CRANFIELD UNIVERSITY

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Flow Cytometry: A Tool for Assessing Drinking Water Quality and Evaluating Chlorine Disinfection Performance

School of Water, Energy and Environment

EngD
Academic Year: 2018 – 2019

Supervisor: Prof. Peter Jarvis
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This thesis is submitted in partial fulfilment of the requirements for the degree of EngD

Abstract

Chlorine disinfection is a process that has been in use for over a century for drinking water treatment; however rare detections of faecal indicator organisms in final treated water still occur. Assessing the performance of the disinfection process in-situ is challenging. Most often this is achieved by monitoring abiotic parameters such as chlorine, turbidity and pH, whereas microbiological sampling takes place daily. Typically, chlorine disinfection occurs within chlorine contact tanks which should be designed to achieve plug flow and minimise short circuiting. In reality, the design of contact tanks vary considerably, and water utilities have inherited many legacy assets that do not conform to modern day design standards. Furthermore, microbiological culture-based data is hard to evaluate when there are sporadic detections of culturable organisms. There is therefore a need to quantify deviation from optimal plug flow design of chlorine contact tanks and a requirement for an alternative microbiological approach to achieve this.

This thesis explores the application of use of flow cytometry (FC), a novel culture independent technique for measuring bacterial cell viability, for disinfection applications. Firstly, an assessment of FC and its value as a monitoring tool for the water industry was carried out. This utilised the largest drinking water FC dataset in the world and concluded that there was no link between coliform detections and FC data, yet coliform detections were shown to be driven by the contact time (Ct) in disinfection, not just the sole parameter of chlorine residual. Secondly, the key process variables of chlorine disinfection were investigated and pilot scale studies demonstrated that

hydraulic efficiency during chlorination impacted upon disinfection efficacy and FC provided insights of bacterial inactivation rates where traditional culture-based methods could not.The findings from this work culminated in an assessment of Ct across Scottish Water and the cost of investment required to bring high risk (large production volume) water treatment works (WTW) up to current standards was estimated. Finally, the implications of this thesis and the learning around chlorine disinfection and the application of FC for the water industry were discussed.

Keywords:

Drinking water, microbiology, chlorine contact tank, disinfection

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Table of Contents

List of Figures

List of Tables

List of Equations

List of Appendices

List of Abbreviations

AWWA	American Water Works Association
CCP	Chlorine contact pipe
CCT	Chlorine contact tank
CFD	Computational Fluid Dynamics
CFU	Colony Forming Unit
CSTR	Continuously Stirred Reactor
Ct	Chlorine exposure (product of concentration and time)
CWS	Clean Water storage
CWT	Clear water tank
DAF	Dissolved air flotation
DBP	Disinfection by-products
DMSO	Dimethyl sulphoxide
DOC	Dissolved Organic Carbon
DWQR	Drinking Water Quality Regulator
FC	Flow Cytometry
FCM	Flow Cytometry monitoring
GAC	Granular activated carbon
GW	Ground Water
HE	Hydraulic efficiency
HEI	Hydraulic efficiency indicator
HNA	High nucleic acid content
HOCl	Hypochlorus acid
HPC	Heterotrophic bacterial count
HRT	Hydraulic retention time
ICC	Intact cell count
LNA	Low nucleic acid content
NGS	Next generation sequencing
OCl	Hypochlorite ion
PCR	Polymerase chain reaction
PF	Plug Flow
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
Re	Reynolds number
RGF	Rapid gravity filtration
RTD	Residence time distribution
SG	SYBR Green I

SGPI	SYBR Green I + propidium iodide
SW	Surface Water
TCC	Total cell count
TOC	Total Organic Carbon
UF	Ultrafiltration
VBNC	Viable but non-culturable
WGS	Whole genome sequencing
WHO	World Health Organisation
WTW	Water Treatment Works
YEA	Yeast Extract Agar

1. Introduction

1.1. Background

Disinfection by chlorine is a worldwide standard as a final barrier to pathogens in drinking water treatment. The first continuous use of chlorine for disinfection in England was in 1905, this resulted following a series of typhoid outbreaks in the city of Lincoln (White, 2010). There is no doubt that the introduction of chlorination to the treatment of water over a century ago has led to one of the greatest improvements in public health in the developed world. Since then, it has remained the most popular disinfection approach principally due to its efficiency, economical cost, convenience and ability to suppress bacteriological growth in distribution (AWWA, 2006). Chlorination is effective against a wide variety of bacteria and viruses often inactivating these organisms after a short contact time. Protozoa on the other hand (e.g. *Cryposporidium* and *Giardia*), are not effectively inactivated during chlorination and require alternative disinfection approaches such as UV or Ozone (Percival *et al.* 2014). The criticality of the disinfection process is illustrated by the Walkerton *E. coli* 0157:H7 outbreak in 2000 that caused illness in more than 2,300 people and left seven members of the public dead. Chlorine dosing was not prioritised following complaints from consumers of a chlorine taste in the water and this coincided with contamination of the groundwater source with manure following heavy rainfall. This is a poignant example of how poor operational management and a misunderstanding of the importance of disinfection can have devastating consequences (Hrudey *et al*., 2003). This outbreak is also a reminder of the importance of an effective multiple barrier approach within drinking water treatment, as typically these outbreaks occurs only in rare circumstances where there is a

multiplicity of failures. Each barrier in the treatment process not only a removes or inactivates pathogens in drinking water but is often essential in ensuring each subsequent treatment stage is effective.

In most cases, chlorine disinfection requires effective pre-treatment of the water. Typically water is treated by conventional treatment processes of coagulation, flocculation and sand filtration. Not only do these pre-treatment stages directly remove a large proportion of the microorganisms, they also remove particles that are able to shield bacteria, as well as organic matter that can react with the chlorine in the system thus reducing the concentration available for disinfection. Operation of the chlorine disinfection processes is based on taking the product of the concentration of free chlorine (C) and the effective residence time (t), to calculate the overall Ct, fundamentally based on the work of Chick (1908):

Ct (mg.min/L) = Concentration of free chlorine (mg/L)(C) x Residence time (min)(T)

Ct values for a given inactivation (e.g. 99% or 2 log reduction) vary widely between organisms, with *E. coli* only requiring a Ct between 0.06 and 3.50 mg.min/L whereas the protozoan *Giardia* is much more tolerant with a Ct around 300 mg.min/L (Stanfield, 2005).

There are four key process variables when considering disinfection with chlorine: *dose, temperature, pH and exposure time.* The temperature and pH of water are both able to increase and decrease the efficiency of free chlorine. The behaviour and speciation of chlorine changes dependent upon the pH. For this reason, considering the Ct alone may not be sufficient for controlling disinfection (Stanfield, 2005). Under acidic pH, the

hypochlorous acid (HOCl) dominates. HOCl is a stronger disinfectant than the hypochlorite ion (OCl^-) which is formed at neutral and higher pH:

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

Decreasing temperature favours formation of HOCl but results in a lower reaction rate and diffusivity of HOCl into the target cells (White, 2010). It is estimated that a 10°C increase in temperature will increase the reaction rate two fold (Stanfield, 2005). An increase in temperature results in increased diffusivity of Cl_2 into the cell, increased reaction with cell components and increased cell metabolic activity, and therefore has an increased toxicity. Maintaining a consistent free chlorine concentration is important to ensure that the correct Ct is applied. Although it is possible to adapt the free chlorine residual for flow, doing the same to compensate for changes in the chlorine demand is a challenge. The chlorine demand of the water is considered as any matter that combines with free chlorine to reduce the disinfection efficiency. By far the biggest demand for chlorine in water typically derives from natural organic matter (NOM) (Jegatheesan *et al*., 2009). The chlorine demand is partially comprised of biological matter. This biological material may be either biofilm on the inner surface of tanks and pipes or planktonic organisms. It is also possible for other constituents of the water such as iron, manganese, ammonia and hydrogen sulphide, to exert a chlorine demand (White, 2010). The discovery of potentially carcinogenic disinfection by-products formed during the reaction of chlorine with dissolved organic carbon (DOC) (Rook, 1974) has led to concerns around the use of chlorine as a primary disinfectant. However, it must be recognised that disinfection should not be compromised whilst there is low confidence

in the risk of chronic exposure to DBPs and there is a known potential acute health risk from pathogens (Hrudey and Fawell, 2015).

The hydraulic performance of chlorine contact tanks has been explored extensively in the past (Falconer, 1986; Marske and Boyle, 1973; Pfeiffer and Barbeau, 2014; Shiono and Teixeira, 2000; Angeloudis *et al*., 2014). However, the majority of these assessments rely on theoretical models, utilising computational fluid dynamics (CFD) to assess process performance. Fundamentally, tracer studies are the basis for much of this work, often following the standard set by Marske and Boyle (1973). A number of pilot scale studies have been undertaken (Rauen, 2005; Angeloudis, 2014) and often the data from these studies is utilised to calibrate CFD models. However, none of these studies have measured actual bacterial inactivation rates. Therefore the link between poor hydraulic performance and disinfection efficacy has not been demonstrated.

Typical microbiological analysis of drinking water is carried out in a low-resolution manner due to the low throughput method of conventional plate counting and the regulations only requiring at most, daily sampling of WTW. Here in the UK, there is no regulatory standard for the general bacteriological quality of drinking water which is monitored via heterotrophic plate counts (HPC). Instead, the primary focus is on indicator organisms *(E.coli*, *Enterococcus*, total coliforms, *Clostridium perfringens,* and *Pseudomonas* aeruginosa*)* that allude to the presence of pathogens with a zero tolerance to any detection of these organisms in final water. There is little evidence to support whether these indicators when identified at WTW correlate with bacteriological failures at the consumer tap (Ellis *et al*., 2012) and as such the actual bacteriological

quality of water the consumer receives often remains unknown. Assessing general bacteriological quality in treatment processes via the HPC method is rarely practiced due to a lead time of between 3 and 7 days from analysis to result. Additionally, there are bacteria in a viable but non-culturable (VBNC) state within drinking water. This VBNC state can be induced in a number of ways such as; sub lethal injury (chlorination, UV disinfection, ozonation), inadequate growth media or conditions, or excess non-heterotrophic organisms (Health Canada, 2012). Observing samples by direct microscopy has also highlighted the fact that less than 1% of the total bacterial population can be enumerated by plate count methods alone (Berney *et al*., 2008). To overcome this discrepancy alternative techniques need to be explored.

Over the last decade, there have been significant advances in non-culture based microbial techniques. The polymerase chain reaction (PCR) is a method that allows for amplification of target fragments of DNA. This method can be useful in the identification of a target organism (e.g. pathogens) whether they be bacteria, virus or protozoa, although each of these may require a sample concentration step. A challenge for any molecular based technique is the understanding of cell viability and whether a detected 'copy' of an organism is from a viable cell. One approach is to use propidium monoazide (PMA) treatment in combination with quantitative PCR (qPCR). For example, Nocker *et al* (2007) showed that the PMA pre-treatment prevents amplification of membrane damaged cells giving a much more realistic count of viable organisms following oxidative stress. Girones *et al.* (2010) provided a comprehensive outline of the pros and cons of molecular techniques. In summary, the authors concluded that these approaches are excellent for identifying specific target organisms, understanding functionality with the

advantage of being sensitive as well as as being rapid techniques. However, these approaches do not provide accurate information on the concentration of microorganisms. This aspect cannot be overlooked when considering disinfection and can lead to overestimates of organism concentrations.

Further detail on the composition of microbial communities in drinking water can be obtained using community approaches. The most commonly used gene marker to establish an understanding of community composition is the 16S rRNA gene (for prokaryotes). As to some parts of this gene being highly conserved and other regions within species displaying variability, thisallows for a distinction of phylogeny between the microorganisms found in drinking water (Douterelo *et al.* 2014). This gene can be targeted in both PCR and sequencing approaches. In recent years there has been a shift away from singular target gene methods towards what is termed next generation sequencing (NGS). NGS allows for the whole genome contained within a sample to be obtained therefore the specificity issues of a targeted region are eliminated, although this same information can still be obtained from the whole genomic data. Prest *et al* (2014) highlighted that 16s rRNA only provides abundance values and not absolute counts of bacterial groups. It is envisioned that utilising the vast amount of data obtained from more advanced sequencing techniques will allow for a clearer correlation between microbial composition, activity and function to water quality.

In contrast to purely DNA based approaches there is still interest in monitoring the physiological responses of microbes in water sources, treatment and distribution. A number of studies have demonstrated the use of monitoring microbial activity, where

enzyme substrate-based fluorescence approaches are useful in establishing intracellular activity (Hoefel *et al*., 2003, 2005; Nebe-von Caron *et al*., 1998; Nocker *et al*., 2011). The quantification of cell bound and extra-cellular adenosine tri-phosphate (ATP) is a rapid and simple technique for assessing general bacterial activity that has been successfully applied in the evaluation of water treatment process performance (Berney *et al*., 2006) and laboratory based disinfection tests(Kong *et al*., 2015). Nescerecka *et al*.(2016) demonstrated once a certain chlorine exposure was reached (0.35 mg/L free chlorine for 5 min) almost all intracellular ATP shifted towards extracellular, and concluded that ATP rapidly leaks from cells with a permeabilised membrane after oxidative stress.

Direct cell quantification is also possible using microscopy in combination with staining techniques such as epifluorescence microscopy and fluorescent in-situ hybridisation (FISH). Essentially fluorescent probes are bound to a target nucleotide sequence allowing visualisation of target organisms. Although a relatively straight forward approach this method of direct cell counting normally suffers from the limitation in sample throughput, as analysis time is long. There must also be consideration of the time consuming optimisation of FISH methods to ensure fluorescent probes hybridise effectively (Douterelo *et al.* 2014). Furthermore, the state of cells must be altered as there is a requirement for samples to be fixed before staining which can result in a misinterpretation of cell viability.

An alternative direct counting method that has become increasingly popular for assessing the microbiological water quality is flow cytometry (FC). FC is a high throughput diagnostic method that is able to count single cells in a turnaround time of

less than 15 min per sample (Nevel *et al*., 2013). FC uses an excitation laser in combination with specific DNA binding dyes and captures the emission in a variety of detectors known as photo multiplier tubes (PMTs). The most commonly used DNA dyes are SYBR Green I for the measurement of total cells and Propidium Iodide to detect membrane permeable (damaged) cells. Unlike the dyes used in FISH, the ones used in FC are similar to the LIVE/DEAD technique used in epifluorescence microscopy and do not require cells to be fixed, therefore cell viability is not affected by the method itself. One of the biggest advantages of FCM in comparison to conventional culture based approaches is that samples are not subject to the confounding error of the VBNC state of organisms, and is able to detect bacterial cells irrespective of the culturable state. FC is gaining popularity for analysis of samples from water treatment works and supply (Gillespie *et al*., 2014; Hammes *et al*., 2008; Prest *et al*., 2013). It has been shown to have a wide variety of applications such as monitoring bacterial numbers throughout treatment (Helmi *et al*., 2014) and distribution (Nescerecka *et al*., 2014); monitoring natural water sources (Besmer *et al*., 2014); and evaluating biological process performance (Arnoldini *et al*., 2013). FC is not considered as being a replacement for culture-based techniques that target specific organisms, instead the method is best thought of as an alternative to HPC. The major benefit of FC is that it provides a much more realistic estimation of the total number of cells (Douterelo *et al.* 2014). Furthermore, it provides the promise of rapid analysis with results available in less than 20 min, and availability in a 96 well plate format for increased throughput. To date, the largest FC dataset that has been collated contained 3,675 data points (Van Nevel *et al*. 2017). There is certainly a requirement for more evidence to be gathered as to the

advantages of FC over traditional HPC methods if this technique is to become a standard method used across the water sector.

In 2013, Scottish Water (SW) were issued with an Information Letter (Id: 2013/3) from the Drinking Water Quality Regulator (DWQR) titled 'Requirement and Expectations for Supply-specific Disinfection Strategies' (DWQR, 2013), as a requirement to improve disinfection and bacteriological performance, ensuring Scottish Water were compliant with regulation 25 of the Public Water Supplies (Scotland) Regulations 2014 (Scottish Parliament, 2014). In summary, this required Scottish Water to demonstrate a site-specific disinfection strategy, an understanding of process performance, and to demonstrate the verification of disinfection efficacy, this was the foundation for this research.

1.2. Research Aim and Objectives

The overall aim of this research was to determine how flow cytometric analysis can be applied in the water industry and specifically how can this method can be utilised to assess the performance of the chlorine disinfection stage of drinking water treatment. To achieve this the following objectives were set:

1. To critically appraise the most appropriate application of flow cytometry for monitoring bacteriological water quality in drinking water production with a focus on disinfection applications.
2. Carry out bench scale disinfection experiments to understand how flow cytometric data responds to typical operational parameters that impact disinfection efficacy.
3. To determine the hydraulic efficiency of chlorine contact tanks of varying design and establish the link between flow behaviour and disinfection efficacy.
4. To assess current disinfection practice across Scottish Water and identify a strategy for improvement where required.

1.3. Thesis Structure

The thesis herein is formatted as a series of chapters as journal papers. All papers were written by the first author, Ryan Cheswick, and edited by the supervisory team of Professor Peter Jarvis and Professor Bruce Jefferson. All experimental work was undertaken by the primary researcher, Ryan Cheswick, with the exception of the water quality analysis in chapters 3 and 4 which were conducted by Scottish Water Scientific Services.

An overall literature review was not conducted for this thesis, instead a critical appraisal of the literature has been made within each chapter. Chapter 2 first explored the most applicable use of flow cytometry within drinking water treatment. (Chapter 2, Paper 1 – published: Cheswick, R., Cartmell, E., Lee, S., Upton, A., Weir, P., Moore, G., Nocker, A., Jefferson, B., Jarvis, P. Comparing flow cytometry with culture-based methods for microbial monitoring and as a diagnostic tool for assessing drinking water treatment processes. Published in Environment International: https://doi.org/10.1016/j.envint.2019.06.003).

Chapter 3 aimed to understand how the key operational parameters of *chlorine dose, contact time, temperature and pH* impact chlorine disinfection efficacy. Here it was also demonstrated that flow cytometry can be successfully applied in disinfection experiments. (Chapter 3, Paper 2 – in preparation: Cheswick, R., Nocker, A., Jefferson, B., Jarvis, P. Understanding how operational conditions impact the efficacy of chlorine disinfection using flow cytometry, *Target journal: Water Research*).

Chapter 4 builds on the findings of the two previous chapters that Ct is critical for ensuring compliance. This pilot scale disinfection study demonstrated the relationship between deviation from ideal plug flow and disinfection efficacy. (Chapter 4, Paper 3 – in preparation. Cheswick, R., Nocker, A., Moore, G., Jefferson, B., Jarvis, P. Disinfection and hydraulic performance in chlorine contact tanks. *Target journal: Environmental Science and Technology*.

Following this work, chapter 5 gives an assessment of 'Ct' across Scottish Waters asset base was carried out. This identified the key risk sites that could not comply with the Drinking Water Quality Regulators (DWQR) and Scottish Water disinfection policy requirements. An assessment of the investment required to bring all chlorine contact tanks up to current specifications was made.

Chapter 6 then provides an overall discussion of the thesis and provides a broader context to the findings of this project for the application in drinking water treatment. Here the the key conclusions are summarised and recommendations are made for further work to improve chlorine disinfection performance and resilience into the future, with an outlook on the application of flow cytometry to the drinking water sector. Table 1.1 summarises the thesis structure in line with the thesis objectives.

Table 1.1. Thesis structure, alignment with objectives and paper status

Chapter	Paper	Objective	Title	Target Journal	Status
2	1	1	Comparing flow cytometry with culture-based methods for microbial monitoring and as a diagnostic tool for assessing drinking water treatment processes	Environmental International	Published (Sept 2019, Vol 130: e104893)
3	2	2	Assessing chlorine disinfection efficacy using flow cytometry and viability staining: the impact of pH, temperature and dose conditions	Water Research	In preparation
4	3	2, 3	Linking chlorine contact tank hydraulic performance and disinfection efficacy using flow cytometry at pilot scale	Environmental Science & Technology	In preparation
5	-	4	Performance of Disinfection Reactors Across Scottish Water: Investment Requirements and Solutions	-	-
6	-	1, 2, 3, 4	Overall Discussion and conclusions	-	-
7	-	-	Further Work	-	-

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2. Comparing flow cytometry with culture-based methods for microbial monitoring and as a diagnostic tool for assessing drinking water treatment processes

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Chapter context

Flow cytometry has been identified as a useful diagnostic tool for monitoring bacteriological water quality. However, to date there is lack of data in the literature from routine water quality monitoring. The following chapter aims to address thesis objective 1:

> *"To critically appraise the most appropriate application of flow cytometry for monitoring bacteriological water quality in drinking water production with a focus on disinfection applications."*

Abstract

Flow cytometry (FCM) and the ability to measure both total and intact cell populations through DNA staining methodologies has rapidly gained attention and consideration across the water sector in the past decade. In this study, water quality monitoring was undertaken over three years across 213 drinking water treatment works (WTW) in the Scottish Water region (Total n = 39,340). Samples subject to routine regulatory microbial analysis using culture-based methods were also analysed using FCM. In addition to final treated water, the bacterial content in raw water was measured over a one-year period. Three WTW were studied in further detail using on-site inter-stage sampling and analysis with FCM. It was demonstrated that there was no clear link between FCM data and the coliform samples taken for regulatory monitoring. The disinfectant Ct value (Ct = mg.min/L) was the driving factor in determining final water cell viability and the proportion of intact cells (intact/total cells) and the frequency of coliform detections in the water leaving the WTW. However, the free chlorine residual, without consideration of treatment time, was shown to have little impact on coliform detections or cell counts. Amongst the three treatment trains monitored in detail, the membrane filtration WTW showed the greatest log removal and robustness in terms of final water intact cell counts. Flow cytometry was shown to provide insights into the bacteriological quality of water that adds significant value over and above that provided by traditional bacterial monitoring.

Key words: flow cytometry, disinfection, drinking water, bacteria

2.1. Introduction

The supply of wholesome, safe drinking water remains the highest priority for all drinking water providers. Ensuring that water is microbiologically safe for the consumer is achieved by regular monitoring of water quality in samples taken as water leaves the water treatment works (WTW) and at the customer's tap. In addition, the microbiological risks presented by the source water and the removal of microorganisms across the water treatment works (WTW) final treated water should be understood. The standard analytical techniques for determining the bacteriological quality of drinking water are culture-based methods. From a regulatory perspective, the primary aim is to monitor for the absence of pathogen indicator organisms such as *Escherichia coli*, total coliforms, *Enterococci* and *Clostridium* using targeted growth media. In the European Union regulatory standards are 0 CFU/100mL in water leaving the WTW and at the customer taps (Drinking Water Inspectorate, 2017). Heterotrophic plate counts (HPC) are also frequently monitored, aiming to provide a general assessment of the microbiological quality of the water, using non-specific media (Allen *et al*., 2004). It is important to note that there is no evidence of a link between HPC and health risk, a point recognised by the World Health Organisation (WHO) (World Health Organisation, 2003) and there are no defined guidelines for monitoring water quality or water treatment performance using HPCs (Chowdhury, 2012). This has resulted in diverse procedures for HPC monitoring, with differences in sample incubation times, temperatures and acceptable critical thresholds applied across the world, with some countries having no specified limits, some adopting a no abnormal change approach, and others accepting up to 500 CFU/mL (Van Nevel *et al*., 2017).

The majority of bacteria, typically <1%, are not culturable under laboratory conditions (Hammes *et al*., 2008). Non-culturability gains additional importance when water is subjected to disinfection processes such as chlorination, as cell damage from oxidation leads to suppression of colony formation (Kong *et al*., 2015) but not always complete cell death (Camper and McFeters, 1979). Such bacteria may then be found in drinking water or contribute to microbial biofilm formation (Liu *et al*., 2014). In the past decade, there have been significant advances in cultivation-independent techniques for monitoring the bacteriological quality of water using fluorescence-based approaches. This has enabled improved understanding of bacterial cell viability in drinking water systems. Flow cytometry (FCM) has emerged as being the most promising method for routine diagnostics due to its ease of operation and the speed of analysis. FCM enables rapid (less than 15 min) and direct cell quantification through the use of fluorescent nucleic acid dyes that bind to individual cells. Commonly, a pairwise set of dyes are used, SYBR Green I (SG) and Propidium Iodide (PI), which enables both the total (TCC) and intact cell count (ICC) to be measured. The differentiation becomes possible as a result of differences in the penetration properties of the two dyes. SG binds irrespective of cell integrity, whereas PI is a membrane impermeant dye, which can only penetrate cells and bind to their DNA once the cell membrane integrity is compromised (for example, following oxidation by chlorine). Stained extracellular DNA does not provide sufficient signal intensity to be captured as a microbe during FCM analysis. It is important to note that damage to the cell membrane does not mean certain death to a bacteria but is a strong indicator of a decrease in the cell viability (Fittipaldi *et al*., 2012).

