taken from the ground water sources were found to be positive for *Acanthamoeba* spp than were found in surface water samples. It is likely that this reflects the lower bacterial loads in aquifer water. It was expected that karstic water sources might have higher bacterial loads than non-karstic ground water sources, and this was the case. It was also thought that this might be associated with slightly higher frequencies of *Acanthamoeba* spp. detection. However, there were no significant differences in the prevalence of *Acanthamoeba* spp. between the two sources. This might be a function of sample sizes and/or that the approach used was not quantitative and that a quantitative approach might have detected differences in the number of amoebae present. Alternatively, it might indicate that contamination of the water from ground water aquifers occurs during the collection of the water rather than in the aquifers, may be due to ingress of surface water into the borehole.

Either way, ground water sources appear to carry a much lower public health risk from *Acanthamoeba* spp. than surface water.

As *Acanthamoeba* spp. were only present in occasional ground water samples, it is perhaps not surprising that they were not isolated at any stage of water processing at WTW A, WTW B or WTW C. However, at WTW D, which handled surface water consistently containing *Acanthamoeba* spp. they were isolated from several part-treated stages, including pre-ozonation, post sedimentation, post flat bottom clarifiers, post rapid gravity filters and GAC inlets. However, there was a reduction in the prevalence at each stage (from 100% raw water samples to around 10% at the GAC inlets) and *Acanthamoeba* spp. were not isolated after the GAC inlet stage of the treatment. Water processing at WTW D involves many more steps than at the other sites studied in order to remove the heavy bacterial loads known to exist in surface water. It would appear that the processing at each site is adequate to remove at least the majority of *Acanthamoeba* spp. This is likely to be due to a combination of physicochemical processes and the removal of bacterial food sources. In the case of WTW D, which takes surface water containing a high prevalence of *Acanthamoeba* spp. there is also extra processing to remove *Cryptosporidium* oocysts, and this probably also helps to remove *Acanthamoeba* spp.

It is also possible, however, that A*canthamoeba* spp. do survive processing as cysts in low numbers, and that they were simply not detected during this study. Again, an optimized PCR might be more sensitive than the culture method to determine this.

*Acanthamoeba* spp. were not isolated at treated water reservoirs before being supplied to consumers, nor in homes to which the water was supplied. *Acanthamoeba* spp. have been detected by PCR in biofilms in water towers in other studies (Stockman *et al.,* 2011). However, it may be that these biofilms rather than being sources of *Acanthamoeba* spp. essentially contain them, thereby preventing the amoebae from appearing in the water itself. The survival of *Acanthamoeba* spp. at these stages, should they survive processing, would be expected at this point as there were still a bacteria present.

Unlike in previous studies of domestic tap water, in the present study *Acanthamoeba* spp. were not isolated from any of the samples from the kitchen taps at domestic properties. However, only kitchen tap water were sampled, and only after careful disinfection of the tap itself, i.e. only water coming directly from the main water supply to the property was sampled. In previous studies (Hoffmann and Michel, 2001; Mahmoudi *et al.,* 2012) few details are given about the sampling, but the reports suggest that it was from a variety of taps, including those supplied by water storage tanks. Furthermore, in some studies there is little detail given on the condition and hygiene of the sample taps, which may have had mould and biofilms growing.

Future work should focus on sources of *Acanthamoeba* spp. within households, comparing water storage systems, pipes and taps, and including perhaps heating and air conditioning units, which are also known sources of Legionnaires' disease (Wang *et al.,* 2012; Wang *et al.,* 2013; WHO, 2007). *Acanthamoeba* spp. are

known to be a major host for *Legionella* (Rowbotham,1980). More work on biofilms, and their stability, would also be valuable.

Despite the WTW apparently efficiently removing *Acanthamoeba* spp. from raw water, AK is an increasing clinical problem. Control of it in the domestic setting is likely to rely on disinfection, particularly in contact lens storage systems. However, there are currently no agreed assays for testing the effectiveness of disinfectants on *Acanthamoeba* spp. An assay was set-up using the stain SRB to test the effectiveness of these isolates to CHX. However with the concentrations tested, the results were highly variable and it was difficult to assess whether the environmental isolates were sensitive or tolerant to CHX. Further modifications to the assay are required in order to reduce the variability in trophozoite counts before it can be used routinely.

In summary, the initial aim of this study was to investigate the prevalence of *Acanthamoeba* species at various stages of the water processing and distribution system, and investigate any factors that might be associated with the presence or absence of, and therefore the risk posed by waterborne *Acanthamoeba* spp.

And to achieve this aim, four objectives were set:

1. To study the prevalence of *Acanthamoeba* spp. in ground and surface raw water sources supplying four WTW.

**This objective was met, and *Acanthamoeba* spp. were found commonly in raw surface water, but only occasionally in ground water.**

2. To investigate the prevalence of *Acanthamoeba* spp. at various stages of the water treatment and distribution process, including in domestic properties served by the WTW.

   **This objective was met. *Acanthamoeba* spp. were found to reduce in prevalence as water passed through the treatment process, such that no *Acanthamoeba* spp. were detected in either the distribution system or at mains supplied taps in domestic properties. This suggests that the *Acanthamoeba* spp. found in domestic settings, and thought to be responsible for most human infection, is likely to originate locally rather than from raw water.**

3. To investigate the genotypes any *Acanthamoeba* spp. detected in raw water and at various stages of the water treatment process.

   **Using PCR and sequencing, all the *Acanthamoeba* spp. detected in this study appeared to be of genotype T4. On the one hand, this is known to be the most widespread of genotypes in both the environment and clinical settings, but on the other, a greater diversity of genotypes had been expected in raw water.**

4. To assess a published assay for testing the effectiveness of disinfectants on environmental *Acanthamoeba* spp.

**A previously published assay, although further developed, proved inconsistent and thus unable to determine any effect of CHX, a commonly used disinfectant, on *Acanthamoeba* trophozoites. More work needs to be done on making this assay more consistent – but also developing assays for cysts.**

## 7.2: Future Studies

The next stage in this research would be to compare the sensitivity of *Acanthamoeba* spp. culture with that of PCR undertaken directly from the filters for the detection of environmental amoebae. Identifying trophozoites and cysts by growing them in culture can take up to two weeks, whereas PCR can give a result on the day the sample is taken. This would be particularly useful if there were an incident and results were required urgently.

Qualitative assays, such as qPCR, would also enable better estimation of the public health risk, and any seasonal variation, as well as enabling studies of which stages in water processing that have the greatest effect on *Acanthamoeba* spp. survival.

The lack of diversity of *Acanthamoeba* genotypes found in this study is interesting. Next steps with the isolates collected would be to carry out whole genome sequencing. Not only would this demonstrate the species found, but it may demonstrate greater diversity between the isolates than found by PCR sequencing ribosomal DNA. Thus it might also identify alternative, i.e. more variable, targets for PCR-sequencing in order to study the molecular ecology of *Acanthamoeba*

spp. Longer term, whole genome sequence of a larger number of *Acanthamoeba* isolates may also enable the identification of sequences associated with pathogenicity.

To date, little work appears to have been done on strain variation and carriage of bacterial pathogens. Again, direct and multiplex PCR might enable pilot studies to be done, although metagenomics approaches would probably be needed to get a better understanding of these communities in water.

Indeed, *Acanthamoeba* spp. may not only carry pathogens, but also protect indicators of faecal contamination during water treatment, and thereby confound current surveillance.

It may be possible to improve the culture method for *Acanthamoeba* spp. In this study, *E.coli* was used as a nutrient source. However other types of bacteria – e.g. *P. aeruginosa* has been used (De Moraes and Alferi, 2008) – may be better for isolating environmental *Acanthamoeba* spp. Little work appears to have been done recently on optimising culture.

Future studies should also look at the systems involved in distributing the water from the treatment plants and water storage reservoirs to domestic properties. This will involve taking samples from domestic properties that are nearest and furthest away from the WTW and analysed for the presence of *Acanthamoeba* spp. It may be that as the concentration of chlorine decreases over distance and time, there may be bacterial growth in the network, which may also support *Acanthamoeba* spp. growth.

This study has demonstrated that WTW are effective at removing environmental *Acanthamoeba* spp. While this is useful in terms of water treatment processes, in public health terms it will be useful to carryout future work focusing on the ecology of *Acanthamoeba* spp. within household water system. Little is known about water microbiology within such systems. However with turnovers, temperatures, materials, flow rates, in addition to routes of entry (e.g. open storage tanks in lofts) the ecology of communities of bacteria and *Acanthamoeba* spp. are likely to complex. It would be interesting to study the diversity of such communities and their interactions and how this influences the water that arrives at the various outlets e.g. bathroom taps, showers in households.

In addition environmental samples or swabs can be taken from the household e.g. soil from the garden and swabs of the taps, pipes and storage systems to find the source of amoebae. Such studies should also include biofilms – indeed much more work on biofilms and *Acanthamoeba* spp. is needed. Of particular interest is to find if there is a relationship between *Pseudomonas,* which is commonly found in biofilms (Qin *et al.,* 2017; Taravaud *et al.,* 2018), and the presence of *Acanthamoeba* spp.

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# Appendices

## Appendix A: Preparation of PYG

PYG was prepared by adding 7.5g proteose peptone, 7.5g yeast extract and 15g glucose. The ingredients were added to a flask and the volume was made up to 1000 ml with deionised water. Once the powder had dissolved in the water, it was transferred into a bottle. Then autoclaved for 15 minutes at 121°C. It was cooled before use.

## Appendix B: Preparation of NNA

25g of agar base was weighed and placed in a flask. Then 1 litre of deionised water was added to the flask. Mix the water with the agar base, and then transfer to a bottle. Then autoclaved for 15 minutes at 121°C. It was cooled to 50°C the poured into 90mm Petri dishes.

## Appendix C: Preparation of *Escherichia coli* cultures

A universal tube containing 10 ml of nutrient broth [nutrient agar base from Oxoid Ltd, Basingstoke, UK] was inoculated with a loop of *Escherichia coli* and incubated at $37^0$C for 24 hours.

## Appendix D: Preparation and viewing of agrose gel

The 1.5% gel was prepared by putting 2.25g of agrose powder in a flask then adding 150 ml of TAE buffer and 7µl eithidium bromide. The flask was placed with the ingredients in the microwave for 30 seconds then the ingredients were mixed and placed in the microwave for another 45 seconds or until all the agrose powder has dissolved. The ingredients were gently mixed to ensure there are no bubbles in the flask. The gel mix was poured in a tray then the comb was placed and the gel was allowed to set. 2 µl of 100bp ladder was pipette into the first well followed by 2µl of the amplified DNA from the samples into each well. The gel was run for 60 minutes. The gel was then viewed under UV transilluminator (Bio-Rad) for the presence of a band between 450bp and 500bp.

## Appendix E: Raw data from samples taken between November 2009 and August 2010 from WTW A

### November 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 11.9 | 12.25 | NM | NM | 7.1 | 712 | 0.20 | 3 | 20 | 9 | 1 | Absent |
| Raw water inlet 2 | 11.8 | 12.29 | NM | NM | 7.1 | 715 | 0.14 | 3 | 38 | 6 | 1 | Absent |
| GAC Inlet 1 | NM | 12.40 | NM | NM | NM | NM | NM | 0 | 7 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 12.50 | NM | NM | NM | NM | NM | 1 | 2 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 12.46 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 12.48 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 12.43 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 12.10 | 0.37 | 0.41 | 7.2 | 730 | 0.13 | 2 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 13.03 | 0.41 | 0.41 | 7.2 | 744 | 0.07 | 2 | 0 | 0 | 0 | Absent |
| Treated water 1 | 11.9 | 13.12 | 0.43 | 0.45 | 7.1 | 718 | 0.12 | 0 | 1 | 0 | 0 | Absent |
| Treated water 2 | 12.0 | 13.17 | 0.39 | 0.50 | 7.1 | 709 | 0.20 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 13.2 | 13.25 | 0.28 | 0.34 | 7.1 | 708 | 0.06 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.9 | 13.52 | 0.22 | 0.22 | 7.2 | 718 | 0.06 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 12.8 | 13.34 | 0.25 | 0.28 | 7.2 | 715 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 12.0 | 13.41 | 0.29 | 0.29 | 7.1 | 720 | 0.09 | 0 | 10 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 12.1 | 13.43 | 0.27 | 0.27 | 7.1 | 724 | 0.05 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 12.1 | 11.59 | 0.10 | 0.20 | 7.2 | 730 | 0.06 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 12.1 | 11.59 | 0.18 | 0.20 | 7.2 | 729 | 0.06 | 0 | 21 | 0 | 0 | Absent |
| Domestic property tap water 1 | 13.1 | 09.50 | 0.23 | 0.33 | 7.4 | 630 | 0.12 | 2 | 0 | 0 | 0 | Absent |
| Domestic property tap water 2 | 11.9 | 11.48 | 0.20 | 0.21 | 7.2 | 709 | 0.17 | 0 | 6 | 0 | 0 | Absent |
| Domestic property tap water 3 | 14.0 | 12.40 | 0.18 | 0.25 | 7.1 | 724 | 0.07 | 0 | 1 | 0 | 0 | Absent |
| Domestic property tap water 4 | 14.5 | 12.00 | 0.27 | 0.34 | 7.1 | 719 | 0.18 | 14 | 15 | 0 | 0 | Absent |
| Domestic property tap water 5 | NM | 15.00 | NM | NM | NM | NM | NM | ~4400 | ~3600 | 225 | 0 | Absent |