The richness of information generated by the FCM provides opportunities for adding value in a variety of applications in the water sector. As a result, these attributes have seen its receptivity within the water sector grow rapidly with applications including: monitoring of microbial concentrations in aquatic environments (Besmer *et al*., 2014), identification of different types of microorganism (Collado *et al.,* 2017), wastewater treatment (Porter *et al*., 1997), drinking water treatment (Hammes and Egli, 2010) and water supply distribution systems (Gillespie *et al*., 2014). One of the major benefits to this approach is that it overcomes the limitation of HPC which can only measure the small proportion of culturable organisms and takes several days to complete. As FCM captures all of the cells in a sample, it provides new insights into the changes in microbial counts across treatment processes where previously HPC had identified no change. Pilot scale systems investigated using FCM include clarification, filtration, ion-exchange, granular activated carbon, membrane and oxidation processes (Hammes *et al*., 2008; Ho *et al*., 2012; elmi *et al*., 2014). However, there is a paucity of robust data available on microbial reductions across live operational drinking water treatment works (WTWs) (Safford and Bischel, 2019). This missing infiormation is important to establish the appropriate data for risk assessments designed to improve the resilience of water supply systems and ensuring the consistent provision of safe drinking water (WHO, 2011).

The WHO recommend that water safety plan (WSP) risk assessments are carried out to effectively understand the risks and effectiveness of treatment barriers (WHO, 2011). As WSP assessments require knowledge of the potential bacterial removal rates at each treatment stage, providing definitive data of the process removal rate can increase the confidence in these estimates. Conventionally, this has been achieved by using culture-

based approaches or surrogate parameters such as *E. coli* counts, turbidity, particle counts or chlorine dose to derive an assumed rate of removal. Such parameters are dis-similar in their nature to the organisms present in natural environments. For example, *E. coli* is much more susceptible to chlorine than many bacteria found in real source waters (Leziart *et al.,* 2019). In addition, other surrogates such as turbidity are not representative of bacteria at all (Schijven *et al*., 2011; Hijnen, 2008). FCM offers the potential for direct rapid measures of bacterial removal rates (denoted as LogR) and hence the ability to populate WSPs with appropriate and dynamic data.

There have been a number of comprehensive reviews outlining the key advantages and suitability of FCM for various areas of application (Van Nevel *et al*., 2017; Safford and Bischel, 2019; Wang *et al*., 2010). Van Nevel *et al*. (2017) demonstrated that there was no conclusive link between HPC and FCM using a large dataset from a variety of drinking water sources (chlorinated and unchlorinated) (n >1,800). However, key questions remain concerning where FCM be used to provide genuine benefit? Accordingly, the aim of this paper was to investigate the potential of flow cytometry in two core areas: (1) the relationship between FCM and regulated indicator organisms and (2) the suitability of FCM as a tool for monitoring bacterial removal and inactivation throughout multiple barriers of treatment. To achieve this water samples from 213 active drinking water sites were sampled over 3 years for indicator organisms, FCM and HPC (n = 39,340). To the authors' knowledge, this is the largest FCM dataset of its kind in the world.

2.2. Materials and Methods

2.2.1. Sampling procedures

Water quality data from final water samples at 213 WTW across Scotland were analysed over a period spanning January 2014 until December 2016. For raw water, microbial water quality data was available between October 2015 and December 2016. These WTW represented a wide range of different source waters, treatment processes and disinfection practices (Table 2.1). In order to understand the variation in process performance, three WTW were selected for further investigation involving inter-stage sampling. These WTW represent the three most common treatment configurations used in Scottish Water, namely conventional coagulation-clarification-filtration (Conventional), direct depth filtration (Direct Filtration) and membrane filtration (Membrane) (Table 2.2). Each sample was collected at least in triplicate and performed according to regulatory sampling procedures. Prior to sampling, sample taps were flushed for 3 min followed by flame sterilisation. A subsequent 30 second flushing step was employed before the sample was collected into a sterile 500 mL sample bottle (Aurora Scientific, Bristol, UK.) containing a pre-aliquoted sodium thiosulphate dose for quenching of chlorine. Samples were refrigerated and transported to the laboratory for analysis within 24 hours of sampling.

Table 2.1. Overview of source water types and disinfection practice for the 213 WTW included in this study.

	Source type			Residual disinfectant	
	Groundwater	Surface water	Mixed supply	Free chlorine	Chloramine
WTW number	22	185	6	173	40
Percentage	10%	87%	3%	81%	19%

Table 2.2. Drinking water treatment trains of the three selected WTW.

	Treatment train sampling points					
Conventional	Surface water (Loch)	Coagulation	Dissolved air flotation (DAF) clarifier	Rapid gravity filtration (RGF)	Chlorination (Ct* = 8 mg.min/L)	Clear water tank (CWT) storage
Direct depth filtration	Surface water (Loch)	Coagulant Flash mix	-	RGF	Chlorination (Ct* = 14 mg.min/L)	CWT storage
Membrane filtration	Surface water (River)	Coagulation	-	Submerged hollow fibre (HF) ultrafiltration (UF) membrane	Chlorination (Ct* = 12 mg.min/L)	Chloramination

**Ct calculated as: Average free chlorine residual at disinfectant stage outlet (mg/L) x residence time at typical flow (as determined by tracer test, AWWA t_{10} methodology) (mins) = Ct (mg.min/L)*

2.2.2. Standard chemical analysis

Free and total chlorine measurements were carried out using the N, N –diethyl-p-phenyldiamine (DPD) colorimetric method and a Hach pocket colorimeter (Hach-Lange, Salford, UK). Total organic carbon was determined using a Formacs high temperature catalytic combustion system (Shimadzu, Milton Keynes, UK). Turbidity analysis was carried out using a Hach-Lange TU5 turbidity meter.

2.2.3. Standard microbiological analysis

Heterotrophic plate counts (HPC) were determined in accordance with the Standing Committee of Analysts (2012) procedure. A 1 mL volume of sample was mixed with 17.5 mL of molten Yeast Extract Agar (YEA, OXOID, UK) and incubated at either 37 ˚C (HPC 37) for 44 hours or 22 ˚C (HPC 22) for 68 hours to give total viable counts at 37 and 22 ˚C (TVC37 and TVC22), respectively. Analyses were performed in duplicate. The determination of coliforms, including *Escherichia coli*, was carried out in accordance with Standing Committee of Analysts (2016) procedures. A 100 mL sample volume was filtered onto a 0.45 µm membrane filter which was transferred to an MLGA plate (OXOID, UK). Plates were incubated at 30 ˚C for 4-5 hours followed by 14-15 hours at 37˚C.

2.2.4. Flow cytometry fluorescence staining and analysis

Flow cytometry analysis was carried out using a BD Accuri C6 flow cytometer with a 488 nm solid-state laser equipped with an auto-sampler (Becton Dickinson UK. Ltd, Oxford, UK). Sample volumes of 25 µL were analysed at a fast flow rate of 66 µL min^{-1} in accordance with the rapid method described previously (Nevel *et al*., 2013). In cases where the event rate was greater than 1500 s^{-1}, the samples were diluted with 0.1 µm

filter sterilized mineral water (Evian, Évian-les-Bains, France). To prevent large particulates entering the FCM system raw water samples were briefly shaken before filtration through 11 µm pore size filters (Grade 1 Whatman filters, GE Healthcare) to remove large particles which may clog the flow cytometer. The green fluorescence was collected in the FL1 channel at 533nm and red florescence in the FL3 channel at 670 nm with the trigger set on FL-1. A signal threshold on the green fluorescence at FL1 = 600 was applied. No compensation was used. All staining parameters were as described elsewhere (Gillespie *et al*., 2014). For the analysis of TCC and ICC the fixed gate described previously (Gatza *et al*., 2013) was used as a template.

2.2.5. Data analysis

Statistical data processing was carried out using MS Excel. Spearmans rank analysis was used to describe monotonic relationship between variables and the Pearsons rank was used to describe linearity. All FCM data analysis were carried out using the BD Accuri C6 software.

2.3. Results and Discussion

2.3.1. Water Quality

Over the sampling period only 92 samples were positive for coliforms resulting in a compliance of 99.76%. Of these samples, 9 were positive for *E. coli* (99.97% compliance) (Table 2.3). The coliform detections occurred at 58 individual WTWs from a variety of treatment configurations: Conventional = 27; Direct filtration = 30; Membrane = 23; Other (including borehole abstraction) = 12. In total, 93% of the coliform detections were associated with surface water (SW) sources.

Table 2.3. Microbial water quality data for all samples between January 2014 and December 2016 (n=39,340).

Parameter	Units	Min	Lower quartile	Median	Upper quartile	Max
Intact cells	Cells/mL	<100	100	240	720	73,322,000
Total cells	Cells/mL	<100	700	6,000	1,054,598	89,912,000
% Intact cells	%	0	1	4	20	100
HPC 22	CFU/mL	0	0	0	0	300
HPC 37	CFU/mL	0	0	0	0	300
Coliform*	CFU/100 mL	1	1	1	2	8,400
*E. coli**	CFU/100 mL	0	0	0	0	10
Enterococci	CFU/100 mL	0	0	0	0	4
Free chlorine**	mg/l	0.03	0.40	0.60	0.74	2.20
Total chlorine**	mg/l	0.03	0.62	0.77	0.97	2.20
pH		5.8	7.7	7.9	8.2	10.8
Post flush temp	˚C	0.0	6.7	9.6	13.0	29.0
Total organic carbon (TOC)***	mgC/l	0.20	0.30	0.70	1.30	31.0
Turbidity***	NTU	0.20	0.20	0.20	0.20	6.10

Only samples where a value >0 were included due to the low number of positive results (coliform n = 92, E.coli n = 9), ** The LoD for chlorine analysis was 0.03 mg/L * The LoD for TOC and turbidity was 0.2 mgC/L and 0.2 NTU respectively.*

Large variations in TCC and ICC in final water were observed from the FCM data, reflected by a broad interquartile range (IQR) of 640 cell/mL for ICC (267% of median) and 1,053,898 cells/mL for TCC (176% of median). This difference was largely a reflection

of the impact chlorine-based disinfection has on cell integrity, resulting in much lower ICC levels. The HPC data was skewed as a significant proportion of the samples did not result in any colony growth, a feature common with culture-based techniques and a limitation in their use for analysing removal processes (Kong *et al*., 2015). Post-flush water temperatures and total organic carbon (TOC) were representative of typical seasonal variations in climate and water quality across Scotland and was similar to that described elsewhere *(*Golea *et al.,* 2017).

2.3.2. Relating FCM data to coliform detections

To elucidate potential links between FCM and regulatory monitoring, only samples containing a positive coliform result were analysed (n = 92) to prevent skewing of the data by the high proportion of blank results observed. Importantly, coliforms were detected in samples that appeared in the lowest cell-count band of the FCM (<1000 ICC/ml), such that it is currently not possible to set an FCM threshold or prescribed value that could be used to replace regulatory sampling. To demonstrate, while the percentage of samples where no coliforms were detected was high at 99.76% for samples where the FCM cell counts were <1,000 ICC/mL (Figure 2.1a), there were still more than 60 samples where coliforms were observed (Figure 2.1b). Five times fewer positive coliform samples were observed for cell counts between 1,000 and 10,000 ICC/mL, but coliform compliance remained at 99.7% overall due to the much lower number of samples in this category. As the ICC concentration increased above 10,000 cells/mL, the frequency of positive coliform samples began to increase relative to the number of samples, where a maximum value of 10.71% of FCM samples resulted in positive coliform detections for the highest recorded intact cell counts (1,000,000-

10,000,000 ICC/mL). Similar observations were seen when the coliform data was considered relative to the proportion of intact cells (ICC/TCC percentage) (Figure 2.1c), although the relationships were less pronounced. Above an ICC/TCC of 50%, the coliform detection rate increased by almost 1.5-fold to 0.31% and for those samples where the intact cell ratio was greater than 75% the relative coliform detections almost doubled to 0.44% compared to samples where the percentage ICC was less than 50% (Figure 2.1c). *E. coli* detections were consistent with the coliform data, but the trend was less pronounced due to the relatively small number of positive samples (*n = 9).* These results show that although the probability of coliform detections increased with increasing cell counts, the highest frequency of coliforms occurred at <1,000 ICC/mL and show that an FCM count threshold is not suitable for regulatory purposes from a classical hygienic perspective.

However, FCM provides positive bacterial counts for all samples, avoiding the large non-detected sample sets with zero culturable counts associated with indicator organism measurements. Relationships between each of the FCM parameters of TCC, ICC, and ICC/TCC% with coliform counts were poorly correlated, with a maximum Pearson correlation coefficient observed of R = 0.13 for the ICC ratio (ICC/TCC%) against coliforms (See Table S1, Supporting Information). Spearman correlations were somewhat higher for the ICC/TCC against coliforms, showing there was a weak correlation (ρ = 0.35), while the ICC and TCC had lower ρ values of 0.32 and 0.23 respectively. The results were comparable to Pearson coefficients obtained by Ellis *et al*. (2013) for culture-based bacteriological failures against surrogates such as average temperature (R = 0.23) and rainfall (R = 0.17), supporting the view that most parameters cannot be accurately linked

to bacterial detections from culture based methods. Given that most regulatory regimes worldwide are heavily reliant on culture methods for bacterial detection this further confirms the view that FCM is not an appropriate replacement, but should rather be considered as an additional diagnostic tool.

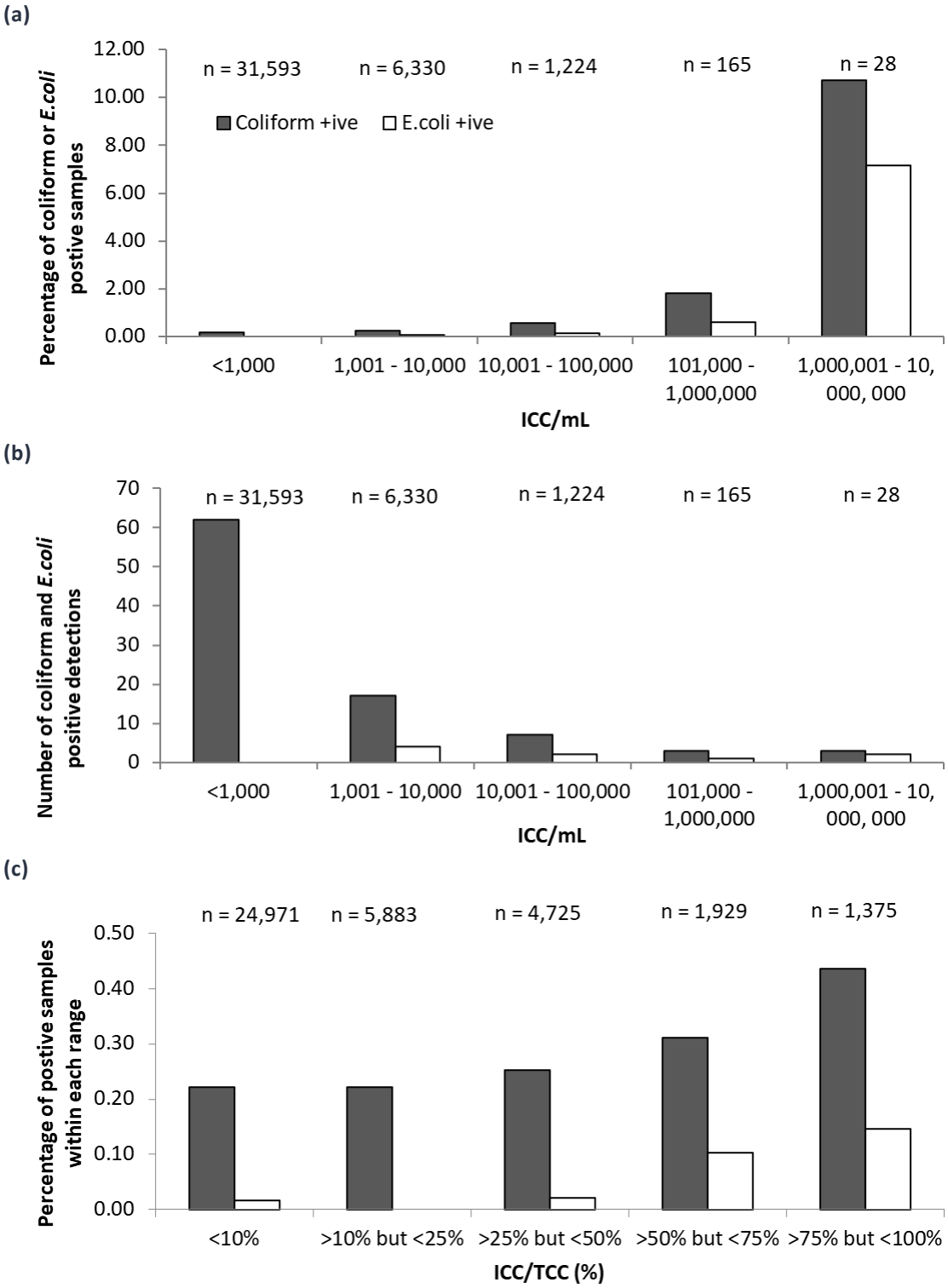


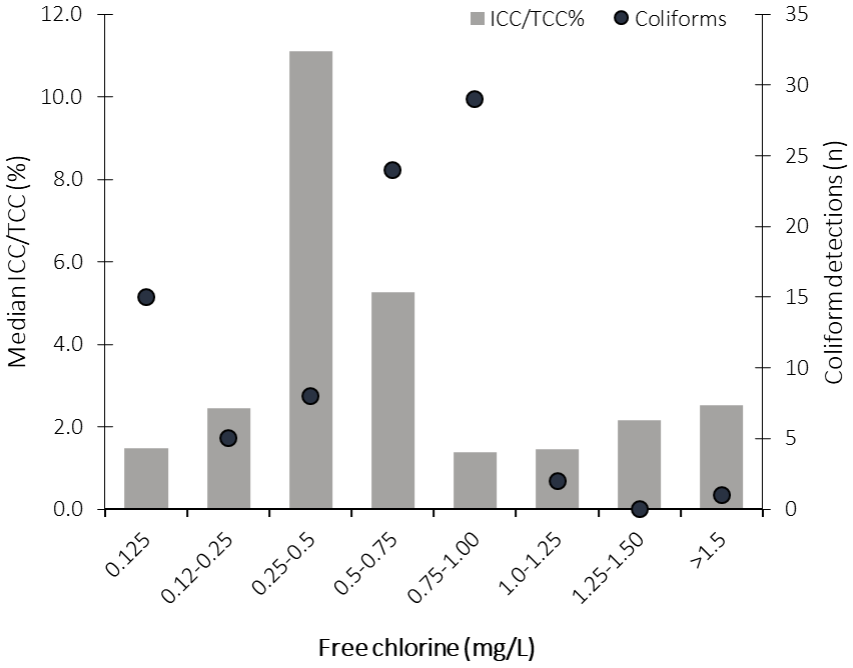
Figure 2.1. (a) Intact cell counts grouped on a logarithmic scale with the percentage of coliform or E. coli detections within each group and (b) the number of positives within each range; (c) The ratio of intact to total cells (ICC/TCC%) with respect to the percentage of coliform and *E.coli* detections. n is the number of samples within each group.

2.3.3. Chlorine residual and contact time

Assessment of the efficacy of disinfection was undertaken by analysing samples that were taken following storage and before the distribution network. These samples therefore represent the water quality leaving the WTWs. Analysis of the relationship between the chlorine residual and positive coliforms showed that free chlorine below 1.0 mg/L was coincidental with nearly all of the positive coliform samples obtained from plate counting (Figure 2.2a). Only three instances were observed where coliform failures occurred above 1.0 mg/L free chlorine (Figure 2.2a), aligning to previously reported instances of coliform growth in elevated chlorine concentrations (Chevallier *et al.*, 1996). From the FCM, a maximum ICC/TCC of 11.1% was observed at a free chlorine concentration of 0.25-0.50 mg/L with a minimum ratio of 1.4% at elevated concentrations of 0.75-1.0 mg/L. This was consistent with previous trials on three WTW with final waters that had chlorine values in the range of 0.5-3.0 mg/L (residual concentration range 0.42-0.51 mg/L) where the ICC/TCC varied between 2.6 and 6.0% (Nescerecka *et al*., 2014). When the distribution network is considered, Gillespie *et al*. (2014) showed that the free chlorine residual had a more obvious influence on ICC/TCC% and a threshold of 0.50 mg/L limited the increase in cell viability. The results also align with the increasing detection of indicator organisms in water distribution systems as the chlorine concentration in the network decreases (Ellis *et al.,* 2013). In these cases, detections at the network extremities were linked to microbial regrowth due to loss of chlorine residual in the network, rather than the direct impact of the disinfection process at the WTWs.

There were stronger relationships between microbes and the product of the chlorine concentration (C) and the disinfection contact time (t), the Ct value. In the present study, bacterial detections were more likely when Ct values were lower. Similarly cell viability (ICC/TCC) increased as the Ct decreased, although the important threshold was different between the FCM and culture data (Figure 2.2b). For coliforms, detections were greatest for sites were Ct was <10 mg.min/L (n=45). As the Ct increased, detections reduced substantially as the Ct range increased to 10-20 mg.min/L (n=19) and more then halved for sites that had Ct between 20-30 mg.min/L (n=8). Above this Ct range, no further reduction in coliform detections took place. When using the FCM, the most significant reduction in cell viability took place when the Ct increased >30 mg.min/L resulting in an ICC/TCC of 1%. At lower Ct values, the ICC/TCC ratio reduced gradually from 3.6 to 4.6%. These data show that significant cell viability was still present between Ct between 20-30 mg.min/L, conditions when culture based coliform detections were minimised. This was a result consistent with laboratory studies where loss of cell culturability has been seen before loss of cell membrane integrity for *E. coli* on exposure to increasing Ct (Virto *et al*., 2005, Nocker *et al*., 2017). The results demonstrate that Ct is the driving factor in determining the efficacy of disinfection and shows that understanding of disinfection is enhanced through the use of FCM. The importance of this from a water safety risk view has yet to be established due to the unknown relationship between potential pathogens and bacterial counts from the FCM. However, the presence of significant viable bacterial populations at Ct below 30 mg.min/L implies that disinfection protocols should be revisited where they have been based on culture-based methods.

(a)



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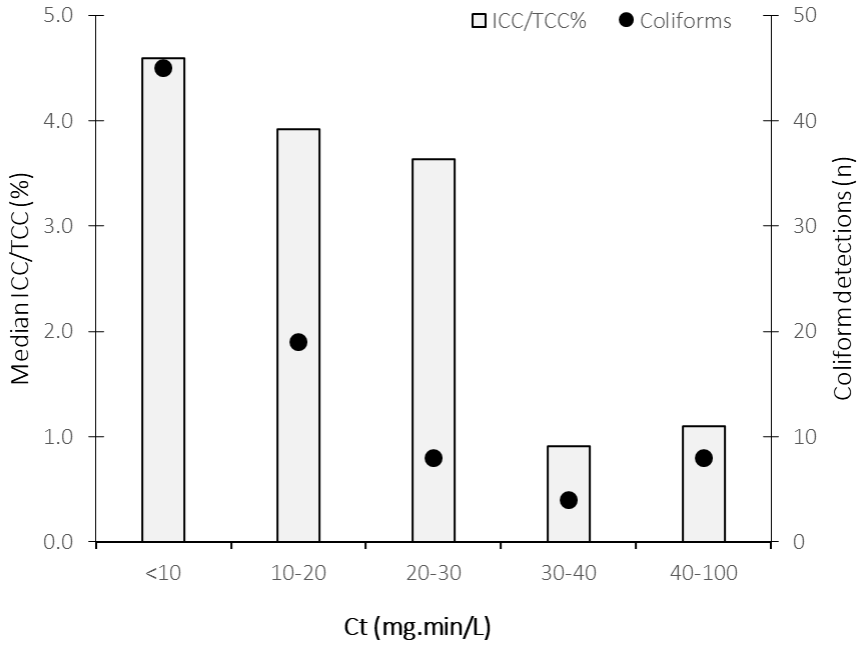


Figure 2.2. (a) The median percentage of intact cells (ICC/TCC%) and positive coliform samples against the residual free chlorine (n = 33,854 - only sites with Ct data); (b) The median ICC/TCC% against the tracer derived Ct at the disinfection stage of each WTW an

2.3.4. Detailed assessment of treatment performance

For each of the WTW types investigated in more depth, the final cell counts were relatively stable irrespective of the raw water inlet counts, showing there was no relationship between the TCC or ICC microbial loading into the WTW and the bacterial levels seen in the final treated water (Figure 2.3a). However, the conventional WTWs received water containing more TCC than for the other two sites, with 84% of samples containing $>1x10^6$ cells, while this was 62 and 67% for the Direct Filtration and Membrane site, respectively. As a result of the higher loading, final TCCs at this WTWs were higher than for the other two WTWs. This was not the case for the ICCs, where again the loading ono the WTWs was highest for the Conventional site, with more samples containing $>1x10^6$ cells. However, ICC in final water were of the same order of magnitude as for the Direct Filtration WTWs, while the Membrane WTWs contained the lowest range of ICCs showing that the treatment processes themselves were controlling bacterial cell. This observation was consistent with that seen by Besmer and Hammes (2016) who have shown that even significant bacterial increases (up to five-fold), measured at higher resolution (20 min online sampling) in the source water, were not seen in the final produced water from the WTW.

The increased granularity of data obtained from the FCM enabled effective assessment of the robustness of performance across the three WTWs (Figure 2.3b). For example, final treated water samples containing less than 1,000 ICC cells/mL were observed in 98.5%, 88.9% and 64.6% of cases for the Membrane, Conventional and Direct Filtration WTW respectively, showing a clear distinction in removal of viable bacteria between the different treatment types. The profile for the TCC in final water was similar as for the

ICC, with the Membrane WTWs resulting in lower cells counts than for the other two WTWs. In this case the 90th percentile was 1000 cells/mL for the membrane site, 15,000 cells/mL for the direct filtration site and 150,000 cells/mL for Conventional WTWs. These results were in-line with the higher loading of TCC onto the Conventional WTWs compared to the Direct Filtration site.

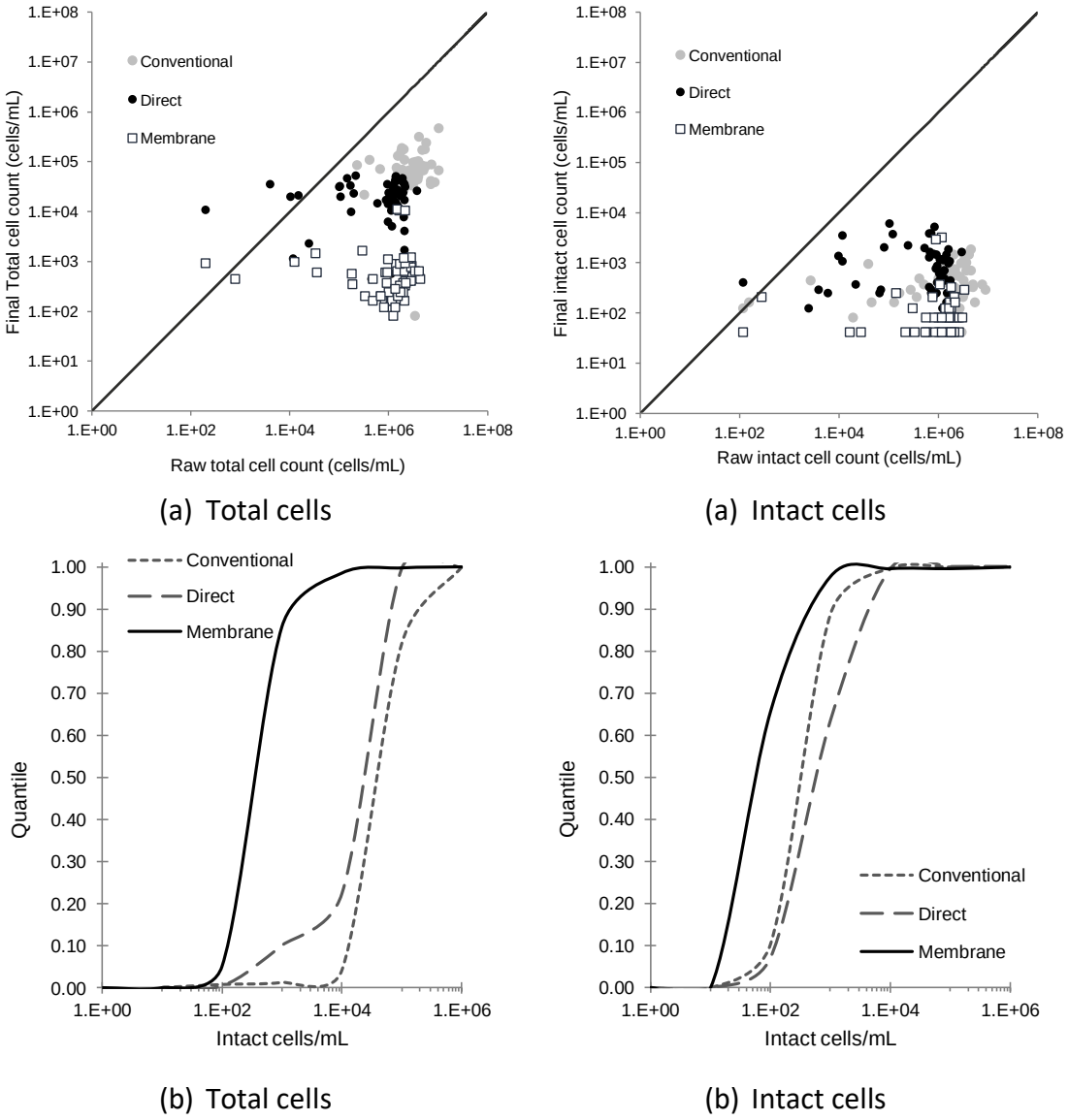


Figure 2.3. (a) Raw and final intact and total cell counts for each of the WTW, solid line represents the y=x; (b) Cumulative frequency distributions of intact cell count at each of the three WTW.

There were a total of nine positive coliform detections seen in treated water over the sampling period across the three WTW: three at the Conventional site; two at the Direct Filtration site; and four at the Membrane site (Figures 2.4-6). At the Conventional WTW, no specific changes in cell concentration were observed for TCC or ICC when the three coliform detections occurred. For the Direct Filtration WTW, there was an increase in the final water ICC after 850 days, increasing from $5x10^2$ to $1x10^4$ cells/mL (Figure 2.5). It was during this period that two failures at this site occurred. At the Membrane WTW, the cell counts in the final treated water were the lowest of the three WTW studied, having median cell counts of <100 cells/mL compared to 360 and 320 cells/mL at the conventional and direct sites respectively (Figures 2.4-6). There was a large spike in the intact cells on day 854 ($1.66x10^5$ cells/mL) in the final water. No coliforms were concurrently detected but 11 days later a positive sample was observed. There were no obvious explanations for the other three detections. The results show that while there were some co-incidental occurrences of higher FCM cell counts with positive culture based samples, most of these detections could not be explained by a higher frequency of cells as measured by the FCM. In fact, seven of the nine coliform events occurred when the ICC concentration was less than 1,000 cell/mL, a result consistent with the observations seen from the overall data (Figure 2.1).

With respect to the HPCs, the data was again sporadically spread, with many non-detected samples. However, the frequency of positive samples was greater than for the coliform detections. The highest frequency of HPC positive detections were at the Membrane WTW = 116 (57 at 22°C, 59 at 37°C); Direct filtration WTW = 59 (31 at 22°C, 28 at 37°C); and Conventional WTW = 48 (25 at 22°C, 23 at 37°C). This was the opposite

order to the cell concentrations observed using FCM and again demonstrates the changing picture that can be developed with the richer data sets generated when using a non-culture-based technique such as FCM. This would, for example, enable investigation into other important features of a WTW system, such as process age, operational practice and process transients. The HPC were also poorly associated with the FCM cell count data, with no trends apparent between the two parameters. Likewise, the coliform and HPC counts were not coincidental, with only one of the coliform detections having a positive corresponding HPC count (this was the first detection at the Conventional WTW on day 530 (Figure 2.4b)).

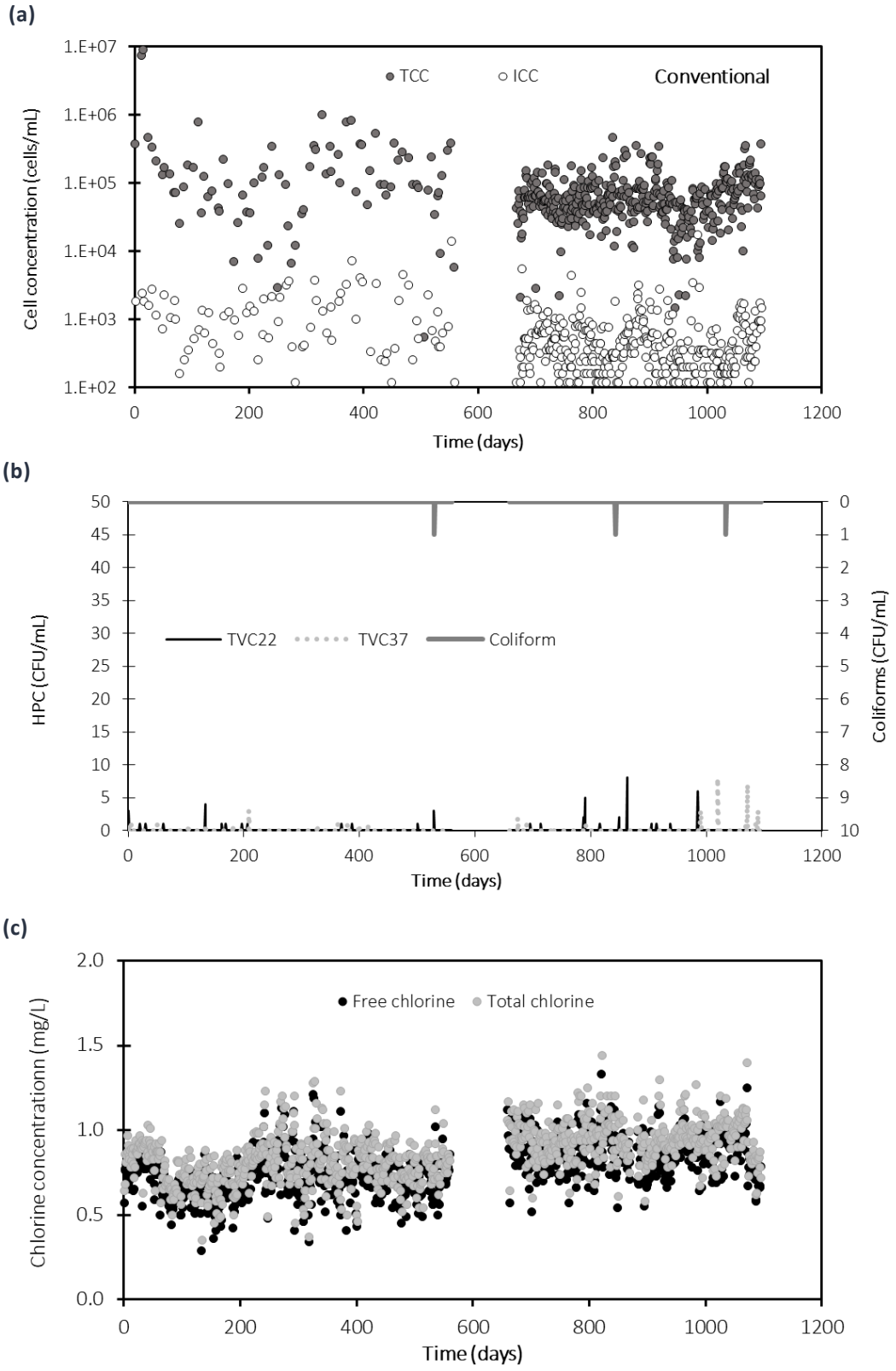


Figure 2.4. Final water quality trends for the Conventional WTW, frequency of Flow Cytometry analysis was increased from every 3 days to daily for the second half of the study period (n=1103) for: (a) Flow cytometric cell concentrations (total and intact); (b) culture derived data, and (c) final water chlorine concentrations.

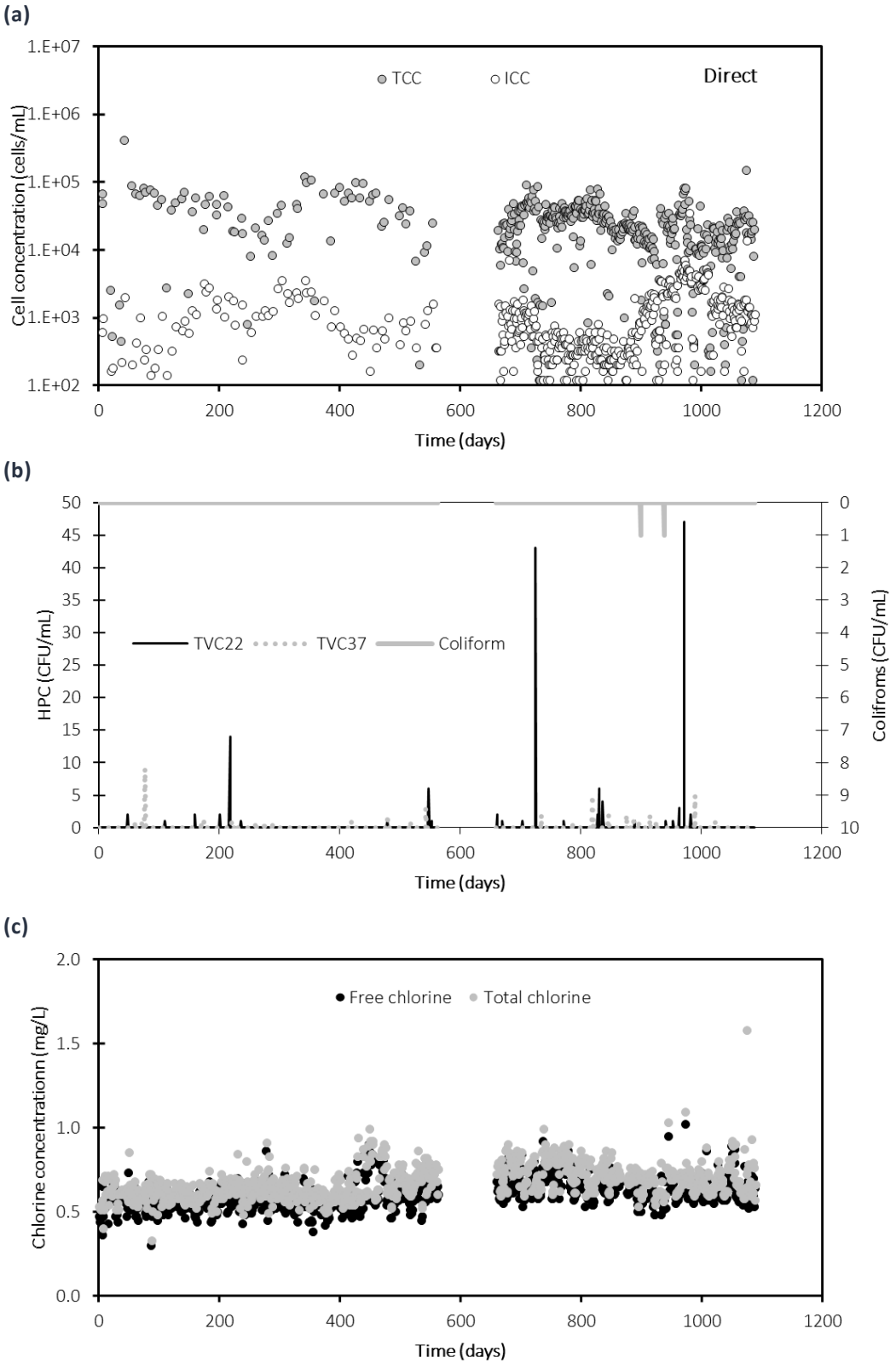


Figure 2.5. Final water quality trends for the Direct filtration WTW, frequency of sampling was increased from every 3 days to daily for the second half of the study period (n=1095) for: (a) Flow cytometric cell concentrations (total and intact); (b) culture derived data, and (c) final water chlorine concentrations.

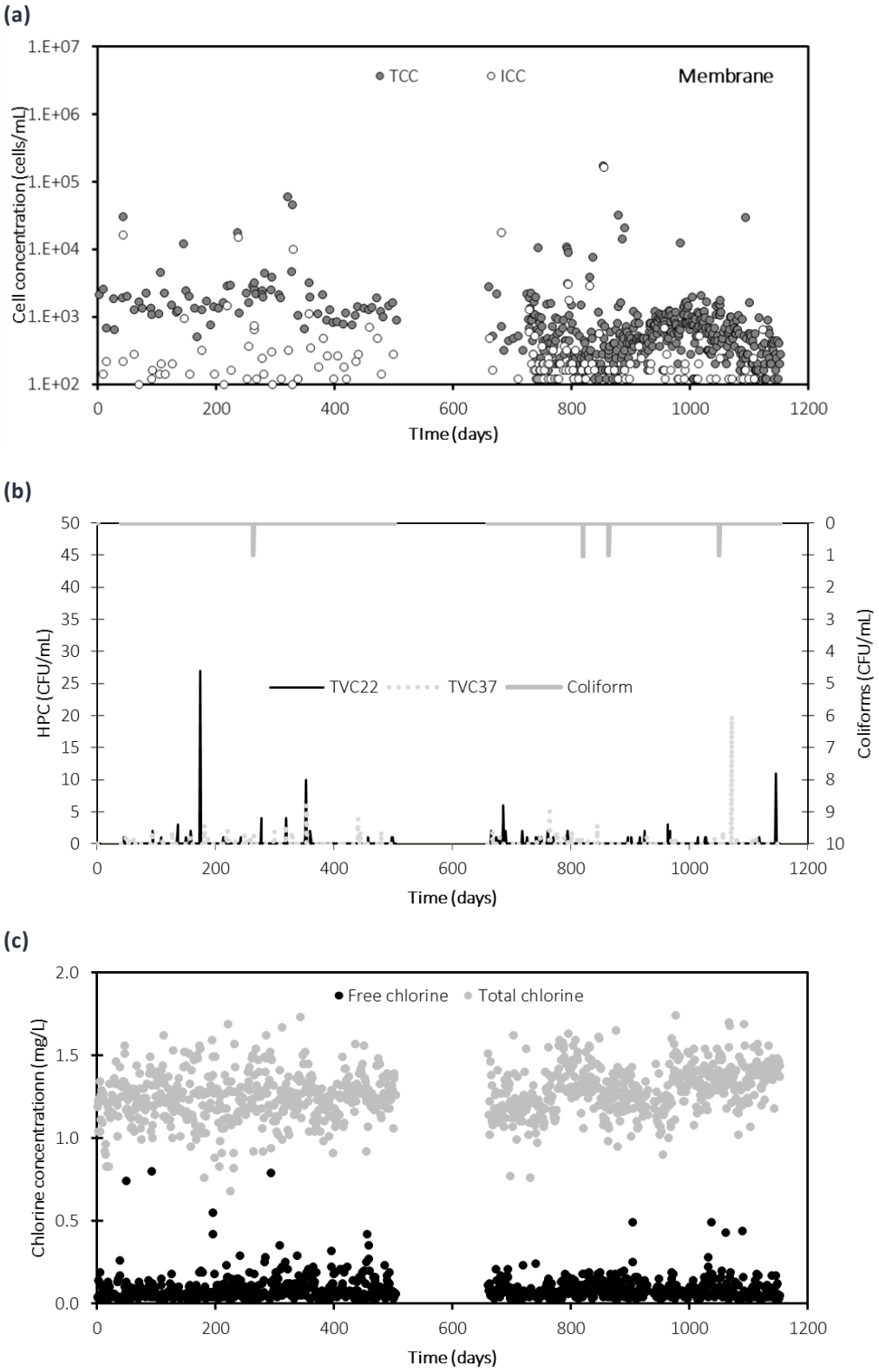


Figure 2.6. Final water quality trends for the Membrane WTW, frequency of sampling was increased from every 3 days to daily for the second half of the study period (n=1203) for: (a) Flow cytometric cell concentrations (total and intact); (b) culture derived data, and, (c) final water chlorine concentrations: note that this site practiced chloramination, hence the difference between the free and total chlorine. TVC22 and TVC37 are total viable counts at 22 and 37°C, respectively.

2.3.5. WTW log removals as an indicator of bacteriological performance

As noted, one of the opportunities provided by FCM is the ability to provide appropriate removal data for water safety plan risk assessments. Bacterial removal rates across whole WTWs and between treatment stages were therefore calculated for the three selected sites. The median TCC log removal (LogR) across the Conventional and Direct Filtration WTW was similar at 1.73 and 1.76 LogR, respectively (Figure 2.7). The ICC removal was much higher at both of these sites with logR of 3.77 and 3.22 for the Conventional and Direct Filtration sites respectively. These data show that at clarification sites, the processes do not provide a complete barrier for removal of cells and rely on the chlorination stage to significantly reduce the viable cell concentration. The presence of the DAF process at the Conventional WTWs facilitated enhanced removal of intact cells, although this was not the case for total cells. While not established directly in this work, this can be attributed to improved capture of viable cells during the extended coagulation/flocculation stage (Ho *et al*., 2012). These results contrast with those seen for the membrane WTWs that provides an absolute barrier to particulates. In this case, both the TCC and ICC LogR was much higher at 3.55 and 4.17, respectively. This data agrees with the rankings established previously when only final water cell counts were considered (Figure 2.3b).

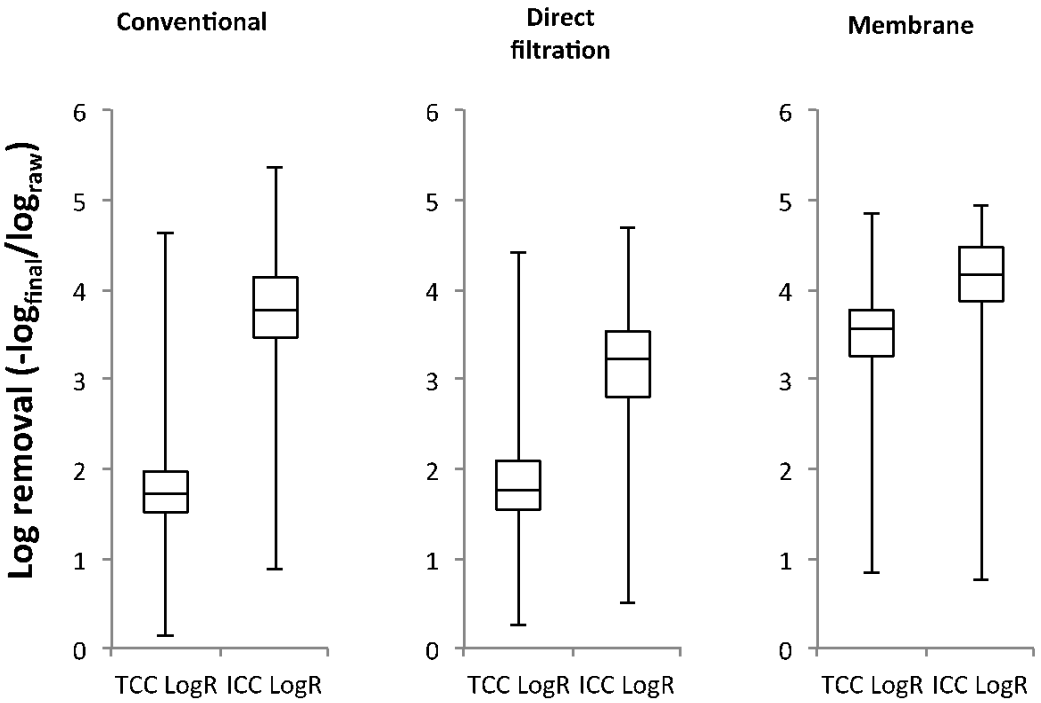


Figure 2.7. Log removal of total and intact cells across three WTW between November 2015 and December 2016.