## December 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 11.0 | 08.16 | NM | NM | 7.1 | 687 | 0.15 | 8 | 43 | 3 | 1 | Absent |
| Raw water inlet 2 | 11.3 | 08.18 | NM | NM | 7.2 | 725 | 0.11 | 2 | 46 | 21 | 11 | Absent |
| GAC Inlet 1 | NM | 08.3 | NM | NM | NM | NM | NM | 1 | 10 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 08.45 | NM | NM | NM | NM | NM | 1 | 4 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 08.45 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 08.47 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 08.32 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 08.02 | 0.51 | 0.54 | 7.2 | 705 | 0.09 | 7 | 2 | 0 | 0 | Absent |
| Post membrane filtration | NM | 09.02 | 0.60 | 0.70 | 7.1 | 697 | 0.16 | 8 | 0 | 0 | 0 | Absent |
| Treated water 1 | 11.5 | 09.22 | 0.50 | 0.61 | 7.1 | 722 | 0.05 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 11.4 | 09.36 | 0.50 | 0.56 | 7.1 | 691 | 0.09 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 11.5 | 10.06 | 0.46 | 0.47 | 7.1 | 684 | 0.06 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 10.2 | 09.51 | 0.27 | 0.30 | 7.1 | 687 | 0.07 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 9.8 | 10.54 | 0.22 | 0.24 | 7.1 | 698 | 0.05 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 11.3 | 10.36 | 0.27 | 0.33 | 7.1 | 700 | 0.05 | 0 | 9 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 11.2 | 10.36 | 0.25 | 0.29 | 7.1 | 694 | 0.05 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 10.8 | 11.59 | 0.21 | 0.24 | 7.2 | 696 | 0.04 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 11.0 | 12.00 | 0.16 | 0.20 | 7.2 | 715 | 0.08 | 0 | 3 | 0 | 0 | Absent |
| Domestic property tap water 1 | 9.7 | 11.05 | 0.26 | 0.30 | 7.3 | 585 | 0.12 | 3 | 2 | 0 | 0 | Absent |
| Domestic property tap water 2 | 10.3 | 11.29 | 0.20 | 0.22 | 7.1 | 721 | 0.07 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 3 | 10.2 | 10.00 | 0.25 | 0.25 | 7.1 | 705 | 0.07 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 4 | 6.7 | 12.19 | 0.12 | 0.20 | 7.1 | 704 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 5 | 13.8 | 10.50 | 0.27 | 0.29 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |

## February 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 12.1 | 12.00 | NM | NM | 7.0 | 719 | 0.14 | 5 | 12 | 11 | 0 | Absent |
| Raw water inlet 2 | 12.1 | 12.55 | NM | NM | 7.1 | 730 | 0.12 | 1 | 18 | 18 | 3 | Absent |
| GAC Inlet 1 | NM | 12.59 | NM | NM | NM | NM | NM | 0 | 3 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 12.41 | NM | NM | NM | NM | NM | 1 | 4 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 11.46 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 12.07 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 12.20 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 12.05 | 0.41 | 0.46 | 7.1 | 720 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 11.20 | 0.41 | 0.49 | 7.0 | 711 | 0.25 | 0 | 0 | 0 | 0 | Absent |
| Treated water 1 | 12.0 | 11.50 | 0.46 | 0.49 | 7.0 | 715 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 12.0 | 10.40 | 0.46 | 0.46 | 6.9 | 707 | 0.08 | 1 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 12.3 | 10.21 | 0.23 | 0.26 | 7.0 | 713 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.2 | 10.37 | 0.21 | 0.23 | 7.0 | 717 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 12.9 | 09.50 | 0.22 | 0.23 | 7.0 | 723 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 12.0 | 10.01 | 0.16 | 0.22 | 7.0 | 723 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 12.0 | 10.10 | 0.20 | 0.22 | 7.0 | 721 | 0.07 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 19.1 | 10.41 | 0.10 | 0.19 | 7.0 | 676 | 0.17 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 19.3 | 10.47 | 0.07 | 0.28 | 7.1 | 680 | 0.16 | 0 | 1 | 0 | 0 | Absent |
| Domestic property tap water 1 | 19.1 | 12.20 | 0.16 | 0.21 | 7.3 | 612 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 2 | 15.9 | 11.00 | 0.15 | 0.18 | 7.1 | 687 | 0.15 | 248 | 291 | 0 | 0 | Absent |
| Domestic property tap water 3 | 17.1 | 11.40 | 0.37 | 0.41 | 7.2 | 724 | 0.12 | 1 | 0 | 0 | 0 | Absent |
| Domestic property tap water 4 | 21.4 | 11.28 | 0.10 | 0.18 | 6.9 | 709 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 5 | 20.2 | 10.23 | 0.53 | 0.55 | 6.9 | 738 | 0.10 | 0 | 1 | 0 | 0 | Absent |

## March 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 10.9 | 11.10 | NM | NM | 7.0 | 702 | 0.16 | 8 | 34 | 6 | 0 | Absent |
| Raw water inlet 2 | 10.9 | 11.15 | NM | NM | 7.1 | 718 | 0.16 | 7 | 45 | 41 | 6 | Absent |
| GAC Inlet 1 | NM | 11.04 | NM | NM | NM | NM | NM | 4 | 8 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 11.40 | NM | NM | NM | NM | NM | 5 | 94 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 11.29 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 11.36 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 11.21 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 12.01 | 0.40 | 0.40 | 7.2 | M | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 11.00 | 0.39 | 0.39 | 7.0 | 708 | 0.15 | 0 | 1 | 0 | 0 | Absent |
| Treated water 1 | 10.9 | 10.40 | 0.41 | 0.50 | 7.0 | 701 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 10.9 | 10.40 | 0.41 | 0.50 | 7.0 | 701 | 0.08 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 10.1 | 10.06 | 0.31 | 0.40 | 7.0 | 686 | 0.08 | 0 | 2 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.1 | 10.19 | 0.19 | 0.25 | 7.0 | 712 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 11.1 | 09.36 | 0.11 | 0.17 | 7.0 | 703 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 10.8 | 09.47 | 0.08 | 0.14 | 7.2 | 710 | 0.09 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 10.8 | 09.51 | 0.05 | 0.11 | 7.1 | 712 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 10.4 | 10.21 | 0.10 | 0.14 | 7.1 | 709 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 10.6 | 13.05 | 0.16 | 0.28 | 7.1 | 737 | 0.31 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 1 | 7.1 | 09.55 | 0.04 | 0.10 | 7.2 | 608 | 0.16 | 196 | 80 | 0 | 0 | Absent |
| Domestic property tap water 2 | 9.4 | 12.00 | 0.20 | 0.24 | 7.1 | 707 | 0.09 | 3 | 23 | 0 | 0 | Absent |
| Domestic property tap water 3 | 9.2 | 11.02 | 0.24 | 0.26 | 7.1 | 697 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 4 | 9.1 | 09.38 | 0.36 | 0.41 | 7.1 | 684 | 0.19 | 34 | 0 | 0 | 0 | Absent |
| Domestic property tap water 5 | 8.7 | 10.01 | 0.46 | 0.46 | 7.1 | 658 | 0.19 | 4 | 0 | 0 | 0 | Absent |

## April 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 10.9 | 11.25 | NM | NM | 7.0 | 712 | 0.16 | 0 | 9 | 3 | 2 | Present |
| Raw water inlet 2 | 10.9 | 11.20 | NM | NM | 7.1 | 714 | 0.14 | 0 | 10 | 1 | 0 | Present |
| GAC Inlet 1 | NM | 13.57 | NM | NM | NM | NM | NM | 0 | 81 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 11.00 | NM | NM | NM | NM | NM | 0 | 8 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 10.40 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 10.05 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 11.40 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 10.00 | 0.26 | 0.31 | 7.2 | 736 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 12.00 | 0.22 | 0.39 | 7.0 | 724 | 0.16 | 0 | 1 | 0 | 0 | Absent |
| Treated water 1 | NM | 12.10 | 0.34 | 0.39 | 7.1 | 720 | 0.07 | 0 | 1 | 0 | 0 | Absent |
| Treated water 2 | NM | 12.15 | 0.33 | 0.39 | 7.0 | 728 | 0.07 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | NM | 12.25 | 0.22 | 0.25 | 7.1 | 731 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.0 | 12.30 | 0.20 | 0.29 | 7.1 | 733 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 12.0 | 12.40 | 0.22 | 0.36 | 7.1 | 733 | 0.19 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 12.1 | 12.50 | 0.30 | 0.34 | 7.2 | 726 | 0.27 | 0 | 11 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 12.2 | 12.55 | 0.30 | 0.33 | 7.1 | 739 | 0.17 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 14.1 | 11.28 | 0.18 | 0.19 | 7.1 | 700 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 13.9 | 11.29 | 0.17 | 0.17 | 7.1 | 700 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 1 | 12.9 | 09.15 | 0.17 | 0.25 | 7.2 | 611 | 0.17 | 1 | 0 | 0 | 0 | Absent |
| Domestic property tap water 2 | 11.7 | 11.29 | 0.19 | 0.24 | 7.1 | 684 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 3 | 11.3 | 10.58 | 0.23 | 0.26 | 7.1 | 733 | 0.11 | 1 | 0 | 0 | 0 | Absent |
| Domestic property tap water 4 | 12.8 | 09.10 | 0.22 | 0.29 | 7.1 | 710 | 0.19 | 2 | 1 | 0 | 0 | Absent |
| Domestic property tap water 5 | 12.8 | 10.00 | 0.21 | 0.27 | 7.2 | 653 | 0.10 | 0 | 0 | 0 | 0 | Absent |

## May 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 12.1 | 10.30 | NM | NM | 7.0 | 710 | 0.15 | 0 | 10 | 51 | 51 | Absent |
| Raw water inlet 2 | 11.9 | 10.40 | NM | NM | 7.1 | 688 | 0.13 | 1 | 13 | 41 | 41 | Absent |
| GAC Inlet 1 | NM | 10.55 | NM | NM | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 10.35 | NM | NM | NM | NM | NM | 1 | 129 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 11.00 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 10.45 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 11.15 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 11.35 | 0.45 | 0.51 | 7.2 | 703 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 12.00 | 0.40 | 0.48 | 7.0 | 707 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Treated water 1 | 11.9 | 12.30 | 0.40 | 0.48 | 7.0 | 713 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 11.4 | 12.25 | 0.45 | 0.53 | 7.0 | 703 | 0.09 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 11.9 | 12.35 | 0.20 | 0.24 | 7.0 | 698 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.9 | 12.45 | 0.21 | 0.21 | 7.1 | 702 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 11.4 | 12.55 | 0.16 | 0.24 | 7.1 | 704 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 12.1 | 13.05 | 0.21 | 0.24 | 7.1 | 701 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 12.4 | 13.10 | 0.22 | 0.22 | 7.1 | 704 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 12.3 | 11.22 | 0.18 | 0.27 | 7.2 | 715 | 0.17 | 0 | 3 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 12.3 | 11.23 | 0.13 | 0.18 | 7.3 | 716 | 0.20 | 0 | 3 | 0 | 0 | Absent |
| Domestic property tap water 1 | 13.0 | 10.30 | 0.10 | 0.11 | 7.2 | 688 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 2 | 11.8 | 11.25 | 0.20 | 0.23 | 7.0 | 725 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 3 | 12.8 | 09.39 | 0.23 | 0.24 | 7.1 | 734 | 0.12 | 0 | 1 | 0 | 0 | Absent |
| Domestic property tap water 4 | 12.8 | 12.10 | 0.28 | 0.34 | 7.0 | 726 | 0.18 | 0 | 14 | 0 | 0 | Absent |
| Domestic property tap water 5 | 13.5 | 12.02 | 0.19 | 0.22 | 7.2 | 668 | 0.20 | 0 | 0 | 0 | 0 | Absent |