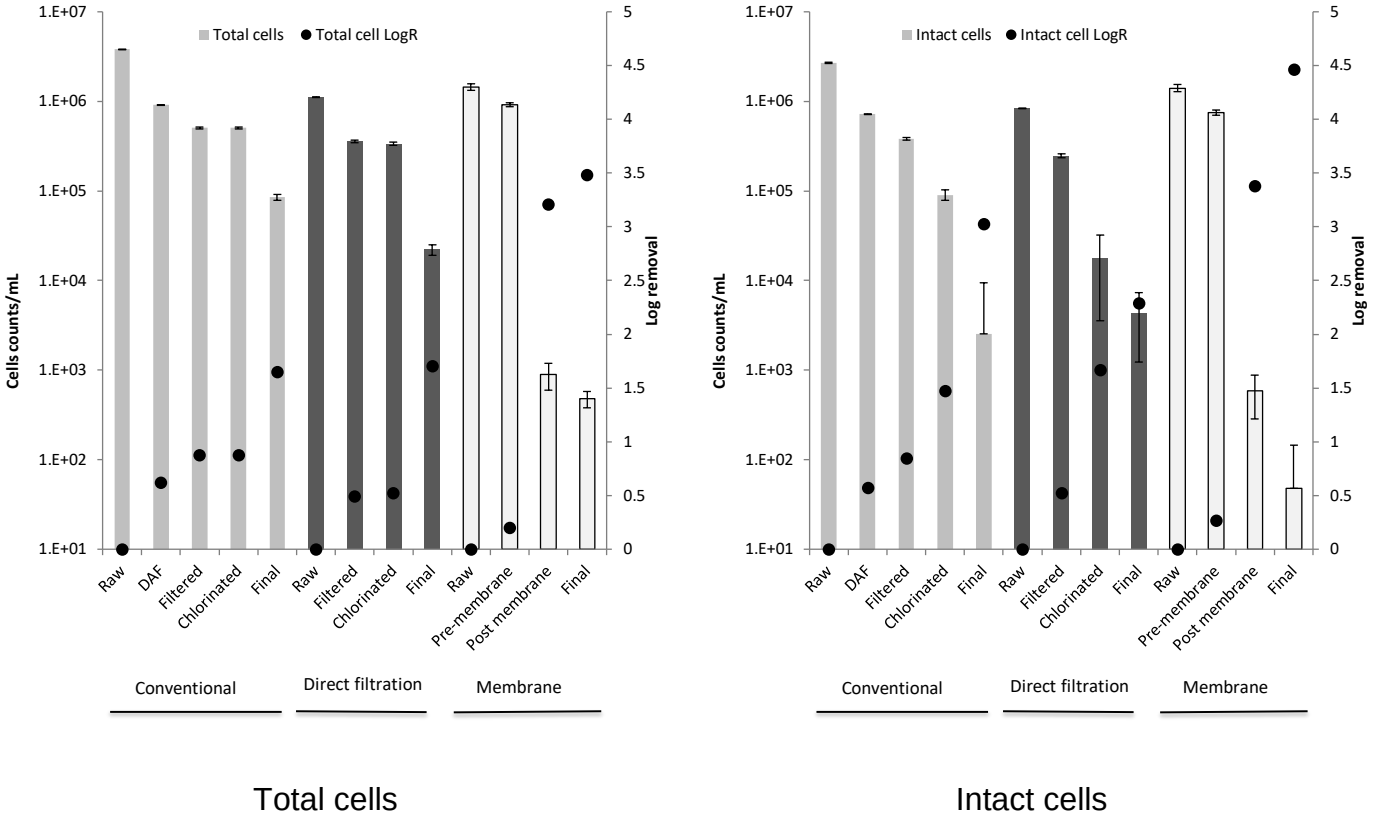
To investigate the microbial removal at these sites in more detail and understand where the microbe removal occurred, inter-stage sampling was carried out (Figure 2.8). Overall, ICC LogR for the Conventional, Direct Filtration and Membrane WTWs were 3.03, 2.29 and 4.47 respectively. Equivalent figures for the TCC logR were 1.6, 1.7 and 3.5. These results were within the ranges shown from the long-term evaluation (Figure 2.7). The removal across the rapid gravity filters at the two depth filtration sites were 0.26 and 0.50 TCC LogR and 0.27 and 0.52 for ICC logR for the Conventional and Direct Filtration WTWs, respectively. However, when the combined removal across the DAF and RGF was considered, overall TCC and ICC LogR were 0.85 and 0.88, a removal greater than that seen for the Direct Filtration site and that there was no apparent difference in the removal of intact and total cells. This shows the beneficial role that effective

coagulation and clarification processes can have in reducing cell and particle loads onto filters, as well as producing particles that are effectively removed by depth filter capture mechanisms (Ho *et al*., 2012). Previous assessment of RGF has shown a higher average LogR of 1.07±0.82 from operational sites (Helmi *et al*., 2014; Hijnen, 2008). The present results also sit at the lower end of the LogR range suggested by WHO for high rate filtration of between 0.2-4.4 (WHO, 2011). Variation in particle and bacteria removal can be expected across processes due to the impacts of contact time, media choice, media arrangement, temperature (changes of which can affect bed expansion), time in run and backwash rates (Hijnen, 2008). However, the current data indicates that previously utilised removal credits may be over estimates and that more work is required especially around the different aspects of the batch operating cycle of filters, a task well suited to FCM.

Cell removal across the membrane at the Membrane WTW was 3.01 and 3.38 for TCC and ICC LogR with cell counts that were below 1000 cells/mL for both parameters, levels that were not seen for the conventional and direct filtration WTWs. These results were comparable to the 3.11 LogR observed by Hammes *et al*. (2008) following UF membrane treatment of GAC permeate. The performance of the membrane was consistent with them acting as an absolute barrier for particles above the pore size of the ultrafiltration membrane, around 0.01 µm. Bacterial cells can be in the size range of 0.5 to 100.0 µm, although typical cells are no larger than 2 µm (Madigan *et al*., 1997). Accordingly, positive cell counts post membrane were attributed to the non-sterile environment downstream of membrane systems (Hammes *et al*., 2008) including biofilm growth in the downstream tanks (Zanetti *et al*., 2010). The other cause of detection would be a

loss of membrane filtration integrity (Krahnstöver *et al*., 2018). The regular monitoring of the permeate by FCM offers great potential as an easy approach for membrane filtration integrity monitoring through the identification of a sudden increase in cells.

The chlorination stage was the most significant contributor to intact cell log reduction at 0.63 and 1.14 ICC LogR for the conventional and direct filtration site respectively. These reductions increased to 1.76 and 2.18 when sampled after the final clean water storage (CWS) tank. While no real change in TCCs occurred when samples were taken after the chlorine contact tank, the LogR of TCC across the CWS was 0.77 and 1.18 for the conventional and direct filtration sites respectively indicating a near total loss of cell integrity, such that many of the remaining cells could not be identified by the FCM. This near complete disintegration of cell structure is known to be the last stage of the chlorine mode of action on bacteria (Xu *et al*., 2018). As the ICC and TCC load onto the disinfection stage was substantively lower at the membrane site than that for the other WTWs, the LogR across disinfection was lower at 1.09 and 0.27. The variation in LogR across the disinfection stage and the CWS tank between the sites were also a reflection of differences in the type of disinfection process used (chlorine and chloramination) residence time, and variations in flow behaviour, factors known to influence disinfection efficacy (Haas and Engelbrecht, 1980; Falconer, 1986).



Total cells Intact cells

Figure 2.8. TCC and ICC with corresponding cumulative log removals (circles) for the three study sites: conventional, direct filtration and membrane WTWs.

2.4. Conclusions

It has been demonstrated in this study that FCM will best benefit the water industry by its use in tandem with current regulatory methods. While there was an increasing probability of coliform detection with increasing cell counts, most positive coliform samples were coincidental with low FCM counts of <1000 ICC/mL. Furthermore, the FCM results did not distinguish between different types of microorganism present in the sample. As a result FCM cannot be used to provide hygienic information about water quality and so is not a suitable replacement for indicator organism monitoring. However, the sporadic detection of bacteria using culture based methods makes FCM the most suitable tool for process monitoring. The 'Ct value' was identified as the key factor for suppression of intact cells and reducing the number of coliform detections in final

drinking water, with Ct >30 mg.min/L being effective at reducing both variables to a minima. FCM was able to provide accurate bacterial cell reduction across treatment processes, providing data that can be used for risk assessments in water safety plans Furthermore, FCM offers opportunities for rapidly diagnosing where critical bacteriological barriers are not performing effectively. It was shown that raw water cell counts were not related to final treated water intact cell numbers. Instead, the final water cell counts were mostly driven by individual treatment processes with membrane treatment having the highest removal efficiency of ICC and TCC. Chlorine disinfection was the main way by which ICC were reduced for the Conventional and Direct Filtration WTWs. This reinforces the importance of maintaining each treatment barrier without reliance on physical or chemical processes alone to deal with microbiological challenges. In the future, it would be beneficial to develop FCM (or align it with other techniques) to be able to quickly and accurately distinguish between different types of bacteria based on their pathogenicity, such that the hygienic risk of the water can be more rapidly assessed.

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Supporting Information

Table S1. Pearson and Spearman correlations of coliform positive samples (n = 92) to FCM parameters.

	Pearson R	Spearman, ρ
ICC vs coliform	-0.0002	0.3215
TCC vs coliform	-0.0095	0.2330
%ICC Ratio vs coliform	0.1254	0.3411

3. Assessing chlorine disinfection efficacy using flow cytometry and viability staining: the impact of pH, temperature and dose conditions

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Chapter context

In chapter 2, it was demonstrated that FC was not comparable to traditional micriobiological culture data. One of the key findings of that chapter was that disinfection drives cell counts obtained by FC. Therefore, it is important to investigate whether FC can be utilised to assess disinfection processes over the typical range of variables. The following chapter aims to address thesis objective 2:

> *"Carry out bench scale disinfection experiments to understand how flow cytometric data responds to typical operational parameters that impact disinfection efficacy."*

Abstract

Monitoring the effectiveness of chlorine disinfection processes is paramount to maintaining wholesome water is supplied to the consumer. In this study the disinfection efficacy of chlorine, over a range of typical water treatment conditions was assessed by flow cytometry (FCM) on both a pure culture (*E. coli)* and a natural water taken from the outlet of a rapid gravity filter (RGF) at an operational WTW. A dose dependent increase in inactivation rate was observed for both test matrices, with k values of -0.03 to -0.26 and -0.32 to –3.14 for the WTW filtrate and *E.coli* respectively. A decrease in disinfection efficacy was observed as temperature decreased from 19°C to 5°C. A series of pH conditions (pH 6, 7, 8) were tested and no change in efficacy was observed across the pH range for the *E.coli,* whilst for the WTW filtrate an increase in efficacy was observed at the alkaline pH and a decreasing in activity at acidic pH. This paper demonstrates that FCM is an effective tool for assessing bacterial inaction, although that there are fundamental mechanisms of chlorine disinfection or methodological alterations required to fully utilize this tool over the range of chlorination conditions observed in an operational environment.

3.1. Introduction

Microbiologically safe drinking water (DW) is a priority in any water supply system and disinfection using chlorine is commonplace for the inactivation of pathogens. Typically, disinfection is the final stage at a drinking water treatment works (WTW), inactivating microorganisms before water enters storage and distribution. The mechanism of action of free chlorine on bacterial cells relates to oxidative damage to membranes, nucleic acids, proteins, amino acids, cell walls and other lipids causing a loss of viability (Haas and Engelbrecht, 1980). Previous studies have demonstrated that the exposure required for a specified inactivation of 3 log (99.9%) varies significantly, with *Staphylococcus epidermis* almost 10 fold more resistant to chlorine than *Escherichia coli* and *Mycobacterium aurum* 1,000 times more resistant to the latter (Helbling and VanBriesen, 2007). Consequently, the operating conditions need to be set appropriately to ensure effective disinfection of the microbial community present within the water at any given moment. There are known to be four main factors affecting the efficiency of disinfection: *Free chlorine concentration, exposure time, temperature and pH.* Operationally, this is delivered by the 'Ct' concept, whereby disinfection is achieved by allowing sufficient contact time (residence time = t) between the water and chlorine disinfectant (Concentration = C). The World Health Organisation recommends a minimum Ct of 15 mg.min/L is required, where the concentration of free chlorine is 0.5 mg/L, the residence time is 30 min, and water pH is less than 8 and the turbidity is less than 1 NTU (World Health Organisation, 2011). When dissolved in water, free chlorine dissociates forming a pH dependent equilibrium between the uncharged HOCl and the charged OCl^- species. At pH <7.5, the HOCl form dominates while at higher pH, OCl^- is

the prevalent ion. HOCl is reported to penetrate through the membrane cell wall more effectively than the charged OCl^- species, a mechanism known as the permeability theory (Haas and Engelbrecht, 1980). Accordingly, established practice is to operate disinfection under lower pH environments to maximise inactivation rates (White, 2010). However, Friberg (1956, 1957) showed an increased cellular uptake of chlorine by *E. coli* in alkaline environments. While the authors did not clarify this mechanism, Haas and Engelbrecht (1980) have hypothesised that there is increased loss of cellular material at low pH leading to reduced cell associated chlorine as a result of increased disinfection in the cell at low pH.

Assessment of the efficacy of disinfection is typically based on the measurement of heterotrophic bacteria (HPC) (Reasoner, 1990), although this has been questioned due to the prevalence of viable but non culturable (VBNC) organisms (Oliver, 2005). It has been estimated that less than 1% of the total bacterial population can be enumerated using plate counting methods and hence 99% exist in the VBNC category (Berney *et al*., 2008). The VBNC state can be induced in bacteria in a number of ways, such as sub lethal injury (chlorination, UV disinfection, ozonation), inadequate growth media or conditions, or an excess non-heterotrophic organisms (Health Canada, 2012).

Flow cytometry (FC) is a modern, high throughput diagnostic method that detects bacterial cells irrespective of the culturable state and so offers an alternative to traditional microbial monitoring approaches (Nevel *et al*., 2013). FC is gaining popularity in the water industry for analysis of water throughout treatment works and in supply systems (Hammes *et al*., 2008; Gillespie *et al*., 2014; Prest *et al*., 2013). FCM has been

shown to have a wide variety of applications such as monitoring bacterial numbers throughout treatment and distribution (Nescerecka *et al*., 2018), monitoring natural water sources (Besmer *et al*., 2016) and evaluating biological process performance (Ziglio *et al*., 2002). Furthermore analysis is rapid with results available in under 20 min, requiring little sample preparation and high throughput can be achieved using well plates that take up to 96 samples (Nevel *et al*., 2013). FC works through the application of dyes that bind to DNA, excitation of these dyes by a laser allows for the fluorescence emission to be captured in a range of photomultiplier tubes (PMTs) with specific wavelength bandpass filters. The most common dyes applied for the measurement of bacterial cells are SYBR Green I (SG) and Propidium Iodide (PI). Application of SG alone allows for quantification of the 'total cells (TCC)' and a combination of SGPI allows 'intact cells (ICC)' to be counted (Hoefel *et al*., 2003). The distinction between total and intact cells is possible due to the fact PI is a membrane impermeable dye, and as such it is only able to bind inside the cell and fluoresce once membrane integrity is compromised (Virto *et al*., 2005).

Total cells are often not considered when assessing disinfection efficacy as the primary concern is around the culturability and viability of organisms, which is more often linked to the integrity of a bacterial cell. In fact, Xu *et al.* (2018) concluded that after exposure to free chlorine, *E. coli* inactivation occurred by the loss of the following attributes in the following order: culturability, membrane integrity (SGPI/ICC), Adenosine Tri Phosphate (ATP, Cellular), mRNA, DNA, complete cell damage (total cell degradation). This would therefore indicate that the mechanism of inactivation of chlorine starts with initial suppression of cultivation followed by increasing damage to cellular functions before

complete cell destruction after prolonged exposure. Therefore, ICC would be a more conservative measure of inactivation whereas TCC is not representative of viability as this is the final step of inactivation.

Whilst applied across a range of application areas, to date no work has been reported utilising flow cytometry to assess chlorination over a typical range of operational conditions. Given FCs ability to measure VBNC and differentiate between intact and total cell numbers (viability) it is posited that the use of FC can provide fresh insights into the chlorination process. Following on from chapter 2, where the suitability of FC as a tool for the water industry and how this approach can be applied was considered(Research objective 1), the observation that Ct drives both coliform detections and the percentage viability of cells warrants further investigation. Accordingly, the current paper critically assesses the impact of dose, pH and temperature on the kinetics and efficacy of disinfection with free chlorine comparing both laboratory cultured and real environmental samples to satisfy Research objective 2.

3.2. Methods and Materials

3.2.1. Demand free glassware

All glassware was prepared to remove all possible chlorine demand during the tests, in accordance with Charnock and Kjønnø (2000). Glassware was initially machine washed at high temperature with detergent, thereafter it was rinsed three times with ultrapure water. These were then filled to the neck with 0.2 M HCl and left to stand overnight. After the acid wash the glassware was rinsed three more times with ultrapure water, air-dried and then capped with foil. The final amounts of trace carbon removal was achieved by heating to 550°C for at least 6h. To remove demand from plasticware a concentrated solution of 1% sodium hypochlorite was left to stand in plasticware overnight, the vessels were then washed three times with ultrapure water, left to air dry and capped with foil prior to use.

3.2.2. Cultivation of E.coli

E.coli (ATCC 25922) was cultured in 10% tryptose soy broth (TSB) for 15 hours at 30°C in order to reach the stationary growth phase (10^9 cells/mL). The bacterial culture was then transferred aseptically to a sterile plastic centrifuge tube and centrifuged at 6000g for 5 min to form a pellet. The supernatant was removed and added to the centrifuge tube with an equal volume of 0.1 µm filtered Phosphate buffer (10 mM, pH 7). The wash step was repeated three times. A final experimental concentration of 10^5 intact cells/mL was used, representative of typical cell counts of drinking water prior to disinfection.

3.2.3. Real water sample

Water samples from two conventional drinking water treatment works (Treatment train = Surface water, coagulation, flocculation, clarification, rapid gravity filtration,

disinfection, storage and distribution) was obtained from the outlet of the rapid gravity filter on two active sites (Named 'WTW Filtrate A' (pH 6.8); 'WTW Filtrate B (pH 6.4)'). The samples were representative of water being presented to the subsequent disinfection stage prior to chlorine addition at the treatment works.

3.2.4. Disinfection by chlorine assay

Experiments were carried out in demand free borosilicate glass beakers in triplicate. Phosphate buffer was added to either ultrapure water or natural water (1 to 10 ratio) to provide a final concentration of 10 mM at the required pH (6, 7 or 8). In addition, for the pure culture tests with *E.coli,* NaCl was added at a final concentration of 0.5% to prevent damage to cells due to osmotic shock.. A 200 mL volume of phosphate buffer at the appropriate pH (*E. coli*) or 200 mL of the natural water was added to the beaker. To this the washed E.coli was added if necessary and prior to chlorine addition, the vessel was left for 30 min to equilibrate to test conditions. Concentration experiments were conducted at pH 7 and a range of chlorine doses of 0.12, 0.25, 0.50, 0.75, 1.00 mg/L for E.coli, the 0.12 mg/L test was omitted for the natural water experiment. The pH variable tests were at either pH 6, 7 or 8 and at room temperature. Temperature tests were conducted at pH 7 and at either room temperature, 5°C or 12°C. Chlorine doses for pH and temperature conditions were 0.12 mg/L for *E. coli* and 0.25 mg/L for the filtrate water. Each experiment was stirred continuously using a magnetic stirrer (150 RPM) and stir bar. At desired timepoints (0, 0.5, 1.0, 1.5, 2, 5, 10, 20, 30 min) a 500 µL sample was taken and added to a microcentrifuge tube containing 5 µL of 0.1 N Sodium thiosulphate to quench any residual chlorine. For the long exposure tests the same procedure was

followed but sampling times were increased to 0, 20, 40, 60, 80 and 120 min and doses were 1.00 and 2.00 mg/L free chlorine. These long exposure tests were not replicated.

3.2.5. Flow cytometry analysis

To quantify total cells (TCC) a 10,000× stock of SYBR Green I (cat. S-7567; Life Technologies Ltd., Paisley, UK) was diluted with dimethyl sulphoxide (DMSO) (Fisher Scientific, Fair Law, NJ) to a working 100× concentration stock and added to samples to achieve a final SYBR Green I concentration of 1×. For the quantification of intact cell concentrations (ICC) a dye mixture was made of SYBR Green I and Propidium iodide (PI) (1 mg ml^{-1}, corresponding to 1.5 mM; cat. P3566; Life Technologies Ltd, Paisley, UK) at a ratio of 5:1 respectively. This mixture was added to the SGPI samples to give a final SYBR Green I concentration of 1× and PI of 3 µM. To each sample a 10% volume of Phosphate buffer (100 mM, pH 7) was added to normalize the pH value prior to incubation and analysis. Once the dyes and sample had been mixed, they were incubated for 10 min at 37°C in a plate incubator. For the analysis, a BD Accuri C6 flow cytometer with a 488 nm solid-state laser (Becton Dickinson UK. Ltd, Oxford, UK) was used. 25 µL of sample was analysed when determining the population of bacteria at a flow rate of 66 µL min^{-1} in accordance with the rapid FC method described elsewhere (Van Nevel *et al*., 2013). For the analysis of TCC and ICC the fixed gate described previously (Gatza *et al*., 2013) was used as a template. HNA/LNA analysis was carried out on a histogram plot of green fluorescence, as populations of bacteria can shift on a sample by sample basis the distinction point of HNA/LNA was always placed between the two clear peaks on the sample prior to disinfectant addition at T = 0. All data processing was carried out using the accompanying Accuri C6 software and Microsoft Excel.

3.2.6. Plate reader analysis of Propidium Iodide

Propidium iodide was added to 1 µg of Calf Thymus DNA (Life Technologies Ltd, Paisley, UK) suspended in filter sterilized ultrapure water at a final concentration of 3 µM. Samples were incubated at 37°C for 10 min. After incubation samples were transferred to a 96 well flat-bottomed black plate where an equal volume of phosphate buffer (10 mM) was added to achieve the desired pH (3, 4, 5, 6, 7, 8 and 9). Fluorescence was then measured using a Tecan plate reader (Tecan Infinite 200 Pro, Männedorf, Switzerland). Instruments settings were as follows: excitation wavelength of 485 nm (excitation bandwidth = 9 nm), emission recorded at both 620 nm and 670 nm (Emission bandwidth = 20 nm) and gain = 120.

3.3. Results and Discussion

3.3.1. Chlorine concentration and temperature

To assess the effect of chlorine exposure on membrane integrity, five concentrations of chlorine dose were applied to either pure culture *E. coli* (N_0 = 1.18 x 10^5 ± 1.5 x 10^4 ICC/mL) or the WTW Filtrate (N_0 = 1.45 x 10^5 ± 2.5 x 10^4 ICC/mL) (Figure 3.1a,b). With regards to the *E. coli* inactivation, at the point of chlorine addition (t = 0.5 min) there was a consistent log reduction of intact cells that was more pronounced at higher chlorine doses. For instance, after one min of exposure, log reductions of greater than 1.0 were detected once the chlorine concentration exceeded 0.25 mg/L. At doses below that the average log reduction after one min was 0.3. However, complete inactivation was still observed after 1.5 min, a result in line with the other doses (Figure 3.1a).

WTW Filtrate A required much higher chlorine exposures to see the same log reduction values as seen with *E. coli* (Figure 2.1a p27). Controlled experiments demonstrated stability of the cells in WTW A filtrate over the test period. At the point of chlorine addition (t = 0.5 min) the maximum log reduction of 0.3 was seen at a dose of 1.00 mg/L whereas the lowest of 0.12 was observed at a dose of 0.25 mg/L. Unlike with *E. coli* it was possible to distinguish the log reduction curves at each chlorine dose throughout the test period. A log reduction of greater than 2 was only observed at chlorine doses of 0.75 and 1.00 mg/L (Figure 3.1a). The stark contrast in susceptibility to chlorine was demonstrated by the inactivation rate constant (k) at each chlorine dose (Figure 3.1b). The rate constant gradually increased as dose increased in the WTW A filtrate tests from k = -0.03 to k = -0.26 whereas the *E. coli* inactivation rapidly increased with increasing dose from k = -0.32 to k = -3.14). The susceptibility of *E. coli* has been demonstrated

previously with a range of 'k' values from -3.75 L/mg.min (Butterfield *et al*., 1943) and -4.71 L/mg.min (Cunningham *et al*., 2008) and even as high as -31.29 L/mg.min (Lee *et al.,* 2010). The contrast in 'k' values obtained between *E. coli* and the real water sample demonstrate the unsuitability of this organism as an overall disinfection efficacy indicator. It was also noted that the inactivation curves for *E.coli* and the natural water showed different curve profiles with a tailing off for the *E.coli* and a shouldering in the natural water, observations which can be attributed to cell clumping (Hiatt, 1964) and a heterogenous bacterial population of variable chlorine susceptibility (Cerf, 1977) (See Appendix A1).