## June 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 11.0 | 12.44 | NM | NM | 7.0 | 712 | 0.20 | 3 | 2 | 1 | 0 | Absent |
| Raw water inlet 2 | 11.1 | 12.38 | NM | NM | 7.1 | 732 | 0.10 | 1 | 12 | 3 | 0 | Absent |
| GAC Inlet 1 | NM | 12.55 | NM | NM | NM | NM | NM | 0 | 12 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 13.05 | NM | NM | NM | NM | NM | 9 | 560 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 13.00 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 13.07 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 12.53 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 13.12 | 0.39 | 0.43 | 7.3 | 731 | 0.29 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 12.36 | 0.35 | 0.48 | 7.0 | 765 | 0.35 | 0 | 0 | 0 | 0 | Absent |
| Treated water 1 | 11.2 | 12.20 | 0.49 | 0.53 | 7.1 | 733 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 11.5 | 12.24 | 0.51 | 0.60 | 7.0 | 744 | 0.13 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 11.4 | 12.00 | 0.14 | 0.18 | 7.0 | 721 | 0.07 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.3 | 12.08 | 0.10 | 0.25 | 7.2 | 726 | 0.09 | 2 | 20 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 11.6 | 11.30 | 0.19 | 0.20 | 7.1 | 725 | 0.12 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 11.2 | 11.41 | 0.11 | 0.13 | 7.1 | 722 | 0.11 | 5 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 11.3 | 11.47 | 0.21 | 0.23 | 7.1 | 725 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 12.0 | 12.20 | 0.11 | 0.22 | 7.1 | 704 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 12.0 | 12.30 | 0.14 | 0.26 | 7.1 | 717 | 0.14 | 0 | 4 | 0 | 0 | Absent |
| Domestic property tap water 1 | 16.4 | 10.10 | 0.04 | 0.10 | 7.3 | 602 | 0.26 | 14 | 51 | 0 | 0 | Absent |
| Domestic property tap water 2 | 13.1 | 11.31 | 0.30 | 0.30 | 7.1 | 717 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 3 | 17.1 | 10.42 | 0.05 | 0.06 | 7.4 | 695 | 0.27 | 6 | 1 | 0 | 0 | Absent |
| Domestic property tap water 4 | 14.2 | 08.37 | 0.23 | 0.24 | 7.0 | 698 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 5 | 15.8 | 11.27 | 0.14 | 0.27 | 7.1 | 686 | 0.10 | 0 | 0 | 0 | 0 | Absent |

## July 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 12.1 | 12.25 | NM | NM | 7.0 | 702 | 0.14 | 1 | 3 | 22 | 1 | Absent |
| Raw water inlet 2 | 12.2 | 12.30 | NM | NM | 7.1 | 735 | 0.13 | 0 | 7 | 6 | 6 | Absent |
| GAC Inlet 1 | NM | 12.00 | NM | NM | NM | NM | NM | 4 | 90 | 6 | 6 | Absent |
| GAC Inlet 2 | NM | 12.20 | NM | NM | NM | NM | NM | 1 | 4 | 2 | 2 | Absent |
| GAC Filters (GAC 1) | NM | 12.10 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 11.55 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 11.50 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 12.40 | 0.33 | 0.34 | 7.2 | 729 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 11.45 | 0.33 | 0.35 | 7.1 | 729 | 0.19 | 0 | 0 | 0 | 0 | Absent |
| Treated water 1 | 11.8 | 11.25 | 0.27 | 0.32 | 7.0 | 719 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 11.8 | 11.20 | 0.33 | 0.37 | 7.0 | 728 | 0.15 | 0 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 11.9 | 11.00 | 0.29 | 0.35 | 7.0 | 684 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.8 | 11.10 | 0.28 | 0.29 | 7.0 | 698 | 0.10 | 133 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 13.0 | 10.25 | 0.18 | 0.22 | 7.2 | 682 | 0.28 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 11.8 | 10.40 | 0.18 | 0.21 | 7.0 | 677 | 0.18 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 11.9 | 10.45 | 0.18 | 0.21 | 7.0 | 687 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 12.5 | 12.10 | 0.19 | 0.24 | 7.0 | 680 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 16.8 | 12.15 | 0.32 | 0.33 | 7.0 | 689 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 1 | 20.6 | 09.17 | 0.29 | 0.35 | 7.4 | 614 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 2 | 16.4 | 10.20 | 0.04 | 0.05 | 7.0 | 698 | 0.11 | 39 | 13 | 0 | 0 | Absent |
| Domestic property tap water 3 | 14.2 | 10.20 | 0.20 | 0.25 | 7.0 | 724 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 4 | 20.1 | 09.05 | 0.20 | 0.27 | 7.1 | 718 | 0.14 | 59 | 0 | 0 | 0 | Absent |
| Domestic property tap water 5 | 19.1 | 09.35 | 0.18 | 0.25 | 7.2 | 662 | 0.12 | 0 | 0 | 0 | 0 | Absent |

## August 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Raw water inlet 1 | 12.1 | 12.00 | NM | NM | 7.0 | 719 | 0.14 | 5 | 12 | 11 | 0 | Absent |
| Raw water inlet 2 | 12.1 | 12.55 | NM | NM | 7.1 | 730 | 0.12 | 1 | 18 | 18 | 3 | Absent |
| GAC Inlet 1 | NM | 12.59 | NM | NM | NM | NM | NM | 0 | 3 | 0 | 0 | Absent |
| GAC Inlet 2 | NM | 12.41 | NM | NM | NM | NM | NM | 1 | 4 | 0 | 0 | Absent |
| GAC Filters (GAC 1) | NM | 11.46 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 1 | NM | 12.07 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC Basin 2 | NM | 12.20 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Pre membrane filtration | NM | 12.05 | 0.41 | 0.46 | 7.1 | 720 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Post membrane filtration | NM | 11.20 | 0.41 | 0.49 | 7.0 | 711 | 0.25 | 0 | 0 | 0 | 0 | Absent |
| Treated water 1 | 12.0 | 11.50 | 0.46 | 0.49 | 7.0 | 715 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Treated water 2 | 12.0 | 10.40 | 0.46 | 0.46 | 6.9 | 707 | 0.08 | 1 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treated water service reservoir 1 | 12.3 | 10.21 | 0.23 | 0.26 | 7.0 | 713 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.2 | 10.37 | 0.21 | 0.23 | 7.0 | 717 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 3 | 12.9 | 09.50 | 0.22 | 0.23 | 7.0 | 723 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 4 | 12.0 | 10.01 | 0.16 | 0.22 | 7.0 | 723 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 5 | 12.0 | 10.10 | 0.20 | 0.22 | 7.0 | 721 | 0.07 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 6 | 19.1 | 10.41 | 0.10 | 0.19 | 7.0 | 676 | 0.17 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 7 | 19.3 | 10.47 | 0.07 | 0.28 | 7.1 | 680 | 0.16 | 0 | 1 | 0 | 0 | Absent |
| Domestic property tap water 1 | 19.1 | 12.20 | 0.16 | 0.21 | 7.3 | 612 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 2 | 15.9 | 11.00 | 0.15 | 0.18 | 7.1 | 687 | 0.15 | 248 | 291 | 0 | 0 | Absent |
| Domestic property tap water 3 | 17.1 | 11.40 | 0.37 | 0.41 | 7.2 | 724 | 0.12 | 1 | 0 | 0 | 0 | Absent |
| Domestic property tap water 4 | 21.4 | 11.28 | 0.10 | 0.18 | 6.9 | 709 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water 5 | 20.2 | 10.23 | 0.53 | 0.55 | 6.9 | 738 | 0.10 | 0 | 1 | 0 | 0 | Absent |

## Appendix F: Raw data from samples taken between November 2009 and August 2010 from WTW B

### November 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | NM | NM | NM | NM | 7.0 | 484 | 0.14 | 0 | 0 | 0 | 0 | Present |
| Final treated water | NM | NM | 0.18 | 0.18 | 7.0 | 490 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Treated water storage tower | NM | NM | 0.06 | 0.08 | 7.1 | 488 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap | NM | 09.00 | 0.13 | 0.15 | 7.4 | 643 | 0.13 | 0 | 0 | 0 | 0 | Absent |

## December 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 10.8 | 11.12 | NM | NM | 7.2 | 520 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 11.0 | 11.15 | 0.22 | 0.24 | 7.1 | 524 | 0.06 | 0 | 0 | 0 | 0 | Absent |
| Treated water storage tower | 10.9 | 11.32 | 0.09 | 0.11 | 7.1 | 538 | 0.07 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap | 11.8 | 11.42 | 0.14 | 0.17 | 7.1 | 522 | 0.06 | 1 | 1 | 0 | 0 | Absent |

## February 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 11.5 | 9.08 | NM | NM | 7.1 | 509 | 0.15 | 1 | 0 | 0 | 0 | Absent |
| Final treated water | 11.6 | 9.08 | 0.23 | 0.25 | 7 | 516 | 0.17 | 0 | 0 | 0 | 0 | Absent |
| Treated water storage tower | 7.0 | 9.28 | 0.16 | 0.18 | 7.1 | 542 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap | 10.4 | 11.23 | 0.14 | 0.17 | 7.1 | 515 | 0.11 | 0 | 0 | 0 | 0 | Absent |

## March 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of Acanthamoeba spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 9.8 | 09.30 | NM | NM | 7.1 | 503 | 0.1 | 0 | 0 | NM | NM | Absent |
| Final treated water | 10.0 | 09.35 | 0.2 | 0.24 | 7 | 517 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Treated water storage tower | 9.9 | 09.12 | 0.11 | 0.14 | 7.2 | 512 | 0.09 | 0 | 1 | 0 | 0 | Absent |
| Domestic property tap | 12.1 | 11.02 | 0.18 | 0.22 | 7.1 | 514 | 0.12 | 0 | 0 | 0 | 0 | Absent |

## April 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 11.9 | 09.55 | NM | NM | 7.1 | 511 | 0.15 | 0 | 1 | 0 | 0 | Absent |
| Final treated water | 13.1 | 09.55 | 0.22 | 0.22 | 7.0 | 511 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water storage | 10.8 | 09.00 | 0.12 | 0.15 | 7.4 | 597 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap | 9.2 | 10.25 | 0.10 | 0.25 | 7.6 | 655 | 0.23 | 1 | 0 | 0 | 0 | Absent |

## May 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Final treated water | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Treated water storage tower | 10.7 | 08.53 | 0.15 | 0.20 | 7.4 | 663 | 0.11 | 2 | 1 | 0 | 0 | Absent |
| Domestic property tap | 13.9 | 11.07 | 0.14 | 0.19 | 7.5 | 684 | 0.11 | 91 | 40 | 0 | 0 | Absent |

## June 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count /ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 12.0 | 11.07 | NM | NM | 7.2 | 498 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 12.0 | 11.09 | 0.16 | 0.16 | 7.1 | 499 | 0.13 | 2 | 0 | 0 | 0 | Absent |
| Treat water storage tower | 16.6 | 11.3 | 0.06 | 0.06 | 7.2 | 564 | 0.2 | 0 | 2 | 0 | 0 | Absent |
| Domestic property tap | 20.0 | 12.57 | 0.1 | 0.14 | 7.1 | 522 | 0.11 | 0 | 0 | 0 | 0 | Absent |

## July 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count /ml) | Total coliforms (cfu/100 ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 11.8 | 10.46 | NM | NM | 7.1 | 482 | 0.34 | 0 | 2 | 0 | 0 | Absent |
| Final treated water | 11.9 | 10.45 | 0.20 | 0.22 | 7.1 | 492 | 0.19 | 0 | 0 | 0 | 0 | Absent |
| Treat water storage tower | 14.0 | 11.08 | 0.04 | 0.08 | 7.3 | 513 | 0.24 | 1 | 0 | 0 | 0 | Absent |
| Domestic property tap | 12.6 | 10.40 | 0.30 | 0.32 | 7.1 | 531 | 0.06 | 0 | 0 | 0 | 0 | Absent |

## August 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/10 0ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole | 11.9 | 10.12 | NM | NM | 7.3 | 488 | 0.23 | 1 | 0 | 0 | 0 | Absent |
| Final treated water | 12.0 | 10.18 | 0.17 | 0.21 | 7.1 | 509 | 0.26 | 1 | 0 | 0 | 0 | Absent |
| Treated water storage | 15.0 | 11.00 | 0.11 | 0.18 | 7.3 | 515 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap | 15.4 | 10.41 | 0.17 | 0.2 | 7.2 | 509 | 0.14 | 0 | 1 | 0 | 0 | Absent |

## Appendix G: Raw data from samples taken between November 2009 and August 2010 from WTW C

### November 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 12.9 | 9.45 | NM | NM | 7.0 | 587 | 0.09 | 1 | 0 | 0 | 0 | Absent |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 12.9 | 9.37 | NM | NM | 7.0 | 598 | 0.08 | 1 | 0 | 0 | 0 | Absent |
| Final treated water | 12.9 | 9.56 | 0.14 | 0.22 | 7.1 | 598 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 11.9 | 8.44 | 0.10 | 0.12 | 7.1 | 0.07 | 0.08 | 0 | 20 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.0 | 8.44 | 0.13 | 0.17 | 7.1 | 610 | 0.07 | 0 | 3 | 0 | 0 | Absent |
| Domestic property tap water | 12.0 | 9.58 | 0.13 | 0.19 | 7.0 | 611 | 0.05 | 0 | 0 | 0 | 0 | Absent |

## December 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 10.9 | 10.39 | NM | NM | 7.1 | 585 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 10.9 | 10.35 | NM | NM | 7 | 590 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 11.4 | 10.42 | 0.30 | 0.36 | 7.1 | 603 | 0.05 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 11.2 | 10.5 | 0.15 | 0.19 | 7.2 | 592 | 0.07 | 0 | 3 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.3 | 10.51 | 0.17 | 0.21 | 7.1 | 599 | 0.10 | 0 | 11 | 0 | 0 | Absent |
| Domestic property tap water | 12.1 | 10.38 | 0.19 | 0.20 | 7 | 615 | 0.05 | 2 | 0 | 0 | 0 | Absent |

## February 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 11.4 | 09.15 | NM | NM | 7.0 | 588 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 11.6 | 09.20 | NM | NM | 7.0 | 598 | 0.15 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 11.1 | 0.2 | 0.20 | 0.28 | 7.0 | 602 | 0.17 | 3 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 9.4 | 0.23 | 0.23 | 0.23 | 7.0 | 586 | 0.11 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 9.4 | 0.26 | 0.26 | 0.26 | 7.0 | 572 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Domestic property tap water | 8.2 | 0.22 | 0.22 | 0.26 | 7.0 | 622 | 0.14 | 1 | 0 | 0 | 0 | Absent |

## March 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (µS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 11.5 | 10.43 | NM | NM | 7.0 | 576 | 0.15 | 0 | 1 | 0 | 0 | Absent |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 10.1 | 10.46 | NM | NM | 7.0 | 576 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 11.1 | 10.51 | 0.27 | 0.33 | 7.0 | 578 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 10.9 | 10.15 | 0.27 | 0.27 | 7.0 | 594 | 0.06 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 10.9 | 10.21 | 0.16 | 0.16 | 7.1 | 595 | 0.07 | 3 | 7 | 0 | 0 | Absent |
| Domestic property tap water | 9.1 | 10.2 | 0.12 | 0.20 | 7.0 | 622 | 0.10 | 0 | 0 | 0 | 0 | Absent |

## April 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 11.8 | 9.17 | NM | NM | 7.0 | 613 | 0.14 | 0 | 0 | 0 | 0 | Present |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 11.8 | 9.3 | NM | NM | 7.0 | 624 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 11.9 | 9.41 | 0.29 | 0.36 | 7.2 | 608 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 12.2 | 8.23 | 0.29 | 0.29 | 7.1 | 580 | 0.08 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.0 | 8.22 | 0.21 | 0.21 | 7.1 | 584 | 0.07 | 0 | 2 | 0 | 0 | Absent |
| Domestic property tap water | 11.6 | 9.23 | 0.17 | 0.22 | 7.0 | 601 | 0.13 | 0 | 0 | 0 | 0 | Absent |

## May 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 11.1 | 10.5 | NM | NM | 7.0 | 593 | 0.17 | 0 | 0 | 0 | 0 | Present |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 11.2 | NM | NM | NM | 7.0 | 593 | 0.13 | 1 | 0 | 0 | 0 | Absent |
| Final treated water | 11.3 | 10.53 | 0.34 | 0.37 | 7.0 | 609 | 0.08 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 12.2 | 8.27 | 0.28 | 0.30 | 7.1 | 596 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 11.9 | 8.26 | 0.29 | 0.34 | 7.1 | 599 | 0.14 | 1 | 6 | 0 | 0 | Absent |
| Domestic property tap water | 12.0 | 10.26 | 0.40 | 0.53 | 6.9 | 597 | 0.43 | 0 | 0 | 0 | 0 | Absent |

## June 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 11.6 | 10.33 | NM | NM | 7.1 | 573 | 0.26 | 1 | 0 | 0 | 0 | Absent |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 11.6 | 10.30 | NM | NM | 7.0 | 567 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 12.0 | 10.35 | 0.27 | 0.34 | 7.1 | 578 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 12.0 | 09.30 | 0.11 | 0.21 | 7.0 | 607 | 0.11 | 0 | 1 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.0 | 09.35 | 0.19 | 0.20 | 7.1 | 598 | 0.10 | 0 | 3 | 0 | 0 | Absent |
| Domestic property tap water | NM | 12.35 | 0.18 | 0.18 | 7.1 | 666 | 0.16 | 0 | 0 | 0 | 0 | Absent |

## July 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 12.0 | 9.2 | NM | NM | 7.0 | 593 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Borehole 2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Borehole 3 | 12.0 | 9.25 | NM | NM | 7.0 | 612 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Final treated water | 12.0 | 9.3 | 0.19 | 0.30 | 7.1 | 596 | 0.10 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 12.8 | 9.2 | 0.10 | 0.21 | 7.1 | 560 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 12.8 | 9.3 | 0.11 | 0.16 | 7.1 | 583 | 0.18 | 0 | 4 | 0 | 0 | Absent |
| Domestic property tap water | 15.8 | 9.27 | 0.20 | 0.20 | 6.9 | 579 | 0.09 | 0 | 0 | 0 | 0 | Absent |

## August 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100 ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Borehole 1 | 11.7 | 10.32 | NM | NM | 7.1 | 577 | 0.21 | 1 | 0 | 0 | 0 | Absent |
| Borehole 2 | 11.6 | 10.37 | NM | NM | 7.1 | 566 | 0.17 | 1 | 3 | 3 | 1 | Absent |
| Borehole 3 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM |
| Final treated water | 12.3 | 10.23 | 0.21 | 0.23 | 7.1 | 600 | 0.08 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 1 | 13.6 | 08.54 | 0.18 | 0.24 | 7.1 | 601 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Treated water service reservoir 2 | 13.3 | 08.54 | 0.20 | 0.20 | 7.1 | 603 | 0.11 | 0 | 2 | 0 | 0 | Absent |
| Domestic property tap water | 16.4 | 10.00 | 0.15 | 0.18 | 7.0 | 610 | 0.11 | 0 | 0 | 0 | 0 | Absent |

## Appendix H: Raw data from samples taken between November 2009 and August 2010 from WTW D and recreational lake

## November 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 12.0 | 12.40 | NM | NM | 7.9 | 593 | 5.2 | NM | NM | NM | NM | Present |
| Raw | NM | 12.38 | NM | NM | 7.9 | 609 | 2.4 | 1300 | ~8400 | 1400 | 0 | Present |
| Pre-ozonation | NM | 12.40 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 12.43 | NM | NM | 7.2 | 613 | 0.52 | NM | NM | NM | NM | Present |
| Flat bottom clarifier1 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 12.45 | NM | NM | 7.2 | 623 | 0.64 | NM | NM | NM | NM | Present |
| Intermediate ozone inlet | NM | 12.16 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC inlet | NM | 12.21 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC outlet | NM | 12.18 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Dechlorination | NM | 12.21 | 0.64 | 0.84 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 11.7 | 12.57 | 0.62 | 0.7 | 7.5 | 627 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Link Main | NM | 8.04 | 0.35 | 0.53 | 7.6 | 619 | 0.3 | 1 | 1 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 12.0 | 10.35 | 0.18 | 0.29 | 7.7 | 665 | 0.12 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 2 | 12.0 | 10.30 | 0.23 | 0.46 | 7.6 | 668 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 11.8 | 10.45 | 0.35 | 0.54 | 7.7 | 647 | 0.09 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 11.3 | 11.09 | 0.03 | 0.12 | 7.5 | 659 | 0.16 | 0 | 4 | 0 | 0 | Absent |
| Reservoir No. 5 | 12.3 | 9.21 | 0.09 | 0.18 | 7.4 | 655 | 0.2 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 6 | 11.5 | 10.30 | 0.29 | 0.47 | 7.5 | 624 | 0.27 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 7 | 11.7 | 10.30 | 0.39 | 0.47 | 7.5 | 632 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 11.5 | 10.34 | 0.31 | 0.46 | 7.5 | 627 | 0.16 | 0 | 1 | 0 | 0 | Absent |
| Domestic Property 1 | 13.0 | 9.37 | 0.45 | 0.64 | 7.5 | 634 | 0.17 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 14.8 | 10.57 | 0.26 | 0.52 | 7.5 | 628 | 0.1 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 3 | 12.6 | 10.18 | 0.5 | 0.82 | 7.5 | 625 | 0.13 | 5 | 0 | 0 | 0 | Absent |
| Recreational Lake | 11.2 | 14.20 | NM | NM | 7.8 | 659 | 0.59 | 30 | 1000 | 29 | 0 | Present |

## December 2009

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 7.7 | 11.36 | NM | NM | 8.0 | 589 | 7.50 | NM | NM | NM | NM | Present |
| Raw | NM | 11.42 | NM | NM | 8.0 | 585 | 6.50 | 720 | 8600 | 2700 | 0 | Present |
| Pre-ozonation | NM | 11.30 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 11.52 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Flat bottom clarifier1 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 11.56 | NM | NM | 7.1 | 591 | 0.72 | NM | NM | NM | NM | Present |
| Intermediate ozone inlet | NM | 11.20 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC inlet | NM | 11.16 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC outlet | NM | 11.18 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Dechlorination | NM | 11.08 | 0.70 | NM | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 7.7 | 10.51 | 0.59 | 0.71 | 7.3 | 601 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Link Main | NM | 12.40 | NM | NM | 7.5 | 575 | 0.14 | 3 | 1 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 9.9 | 09.50 | 0.10 | 0.22 | 7.5 | 579 | 0.13 | 4 | 2 | 0 | 0 | Absent |
| Reservoir No. 2 | 9.9 | 09.55 | 0.37 | 0.41 | 7.4 | 578 | 0.22 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 3 | 9.4 | 10.05 | 0.35 | 0.54 | 7.5 | 564 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 10.0 | 09.45 | 0.02 | 0.10 | 7.4 | 583 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 5 | 9.3 | 08.25 | 0.13 | 0.21 | 7.3 | 588 | 0.15 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 6 | 7.9 | 09.05 | 0.36 | 0.64 | 7.2 | 580 | 0.13 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 7 | 7.9 | 09.10 | 0.26 | 0.54 | 7.2 | 585 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 7.3 | 09.15 | 0.29 | 0.50 | 7.2 | 596 | 0.16 | 0 | 2 | 0 | 0 | Absent |
| Domestic Property 1 | 10.9 | 10.35 | 0.42 | 0.56 | 7.2 | 576 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 9.2 | 10.15 | 0.13 | 0.41 | 7.2 | 597 | 0.14 | 0 | 1 | 0 | 0 | Absent |
| Domestic Property 3 | 9.5 | 10.43 | 0.53 | 0.66 | 7.3 | 612 | 0.22 | 28 | 8 | 0 | 0 | Absent |
| Recreational Lake | 5.0 | 08.30 | NM | NM | 7.9 | 652 | 0.87 | <10 | 170 | 14 | 9 | Present |

## February 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 6.4 | 10.36 | NM | NM | 7.9 | 563 | 8 | NM | NM | NM | NM | Present |
| Raw | NM | 11.00 | NM | NM | 7.9 | 564 | 3 | 59 | ~14000 | 100 | 100 | Present |
| Pre-ozonation | NM | 10.40 | NM | NM | NM | NM | NM | 370 | 620 | 20 | 20 | Present |
| Sedimentation plant outlet | NM | 11.08 | NM | NM | 7.1 | 566 | 0.71 | 20 | 9 | 0 | 0 | Present |
| Flat bottom clarifier1 | NM | 11.12 | NM | NM | 7.1 | 567 | 0.81 | 24 | 151 | 0 | 0 | Absent |
| Flat bottom clarifier2 | NM | 11.15 | NM | NM | 7.1 | 577 | 0.76 | 25 | 20 | 0 | 0 | Absent |
| Intermediate ozone inlet | NM | 11.59 | NM | NM | NM | NM | NM | 3 | 0 | 0 | 0 | Absent |
| GAC inlet | NM | 12.00 | NM | NM | NM | NM | NM | 3 | ~580 | 0 | 0 | Absent |
| GAC outlet | NM | 11.56 | NM | NM | NM | NM | NM | 3 | ~2000 | 0 | 0 | Absent |
| Dechlorination | NM | 11.54 | 0.65 | 0.83 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 5.3 | 11.30 | 0.60 | 0.80 | 7.3 | 605 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Link Main | NM | 09.10 | 0.45 | 0.52 | 7.3 | 587 | 0.21 | 0 | 1 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 6.4 | 10.00 | 0.10 | 0.25 | 7.5 | 566 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 2 | 6.4 | 09.55 | 0.40 | 0.50 | 7.4 | 574 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 6.2 | 10.10 | 0.49 | 0.60 | 7.5 | 568 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 6.4 | 10.15 | 0.03 | 0.14 | 7.4 | 604 | 0.17 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 5 | 5.8 | 08.05 | 0.04 | 0.15 | 7.2 | 595 | 0.24 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 6 | 5.2 | 09.19 | 0.21 | 0.41 | 7.2 | 608 | 0.15 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 7 | 5.2 | 09.05 | 0.24 | 0.43 | 7.3 | 614 | 0.47 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 5.3 | 09.15 | 0.38 | 0.48 | 7.2 | 608 | 0.2 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 1 | 7.4 | 10.30 | 0.26 | 0.30 | 7.4 | 608 | 0.2 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 6.4 | 12.05 | 0.18 | 0.24 | 7.3 | 613 | 0.18 | 2 | 0 | 0 | 0 | Absent |
| Domestic Property 3 | 6.4 | 11.15 | 0.45 | 0.54 | 7.2 | 618 | 0.23 | 0 | 0 | 0 | 0 | Absent |
| Recreational Lake | 5.6 | 12.20 | NM | NM | 7.8 | 665 | 1.1 | 30 | 110 | 3 | 2 | Present |