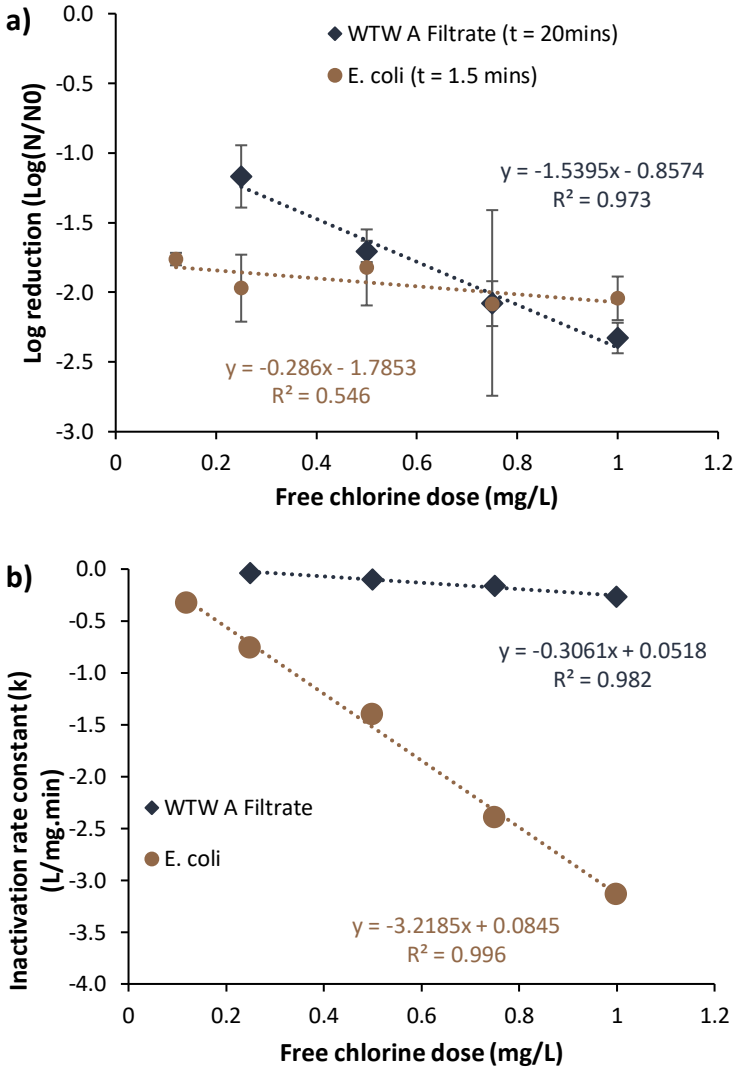

Figure 3.1. Log reduction of *E. coli* (1.5 min exposure) and WTW A filtrate (20 min exposure), SYBR Green I + Propidium iodide (Intact) cells exposed to chlorine dose of 0.12, 0.25, 0.50, 0.75 and 1.00 mg/L. All tests were carried out at pH 7 at room temperature. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats.

A reduction in the rate of inactivation was observed as a function of temperature when comparing trials at 5°C, 12°C and 20°C (room temperature) for both *E.coli* and the treatment works sample (Figure 3.2a, b). At 5°C, log reductions did not exceed 1.6 and 0.73 for *E. coli* and WTW A filtrate, respectively. In comparison, the efficacy of chlorine

inactivation was seen to improve over the first two min at 12°C for the *E.coli* test but then plateaued thereafter leading to no significant difference to that at 5°C after 5 min (Figure 3.2a). In contrast, a significant increase in efficacy was observed at 20°C compared to the two lower temperatures with a maximum log reduction of 2.13 after 5 min. A different result was observed when testing the real water sample whereby no significant difference was observed between 12°C and 20°C, with log reduction levels of 2.13 after 5 min. In this case, a difference was only observed between the 5°C test and the two other higher temperatures, with the colder conditions resulting in a lower log reduction of 0.72 (Figure 3.2b).

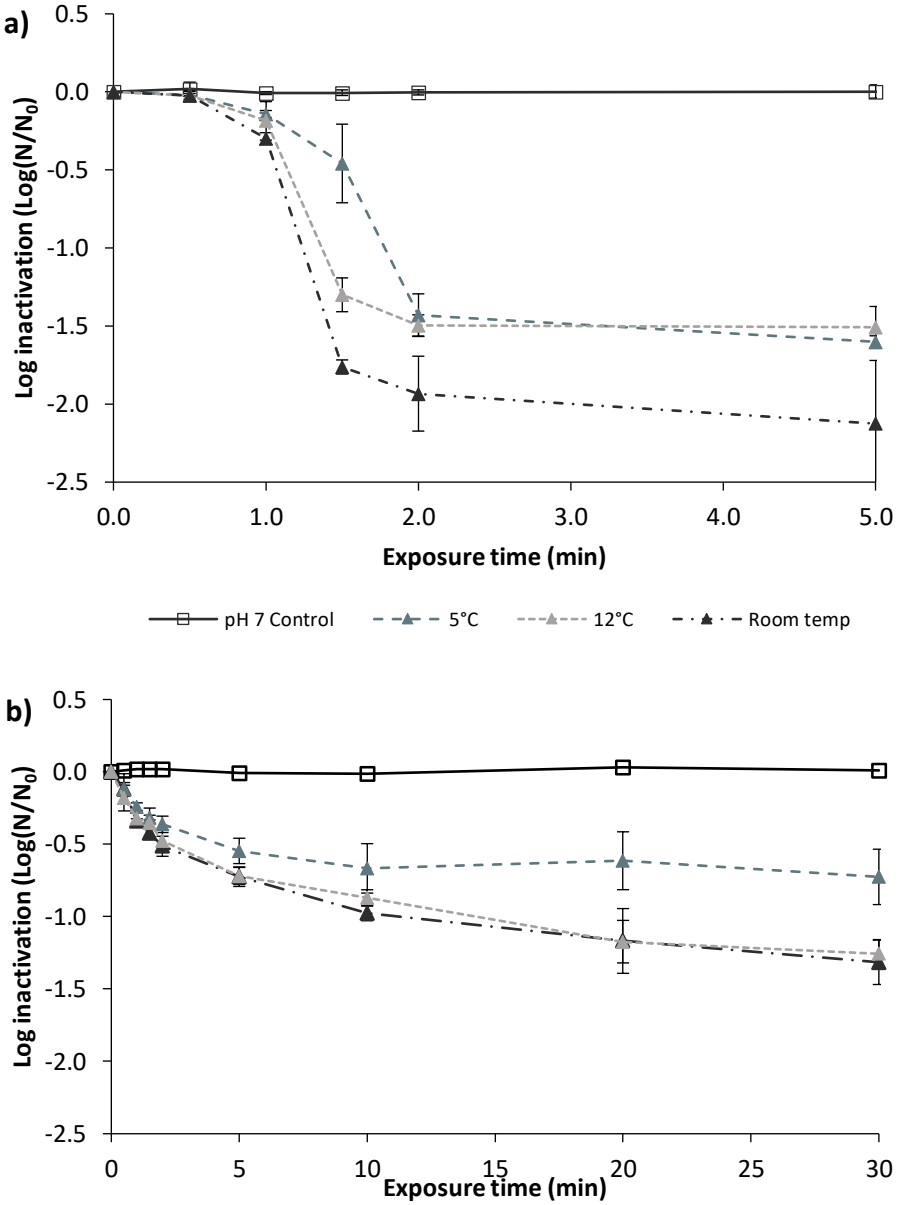

Figure 3.2. Log inactivation of *E. coli* cells exposed to a chlorine dose of 0.12 mg/L across a temperature range (room temperature, 6°C and 12°C) (a). Log inactivation of cells in filtrate water from WTW A exposed to a chlorine dose of 0.25 mg/L at varying temperatures (room temperature, 6°C and 12°C) (b). All tests were carried out at pH 7. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats.

3.3.2. Total cell counts and HNA/LNA responses to chlorination

Unlike ICC measurements, TCC determination only uses SYBR Green I and as such is not expected to be impacted by loss of membrane integrity caused by disinfectants over typical contact periods (30 min). Considering that drinking WTW which apply chlorine typically have at least four hours of contact with the disinfectant based on a typical contact time and subsequent storage before distribution of around 3.5 hours), the impact of this extended chlorine exposure on the TCC was investigated.

Observations from the experiments within this study indicated that *E.coli* cells exposed to a range of chlorine concentrations (Figure 3.3) over 30 min had the highest TCC log reduction of 0.25 at a free chlorine dose of 1.00 mg/L. In the WTW A filtrate only a small decrease in total cells was observed at a chlorine dose of 0.25 mg/L (LogR 0.04), in all other test conditions there was a slight increase in the TCC (See Appendix A1). To further elucidate whether extended chlorine exposure would lead to a decrease in TCC, a second sample from WTW A was subjected to higher chlorine exposures of 1.00 and 2.00 mg/L for 120 min (Figure 3.4). In this second sample there was an increased log reduction in TCC after 20 min exposure at 1.00 mg/L (LogR = 0.14) in contrast to that observed previously (Figure 3.4). After 40 min of exposure TCC began to decrease more rapidly and continued at a similar rate until 80 min exposure. After 120 min a maximum log reduction of 1.76 was recorded. At the higher concentration of 2.00 mg/L the loss of TCC was more prevalent with a maximum log reduction of 1.77 seen after 60 min. This decrease in total cells has been reported previously when chlorine exposures exceeded 0.3 mg/L (Song *et al*., 2019) and is further supported by Phe *et al*., (2005) who suggested

that after 80 min of chlorine exposure the nucleic acids become sufficiently damaged that the binding of the stain is reduced, either due to DNA damage or cell lysis.

FC is not only able to count discrete events ('cells') but also the intensity of fluorescence from each detected event. This fluorescence intensity can be split into two clusters of data, often termed High Nucleic acid content (HNA) or Low nucleic acid content (LNA) based on high or low green fluorescence respectively.

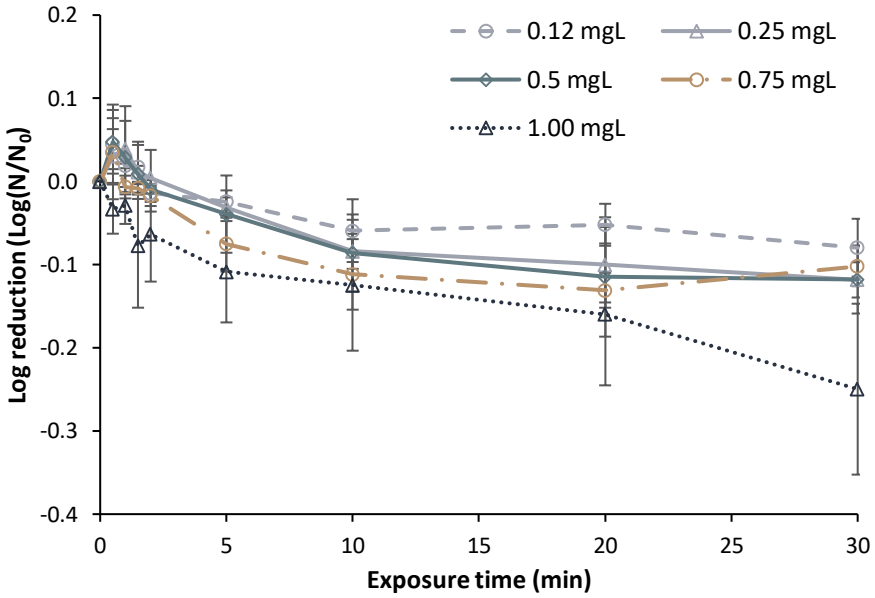

Figure 3.3. Log reduction values for total cell counts of *E. coli* exposed to chlorine concentrations of 0.12, 0.25, 0.50, 0.75 and 1.00 mg/L. Experiments were conducted in chlorine demand free phosphate buffer (pH 7) at room temperature. Error bars represent standard deviation of three independent experimental repeats.

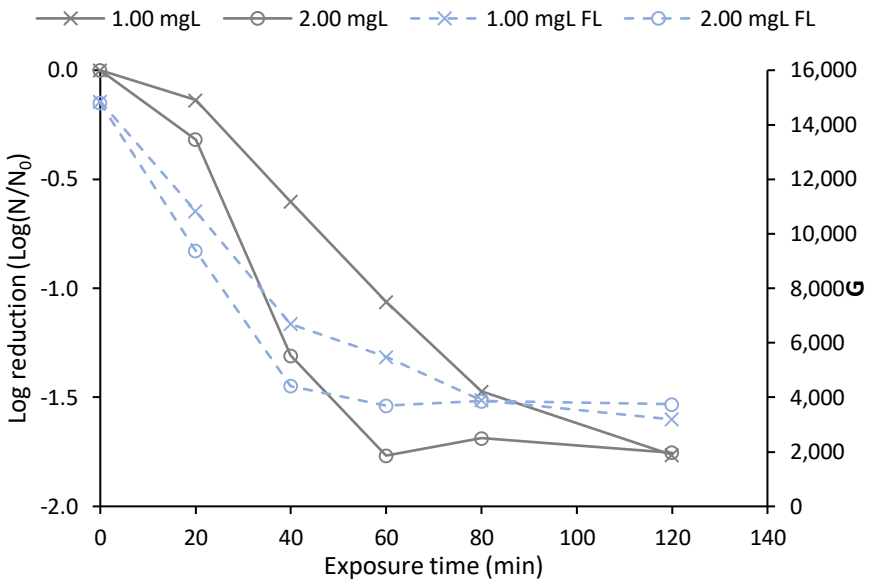

Figure 3.4. Log reduction values for total cell counts of filtrate water from WTW A exposed to chlorine concentrations of 1.00 and 2.00 mg/L. Experiments were conducted at pH 7 and room temperature. Data represents single measured values.

HNA/LNA analysis of total cells (SYBR Green I only) showed that both HNA% and LNA% remained stable up to 60 min exposure at 1.00 mg/L, after which HNA% began to increase with a commensurate reduction in the LNA% (Figure3.5a). At a higher dose of 2.00 mg/L the same pattern occurred after 40 min of exposure and prior to this point the HNA/LNA ratio remained consistent. Log reductions were calculated for each of the HNA and LNA fractions relative to t_0 (Figure3.5b) and showed that there was a greater susceptibility of LNA bacteria to chlorine than HNA. A similar result was observed when considering only intact cells with log reductions of 2.87 and 3.28 for LNA bacteria and 2.10 and 2.08 for HNA bacteria at the 1.00 and 2.00 mg/L chlorine doses respectively (Figure 3.6).

In the current study LNA bacteria were found to be the most sensitive to chlorination. In contrast, Ramseier *et al*. (2010) found HNA bacteria to be slightly more susceptible to

free chlorine at Ct of less than 15 mg.min/L but at 20 mg.min/L the inactivation rate of each group was almost equal.

Looking at the FC dot plots in the present study, it was observed that for these samples there was a common cluster of cells with an approximate green fluorescence value between 4 to 6 x 10^4 (greater than the HNA threshold of 1 x 10^4) that were able to persist even after 120 min of exposure suggesting these are highly chlorine tolerable organisms. This remaining cluster of organisms represented around 2 x 10^3 total cells/mL and 5 x 10^2 intact cells/mL (1.7% and 0.6% of the original count respectively). Such a finding aligns with the fact that some organisms are highly tolerant to chlorine. For example, *Mycobacterium avium* requires a Ct greater than 100 mg.min/L to deliver a 2 log inactivation (Luh and Mariñas, 2007). Considering the natural water in this study was of an unknown heterogenous environmental composition it is plausible that the remaining organisms are chlorine tolerant bacteria, much like the *Flavobacterium* identified in past chlorine disinfection studies of natural drinking water (Wolfe *et al.*, 1985).

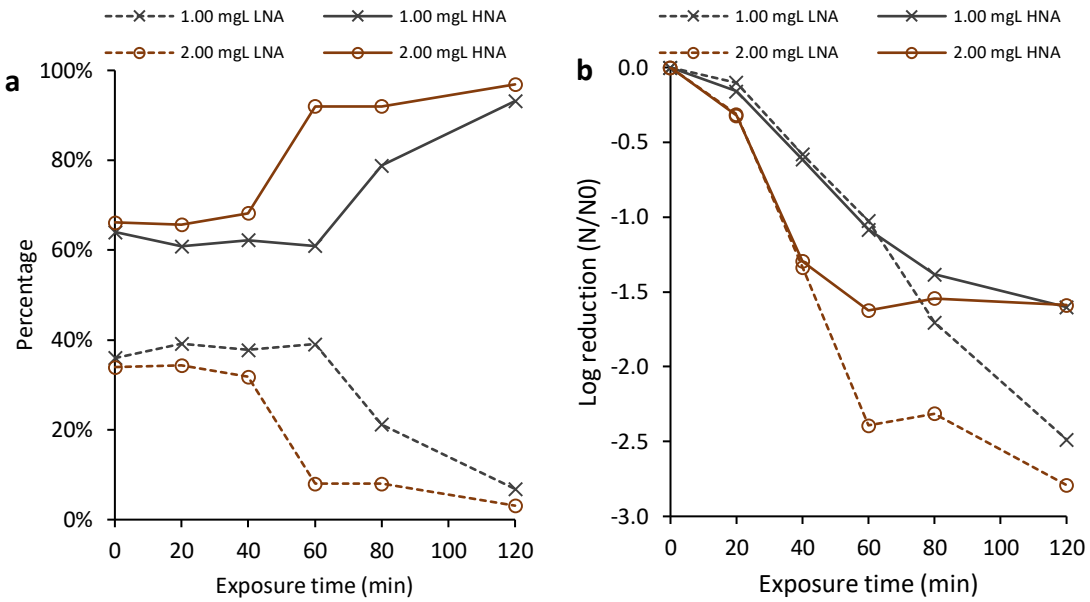

Figure3.5. Total HNA and LNA in filtrate water from WTW A at 1 and 2 mg/L chlorine. Data represents single measured values.

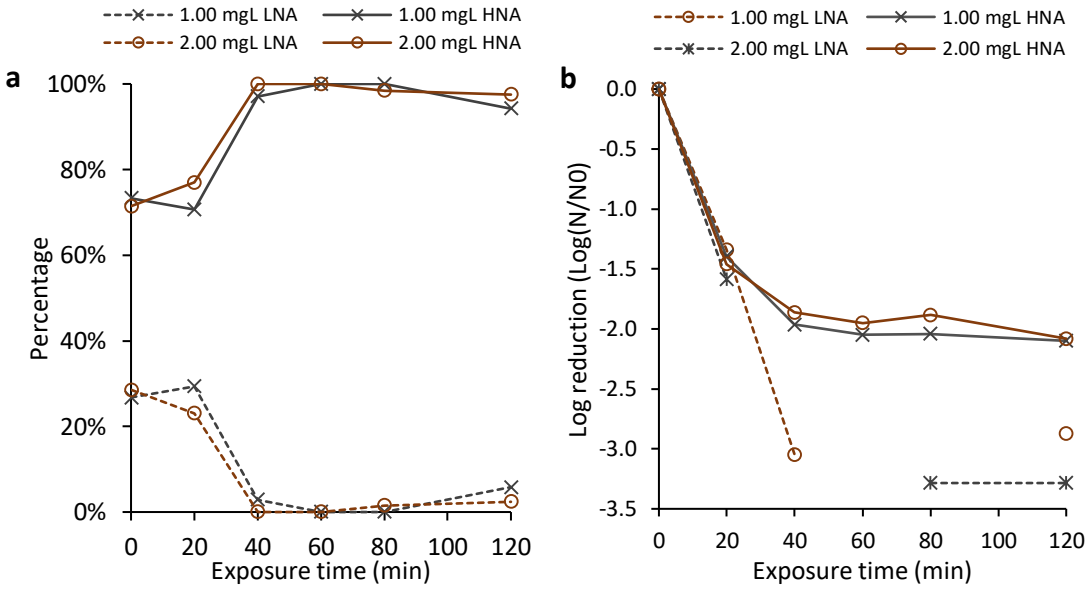

Figure 3.6. Total HNA and LNA from SYBR Green (I) + Propidium Iodide staining (Intact cell) in filtrate water from WTW A at chlorine exposures of 1.00 and 2.00 mg/L. Percentage of HNA/LNA at each time step (a) and log reduction rates for HNA/LNA (b) are displayed. Data represents single measured values.

3.3.3. Understanding the interaction between pH, chlorination and membrane integrity

The impact of pH was assessed at low chlorine doses of 0.12 mg/L (*E. coli*) and 0.25 mg/L (genuine water) to maximise the potential of the observed impacts. The control tests (without chlorine) demonstrated stable responses, indicating that any observed result was due to the addition of chlorine. Overall there was no clear significant difference observed with regards to disinfection efficacy as a function of pH (Figure 3.7). To illustrate, after 2 min of exposure the observed log reductions were 1.93, 1.80, 1.57 for pH 7, 6 and 8, respectively. However, after 5 min there was further reduction to a total of 2.13 log for the pH 7 trials whereas the pH 6 and 8 experiments stabilised to a value of 1.74. In contrast, a clear difference was observed with the real water trial with log reductions of 0.70, 1.15 and 1.32 after 30 min for pH of 6, 8 and 7 respectively (Figure 3.8). A second experiment using a different real water confirmed the observed difference with pH, but with a different sequence such that log reductions of 0.56, 1.31 and 1.63 were seen after 30 min for pH of 6, 7 and 8, respectively (Figure 3.9).

The observed results were unexpected in that disinfection efficacy was enhanced under alkaline conditions. Consequently, additional experiments were undertaken to assess whether the results were an artefact of the method used. The differentiation between intact cells and those with compromised cell walls is through the comparison of the uptake of two dyes. Intact cells repel propidium iodide and are thus stained with SYBR Green I. In comparison, damaged cells will allow propidium iodide into the cell and lead to subsequent displacement of SYBR Green I from the nucleic acids, altering the fluorescence from green to red. To assess whether pH impacts the uptake of PI, the dye

was added to pure Calf thymus DNA, and stained following which the pH was then adjusted (Figure 3.10). Emission at both 670 nm (which replicates the FL3 670/LP bandpass filter of the Accuri C6 FC) and 620 nm (which is the optimum emission point for PI) revealed no direct impact as a function of pH across the range of interest. Similar results have previously been reported for SYBR Green I, indicating that neither of the dyes fluorescence intensity appears impacted by the pH range observed in drinking water analysis and is therefore not an artefact of the applied method.

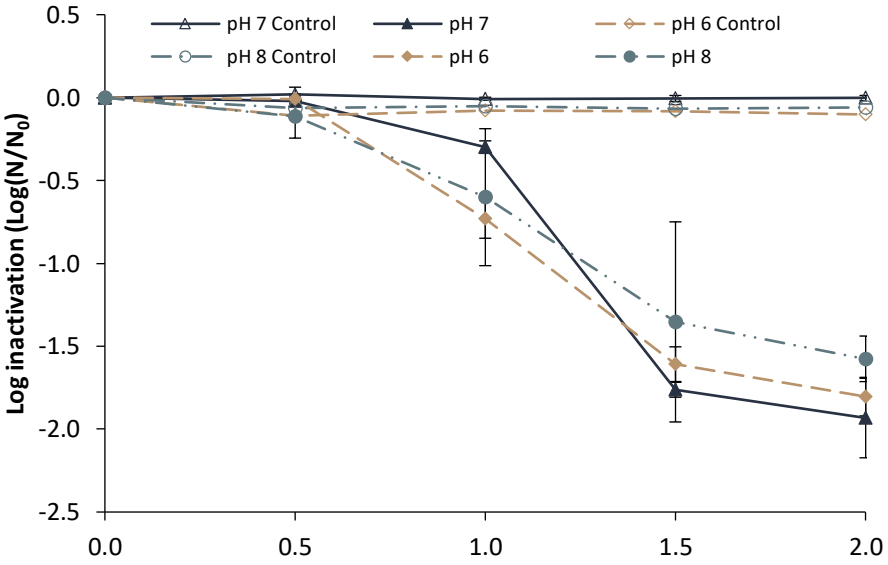

Figure 3.7. Log inactivation of *E. coli* cells exposed to a chlorine dose of 0.12 mg/L at varying pH values (pH 6, 7, 8) at room temperature. All tests were carried out in demand free phosphate buffer. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats.