## March 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 8.2 | 10.37 | NM | NM | 8.0 | 591 | 5.10 | NM | NM | NM | NM | Present |
| Raw | NM | 10.54 | NM | NM | 8.0 | 604 | 1.60 | 430 | 2860 | 1800 | 400 | Present |
| Pre-ozonation | NM | 10.40 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 10.32 | NM | NM | 7.2 | 621 | 0.61 | NM | NM | NM | NM | Present |
| Flat bottom clarifier1 | NM | 10.32 | NM | NM | 7.2 | 618 | 1.60 | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 11.35 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC inlet | NM | 11.36 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC outlet | NM | 11.38 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Dechlorination | NM | 11.28 | 0.61 | 0.72 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 6.8 | 11.00 | 0.60 | 0.73 | 7.2 | 614 | 0.10 | 0 | 4 | 0 | 0 | Absent |
| Link Main | NM | 12.15 | 0.34 | 0.64 | 7.5 | 558 | 0.16 | 0 | 1 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 7.0 | 09.45 | 0.10 | 0.19 | 7.5 | 585 | 0.21 | 0 | 14 | 0 | 0 | Absent |
| Reservoir No. 2 | 7.5 | 09.36 | 0.11 | 0.26 | 7.5 | 593 | 0.13 | 0 | 14 | 0 | 0 | Absent |
| Reservoir No. 3 | 7.8 | 09.36 | 0.13 | 0.29 | 7.5 | 579 | 0.25 | 1 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 7.3 | 10.05 | 0.05 | 0.10 | 7.3 | 594 | 0.17 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 5 | 7.2 | 08.25 | 0.10 | 0.16 | 7.3 | 603 | 0.11 | 0 | 4 | 0 | 0 | Absent |
| Reservoir No. 6 | 7.2 | 09.05 | 0.35 | 0.50 | 7.3 | 625 | 0.32 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 7 | 7.2 | 09.10 | 0.39 | 0.51 | 7.3 | 624 | 0.34 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 8 | 7.0 | 09.15 | 0.41 | 0.52 | 7.3 | 617 | 0.21 | 2 | 0 | 0 | 0 | Absent |
| Domestic Property 1 | 7.8 | 09.29 | 0.43 | 0.61 | 7.2 | 605 | 0.13 | 1 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 7.6 | 10.05 | 0.32 | 0.47 | 7.2 | 614 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 3 | 8.7 | 09.55 | 0.27 | 0.47 | 7.3 | 607 | 0.16 | 0 | 2 | 1 | 0 | Absent |
| Recreational Lake | 10.6 | 08.05 | NM | NM | 8.3 | 650 | 2.80 | 30 | 20 | 0 | 0 | Present |

## April 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 10.1 | 10.55 | NM | NM | 8.2 | 606 | 4.10 | NM | NM | NM | NM | Present |
| Raw | NM | 10.45 | NM | NM | 8.2 | 585 | 3.50 | 430 | ~4000 | 1450 | 430 | Present |
| Pre-ozonation | NM | 10.30 | NM | NM | NM | NM | NM | 130 | ~220 | 83 | 8 | Present |
| Sedimentation plant outlet | NM | 10.25 | NM | NM | 7.3 | 596 | 0.55 | 92 | ~420 | 1 | 0 | Present |
| Flat bottom clarifier1 | NM | NM | NM | NM | 7.3 | 592 | 0.42 | 17 | 18 | 1 | 0 | Present |
| Flat bottom clarifier2 | NM | 11.40 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 11.50 | NM | NM | NM | NM | NM | 1 | 150 | 0 | 0 | Present |
| GAC inlet | NM | 11.45 | NM | NM | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| GAC outlet | NM | 11.20 | NM | NM | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Dechlorination | NM | 11.20 | 0.54 | 0.64 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 12.1 | 11.15 | 0.66 | 0.86 | 7.3 | 603 | 0.19 | 0 | 1 | 0 | 0 | Absent |
| Link Main | NM | 12.22 | 0.57 | 0.60 | 7.6 | 553 | 0.10 | 66 | 1 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count /ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 10.4 | 11.25 | 0.33 | 0.51 | 7.7 | 579 | 0.19 | 1 | 0 | 0 | 0 | Absent |
| Reservoir No. 2 | 10.7 | 11.25 | 0.46 | 0.61 | 7.6 | 582 | 0.15 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 10.9 | 11.11 | 0.29 | 0.51 | 7.6 | 573 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 11.4 | 09.45 | 0.02 | 0.12 | 7.5 | 613 | 0.17 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 5 | 11.0 | 08.26 | 0.07 | 0.17 | 7.4 | 611 | 0.15 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 6 | 11.5 | 08.57 | 0.31 | 0.37 | 7.4 | 608 | 0.19 | 1 | 1 | 0 | 0 | Absent |
| Reservoir No. 7 | 11.9 | 08.57 | 0.40 | 0.47 | 7.4 | 608 | 0.17 | 0 | 18 | 0 | 0 | Absent |
| Reservoir No. 8 | 11.7 | 09.07 | 0.41 | 0.50 | 7.3 | 611 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 1 | 11.4 | 09.41 | 0.49 | 0.62 | 7.4 | 594 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 11.2 | 10.17 | 0.30 | 0.46 | 7.4 | 607 | 0.15 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 3 | 13.4 | 09.15 | 0.32 | 0.45 | 7.4 | 591 | 0.17 | 6 | 2 | 0 | 0 | Absent |
| Recreational Lake | 14.1 | 08..10 | NM | NM | 8.2 | 660 | 0.52 | 10 | 70 | 2 | 2 | Present |

## May 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 13.6 | 11.15 | NM | NM | 8.1 | 572 | 2.20 | NM | NM | NM | NM | Present |
| Raw | NM | 11.05 | NM | NM | 8.1 | 575 | 1.80 | 590 | ~20000 | 1650 | 360 | Present |
| Pre-ozonation | NM | 11.20 | NM | NM | NM | NM | NM | 100 | 350 | 165 | 32 | Present |
| Sedimentation plant outlet | NM | 11.00 | NM | NM | 7.2 | 588 | 0.49 | ~400 | ~1400 | 9 | 0 | Present |
| Flat bottom clarifier1 | NM | 10.50 | NM | NM | 7.2 | 587 | 0.62 | ~460 | ~2700 | 8 | 1 | Present |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 11.55 | NM | NM | NM | NM | NM | ~300 | ~1500 | 8 | 0 | Present |
| GAC inlet | NM | 11.50 | NM | NM | NM | NM | NM | 0 | 1 | 0 | 0 | Absent |
| GAC outlet | NM | 11.45 | NM | NM | NM | NM | NM | 0 | 120 | 0 | 0 | Absent |
| Dechlorination | NM | 12.00 | 0.79 | 0.91 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 12.3 | 12.03 | 0.68 | 0.76 | 7.3 | 594 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Link Main | NM | 10.35 | 0.35 | 0.56 | 7.6 | 600 | 0.12 | 13 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 12.5 | 09.15 | 0.34 | 0.50 | 7.7 | 599 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 2 | 12.5 | 09.10 | 0.26 | 0.49 | 7.6 | 595 | 0.34 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 12.1 | 09.05 | 0.24 | 0.38 | 7.6 | 601 | 0.22 | 1 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 12.6 | 09.1 | 0.09 | 0.13 | 7.4 | 548 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 5 | 13.4 | 11.24 | 0.08 | 0.16 | 7.3 | 592 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 6 | 12.5 | 10.12 | 0.27 | 0.40 | 7.3 | 594 | 0.23 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 7 | 12.5 | 10.12 | 0.35 | 0.47 | 7.3 | 594 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 12.5 | 10.22 | 0.35 | 0.48 | 7.3 | 593 | 0.15 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 1 | 12.8 | 10.05 | 0.30 | 0.47 | 7.3 | 601 | 0.24 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 13.0 | 10.10 | 0.45 | 0.55 | 7.2 | 608 | 0.14 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 3 | 12.6 | 09.40 | 0.25 | 0.30 | 7.2 | 623 | 0.19 | 5 | 0 | 0 | 0 | Absent |
| Recreational Lake | 14.0 | 09.49 | NM | NM | 8.1 | 629 | 0.60 | 0 | 0 | 0 | 0 | Present |

## June 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 19.2 | 11.20 | NM | NM | 7.9 | 624 | 3.00 | NM | NM | NM | NM | Present |
| Raw | NM | 11.10 | NM | NM | 7.9 | 603 | 1.40 | ~90000 | ~6400 | 20100 | 1000 | Present |
| Pre-ozonation | NM | 11.15 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 11.28 | NM | NM | 7.1 | 634 | 0.48 | NM | NM | NM | NM | Present |
| Flat bottom clarifier1 | NM | 11.52 | NM | NM | 7.0 | 627 | 0.58 | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 11.34 | NM | NM | 7.0 | 614 | 0.48 | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 12.00 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC inlet | NM | 12.02 | NM | NM | NM | NM | NM | 1 | 24 | 1 | 1 | Absent |
| GAC outlet | NM | 12.05 | NM | NM | NM | NM | NM | 4 | 39 | 18 | 0 | Absent |
| Dechlorination | NM | 11.50 | 0.83 | 1.01 | NM | NM | NM | 4 | 3 | 0 | 0 | Absent |
| Main | 18.2 | 11.44 | 0.64 | 0.82 | 7.4 | 627 | 0.20 | 1 | 6 | 0 | 0 | Absent |
| Link Main | NM | 08.25 | 0.60 | 0.75 | 7.4 | 579 | 0.19 | 54 | 1 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 17.2 | 11.20 | 0.04 | 0.20 | 7.7 | 593 | 0.20 | 12 | 8 | 0 | 0 | Absent |
| Reservoir No. 2 | 17.5 | 11.25 | 0.12 | 0.22 | 7.6 | 610 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 17.1 | 11.35 | 0.10 | 0.24 | 7.6 | 605 | 0.19 | 1 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 17.2 | 08.15 | 0.05 | 0.15 | 7.5 | 625 | 0.37 | 1 | 6 | 0 | 0 | Absent |
| Reservoir No. 5 | 17.4 | 06.50 | 0.06 | 0.16 | 7.4 | 621 | 0.34 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 6 | 17.2 | 07.20 | 0.34 | 0.42 | 7.5 | 628 | 0.23 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 7 | 16.8 | 07.30 | 0.30 | 0.46 | 7.5 | 633 | 0.27 | 1 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 16.6 | 07.35 | 0.33 | 0.44 | 7.5 | 628 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 1 | 18.5 | 08.50 | 0.37 | 0.52 | 7.5 | 627 | 0.18 | 2 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 17.3 | 09.20 | 0.41 | 0.70 | 7.4 | 625 | 0.16 | 6 | 7 | 0 | 0 | Absent |
| Domestic Property 3 | 18.6 | 08.00 | 0.60 | 0.64 | 7.4 | 622 | 0.15 | 1 | 0 | 0 | 0 | Absent |
| Recreational Lake | 19.8 | 12.12 | NM | NM | 8.2 | 647 | 1.70 | 60 | 2180 | 21 | 8 | Present |

## July 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 19.2 | 11.20 | NM | NM | 7.9 | 624 | 3.00 | NM | NM | NM | NM | Present |
| Raw | NM | 11.20 | NM | NM | 8.1 | 610 | 1.60 | 2300 | ~5100 | 1600 | 120 | Present |
| Pre-ozonation | NM | 11.35 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 11.10 | NM | NM | 7.1 | 631 | 0.50 | NM | NM | NM | NM | Present |
| Flat bottom clarifier1 | NM | 11.05 | NM | NM | 7.0 | 635 | 0.39 | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 11.00 | NM | NM | 7.0 | 628 | 0.32 | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 12.00 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC inlet | NM | 12.10 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC outlet | NM | 12.05 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Dechlorination | NM | 11.50 | 0.59 | 0.82 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 21.8 | 11.40 | 0.43 | 0.52 | 7.3 | 630 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Link Main | NM | 08.20 | 0.58 | 0.65 | 7.4 | 599 | 0.09 | 119 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | Electrical conductivity @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | 20.9 | 09.30 | 0.10 | 0.18 | 7.8 | 653 | 0.20 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 2 | 21.1 | 09.35 | 0.23 | 0.34 | 7.7 | 650 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 20.9 | 09.15 | 0.06 | 0.13 | 7.6 | 659 | 0.17 | 0 | 1 | 0 | 0 | Absent |
| Reservoir No. 4 | 21.7 | 11.20 | 0.22 | 0.1 | 7.6 | 637 | 0.14 | 0 | 12 | 0 | 0 | Absent |
| Reservoir No. 5 | 21 | 09.46 | 0.03 | 0.11 | 7.4 | 660 | 0.13 | 0 | 6 | 0 | 0 | Absent |
| Reservoir No. 6 | 21.7 | 10.20 | 0.29 | 0.32 | 7.4 | 677 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 7 | 21.8 | 10.25 | 0.32 | 0.33 | 7.4 | 663 | 0.12 | 1 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 21.8 | 10.31 | 0.35 | 0.39 | 7.4 | 677 | 0.12 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 1 | 20.1 | 10.40 | 0.33 | 0.47 | 7.4 | 653 | 0.11 | 0 | 1 | 0 | 0 | Absent |
| Domestic Property 2 | 17.3 | 11.25 | 0.08 | 0.14 | 7.5 | 621 | 0.18 | 199 | 124 | 0 | 0 | Absent |
| Domestic Property 3 | 21.1 | 11.10 | 0.38 | 0.50 | 7.3 | 660 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Recreational Lake | 22.6 | 12.15 | NM | NM | 8.3 | 590 | 2.60 | 100 | 710 | 130 | 0 | Present |