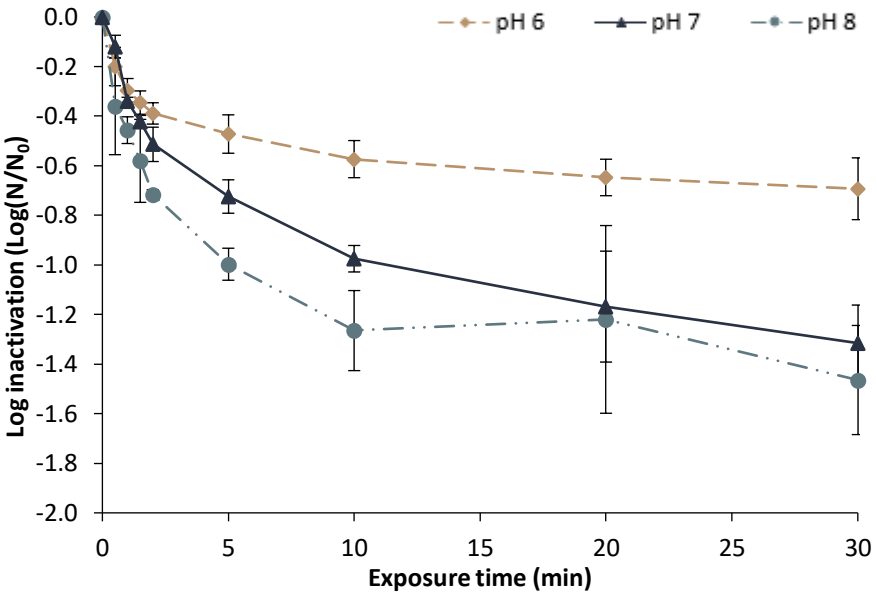

Figure 3.8. Log inactivation of filtrate water from WTW A exposed to a chlorine dose of 0.25 mg/L at varying pH values (pH 6, 7, 8) at room temperature. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats.

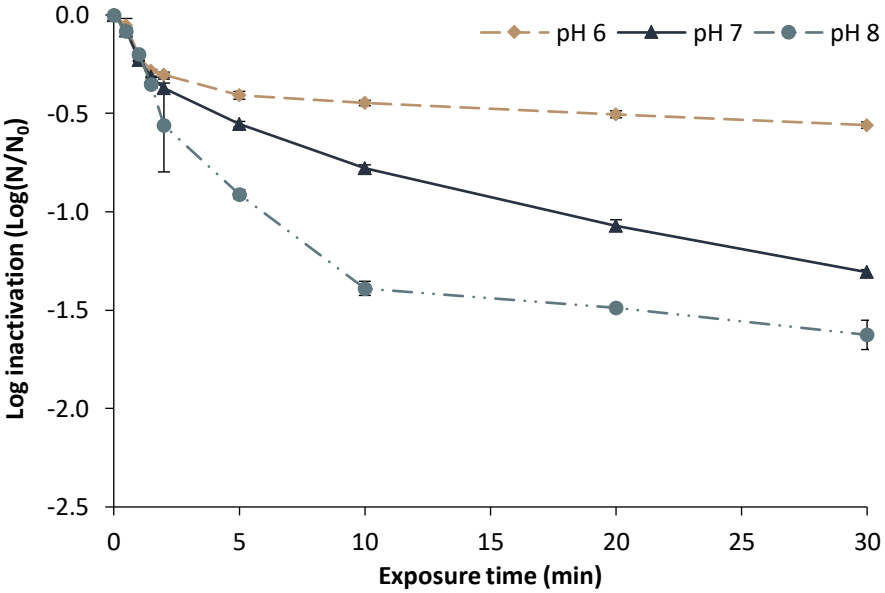

Figure 3.9. Log inactivation of filtrate water from WTW B exposed to a chlorine dose of 0.25 mg/L at varying pH values (pH 6, 7, 8) at room temperature. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats

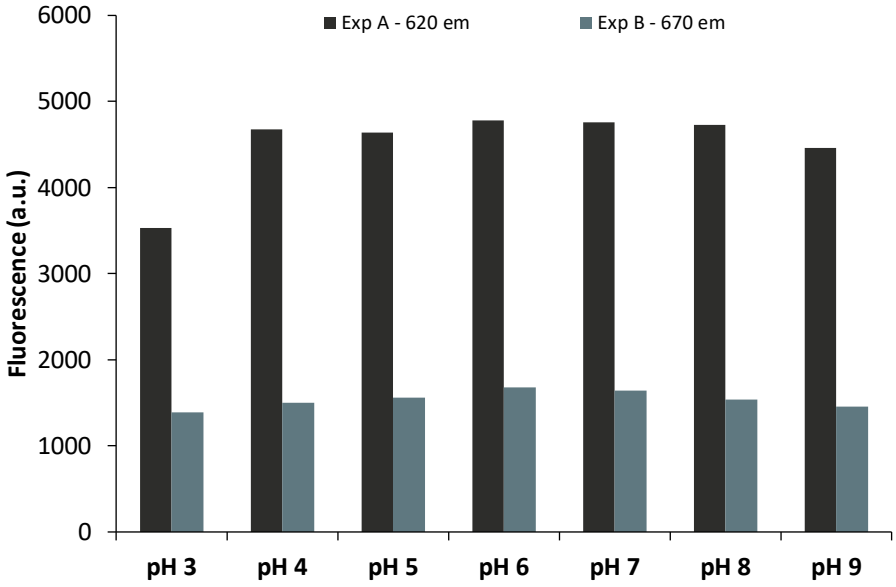

Figure 3.10. Fluorescence intensity of Propidium Iodide over a range of pH values. Dye was added to Calf Thymus DNA at the same concentration and for the same incubation period as with FC analysis. Experiments were repeated in duplicate and data was recorded at two emission points, the optimum for PI (620 nm) and the wavelength of the FL3 channel on the Accuri C6 FC (670 nm). Data represents single measured values.

While in traditional culture tests, low pH results in a higher disinfection efficacy it was the case with intact cell measurements measured by FC that at different pH there was no significant difference (for *E. coli*) or an inverse to the expected result was observed where high pH disinfection was more effective than low pH (WTW A filtrate). To the authors knowledge there are only two other studies that have assessed FC for assessing chlorine disinfection over a pH range. A study by Howard and Inglis (2003) found that *Burkholderia pseudomallei* was inactivated at a greater rate at pH 7 and 8, compared to lower pH values of 4, 5, and 6 where inactivation was reduced. The authors concluded that *B. pseudomallei* must employ survival strategies at low pH or is able to exclude chlorine under these conditions. The second study by Ramseier *et al.* (2010) included data for chlorine exposure of drinking water (from an unchlorinated distribution) over a

pH range. Extracting this data yielded an interesting observation in that the data was clustered into two categories of high and low pH. The data agreed with the conventional theory that low pH increased the rate of inactivation. However, data at pH 6 and 7 was clustered together, likewise the data from pH 8 and 9 was grouped and there was no inactivation difference within each cluster.

It would therefore appear that in all cases where FC has been applied in the assessment of chlorine disinfection efficacy over a range a pH values the results have not been conclusive, or in total agreement with the established 'permeability theory' of increased efficacy at low pH. This then raises the question as to whether these FC studies have highlighted an important component of the mechanism of action by chlorine on bacterial cells. Supporting evidence is provided by previous work investigating the impacts of pH on *E. coli* using radioactively labelled chlorine where an increased uptake rate was observed at high pH (Friberg, 1956, 1957). However, other studies have reported data consistent with improved inactivation efficacy at pH 5 compared to pH 8.5 for *Entamoeba histolytica* cysts (Chang, 1944). Consequently, there appears uncertainity in the established position that warrants further investigation. The ability of operating over a potentially broader pH range enables better control associated issues such as disinfection by product formation (Bougeard *et al*., 2008) and plumbosolvency (Jarvis *et al*., 2018).

3.4. Conclusions

FC and the SG/SGPI staining methodologies were shown to be suitable at assessing cell damage caused by chlorine over a range of dose and exposure times and this was found to be repeatable in both controlled (pure culture) and practical (WTW filtrate) scenarios. Likewise, the effects of reducing temperature can be seen by the reduced effectiveness of chlorine. Chlorine not only leads to a reduction in membrane intact cells (ICC) but prolonged exposure caused both a reduction in the TCC and fluorescence values of each event, indicating DNA was becoming damaged and the cells were undergoing complete lysis. We also identified a chlorine tolerant population in the filtrate sample that profiled as 'HNA', highlighting that not only LNA bacteria are more chlorine tolerant.

An unexpected observation was seen that showed that higher pH led to an increased loss of membrane integrity when compared to low pH. This was postulated to be a difference in the mechanistic action of chlorine on the bacterial cell membrane at higher pH. Overall the use of FC has raised some questions concerning established practice that warrant further exploration as they provide potential added benefits related to DBPs and lead plumbosolvency control.

The current chapter satisfies the requirements of Research objective 2, the key variables of chlorine disinfection were explored, and it was demonstrated where FC can be utilised and where there are still research opportunities. Knowing that pH is a variable that could skew interpretation of FC data,the subsequent chapter focuses on the process variables of disinfectant dose and contact time at pilot scale.

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4. Linking chlorine contact tank hydraulic performance and disinfection efficacy using flow cytometry at pilot scale

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Chapter context

Chapter 3 demonstrated both the advantages and disadvantages of FC. There is clearly further work required to understand the impact of pH on FC analysis when assessing chlorine disinfection efficacy and for this reason the pH variable was controlled in the experiments carried out in this chapter. The variables of dose and retention time could then be studied to address thesis objective 3:

> *"To determine the hydraulic efficiency of chlorine contact tanks of varying design and establish the link between flow behaviour and disinfection efficacy"*

Abstract

A pilot scale chlorine contact tank (CCT), with the feasibility of altering baffle arrangements, was installed at an operational WTW taking a direct feed from the outlet of the rapid gravity filters (RGF) and a range disinfection conditions were tested. The variables of *dose*, *time*, and *hydraulic efficiency* (short circuiting and dispersion) were explored. Tracer tests were conducted to determine residence time distributions (RTD) and from these hydraulic efficiency indicators (HEI) could be derived. Inactivation through the CCT was quantified using both standard heterotrophic plate counts (HPC) and flow cytometry (FCM) (intact and total cells) methods. FCM allowed for distinction between changes in performance where HPC monitoring could not. In agreement with the literature the t_{10} was found to be most effective at describing short circuiting within the reactor. Adjusting the Ct (product of concentration and residence time) for the mean tracer time ($Ct_{\tilde{x}}$) was found to correlate the most strongly with the survival ratio of organisms. Results highlight the importance of well-designed and operated CCT and that FCM offers the potential for in-situ quantification of inactivation efficacy that can be extrapolated to various treatment processes.

4.1 Introduction

Disinfection by chlorine remains the predominant final barrier in drinking water treatment before water enters distribution and is still the most prominent disinfection method used across the world. This process operates on the Ct concept, which is summarised as the product of the chlorine concentration (C) and contact time (t) (Equation 4.1).

Equation 4.1

$$Ct\ (mg.min/L) = Chlorine\ residual\ (mg/L) \times Hydraulic\ residence\ time\ (minutes)$$

Ct values for a required log inactivation (e.g. 99%, 2 -log) of specific organisms, such as pathogens, can be obtained from 'Ct tables' (AWWA, 2006). These Ct tables cover a range of typical pH and temperatures experienced in water treatment and are typically derived from laboratory studies of chlorine demand free systems that are not representative of the typical drinking water organisms or specific chemistry (Haas *et al*., 1996). This serves as a guideline for water utilities to appropriately design and operate their chlorination disinfection processes. The efficacy of chlorine is also determined by the pH and temperature of the water. Chlorine dissociates between the more effective hypochlorous acid (HOCl) at low pH to the less effective hypochlorite ion (OCl^-) at high pH. Temperature also impacts on the process in relation to both the dissociation of chlorine and the reaction rate or either species.

The reactors used for disinfection are termed 'chlorine contact tanks' (CCT) and are found in a variety of geometric configurations. The desired flow conditions in CCT should be as close to plug flow as possible to ensure the full contact time is experienced by all portions of the water being treated. True plug flow is seldom achieved due to

incompatible geometries (the ideal is a narrow channels with a length to width (L/W) ratio greater than 40) or increased dispersion due to turbulence caused by friction effects with the walls and baffles (Rauen *et al*., 2012). Non-ideal flow conditions are detrimental to the performance of CCTs, leading to short-circuiting and unwanted recirculation within the reactor. Short circuiting occurs when the fluid can by-pass much of the available reactor volume and water exits the reactor sooner than the theoretical hydraulic retention time (HRT), which in turn can lead to insufficient inactivation of microorganisms. Recirculation leads to excessive contact time in 'dead zones' of the reactor and further promotes short circuiting due the reduction in the effective operating proportion of the tank. The importance of having as close to ideal flow conditions in CCTs has been demonstrated previously using both empirical and theoretical testing (Angeloudis *et al*., 2014; Teixeira *et al*., 2008; Rauen *et al*., 2008). The effectiveness of a CCT can be described with hydraulic efficiency indicators (HEIs) that are derived from residence time distributions (Teixeira *et al*., 2008).

The fact that many CCT in operation do not achieve close to plug flow has led to the 'Ct10 concept' for Ct calculations. The t_{10} value is the most accepted value for assessing the true residence time of a reactor as it represents the time taken for 10% of the flow to pass the outlet, meaning 90% of the water remains in the tank for at least this period of time (AWWA, 2006). The ratio of the t_{10}/T gives a measure of the efficiency of the tank and from this a more conservative estimation of the operational Ct value can then be derived, the Ct_{10} (Equation 4.2):

Equation 4.2

$$Ct_{10}(mg.min/L) = Chlorine\ concentration\ (mg/L) \times t_{10}\ (mins)$$

HEIs can also be derived via computational fluid dynamics (CFD), a simulation approach that does not require physical experimental testing. Furthermore, physical models are not able to scale the turbulence within the reactor (Rauen *et al*., 2012), an important factor to consider if the a pilot scale reactor is based on a currently operational reactor. However, calibration of CFD models is required and where possible it is preferential to carry out physical testing to best calibrate the simulations (Falconer, 1986). It is rare for physical testing of CCTs to be a) carried out on-site with the appropriate feed water or b) include microbiological and chemical testing of the water under variable disinfection conditions. Previous work (Angeloudis *et al.* 2014) has used established disinfection inactivation kinetic calculations (e.g. Chick-Watson law) and DBP formation kinetics (Brown *et al*., 2011) to estimate the disinfection efficacy. However, these experiments did not record real microbial inactivation rates and, as Asraf-Snir and Gitis (2011) pointed out using tracers of fluorescently labelled cells, organisms of a different size each produce a unique RTD. Research is ongoing as to which model best describes the transport of particles (in this case bacteria) through the reactor and the most appropriate disinfection kinetic model for CFD approaches (Angeloudis, 2014).

Monitoring of disinfection efficacy can present its own challenges, pathogen surrogates (coliforms, *E.coli* or Enterococci) are not suitable performance indicators due to the rare occurrence of these organisms in disinfected water. Instead, the alternative is to try and monitor the heterotrophic bacteria using heterotrophic plate counting (HPC). These bacteria are typically abundant in drinking water and are ever present with higher recovery rates following disinfection, therefore making them a more suitable process indicator (Hijnen, 2008). However, chlorination can lead to the loss of bacterial

culturability but not viability which can lead to skewed representations of inactivation (Berney *et al*., 2006). An alternative approach, that has been applied in this study, is the use of flow cytometry (FCM) for quantifying the bacterial population in drinking water. There have been many demonstrations of the benefits of this technique over traditional culture based methods (Berney *et al*., 2007; Van Nevel *et al*., 2017; Hoefel *et al*., 2005; Ho *et al*., 2012). Successful application has been shown in laboratory studies investigating various oxidant kinetics (Ramseier *et al*., 2010) and real-time monitoring of biocide kinetics (heat and ciprofloxacin exposure) (Arnoldini *et al*., 2013). This technology has also gained significant attention as a tool for assessing operational water treatment works (WTW) (Helmi *et al*., 2014), distribution systems (Gillespie *et al*., 2014; Nescerecka *et al*., 2018) as well as an option for directly quantifying biomass in waste water treatment (Foladori *et al*., 2010) and water reuse (Safford and Bischel, 2018; Whitton *et al*., 2018). More recent developments include online automated monitoring (Besmer and Hammes, 2016; Besmer *et al*., 2014; Broger *et al*., 2011) which has shown in much greater detail the variability of short and long term process dynamics that impact bacterial abundance.

Following the outcomes of chapter 3 and the understanding of the advantages and limitations of FC, the work contained within this chapter focused on the variables of the disinfection process that could be effectively controlled, namely dose, temperature and contact time. In this study we have assessed the bacteriological water quality produced by a pilot scale CCT reactor at an operational drinking water treatment works (WTW) in Scotland. The aim of this work was to understand how inactivation during chlorine disinfection is impacted by variations in effective contact times and determine the

extent of the impact of non-ideal flow conditions. FC has been applied to provide empirical inactivation data in a filtered water supply that is representative of typical water presented to chlorination processes during day-to-day operation.

4.2 Material and Methods

4.2.1 Experimental conditions

A pilot scale CCT was constructed in a container that allowed for the pilot plant to be transported on site. The WTW was a conventional surface water treatment plant (Figure 4.1a). The pilot plant was supplied with water from the WTW following filtration, before any chlorine addition or pH correction, and was therefore representative of real water presented to disinfection. The pilot CCT reactor mimicked a typical serpentine baffled CCT that had the option of removing baffles to change the hydraulic conditions (Figure 4.1b). This pilot CCT was 1.8 m long, 1.3 m wide and depth in the tank was fixed at a height of 0.41 m. The inlet to the reactor was via a 100 mm diameter pipe, this pipe fed into a small inlet section of the tank (width 0.23 m, length 0.29 m). The minimal channel width was 0.23 m when fully baffled and 1.3 m when unbaffled. Flow rates could be varied using a pump supply from the filtered water channel. The flow rate was monitored by a Siemens SITRANS F M MAG flow meter (Siemens, Nordborg, Denmark). Sodium hypochlorite was dosed via a Grundfus Digital Dosing Control (DDC) pump (Grundfos Pumps Ltd, Bedfordshire, UK) prior to an inline static mixer. Downstream of this mixer was an AMI Turbiwell turbidity meter (Swan Analytical USA Inc, Illinois, USA) and a Hach Cl17 free chlorine monitor (Hach, Manchester, UK). At the outlet of the tank was a CYCLOPS-7 rhodamine sensor with internal data logger (Turner Designs, California, USA) for the tracer studies and a second Hach Cl17 free chlorine monitor was fed from the outlet pipe. A programmable logic controller (PLC) allowed for outlet chlorine concentrations to be controlled.

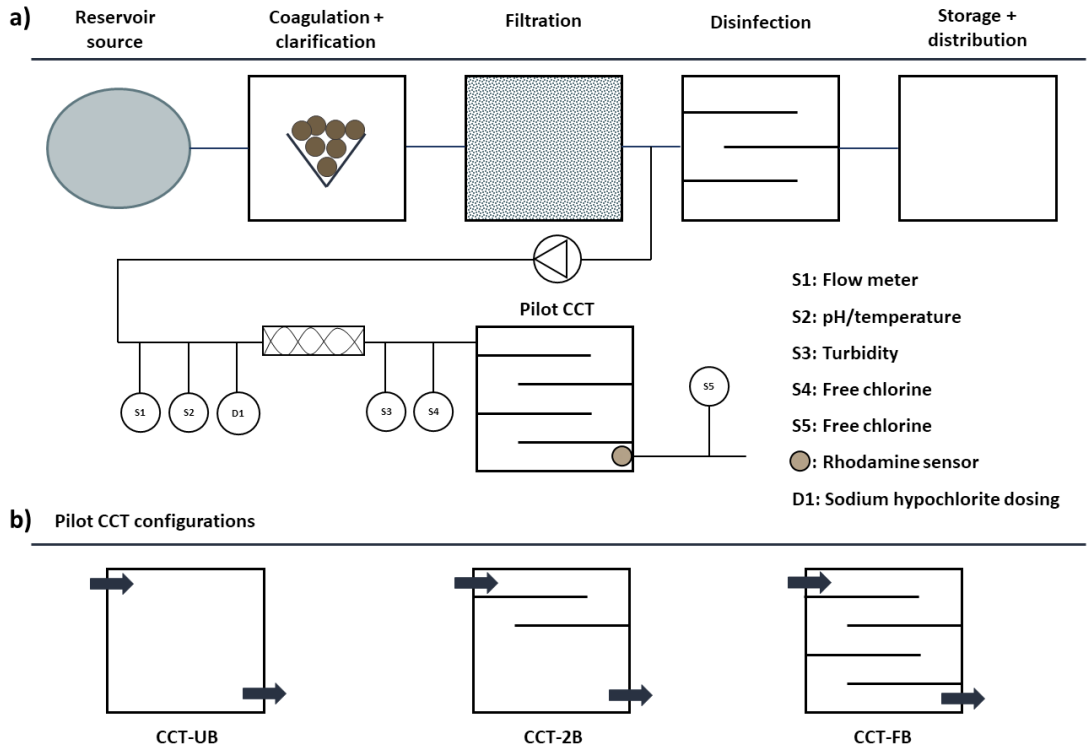

Figure 4.1. Schematic drawing of the WTW used to supply the filtered water for this study and arrangement of sensor and dosing points (a), and pilot CCT baffle configurations; UB = Unbaffled, 2B = Two baffles, FB = Fully baffled (b)

A total of 18 disinfection tests were carried out at a range of flow rates, chlorine doses and baffling conditions (Table 4.1) that best represent the range of disinfection conditions seen in operational practice.

Table 4.1. Experimental conditions and reactor parameters

CCT configuration	No. of baffles (n)	Reactor volume (m^3)	Flow rates tested (L/min)/HRT (min)	Chlorine doses (mg/L)		Reynolds number (Re)*
CCT-UB	0	0.924	92.4 (10)	0.5	1.0	3,020
			46.2 (20)			1,510
			23.1 (40)			755
CCT-2B	2	0.924	92.4 (10)	0.5	1.0	5,131
			46.2 (20)			2,569
			23.1 (40)			1,284
CCT-FB	4	0.924	92.4 (10)	0.5	1.0	5,757
			46.2 (20)			2,879
			23.1 (40)			1,439

**Reynolds number >2000 for open channel flow is considered turbulent*

4.2.2 Tracer experiments

All tracer experiments in this study were carried out using the pulse trace methodology using Rhodamine WT (Acros Organics, Geel, Belgium). A submersible fluorometer, Turner Design CYCLOPS-7 sensor and logger, (Precision Measurement Engineering Inc, California, USA) at a maximum resolution of Δt = 3s was used for the detection of the rhodamine trace. Each tracer experiment lasted for a minimum of three theoretical retention times and subsequent tests were only carried out after a fourth retention time had passed to allow complete turnover of the water within the reactor. The tracer was added to the inlet of the reactor via a manual syringe injection of Rhodamine WT solution (20 mL at 35 mg/L, mass of rhodamine added = 7,000 µg). This injection time was within that recommended by Marske and Boyle (1973) and was no more than 1/50th of the reactor retention time. Each tracer test was carried out once. To effectively describe the residence time distribution (RTD) the results were normalised using the E-curve method (Levenspeil, 2012) (See Appendix B). This normalisation step allows for interpretation of the hydraulic performance across various flow rates. Time specific tracer parameters were recorded and HEIs were calculated using the RTD results (Table 4.2)and were classified from poor to excellent (

Table 4.3).

Table 4.2. Commonly used time specific and hydraulic efficiency indicators (HEIs) for describing residence time distributions (RTDs) of tracer tests (adapted from (Wang and A. Falconer, 1998; Teixeira *et al*., 2008).