## August 2010

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 19.2 | 11.55 | NM | NM | 8.1 | 637 | 1.20 | NM | NM | NM | NM | Present |
| Raw | NM | 12.10 | NM | NM | 8.0 | 632 | 1.20 | 320 | ~3600 | 1500 | 320 | Present |
| Pre-ozonation | NM | 12.00 | NM | NM | NM | NM | NM | 40 | 110 | 31 | 2 | Present |
| Sedimentation plant outlet | NM | 11.50 | NM | NM | 7.3 | 639 | 0.28 | 55 | ~1300 | 2 | 0 | Present |
| Flat bottom clarifier1 | NM | 11.42 | NM | NM | 7.2 | 643 | 0.30 | 65 | ~1600 | 8 | 0 | Absent |
| Flat bottom clarifier2 | NM | 11.35 | NM | NM | 7.1 | 641 | 0.24 | 40 | ~1300 | 4 | 2 | Absent |
| Intermediate ozone inlet | NM | 12.42 | NM | NM | NM | NM | NM | 42 | ~1300 | 3 | 0 | Absent |
| GAC inlet | NM | 12.36 | NM | NM | NM | NM | NM | 7 | ~1300 | 1 | 0 | Absent |
| GAC outlet | NM | 12.45 | NM | NM | NM | NM | NM | 158 | ~1400 | 0 | 0 | Absent |
| Dechlorination | NM | 12.53 | 0.62 | 0.83 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 19.0 | 12.58 | 0.68 | 0.86 | 7.4 | 667 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Link Main | NM | 10.13 | 0.55 | 0.69 | 7.5 | 595 | 0.25 | 9 | 0 | 0 | 0 | Absent |

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reservoir No. 1 | NM | 09.40 | 0.09 | 0.32 | 7.7 | 688 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 2 | 19.5 | 09.45 | 0.34 | 0.40 | 7.7 | 693 | 0.16 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 3 | 19.2 | 09.50 | 0.36 | 0.41 | 7.6 | 683 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 4 | 18.9 | 12.28 | 0.02 | 0.06 | 7.5 | 683 | 0.12 | 0 | 2 | 0 | 0 | Absent |
| Reservoir No. 5 | 19.5 | 08.52 | 0.03 | 0.08 | 7.4 | 629 | 0.19 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 6 | 18.2 | 09.22 | 0.24 | 0.44 | 7.5 | 635 | 0.25 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 7 | 18.2 | 09.22 | 0.31 | 0.50 | 7.4 | 677 | 0.18 | 0 | 0 | 0 | 0 | Absent |
| Reservoir No. 8 | 18.6 | 09.34 | 0.34 | 0.49 | 7.4 | 638 | 0.16 | 0 | 2 | 0 | 0 | Absent |
| Domestic Property 1 | 19.9 | 09.10 | 0.40 | 0.57 | 7.4 | 674 | 0.11 | 0 | 0 | 0 | 0 | Absent |
| Domestic Property 2 | 18.6 | 11.10 | 0.11 | 0.19 | 7.5 | 693 | 0.12 | 0 | 2 | 0 | 0 | Absent |
| Domestic Property 3 | 21.0 | 10.55 | 0.16 | 0.25 | 7.3 | 683 | 0.13 | 0 | 0 | 0 | 0 | Absent |
| Recreational Lake | 18.5 | 14.48 | NM | NM | 8.1 | 606 | 1.10 | 360 | 1750 | 145 | 14 | Present |

## Appendix I: Raw data from samples taken between February 2017 and January 2018 from WTW D

## February 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | NM | 12.02 | NM | NM | 8 | 603 | 5.6 | NM | NM | 1300 | 308 | Present |
| Raw | NM | 12.10 | NM | NM | 8.1 | 607 | 4.9 | 141 | 1000 | 2420 | 548 | Present |
| Pre-ozonation | NM | 12.38 | NM | NM | NM | NM | NM | 126 | 91 | 8 | 0 | Absent |
| Sedimentation plant outlet | NM | 12.21 | NM | NM | 7 | 6.19 | 0.49 | 9 | 10 | 0 | 0 | Absent |
| Flat bottom clarifier1 | NM | 12.34 | NM | NM | 7 | 611 | 1.3 | 25 | 45 | 0 | 0 | Absent |
| Flat bottom clarifier2 | NM | 12.26 | NM | NM | 7.1 | 613 | 0.65 | 9 | 10 | 0 | 0 | Absent |
| Intermediate ozone inlet | NM | 12.00 | NM | NM | NM | NM | NM | 0 | 15 | 0 | 0 | Absent |
| GAC inlet | NM | 12.29 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| GAC outlet | NM | 12.29 | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Dechlorination | NM | 10.42 | 0.9 | 1.05 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 10.1 | 10.35 | 0.79 | 0.94 | 7.2 | 638 | 0.12 | 0 | 0 | 0 | 0 | Absent |

## March 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of Acanthamoeba spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 9.9 | 10.38 | NM | NM | 8 | 600 | 19 | NM | NM | NM | NM | Present |
| Raw | NM | 10.4 | NM | NM | 8 | 603 | 10 | NM | NM | 3450 | 1200 | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 10.48 | NM | NM | 7.3 | 619 | 1.1 | 8 | 12 | 0 | 0 | Absent |
| Flat bottom clarifier1 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 10.45 | NM | NM | 7.3 | 6.18 | 1.3 | 36 | 56 | 0 | 0 | Absent |
| Intermediate ozone inlet | NM | 11.03 | NM | NM | NM | NM | NM | 3 | 11 | 0 | 0 | Absent |
| GAC inlet | NM | 10.59 | NM | NM | NM | NM | NM | 0 | 2 | 0 | 0 | Absent |
| GAC outlet | NM | 10.54 | NM | NM | NM | NM | NM | 5 | 4 | 0 | 0 | Absent |
| Dechlorination | NM | 11.07 | 0.85 | 1.04 | NM | NM | NM | 3 | 0 | 0 | 0 | Absent |
| Main | 10.1 | 11.2 | 0.74 | 0.92 | 7.4 | 628 | 0.11 | 2 | 0 | 0 | 0 | Absent |

## April 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 13.8 | 9.16 | NM | NM | 7.8 | 622 | 2 | NM | NM | NM | NM | Present |
| Raw | NM | 9.2 | NM | NM | 7.9 | 628 | 2 | NM | NM | 4350 | 670 | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 9.06 | NM | NM | 7.3 | 651 | 0.36 | 4 | 10 | 0 | 0 | Absent |
| Flat bottom clarifier1 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 9.11 | NM | NM | 7.2 | 632 | 0.64 | 14 | 10 | 1 | 0 | Absent |
| Intermediate ozone inlet | NM | 9.33 | NM | NM | NM | NM | NM | 0 | 12 | 0 | 0 | Absent |
| GAC inlet | NM | 9.3 | NM | NM | NM | NM | NM | 0 | 13 | 0 | 0 | Absent |
| GAC outlet | NM | 9.38 | NM | NM | NM | NM | NM | 22 | 51 | 0 | 0 | Absent |
| Dechlorination | NM | 9.56 | 0.65 | 0.89 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 14.2 | 10.03 | 0.51 | 0.51 | 7.3 | 660 | 0.11 | 1 | 0 | 0 | 0 | Absent |

## May 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 18 | 11.05 | NM | NM | 7.9 | 569 | 2.6 | NM | NM | NM | NM | Present |
| Raw | NM | 11.04 | NM | NM | 7.9 | 570 | 4.1 | NM | NM | NM | NM | Pressent |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Sedimentation plant outlet | NM | 11.11 | NM | NM | 7.3 | 582 | 0.43 | 18 | 3500 | 3 | 0 | Absent |
| Flat bottom clarifier1 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Flat bottom clarifier2 | NM | 11.16 | NM | NM | 7.2 | 581 | 0.48 | 12 | 156 | 7 | 0 | Absent |
| Intermediate ozone inlet | NM | 11.30 | NM | NM | NM | NM | NM | 12 | 800 | 7 | 2 | Absent |
| GAC inlet | NM | 11.34 | NM | NM | NM | NM | NM | 1 | 0 | 0 | 0 | Absent |
| GAC outlet | NM | 11.37 | NM | NM | NM | NM | NM | 1 | 34 | 18 | 0 | Absent |
| Dechlorination | NM | 11.11 | 0.81 | 1.02 | NM | NM | NM | 4 | 0 | 0 | 0 | Absent |
| Main | 17 | 11.05 | 0.74 | 0.90 | 7.4 | 612 | 0.13 | 0 | 0 | 0 | 0 | Absent |

## June 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | Electrical conductivity @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 17.8 | 8.07 | NM | NM | 8 | 632 | 1.8 | NM | NM | NM | NM | Present |
| Raw | NM | 8.05 | NM | NM | 8.1 | 639 | 1.3 | NM | NM | 2420 | 365 | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Sedimentation plant outlet | NM | 8.05 | NM | NM | 7.3 | 646 | 0.25 | 9 | 1300 | 10 | 0 | Absent |
| Flat bottom clarifier1 | NM | 7.55 | NM | NM | 7.4 | 646 | 0.47 | 13 | 1100 | 3 | 0 | Absent |
| Flat bottom clarifier2 | NM | 7.50 | NM | NM | 7.2 | 636 | 0.3 | 7 | 2700 | 24 | 0 | Absent |
| Intermediate ozone inlet | NM | 8.33 | NM | NM | NM | NM | NM | 9 | 58 | 6 | 0 | Absent |
| GAC inlet | NM | 8.22 | NM | NM | NM | NM | NM | 2 | 10 | 0 | 0 | Absent |
| GAC outlet | NM | 8.29 | NM | NM | NM | NM | NM | 2 | 72 | 2 | 0 | Absent |
| Dechlorination | NM | 8.15 | 0.98 | 1.07 | NM | NM | NM | 2 | 1 | 0 | 0 | Absent |
| Main | 19.9 | 8.45 | 0.77 | 0.98 | 7.3 | 662 | 0.2 | 1 | 0 | 0 | 0 | Absent |

## July 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 22.1 | 12.2 | NM | NM | 8 | 625 | 1.8 | NM | NM | NM | NM | Present |
| Raw | NM | 12.23 | NM | NM | 8 | 628 | 2 | NM | NM | 4350 | 1046 | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Sedimentation plant outlet | NM | 12.15 | NM | NM | 7.2 | 634 | 0.44 | 205 | 600 | 51 | 1 | Absent |
| Flat bottom clarifier1 | NM | 12.14 | NM | NM | 7.2 | 640 | 0.13 | 131 | 165 | 29 | 2 | Absent |
| Flat bottom clarifier2 | NM | 12.18 | NM | NM | 7.3 | 637 | 0.42 | 70 | 125 | 28 | 0 | Absent |
| Intermediate ozone inlet | NM | 13.02 | NM | NM | NM | NM | NM | 65 | 150 | 17 | 0 | Absent |
| GAC inlet | NM | 13.01 | NM | NM | NM | NM | NM | 93 | 700 | 0 | 0 | Absent |
| GAC outlet | NM | 12.59 | NM | NM | NM | NM | NM | 38 | 111 | 5 | 0 | Absent |
| Dechlorination | NM | 13.05 | 0.88 | 1 | NM | NM | NM | 1 | 0 | 0 | 0 | Absent |
| Main | 22.1 | 12.2 | NM | NM | 8 | 625 | 0.12 | NM | NM | NM | NM | Absent |

## August 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | NM | 9.30 | NM | NM | 7.9 | 615 | 1.9 | NM | NM | NM | NM | Present |
| Raw | NM | 9.35 | NM | NM | 7.9 | 612 | 1.8 | NM | NM | 2480 | 270 | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 9.44 | NM | NM | 7.3 | 631 | 0.33 | 68 | 400 | 10 | 0 | Absent |
| Flat bottom clarifier1 | NM | 9.49 | NM | NM | 7.3 | 628 | 0.7 | 91 | 146 | 4 | 4 | Absent |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 10.04 | NM | NM | NM | NM | NM | 26 | 71 | 5 | 0 | Absent |
| GAC inlet | NM | 10.00 | NM | NM | NM | NM | NM | 16 | 51 | 0 | 0 | Absent |
| GAC outlet | NM | 10.10 | NM | NM | NM | NM | NM | 70 | 103 | 0 | 0 | Absent |
| Dechlorination | NM | 10.13 | 0.84 | 1.03 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 19.2 | 10.27 | 0.6 | 0.77 | 7.4 | 650 | 0.38 | 0 | 0 | 0 | 0 | Absent |

## September 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC conductivity @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 15.3 | 12.39 | NM | NM | 8 | 624 | 1.1 | NM | NM | NM | NM | Present |
| Raw | NM | 12.42 | NM | NM | 8 | 626 | 1.3 | NM | NM | 727 | 110 | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Present |
| Sedimentation plant outlet | NM | 12.47 | NM | NM | 7.3 | 645 | 0.33 | 18 | 25 | 1 | 0 | Absent |
| Flat bottom clarifier1 | NM | 12.5 | NM | NM | 7.2 | 649 | 0.69 | 7 | 27 | 1 | 0 | Absent |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 11.59 | NM | NM | NM | NM | NM | 68 | 68 | 0 | 0 | Absent |
| GAC inlet | NM | 12.01 | NM | NM | NM | NM | NM | 57 | 87 | 1 | 0 | Absent |
| GAC outlet | NM | 12.10 | NM | NM | NM | NM | NM | 4 | 73 | 0 | 0 | Absent |
| Dechlorination | NM | 11.46 | 0.78 | 0.89 | NM | NM | NM | 0 | 1 | 0 | 0 | Absent |
| Main | 16 | 12.15 | 0.72 | 0.73 | 7.3 | 662 | <0.10 | n/a | 0 | 0 | 0 | Absent |

## October 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 14.1 | 9.50 | NM | NM | 8 | 655 | 1.2 | NM | NM | 860 | 170 | Present |
| Raw | NM | 10.00 | NM | NM | 7.9 | 659 | 1.3 | 1 | 2600 | NM | NM | Present |
| Pre-ozonation | NM | 10.2 | NM | NM | NM | NM | NM | 2600 | 2600 | NM | NM | Absent |
| Sedimentation plant outlet | NM | 10.12 | NM | NM | 7.5 | 669 | 0.38 | 10 | 171 | 3 | 0 | Absent |
| Flat bottom clarifier1 | NM | 10.16 | NM | NM | 7.5 | 650 | 0.59 | 11 | 57 | 5 | 0 | Absent |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 10.36 | NM | NM | NM | NM | NM | 5 | 96 | 1 | 0 | Absent |
| GAC inlet | NM | 10.34 | NM | NM | NM | NM | NM | 104 | 0 | 36 | 0 | Absent |
| GAC outlet | NM | 10.39 | NM | NM | NM | NM | NM | 114 | 129 | 4 | 0 | Absent |
| Dechlorination | NM | 10.45 | 0.81 | 1.01 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 15 | 11.17 | 0.71 | 0.94 | 7.3 | 677 | <0.01 | 0 | 0 | 0 | 0 | Absent |

## November 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/m l) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | NM | 11.52 | NM | NM | 7.9 | 674 | 1.1 | NM | NM | NM | NM | Present |
| Raw | NM | 11.54 | NM | NM | 7.9 | 673 | 1.8 | NM | NM | NM | NM | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Sedimentation plant outlet | NM | 11.50 | NM | NM | 7.3 | 686 | 0.49 | 0 | 71 | 3 | 1 | Absent |
| Flat bottom clarifier1 | NM | 12.00 | NM | NM | 7.3 | 685 | 0.73 | 1 | 29 | 0 | 0 | Absent |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 12.57 | NM | NM | NM | NM | NM | 0 | 14 | 0 | 0 | Absent |
| GAC inlet | NM | 12.51 | NM | NM | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| GAC outlet | NM | 12.58 | NM | NM | NM | NM | NM | 0 | 61 | 5 | 0 | Absent |
| Dechlorination | NM | 12.27 | 0.86 | 1.1 | NM | NM | NM | 0 | 1 | 0 | 0 | Absent |
| Main | 10.4 | 12.14 | 0.71 | 0.96 | 7.3 | 699 | 0.15 | n/a | 0 | 0 | 0 | Absent |

## December 2017

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 6.2 | 10.10 | NM | NM | 7.8 | 623 | 18 | NM | NM | NM | NM | Present |
| Raw | NM | 10.21 | NM | NM | 7.8 | 629 | 26 | NM | NM | NM | NM | Present |
| Pre-ozonation | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Sedimentation plant outlet | NM | 10.30 | NM | NM | 7.2 | 643 | 1.2 | 12 | 19 | 0 | 0 | Absent |
| Flat bottom clarifier1 | NM | 10.35 | NM | NM | 7.1 | 635 | 1.4 | 22 | 28 | 1 | 0 | Absent |
| Flat bottom clarifier2 | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | NM | Absent |
| Intermediate ozone inlet | NM | 11.20 | NM | NM | NM | NM | NM | 1 | 2 | 0 | 0 | Absent |
| GAC inlet | NM | 11.3 | NM | NM | NM | NM | NM | 0 | 3 | 0 | 0 | Absent |
| GAC outlet | NM | 11.25 | NM | NM | NM | NM | NM | 0 | 6 | 0 | 0 | Absent |
| Dechlorination | NM | 10.51 | 0.78 | 1.34 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 5.1 | 10.41 | 0.73 | 0.93 | 7.2 | 678 | 0.15 | n/a | 0 | 0 | 0 | Absent |

## January 2018

| Sample Point | Temperature of sample (°C) | Time of sample collection | Level of free chlorine (mg/l) | Level of total chlorine (mg/l) | pH | EC @ 20°C (uS/cm) | Turbidity (NTU) | HPC 2D37 (count/ ml) | HPC 2D22 (count/ml) | Total coliforms (cfu/100ml) | E.coli (cfu/100ml) | Presence of *Acanthamoeba* spp. by microscopy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| River | 7.5 | 8.45 | NM | NM | 7.9 | 548 | 38 | NM | NM | 24200 | 4610 | Present |
| Raw | NM | 8.52 | NM | NM | 7.7 | 557 | 43 | 930 | 5900 | 17330 | 7270 | Present |
| Pre-ozonation | NM | 8.25 | NM | NM | NM | NM | NM | 286 | 400 | 326 | 44 | Absent |
| Sedimentation plant outlet | NM | 9.00 | NM | NM | 7.2 | 583 | 0.5 | 7 | 20 | 0 | 0 | Absent |
| Flat bottom clarifier1 | NM | 9.15 | NM | NM | 7.3 | 589 | 1.1 | 10 | 28 | 1 | 0 | Absent |
| Flat bottom clarifier2 | NM | 9.10 | NM | NM | 7.3 | 585 | 0.97 | 25 | 34 | 0 | 0 | Absent |
| Intermediate ozone inlet | NM | 9.50 | NM | NM | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| GAC inlet | NM | 10.01 | NM | NM | NM | NM | NM | 0 | 2 | 0 | 0 | Absent |
| GAC outlet | NM | 9.55 | NM | NM | NM | NM | NM | 0 | 8 | 0 | 0 | Absent |
| Dechlorination | NM | 9.31 | 0.91 | 1.08 | NM | NM | NM | 0 | 0 | 0 | 0 | Absent |
| Main | 6.7 | 9.20 | 0.87 | 1.01 | 7.3 | 607 | <0.10 | 0 | 0 | 0 | 0 | Absent |

## Appendix J: Sequences for Blast search

| Sample number | Month of sampling | Sampling point | Sequence used for Blast search |
|---|---|---|---|
| A9 | Feb-17 | River | TATCNNTATTAATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGC |
| A8 | Feb-17 | Raw | TATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTG |
| JB1 | Mar-17 | River | CNACTATCCNATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCA |
| JB3 | Mar-17 | Pre-ozone | TTATCATTACCCTAGTCCTCGCGCTGCCNAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCAA |
| JR3 | Apr-17 | River | ATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCA |

| | | | |
|---|---|---|---|
| JB4 | Apr-17 | Raw | TAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCA TTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTT CACGGTAAACGATCTGGGCCAAAA |
| JR1 | Apr-17 | Pre-ozone | CNATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATT CCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTT TTTCACGGTAAANGATCTGGGCCAA |
| JR2 | Apr-17 | Pre-ozone | ATCNTTACCCTAGTCCTCGCGCTGCCNAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATT ATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCA CGGTAAACGATCTGGGCNA |
| JB5 | May-17 | River | CCNATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTAT TCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATT TTTTCACGGTAAACGATCTGGG |
| JB6 | May-17 | Raw | ATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCC ATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTT TCACGGTAAACGATCTGGGCCAN |
| JB7 | Jun-17 | River | TATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCAT TATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTC ACGGTAAACGATCTGGGCCAA |

| | | | |
|---|---|---|---|
| JB8 | Jun-17 | Raw | CNATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCAA |
| JB9 | Jul-17 | River | TCNNATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGC |
| JB10 | Jul-17 | Raw | ACCAATTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAAAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCAACACCCCACGCNCGCGNGGGCTGTTTTCCCNGCTGGCAGCGATGAAAGACCCCCCCCACCANCTTG |
| JB11 | Aug-17 | River | NATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCAANN |
| JB12 | Aug-17 | Raw | NCGCTACCCNAGTCCTCAAACAGCAAGNNAAACTGATATANGANGACNNGCTCCTATTCCCTTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTTNCNGTAAANNATCTGGGCCAGAA |
| A2 | Aug-17 | Pre-ozone | TCCTATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCAANNNNN |

| | | | |
|---|---|---|---|
| JR4 | Sep-17 | River | TTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCA TTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTT CACGGTAAACGATCTGGGCCAA |
| JB13 | Sep-17 | Raw | ATCCNATTATCNTTACCCTAGTCCTCGCGCTGCCNAAACCAACTGAAAATAGGAGGACAGGGTCC TATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTA ATTTTTTCACGGTAAACGATCTGGGCC |
| A1 | Sep-17 | Pre-ozone | GCCAAGGACGACCGCGCCGATGGTGGTGTTTTGTATTCAACGTCTCCTAATCGCTGGTCGGCATC GTTTATGGTTAAGACTACGACGGTATCTGATCGTCTTCGATCCCCTAACTTTCGTTCTTGATTAATG AAAACNTCCT |
| JR5 | Oct-17 | River | CCTATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTAT TCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATT TTTTCACGGTAAACGATCTGGGC |
| JB14 | Oct-17 | Raw | ACNNTCNNTATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGG GTCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACAC TCTAATTTTTTCACGGTAAACGATCTGGGCCA |
| JR6 | Nov-17 | River | TCCNATTATCNTTACCCTAGTCCTCGCGCTGCCNAAACCAACTGAAAATAGGAGGACAGGGTCCT ATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAA TTTTTTCACGGTAAACGATCTGGGCC |

| | | | |
|---|---|---|---|
| JR7 | Nov-17 | River | ATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCC<br>ATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTT<br>TCACGGTAAACGATCTGGGCCA |
| JB15 | Nov-17 | Raw | TTATCNTTACCCTAGTCCTCGCGCTGCCAAAACNNNNTGAAAATAGGAGGACAGGGTCCTATTCN<br>ATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGNCTGCTTTGAACACTCTAATTTTT<br>TCACGGTAAACGATCNGGGCC |
| JR8 | Dec-17 | River | TATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCAT<br>TATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTC<br>ACGGTAAACGATCTGGGCCA |
| JR9 | Dec-17 | River | CAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCNTTATCCCATGCTAATGTATTCGGTGGCA<br>GAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAAACGATCTGGGCCAAAAGTC<br>CACCNCACGCGCGGGAGGGGTAANTCCCCCGG |
| JR10 | Dec-17 | River | TTATNATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCA<br>TTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTT<br>CACGGTAAACGATCTGGGCCAAANTNATGNNANNNTTGNTTNNNNNNTNNCNNCTGCNAAGNNTG<br>TNTNCANTNNNNGAGNNNNACNGTTGAGNNATNNANNNNNATGNNANNCCNTNNNNCTNTTAANC<br>ATTAACGATGCCNNCCNGCNN |
| A3 | Dec-17 | River | ATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCC<br>ATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTT<br>TCACGGTAAACGATCTGGGCCAAN |
| A4 | Dec-17 | River | NATTATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTC<br>CATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTT<br>TTCACGGTAAACGATCTGGGCCA |

| | | | |
|---|---|---|---|
| A5 | Dec-17 | River | TTAATCNTTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCC ATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTT TCACGGTAAACGATCTGGGCCAA |
| JB16 | Dec-17 | Raw | ATCCNATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTC CTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCT AATTTTTTCACGGTAAACGATCTGGGCCA |
| JR11 | Jan-18 | River | CCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTATTCCATTATCCCA TGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTTTCACGGTAA ACGATCTGGGCCANANTNATGAAANNNNNNCTTNNGNNANNGCATCNGCCAAGNNTGTTNTCATT AANNNAGANNNAANNNTNNNGNNATNNAANNNNATNAGATACCGTNNNAGTCTTAACCATAAACG ATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGCGCGGTCGT |
| A6 | Jan-18 | River | ATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGANAGGGTCCTATTCC ATTATCCNATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATTTTT TCACGGTAAACGATCTGGGCC |
| A7 | Jan-18 | Raw | CCNATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCTAT TCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACACTCTAATT TTTTCACGGTAAACGATCTGGGCCA |

| Sample number | Month of sampling | Sampling point | Sequence used for Blast search |
|---|---|---|---|
| JB20 | **Jun-18** | **River** | CNATTAATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGGTCCT ATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGGATCTGCCTGCTTTGAACACTCT AATTTTTTCACGGTAAACGATCTGGNCNN |
| JB21 | **Jun-18** | **Treated** | NTATCCNATTATCATTACCCTAGTCCTCGCGCTGCCAAAACCAACTGAAAATAGGAGGACAGGG TCCTATTCCATTATCCCATGCTAATGTATTCGGTGGCAGAAAATTGAATCTGCCTGCTTTGAACA CTCTAATTTTTTCACGGTAAACGATCTGGGC |