Class	Name	Description
Time specific parameters	T	Theoretical hydraulic retention time
	t_i	Time for initial tracer front to pass the outlet
	t_{10}	Time taken for 10% mass of tracer to pass the outlet
	t_{50}	Time taken for 50% of the tracer mass to pass the outlet
	t_P	The maximum tracer concentration arrival time
	$t_{\bar{x}}$	Tracer mean.
Short circuiting indices	t_{10}/T	Relative time taken for 10% of the tracer to pass. Standard value for assessing short circuiting within reactors.
	T_{90}/T	Relative time taken for 90% of the tracer to pass. A measure of the recirculation within the tank
	$t_{\bar{x}}/T$	Relative tracer mean. Used to identify if there are dead spaces/stagnation within the reactor in conjunction with T. If $t_{\bar{x}} = T$ then all of the tank volume is in use by the fluid. If $t_{\bar{x}} < T$ then some of the vessel is not being used (indicating dead regions) and finally if $t_{\bar{x}} > T$ this indicates there is recirculation within the reactor holding the tracer back.
Mixing indexes	Mo (Morril index)	The Morril index is a ratio between the time taken for 90 and 10% of the tracer to pass the outlet (t_{90}/t_{10})
	σ^2 (Dispersion index)	The dispersion index is a measure of the variance of the RTD function
	t_{90}-t_{10}	The time elapsed between 10 and 90% of the tracer leaving the vessel.

Table 4.3. HEI used in this study and the target values (AWWA, 2006; Angeloudis, Stoesser, Falconer, *et al*., 2014)*

HEI	Target	Poor	Compromising	Acceptable	Excellent
t_{10}/T	1	0.3	0.5	0.7	1.0
Mo	1	25	10	5	2
σ^2	0	1.0	0.8	0.2	0.1

*It is important to state that the classification of HEIs are based upon hydraulic efficiency alone and do not consider overall process performance.

Table 4.4. Baffling factor (BF) classifications from AWWA (2006) Ct10 guidance

Baffling condition	Hydraulic efficiency (HE) (t_{10}/T)	Description
Unbaffled	0.1	No baffling, agitated basin, very low length-to-width ratio, high inlet and outlet velocities
Poor	0.3	Single or multiple unbaffled inlets and outlets, no baffles
Average	0.5	Baffled inlet or outlet with some intrabasin baffles
Good	0.7	Perforated inlet baffle, serpentine or perforated intrabasin baffles, outlet weir or perforated launders, filters
Perfect (Plug flow)	1.0	Very high length-to-width ratio, perforated inlet, outlet, and intrabasin baffles, pipelines, not tanks

4.2.3 Water quality measurements

Microbiological analysis for the determination of heterotrophic plate counts was determined by mixing 1 mL of sample into 18 mL of molten Yeast Extract Agar (YEA) (Oxoid, ThermoFisher Scientific, UK.). These plates were duplicated with one incubated at 37°C for 48 hours and the second at 22°C for 72 hours, in accordance with standard methods. The determination of coliforms and *Escherichia coli* was carried out by membrane filtration. 100 mL of sample was passed through a 0.45 μm membrane filter. Plates were incubated at 30°C for 4 hours and then 37°C for 14 hours. Flow cytometry analysis for intact and total cells was carried out as described previously (Gillespie *et al*., 2014) with the amendment of 25 μL sample volume in accordance with the rapid method described by Nevel *et al.* (2013).

4.3 Results and Discussion

4.3.1 General Water quality data

Water quality entering the pilot from the operational WTW remained constant throughout the trial period resulting in a consistent chlorine demand of 0.18 ± 0.05 mg/L across the trial period (Table 4.5). The pH of the feed water was constant throughout the trial (pH = 6.4 ± 0.1) as was the water temperature (12.7 ± 0.5°C) and DOC (2.7 ± 0.3 mg/L). The consistency of the water quality indicated that the water characteristics were effectively controlled throughout the trials enabling the inactivation data to be directly related to the two components of chlorine dose and hydraulics (White, 2010; Haas and Engelbrecht, 1980). With regards to microbiological parameters, HPC 22°C at the inlet showed a large variation and low counts (minimum = non-detectable; and maximum = >300 CFU/mL) meaning accurate inactivation rates would be challenging to elucidate. In contrast, the FCM Intact cell count remained reasonably constant throughout the trial ($5.20 \times 10^5 \pm 3.36 \times 10^4$ cells/mL) and values were between 3 and 4 orders of magnitude higher than culture-based data therefore allowing more accurate calculations of inactivation rates.

Table 4.5. Inlet water quality data throughout trial period.

		Inlet			
Parameter	**Units**	**Avg.**	**S.d.**	**Min**	**Max**
Chlorine demand*	mg/L	0.18	0.05	0.10	0.32
Turbidity	NTU	0.06	0.03	0.03	0.17
Temperature	°C	12.7	0.5	12.2	13.5
pH	n/a	6.4	0.1	6.3	6.8
Total Organic Carbon (TOC)	mgC/L	2.8	0.4	2.3	4.4
Dissolved Organic Carbon (DOC)	mgC/L	2.7	0.3	2.3	3.8
Manganese	µg/L	2.9	0.7	1.6	3.9
UV_{254} Transmittance	n/a	85.6	1.8	78.2	88.1
HPC (22˚C)	CFU/mL	40	55	0	>300**
HPC (37˚C)	CFU/mL	0	n/a	0	4
Total Coliforms	CFU/100 mL	0	1	0	4
Total cells	Cells/mL	6.38×10^5	8.23×10^4	5.10×10^5	8.67×10^5
Intact cells	Cells/mL	5.20×10^5	3.36×10^4	1.71×10^4	6.34×10^5

*Chlorine demand was calculated as the difference between the dosed chlorine and the outlet chlorine concentration, the outlet concentration was always maintained irrespective of changing chlorine demand.

**Maximum countable colonies per plate, subsequent serial dilutions were not carried out.

4.3.2 Hydraulic performance

The hydraulic performance of the pilot CCT was improved as baffling conditions increased although each baffle arrangement showed deviation from plug flow, a factor that is not considered when Ct is not adjusted to account for hydraulic efficiency (i.e. the theoretical HRT). The un-baffled condition (CCT-UB) showed a large spike of tracer leaving the reactor shortly after injection due to streaming across the tank. This was followed by a long tail of the trace caused by the large recirculation zone in the centre of the tank (Figure 4.2). This profile is typical of unbaffled reactors and is similar to that of a continuously stirred reactor (CSTR) rather than the desired plug flow (PF) conditions.

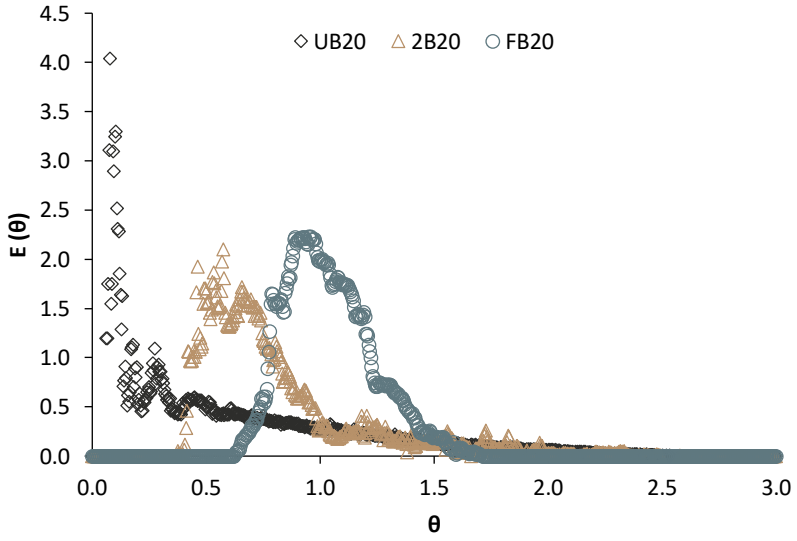

Figure 4.2. Normalised residence time distributions (RTDs) for each of the baffling conditions at a theoretical retention time of 20 min.

Analysis of this RTD at the theoretical HRT=20 min gave a t_{10}/T of 0.09 showing in this case that 10% of the tracer had exited the reactor within 1.8 min of the injection. With regards to mixing in the tank, both approaches to quantify this using the dispersion index ($\sigma^2 = 0.74$) and the Morril index (Mo = 15.41) resulted in values classified as *poor* by the

HEI descriptors (Table 4.3). With the introduction of two baffles into the tank the RTD profile improved. The tracer front was held back from short circuiting the reactor rapidly, and the tail of the RTD was shortened. The introduction of these channels reduced the dispersion and improved the value of the dispersion index to a level associated with an HEI descriptor of *acceptable* ($\sigma^2 = 0.19$). Similarly, the Morril Index improved from an HEI descriptor of *compromising* to one classified as *acceptable* (Mo = 2.71) (

Table 4.3). Short circuiting was reduced ($t_{10}/T = 0.47$) but was still evident due to 50% of the tank volume remaining un-baffled, which allowed for streaming in the final compartment. It was visually observed from the tracer that recirculation was eminent in this final section of the tank also (Figure 4.3). When fully baffled the RTD resembled a normally distributed curve (Figure 4.2), centred around the theoretical residence time ($\theta = 1$). Short circuiting was significantly improved to a value associated with an HEI descriptor of *excellent* for serpentine CCT reactors ($t_{10}/T = 0.81$), and dispersion was brought down to a level commensurate with the descriptor *excellent* ($\sigma^2 = 0.03$). This was visually confirmed by a compact tracer moving through the tank (Figure 4.3).

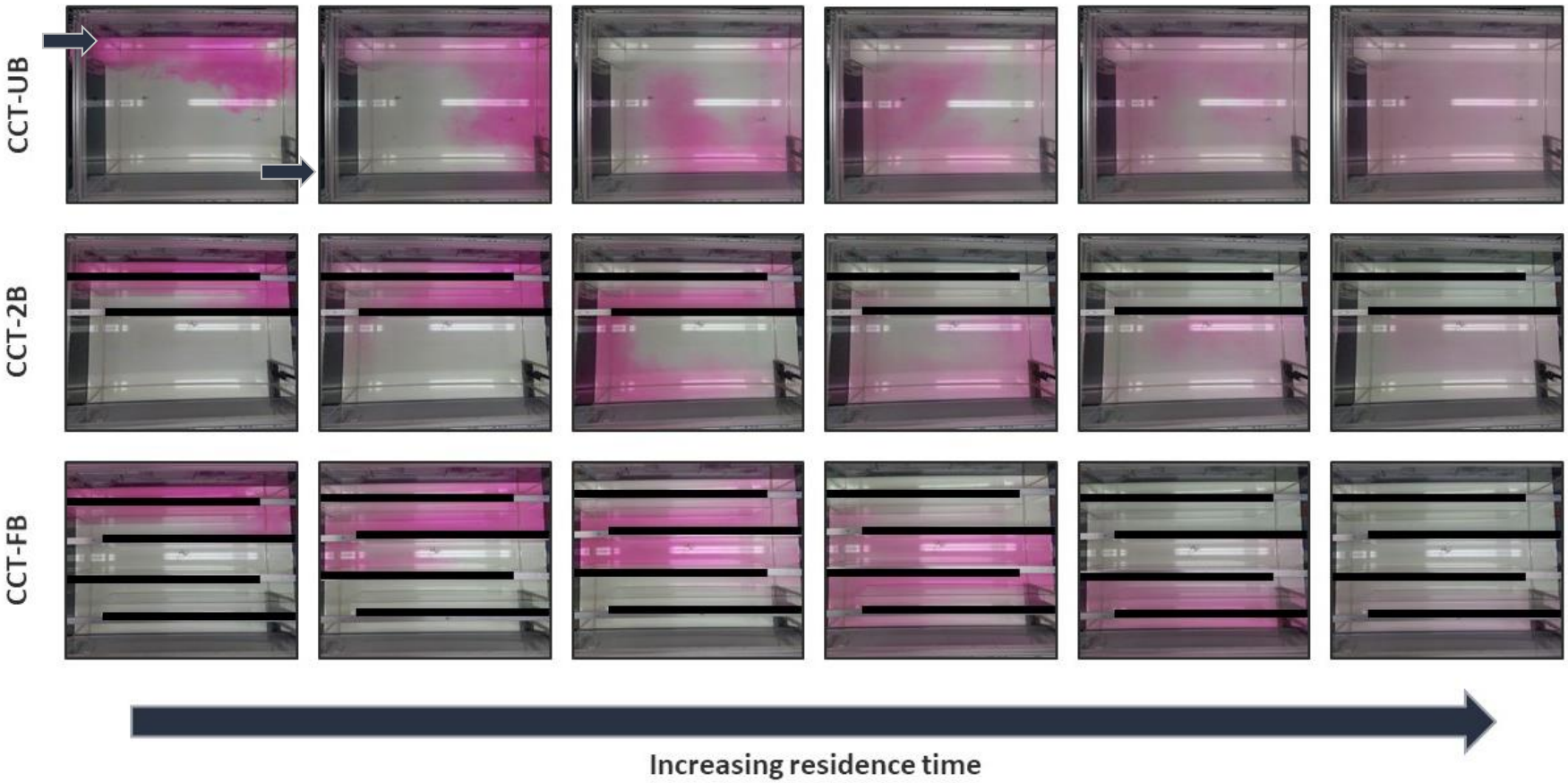

Figure 4.3. Images of the rhodamine tracer in the pilot CCT captured over the theoretical residence time. Inlet and outlet locations are indicated by arrows and are in the same location for each test.

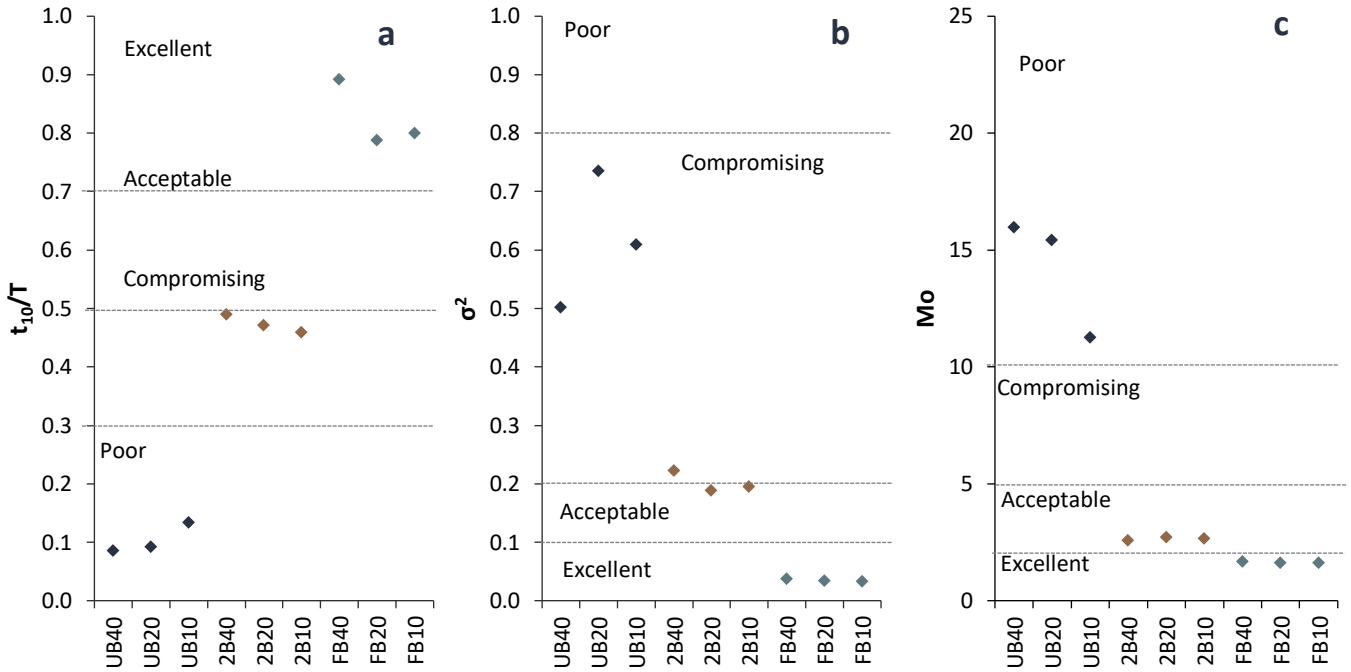

Figure 4.4. Key hydraulic efficiency indices (HEIs) to describe performance for each experimental condition of baffling and flow rates. Thresholds of poor, acceptable, compromising and excellent were adapted from (Angeloudis *et al.*, 2014) for σ2 and Mo, whereas the t10 were taken from (AWWA, 2006). Baffling conditions and target HRT are specified on the horizontal axis. Data points represent data from singular tracer tests.

Comparison of the hydraulic efficiency of the different baffle arrangements was assessed at three HRTs and compared in relation to t_{10}/T, σ^2 and Mo (Table 4.4, Figure 4.4). The normalised HEIs indicated only small variation as a function of HRT as illustrated through the t_{10}/T ranges which were 0.09-0.13, 0.48-0.52 and 0.81 -0.93 for the unbaffled, two baffles and fully baffled tanks respectively. The overall picture indicated that irrespective of HRT, the inclusion of more baffles leads to more plug flow like conditions. This observation was not so defined for comparison of the two mixing indices (σ^2 and Mo), which indicated performance was defined as *compromising* or *poor* in the unbaffled reactor, yet the two baffled reactor showed an *acceptable* dispersion value of 0.205 whilst the Morril index was 2.64 which graded it as *acceptable* yet close to the Morril index for the fully baffled case which was 1.63. The introduction of the two baffles (38% tank volume) led to three channels within the tank and much of the tracer was transported to the end of the tank rapidly after injection (Figure 4.3), condensing the t_{10} and t_{90} values. The relative value of t_{90}/T showed little difference over each of the test conditions (See Appendix B). The σ^2 on the other hand was impacted significantly by changing of baffle conditions which is representative of this arrangement of longitudinal baffling. Overall, baffling had a larger impact on HEI's than the flow rate, a finding supported by Zhang *et al.* (2014) who also demonstrated that over three flow ranges, the t_{10}/T and Morril index were unaffected by changing flow rates.

The comparison in Figure 4.4 has shown that the different tank arrangements are best distinguished by the t_{10}/T as this provides the greatest separation. Neither of the two mixing HEIs should be assessed alone, as an *acceptable* σ^2 and *excellent* Mo could be

classified as borderline *compromising* with regards to t_{10}/T. When considering mixing, the σ^2 was reported to be the most suitable mixing index for assessment of CCTs as it considers the whole RTD function, such that the Mo does not add any further information (Teixeira *et al.,* 2008). However, in the current study the Mo was more reproducible under poor hydraulic conditions and therefore would be worth consideration if σ^2 was not producing reliable results.

4.3.3 Inactivation efficiency

In order to assess the efficacy of disinfection, samples were taken from both the inlet and outlet of the CCT for each experimental assessment of baffling and HRT condition. Analysis of total bacteria was carried out using both the culture-based HPC and the culture independent flow cytometric method. The plate count data subsequent to chlorine addition was limited, due to the non-culturability of these cells following disinfection (Figure 4.5a). In contrast, there was much greater information available from the flow cytometric analysis because more bacteria could be enumerated both at the inlet and outlet of the CCT (Figure 4.5b). At the inlet prior to chlorine addition there was a median HPC count of 25 CFU/mL, with the minimum reported value as 'non-detectable' and a maximum reported as >300 CFU/mL (n=2, laboratory protocol was to not carry out dilutions on these sample). This variation is typical of the total viable count method and routinely seen in operational practice, where 92% of samples in chlorinated water are returned blank (see Chapter 3). Only three HPC positive results were observed after chlorine addition from the samples taken at the reactor outlet (maximum CFU/mL =3) and these did not relate to any ICC or TCC changes (data not shown). All other samples (n= 87) were reported as 0 CFU/mL (non-detectable). Irrespective of the disinfection conditions, only a single coliform colony was detected by standard microbiological analysis (Table 4.5). Furthermore no *E. coli* were detected throughout the entirety of the study. In contrast, the results from FCM analysis carried out on the same sample offer more insights into process performance. At the inlet to the CCT there was a median TCC observed of 610,320 cells/mL, reaching a maximum of 867,200 cells/mL and a minimum of 510,160 cells/mL. Intact cells represent only those organisms

with membrane integrity and always appear lower than total cell counts, even prior to disinfectant addition. The median ICC at the inlet was 86% of the TCC median (525,760 cells/mL). At the outlet of the reactor there was a much wider variation in the total cell counts than those observed at the inlet. The median TCC was 4% lower in the outlet than the TCC at the inlet (584,400 cells/mL) whilst the range of TCC went from 23,600 to 835,200 cells/mL. The median ICC was 26,540 cells/mL (range 7,080 to 50,000 cells/mL).

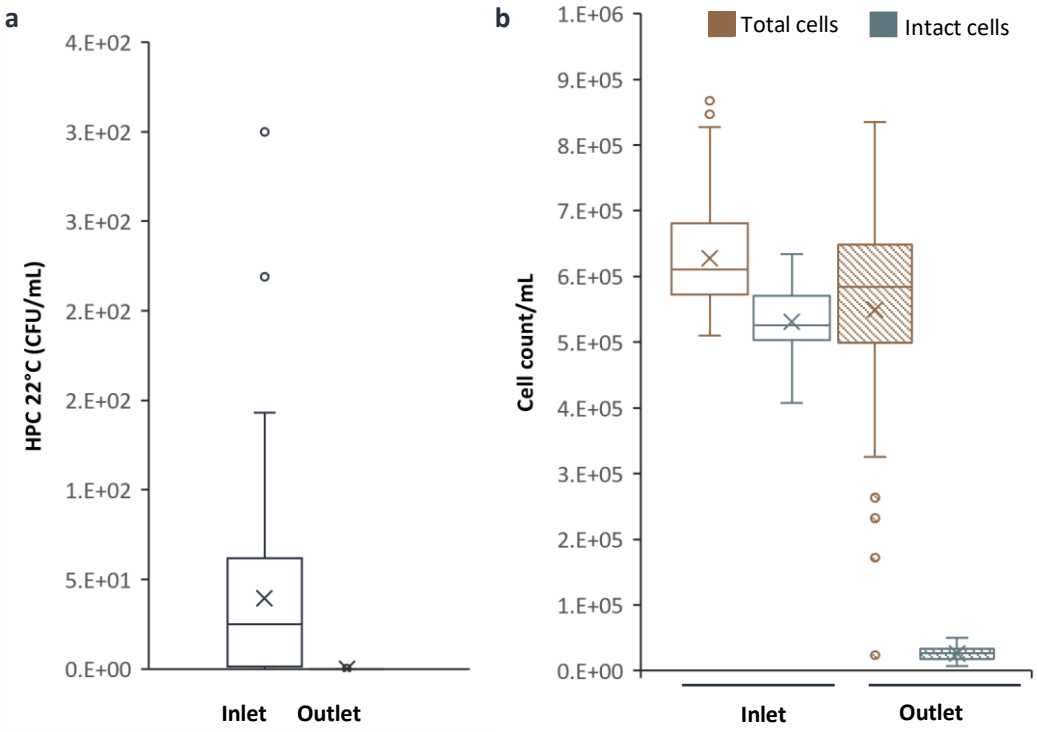

Figure 4.5. Box whisker plots for inlet (n=89) and outlet (n=90) microbiological monitoring throughout the trial period. This data has been pooled into one sample set irrespective of the disinfection conditions for comparison of methods. Heterotrophic plate counts (HPC) are shown (a) and flow cytometric data for intact and total cells are included (b).