## Appendix K: Raw data for the effectiveness of CHX on *Acanthamoeba* spp. isolates

| Parameter | Row | Plate 1 | | Plate 2 | | Plate 3 | | Plate 4 | | Plate 5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm |
| Amoeba Only | 2 | 0.29312 | 0.29644 | 0.27646 | 0.27494 | 0.25719 | 0.26041 | 0.31064 | 0.30674 | 0.38739 | 0.30799 |
| Amoeba Only | 2 | 0.31614 | 0.30282 | 0.28001 | 0.26153 | 0.26340 | 0.25475 | 0.28109 | 0.25749 | 0.35307 | 0.27938 |
| Amoeba Only | 2 | 0.29778 | 0.28381 | 0.38176 | 0.26393 | 0.31635 | 0.21796 | 0.32206 | 0.24689 | 0.34779 | 0.25308 |
| Amoeba Only | 2 | 0.28263 | 0.27170 | 0.30618 | 0.28724 | 0.23151 | 0.23453 | 0.38704 | 0.26972 | 0.37377 | 0.26741 |
| Amoeba Only | 2 | 0.26266 | 0.25742 | 0.29485 | 0.24606 | 0.23172 | 0.22555 | 0.30578 | 0.23959 | 0.23591 | 0.22816 |
| Amoeba Only | 2 | 0.29155 | 0.26508 | 0.30970 | 0.29707 | 0.31621 | 0.28912 | 0.29581 | 0.29951 | 0.30970 | 0.27938 |
| Amoeba Only | 3 | 0.27528 | 0.27727 | 0.32372 | 0.26248 | 0.58340 | 0.28014 | 0.27903 | 0.27216 | 0.30949 | 0.26753 |
| Amoeba Only | 3 | 0.30971 | 0.30476 | 0.37132 | 0.26528 | 0.25005 | 0.25380 | 0.25882 | 0.25987 | 0.27060 | 0.25333 |
| Amoeba Only | 3 | 0.29250 | 0.27302 | 0.51916 | 0.27000 | 0.27573 | 0.28360 | 0.28546 | 0.28548 | 0.31308 | 0.29602 |
| Amoeba Only | 3 | 0.29607 | 0.26679 | 0.29303 | 0.24330 | 0.27293 | 0.25174 | 0.25671 | 0.24503 | 0.24831 | 0.24252 |
| Amoeba Only | 3 | 0.27066 | 0.27373 | 0.30910 | 0.29541 | 0.33246 | 0.30786 | 0.28435 | 0.28291 | 0.36131 | 0.30434 |
| Amoeba Only | 3 | 0.22937 | 0.23161 | 0.28956 | 0.26405 | 0.35837 | 0.28680 | 0.29550 | 0.25628 | 0.30429 | 0.27521 |
| 0.6µm CHX | 4 | 0.25608 | 0.26345 | 0.33413 | 0.25785 | 0.25901 | 0.25045 | 0.25258 | 0.24032 | 0.28003 | 0.24727 |
| 0.6µm CHX | 4 | 0.35655 | 0.29650 | 0.32646 | 0.27118 | 0.25100 | 0.24246 | 0.26802 | 0.26585 | 0.36356 | 0.28337 |
| 0.6µm CHX | 4 | 0.34179 | 0.27837 | 0.28250 | 0.23028 | 0.25355 | 0.24869 | 0.28988 | 0.23739 | 0.28100 | 0.25111 |
| 0.6µm CHX | 4 | 0.33063 | 0.24426 | 0.33707 | 0.27709 | 0.29232 | 0.26488 | 0.27957 | 0.27572 | 0.27311 | 0.24005 |
| 0.6µm CHX | 4 | 0.29155 | 0.26508 | 0.29034 | 0.24344 | 0.43511 | 0.24244 | 0.24870 | 0.24166 | 0.31926 | 0.23509 |
| 0.6µm CHX | 4 | 0.25408 | 0.25272 | 0.36475 | 0.24626 | 0.25650 | 0.22184 | 0.24493 | 0.24471 | 0.23708 | 0.22366 |
| 1.25µm CHX | 5 | 0.27355 | 0.26654 | 0.33124 | 0.24865 | 0.29168 | 0.27582 | 0.26160 | 0.26523 | 0.26524 | 0.25210 |
| 1.25µm CHX | 5 | 0.39748 | 0.31157 | 0.28000 | 0.22108 | 0.24263 | 0.22593 | 0.22143 | 0.21971 | 0.24833 | 0.21796 |
| 1.25µm CHX | 5 | 0.37514 | 0.26804 | 0.28023 | 0.26079 | 0.38744 | 0.28270 | 0.26039 | 0.25174 | 0.25950 | 0.26116 |
| 1.25µm CHX | 5 | 0.34746 | 0.24980 | 0.28258 | 0.22628 | 0.59140 | 0.25369 | 0.22260 | 0.21010 | 0.28013 | 0.25944 |
| 1.25µm CHX | 5 | 0.28760 | 0.25922 | 0.29779 | 0.22664 | 0.22939 | 0.21525 | 0.22575 | 0.21554 | 0.27649 | 0.21973 |
| 1.25µm CHX | 5 | 0.25797 | 0.24643 | 0.32966 | 0.25023 | 0.27873 | 0.27019 | 0.28331 | 0.24162 | 0.45005 | 0.24639 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.5µm CHX | 6 | 0.27763 | 0.25894 | 0.30420 | 0.22689 | 0.25449 | 0.24177 | 0.21610 | 0.20925 | 0.28031 | 0.24999 |
| 2.5µm CHX | 6 | 0.53573 | 0.27884 | 0.28925 | 0.26676 | 0.28221 | 0.25476 | 0.22913 | 0.23265 | 0.28738 | 0.27903 |
| 2.5µm CHX | 6 | 0.26198 | 0.24394 | 0.25930 | 0.23833 | 0.35739 | 0.24451 | 0.24114 | 0.21612 | 0.24825 | 0.22636 |
| 2.5µm CHX | 6 | 0.28071 | 0.24436 | 0.23731 | 0.23246 | 0.27944 | 0.22413 | 0.28696 | 0.25844 | 0.24378 | 0.22489 |
| 2.5µm CHX | 6 | 0.26679 | 0.26087 | 0.26198 | 0.25074 | 0.26181 | 0.24723 | 0.26612 | 0.25452 | 0.26542 | 0.25197 |
| 2.5µm CHX | 6 | 0.42515 | 0.21889 | 0.21379 | 0.20708 | 0.24354 | 0.22296 | 0.22081 | 0.19878 | 0.35874 | 0.22069 |
| 5µm CHX | 7 | 0.29382 | 0.27306 | 0.25829 | 0.25206 | 0.28933 | 0.27342 | 0.26685 | 0.25097 | 0.43513 | 0.27099 |
| 5µm CHX | 7 | 0.32397 | 0.29621 | 0.24635 | 0.22842 | 0.48138 | 0.24255 | 0.32225 | 0.21331 | 0.30179 | 0.23775 |
| 5µm CHX | 7 | 0.32114 | 0.27347 | 0.23558 | 0.22084 | 0.26769 | 0.23999 | 0.30321 | 0.23306 | 0.27940 | 0.23638 |
| 5µm CHX | 7 | 0.36890 | 0.25885 | 0.25863 | 0.24418 | 0.42144 | 0.26090 | 0.30952 | 0.24059 | 0.25940 | 0.24286 |
| 5µm CHX | 7 | 0.42986 | 0.26852 | 0.21653 | 0.20753 | 0.29079 | 0.20120 | 0.30515 | 0.19669 | 0.23288 | 0.20926 |
| 5µm CHX | 7 | 1.14236 | 0.25649 | 0.25771 | 0.24777 | 0.30038 | 0.27565 | 0.22787 | 0.23480 | 0.28726 | 0.27193 |
| 10µm CHX | 8 | 0.28991 | 0.25607 | 0.25687 | 0.23077 | 0.26788 | 0.25174 | 0.24769 | 0.21809 | 0.27635 | 0.23187 |
| 10µm CHX | 8 | 0.32219 | 0.28080 | 0.25355 | 0.22995 | 0.25544 | 0.23736 | 0.21511 | 0.20734 | 0.24895 | 0.23253 |
| 10µm CHX | 8 | 0.28395 | 0.24160 | 0.26687 | 0.24280 | 0.37828 | 0.26897 | 0.22076 | 0.22669 | 0.25776 | 0.25197 |
| 10µm CHX | 8 | 0.33323 | 0.23827 | 0.20549 | 0.20247 | 0.28158 | 0.24599 | 0.20145 | 0.19458 | 0.24659 | 0.21454 |
| 10µm CHX | 8 | 0.36689 | 0.25827 | 0.21562 | 0.19095 | 0.26290 | 0.22195 | 0.22502 | 0.21088 | 0.22399 | 0.19324 |
| 10µm CHX | 8 | 0.79288 | 0.21917 | 0.27592 | 0.23952 | 0.28455 | 0.24997 | 0.26240 | 0.22477 | 0.24318 | 0.23432 |
| 20µm CHX | 9 | 0.26016 | 0.26209 | 0.32610 | 0.22144 | 0.41557 | 0.24157 | 0.24856 | 0.22429 | 0.23971 | 0.22669 |
| 20µm CHX | 9 | 0.28928 | 0.28109 | 0.26893 | 0.22495 | 0.33243 | 0.25730 | 0.27809 | 0.23361 | 0.23745 | 0.21633 |
| 20µm CHX | 9 | 0.26480 | 0.23489 | 0.25522 | 0.24454 | 0.33620 | 0.27679 | 0.28989 | 0.26747 | 0.25077 | 0.23213 |
| 20µm CHX | 9 | 0.43018 | 0.24026 | 0.23882 | 0.22657 | 0.34614 | 0.27495 | 0.28194 | 0.22534 | 0.28507 | 0.25032 |
| 20µm CHX | 9 | 0.25588 | 0.25286 | 0.20780 | 0.19371 | 0.39360 | 0.29422 | 0.23361 | 0.21085 | 0.27332 | 0.24237 |
| 20µm CHX | 9 | 0.24299 | 0.21731 | 0.21111 | 0.20446 | 0.28102 | 0.21999 | 0.24217 | 0.20577 | 0.30499 | 0.19629 |

**Averages**

| Wells | Row | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Amoeba Only | 2 | 0.29065 | 0.27955 | 0.30816 | 0.27180 | 0.26940 | 0.24705 | 0.31707 | 0.26999 | 0.33460 | 0.26923 |
| Amoeba Only | 3 | 0.27893 | 0.27120 | 0.35098 | 0.26675 | 0.34549 | 0.27732 | 0.27665 | 0.26696 | 0.30118 | 0.27316 |
| 0.6µm CHX | 4 | 0.30511 | 0.26673 | 0.32254 | 0.25435 | 0.29125 | 0.24513 | 0.26395 | 0.25094 | 0.29234 | 0.24676 |
| 1.25µm CHX | 5 | 0.32320 | 0.26693 | 0.30025 | 0.23894 | 0.33688 | 0.25393 | 0.24585 | 0.23399 | 0.29662 | 0.24280 |
| 2.5µm CHX | 6 | 0.34133 | 0.25097 | 0.26097 | 0.23704 | 0.27981 | 0.23923 | 0.24338 | 0.22829 | 0.28065 | 0.24216 |
| 5µm CHX | 7 | 0.48001 | 0.27110 | 0.24551 | 0.23347 | 0.34183 | 0.24895 | 0.28914 | 0.22824 | 0.29931 | 0.24486 |
| 10µm CHX | 8 | 0.39817 | 0.24903 | 0.24572 | 0.22274 | 0.28844 | 0.24600 | 0.22874 | 0.21373 | 0.24947 | 0.22641 |
| 20µm CHX | 9 | 0.29055 | 0.24808 | 0.25133 | 0.21928 | 0.35083 | 0.26080 | 0.26237 | 0.22789 | 0.26522 | 0.22736 |

**Standard Deviation**

| Wells | Row | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm | PYG 492nm | PYG 630nm |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Amoeba Only | 2 | 0.01764 | 0.01792 | 0.03847 | 0.01853 | 0.03856 | 0.02635 | 0.03698 | 0.02770 | 0.05513 | 0.02705 |
| Amoeba Only | 3 | 0.02815 | 0.02349 | 0.08755 | 0.01679 | 0.12343 | 0.02134 | 0.01558 | 0.01593 | 0.03890 | 0.02393 |
| 0.6µm CHX | 4 | 0.04435 | 0.01864 | 0.03093 | 0.01775 | 0.07209 | 0.01406 | 0.01820 | 0.01586 | 0.04359 | 0.02037 |
| 1.25µm CHX | 5 | 0.05795 | 0.02353 | 0.02431 | 0.01631 | 0.13653 | 0.02775 | 0.02609 | 0.02220 | 0.07604 | 0.01930 |
| 2.5µm CHX | 6 | 0.11349 | 0.02030 | 0.03304 | 0.02047 | 0.04075 | 0.01290 | 0.02785 | 0.02449 | 0.04192 | 0.02248 |
| 5µm CHX | 7 | 0.32797 | 0.01421 | 0.01687 | 0.01745 | 0.08762 | 0.02777 | 0.03526 | 0.01977 | 0.07071 | 0.02371 |
| 10µm CHX | 8 | 0.19572 | 0.02101 | 0.02853 | 0.02108 | 0.04538 | 0.01568 | 0.02236 | 0.01204 | 0.01725 | 0.02012 |
| 20µm CHX | 9 | 0.07007 | 0.02232 | 0.04378 | 0.01790 | 0.04789 | 0.02691 | 0.02372 | 0.02190 | 0.02710 | 0.01930 |