To elucidate the impacts of improper plug flow on the efficacy of disinfection, the survival ratio was correlated to the various HEI adjusted Ct values (Figure 4.6). The adjustment of Ct to Ct_{10} was described previously, and the adjustment for the mean of the RTD curve was calculated in the same manner using the $t_{\bar{x}}$ ($Ct_{\bar{x}}$). The inactivation data shows a stronger correlation with the $Ct_{\bar{x}}$ data (Figure 4.6a) when compared to the Ct_{10} (Figure 4.6b), suggesting this $Ct_{\bar{x}}$ parameter from the RTD mean is more representative of the inactivation rate. As the Ct_{10} value increased there was a decrease in the survival of intact cells as the survival ratio went from 0.075 to 0.02 for Ct_{10} between 1 and 20 mg.min/L (Figure 4.6b). For the UB conditions there was a sharp linear drop in the survival ratio with little change in the Ct_{10} from (0.3 -3.6 mg.min/L). The results for the 2B conditions show a similar linear trend to the UB conditions, with a tailing off of results above 10 mg.min/L. This tailing off was evident in almost all tests and can be attributed to both the chlorine demand and heterogenous bacterial population with varying chlorine tolerances (Cerf, 1977). For the FB tests the data was similar to that obtained from the 2B studies although a more pronounced tailing profile was apparent congruent with further inactivation being difficult to achieve without a major increase in the overall Ct. With regards to the dispersion index, an *excellent* HEI value (σ^2 = 0.1) did not guarantee the most effective disinfection, although higher dispersion indices did result in poorer performance (Figure 4.6d). The weakest correlation between the survival ratio of bacteria and each Ct calculation was in relation to the unbaffled configuration (r^2 = 0.55 – 0.79) whereas the two baffled and fully baffled arrangements were more strongly correlated, with r^2 levels of 0.96-0.98 and 0.87, respectively.

Assessments of the most suitable HEI to describe both short circuiting and mixing conditions have often come to the conclusion that no single HEI is the best choice but that the t_{10}/T and dispersion index would be the most suitable for describing the disinfection process (Teixeira *et al.*, 2008). In this study it was found that although the t_{10}/T index was the most appropriate for ranking short circuiting, and the BF derived by the AWWA agree with the t_{10}/T, the $t_{\bar{x}}/T$ correlated most strongly with bacterial inactivation and was most likely as a result of the increased contact time the $t_{\bar{x}}/T$ represents.

The log inactivation rates observed in this study achieved the 2-log threshold that would be expected of a chlorine disinfection process based on World Health Organisation (WHO) guidelines (WHO, 2008). As a comparison, laboratory studies by Ramseier *et al.* (2010) explored the kinetics of membrane damage caused by a variety of disinfectants (chlorine, ozone, chlorine dioxide, monochloramine, ferrate (IV) and permanganate). They found up to 1 log inactivation by free chlorine at a Ct of 20 mg.min/L at an initial bacterial concentration of 14×10^7 TCC/mL, whereas in this study at an equivalent Ct, the log reduction was calculated as 1.6 (initial cell concentration = 6.38×10^5 TCC/mL). This difference in inactivation rates can most likely be attributed to the higher cell concentrations which exert an increased amount of chlorine demand, meaning less is available for inactivation (Helbling and VanBriesen, 2007).

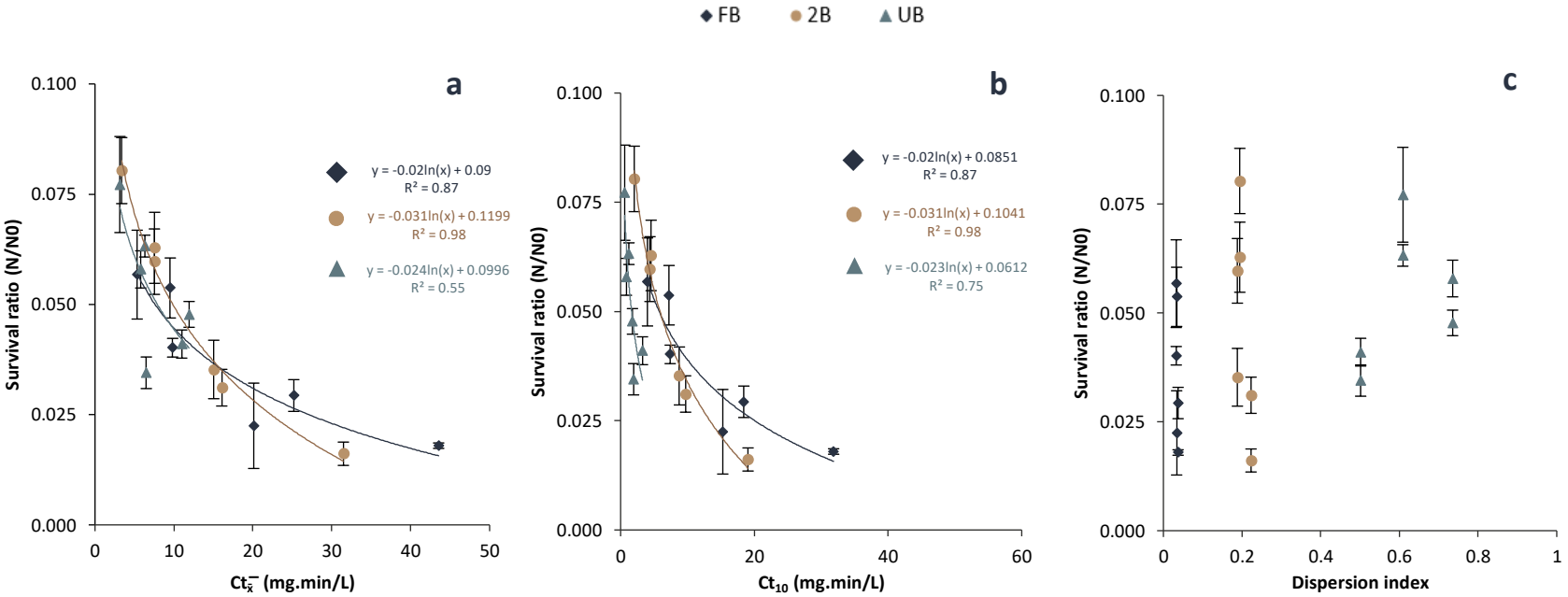

Figure 4.6. Survival ratio curves of intact cell counts calculated from the inlet and outlet of the CCT against three HEI converted Ct values, the $Ct_{\bar{x}}$ (a), Ct_{10} (b) and the Dispersion Index (σ^2) (c). FB = Fully baffled, 2B = 2 baffles, UB = Unbaffled. Error bars represent standard deviation of triplicate sampling over each test period.

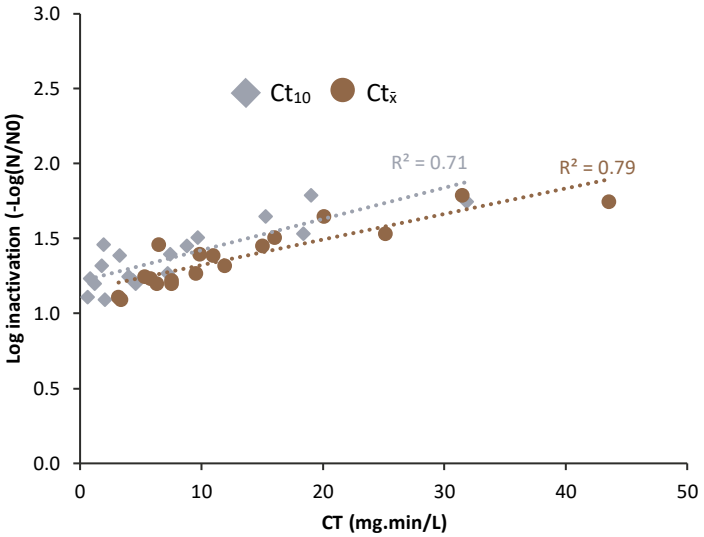

Figure 4.7. Log inactivation (-Log(N/N_0) plot for all experimental conditions calculated for different HEIs, the Ct_{10} and $Ct_{\bar{x}}$

Results of this study suggest that the Ct_{10} is a suitable indicator for inactivation (r^2 = 0.71) but the strongest correlation was observed for the $Ct_{\bar{x}}$ (0.79) (Figure 4.7). The standard method for assessing Ct is to use the t_{10} value because this is the most conservative approach, as it considers the fact this HEI describes the disinfection conditions that 90% microbes are exposed to. The t_{10} is always a shorter exposure time than the $t_{\bar{x}}$ and is impacted more so as deviation from plug flow occurs (i.e. increasing dispersion). This indicates that the inactivation is more strongly linked to the bulk flow which the $t_{\bar{x}}$ offers a good representation of, rather than the initial tracer front which the t_{10} is more aligned with.

4.3.4 Future perspectives of FC as monitoring technique for disinfection efficacy

Methods such as FCM for total bacterial counts are not a replacement for detecting the presence of indicator organisms (*E.coli*, total coliforms, Enterococci etc.) but instead should be applied as an effective tool for assessing process performance in relation to

bacteria. Suitable process indicators are those that are continuously present in the source, detectable at all stages of treatment and must be eliminated at an equivalent rate to potential pathogens of interest (Hijnen *et al*., 2004). Commonly used indicator organisms, such as *E.coli* (and other coliforms), are often utilised in both laboratory and theoretical testing of disinfection processes. However, this organism is not representative of most of the bacterial community and has been shown to be highly susceptible to small doses of free chlorine (<0.1 mgL), with typical Ct values ranging from 0.01 – 1 mg.min/L (Stanfield, 2005). In addition, this organism may not be found in significant concentrations in water sources, although coliforms are not assumed to be *E.coli*, they are an indicator of its presence and as an example they were only detected in 13% of the inlet samples at the pilot CCT. Although FCM log reductions are typically lower than those observed by culture methods (Nocker *et al*., 2016), this study also showed that *E.coli* (10^5 cells/mL) exposed to chlorine lost culturability as soon as the fluorescence signal changed due to uptake of PI through the damaged membrane. This observed loss of culturability supports the work of Wang *et al*. (2010) in that chlorination induces a viable but not culturable (VBNC) state of the bacterial cells and explains why all but 3 HPC samples in the current study returned a 'non-detectable' results. FCM has enabled interpretation of the in-situ inactivation rates of a CCT over a wide range of Ct values and gave the granularity in the data to distinguish between small changes in the hydraulic configuration. All of this was possible with a small volume of sample and processing time, meaning this technique is ideally suited for process investigations such as route cause analysis. With the development of online flow cytometry and the increasing uptake in the application of this approach for monitoring treatment process

dynamics (Besmer *et al*., 2016) it is likely that FCM could be applied in near real time for active disinfection monitoring.

4.4 Conclusions

Survival ratios of a typical drinking water population were successfully correlated with HEIs and efficiency adjusted Ct values. Improvements in baffling led to an increased hydraulic efficiency and was critical in ensuring effective disinfection. It was deemed that the most appropriate hydraulic parameter to quantify short circuiting was the t_{10}, but the dispersion index must also be calculated for determining the variance of the RTD. A comparison of the HEI adjusted Ct revealed that the $Ct_{\tilde{x}}$ was most strongly correlated with inactivation rates, suggesting this value best represents inactivation through the reactor. The above conclusions of this work successfully achieve Research objective 3. Whereby, with the use of flow cytometry and the measurement of total and intact cells inactivation rates can be derived. Demonstrating the added value this type of analysis could bring to the operational monitoring and optimisation of CCTs.

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5. Performance of disinfection reactors across Scottish Water: Investment requirements and solutions

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Chapter context

With the understanding developed in chapter 4, an approach towards assessing disinfection performance across Scottish Water could be made. Within this chapter the disinfection processes across Scottish Water were investigated to satisfy the final thesis objective:

> *"To assess current disinfection practice across Scottish Water and identify a strategy for improvement where required"*

Abstract

A range of disinfection reactors across Scottish Water (SW) were assessed in terms of hydraulic efficiency (HE) and operational Ct in response the Drinking Water Quality Regulator (DWQR) requirements for Scottish Water to fully understand chlorine contact time across the asset base. An assessment of the Ct at high risk sites, those responsible for producing 88% of SW daily drinking water production, was undertaken. These larger production sites use chlorine contact tanks (CCT) for the disinfection process and using tracer data the HE of these reactors was assessed. This assessment led to a classification of investment requirements for SW CCTs. This identified 12 sites that would benefit from additional baffling to meet the required 20-min residence time at a cost of £ 498,558 and 14 sites requiring a new tank at a cost of £ 17,508,370. Finally, a decision tree for determining the optimum investment route for CCT reactors was produced to identify where amendment to existing or new CCT where required.

5.1 Introduction

In October 2013, the Drinking Water Quality Inspectorate (DWQR) for Scotland issued Scottish Water (SW) with Information Letter 3/2013 'Requirement and Expectations for Supply-specific Disinfection Strategies' (DWQR, 2013). This was issued to ensure that SW was fully compliant with regulation 25 of the Public Water Supplies (Scotland) Regulations 2014 (Scottish Parliament, 2014), which requires that all water be disinfected before it is supplied to consumers. As part of improvements to the SW disinfection strategy and to gain further understanding of chlorine disinfection, research was undertaken to enable successful delivery of their disinfection policy. This was achieved through determining the best approach for monitoring micro-organisms (Chapter 2 and 3) and validating the efficacy of chlorine contact tank design with respect to disinfection efficacy (Chapter 4). The following chapter considers how SW can implement this new knowledge and understanding, specifically, in relation to one of the specific requirements of the DWQR:

> *'14. Contact times must be specified under full range of flow conditions at WTW and there must be a demonstration of the understanding of flow characteristics'*
>
> *(DWQR, 2013)*

SW have a recently updated disinfection policy which requires that the water presented to disinfection of the appropriate quality. Turbidity prior to disinfection must be less than 0.2 NTU 100% of the time and less than 0.1 NTU 95% of the time. In addition, total organic carbon (TOC) must be low enough to prevent trihalomethane formation exceeding 40 µg/L and at new water treatment works (WTW), the TOC of water entering disinfection should be 1.0 mg/L or less. There must also be a consideration of the pH in

the water presented to disinfection and the effective HOCl%. Although lower temperatures favour HOCl formation, this results in a lower reaction rate and diffusivity of HOCl into the target cells (Haas and Engelbrecht, 1980; White, 2010). Increasing temperature leads to increased diffusivity of Cl_2 into bacterial cells, increased reaction with cell components and increased metabolic activity leading to an increase in toxicity. It is estimated that a 10°C increase in temperature will increase the reaction rate two-fold (Stanfield, 2005). There is a consensus that maintain disinfection pH of less than 7.5 is appropriate for chlorine disinfection.

The minimum Ct requirement for SW varies depending on the type of water presented to disinfection (Table 5.1). For example, simple disinfection of surface water has a Ct of 30 mg.min/L, while a full chemical treated water only requires an Ct of 15 mg.min/L. The minimum hydraulic efficiency (HE , sometimes termed 'plug flow factor') for a contact tank is specified as 70% (AWWA, 2006) and the Ct must be achieved within a dedicated contact structure of either a tank chlorine contact tank (CCT) or pipe (CCP) and must have a minimum hydraulic residence time (HRT) of 20 min.

Table 5.1. Ct standards for Scottish Water WTW

Treatment description	Ct (mg.min/L)
Simple disinfection of surface waters	30
Physical filtration of surface waters without coagulation or groundwater / river gravel supplies	22.5
Full chemical treatment or membrane filtration providing a minimum of Log 2 removal of viruses and bacteria	15

There was an awareness when this policy was developed that SW was lacking information on the design and operational Ct of disinfection processes across the water asset base. This understanding has since been improved with various disinfection policy driven projects, yet there is still uncertainty around the actual hydraulic performance at each water treatment works (WTW). These hydraulic conditions are important to consider as deviation from the plug flow, in terms of short circuiting and dispersion during disinfection can lead to inefficient disinfection (see Chapter 4).

The variation in design and specification of water treatment processes arises from the fact that these assets have an expected lifetime of at least 40 years, but many across the UK are much older. This means many of the inherited, legacy treatment processes may not meet modern day standards and specifications and there are therefore two options for resolving this issue. The first is capital investment and complete replacement of the asset, and the second is enhancement of the current process.

Construction of chlorine contact tanks for disinfection can be costly (> £1 million) but is only required where the tank volume for the given flow rate is a limiting factor in achieving the desired HRT. For tanks where volume is sufficient, it may be that hydraulic efficiency can be improved by minimising dispersion and short circuiting, through the use of baffles to improve the overall length-to-width (L:W) of the tank (Marske and Boyle, 1973). Physical optimisation of CCT after installation is a challenge, as often these assets are buried underground or under buildings with the only access being through manhole hatches. In these situations, the only suitable option for modification of hydraulic efficiency is using baffle curtains. These curtains can be folded so that they fit through hatches in the tanks and are unfolded inside for installation. The curtains are then fixed to the floor, walls, roof and supporting pillars of the tank to ensure that they remain in place whilst under stress from the water in the tank (Figure 5.1). These baffles are typically made of materials that have approval by the Water Regulations Advisory Scheme (WRAS) and are typically waterproof plastic membrane materials (e.g. FLAGON GEOP AT, Soprema, Witham, UK). Improved baffling of tanks leads to an increase in hydraulic efficiency and is a simple cost-effective solution that will lead to improvements in reactor performance.

Figure 5.1. Examples of baffle curtain installations showing anchoring. (https://www.water-lines.co.uk/baffle-curtains).

The aim of this chapter was to understand the distribution of disinfection reactor classification across Scottish Water and build a picture of operational CT across the company. In turn, this enabled cost estimation of the investment required (capital or enhancement) for SW to align the chlorine disinfection assets with the current disinfection policy standards and DWQR requirements.

5.2 Methods

5.2.1 Ct survey

In response to DWQR Information Letter 2013/3, a large sample (n = 173) of Scottish WTWs were surveyed to determine the range of operational Ct seen across the business. This was achieved by carrying out tracer tests using a spike of increased chlorine dosing into the reactor to determine the approximate residence time at each site, as well as gather information on disinfection practice. Information was collected on the disinfection pH, disinfectant type (chlorine gas or sodium hypochlorite), tank geometries and layout, as well as operational and maximum flow rates. The observed Ct were compared against a target Ct of 15 mg.min/L and hydraulic residence time (HRT) of 20 min based upon company standards. While chlorine is not an ideal tracer, due to its reactivity and the difficulty in measuring it at low concentrations, it is an easy way by which water companies can gain a more accurate estimation of the actual HRT within a contact vessel. This is because chlorine is a commonly utilised chemical in the disinfection of water and therefore has regulatory approval for use in drinking water. Moreover, all WTW in this study were equipped with online chlorine measurement instruments at both the inlet and outlet of the disinfection stage making it a practical tracer choice.

The flow rate at the time of the tracer test and the maximum design flow were used to calculate the theoretical HRT (Equation 5.1).

Equation 5.1

$$HRT\ (mins) = \frac{Tank\ volume, V\ (m^3)}{Flow\ rate, Q\ (m^3/min)}$$

From the tracer testing, the hydraulic efficiency (HE) of each chlorine contact system was calculated from Equation 5.2:

Equation 5.2

$$Hydraulic\ efficiency\ (HE) = \frac{Tracer\ measured\ HRT\ (mins)}{Theoretical\ HRT\ (mins)}$$

In theory, a tank efficiency greater than 0.7 should be achieved by an appropriately designed CCT and efficiencies greater than 0.8 should be achieved for CCPs (AWWA, 2006). Following this HE calculation it was then possible to calculate the expected true residence time at max flow (Equation 5.3), as chapter 4 demonstrated that HE does not change where the tank depth remains the same and for the purpose of this study depths were assumed to be static.

Equation 5.3

$$HE\ adjusted\ HRT\ (mins) = HE\ \times HRT\ \max flow\ (mins)$$

The Ct parameter could then be calculated using either the tracer HRT, theoretical HRT or the HE adjusted HRT (Equation 5.4).

Equation 5.4

$$Ct\ (mg.min/L) = Chlorine\ concentration\ (mg/L) \times HRT(mins)$$

5.2.2 Financial assessment of Ct remediation strategies

The financial costs of ensuring that WTW in Scotland could meet the requirements of the disinfection policy in relation to Ct were calculated using a variety of industry standard and SW specific cost calculations. As part of this assessment several assumptions have been made when calculating costs and are detailed below (Table 5.2).

Table 5.2. Assumptions made when estimating costs for Ct upgrades across Scottish Water.

Assumption	Description	Reference
1	Required HRT is not adjusted by pH or temperature. The HRT has been calculated to achieve the required Ct at a dose of 1.00 mg/L free chlorine.	-
2	HRT should not be less than 20 min	SW Disinfection Policy
3	A Lang Factor of 2.6 was used to encompass additional costs not factored into the construction costs and is deemed an industry standard value	(Whitton, 2016)
4	The Power Factor Model was used to account for economies of scale based upon internal SW costing of a tank 800 m^3 in volume. A power factor (pf) of 0.67 was applied as recommended in the literature. $(Capital\ cost\ of\ new\ project) = \left(\frac{Size\ of\ new\ project}{Size\ of\ old\ project}\right)^{pf} \cdot (Capital\ cost\ of\ old\ project)$	(Petley, 1997; Remer and Chai, 1990)
5	A gross cost was estimated from the net cost using a factor of 2.19.	Pers comms. C. Murray, Scottish Water, Feb 2019
6	Costing of baffles was based upon the quoted price for replacement of 94 m^2 of baffles (see below)	Pers comms. Water-lines solutions Ltd. Jan 2019.

Item	*Value*
Length of baffles (m)	*8*
Depth of baffles (m)	*3.93*
Baffle area (m^2)	*31.44*
Number of baffles	*3*
Total baffle area (m^2)	*94.32*
Total cost (£)	*7,500*

5.3 Results and discussion

5.3.1 Classification of disinfection reactor type

Information gained from site visits to confirm disinfection points allowed for classification of disinfection reactors across the company (Figure 5.2). Reactors could be assigned to six categories, with the largest proportion (86% total) falling into categories of chlorine contact pipes (CCP), limestone contactors (LCT) and chlorine contact tanks (CCT). The majority of sites with LCTs were low output membrane sites (Max site output = 15.6 ML/d) where water passes through an LCT directly after chlorine dosing. Similarly, those sites with CCP reactors were also of low output (Max site output = 6.9 ML/d). In contrast there was a high production volume at treatment works with CCTs (Max site output = 364 ML/d) (Figure 5.2b). Of a maximum possible production volume of 2,623 ML/d, sites with CCTs can produce up to 2,281 ML/d (88.5% of the total). The large production volume associated with CCTs demonstrates the high risk of these sites due to the large number of customers served by these WTWs and justifies why extra consideration must be taken to ensure these processes perform optimally. As a result, these high risk CCT sites and those with no dedicated CCT that utilise a CWT for disinfection credit were the only sites considered in the rest of this assessment.

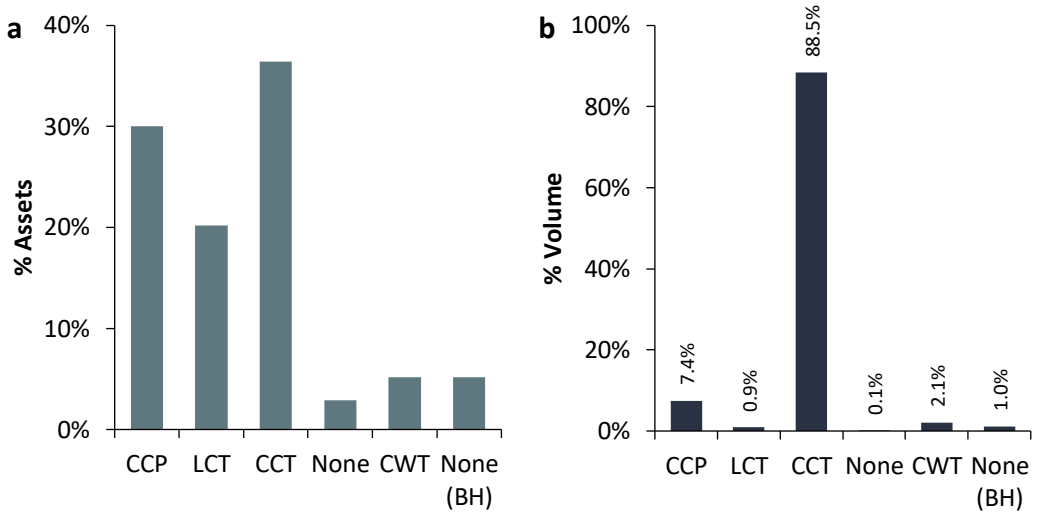

Figure 5.2. Distribution of chlorine disinfection reactor type as a percentage of asset base (a) and the percentage volume of water treated by each category per day (b) (n = 173) CCP = chlorine contact pipe, LCT = Limestone contactor, CCT = chlorine contact tank, None = No disinfection stage, CWT = clear water tank, None (BH) = Borehole site with no disinfection stage.

5.3.2 Scottish Water CCT Performance

The hydraulic residence time of a reactor can vary greatly depending on design characteristics. Poor design most commonly results in reactors that short circuit, whereby the true residence time is significantly shorter than that which is theoretically calculated. This is covered in more detail in Chapter 4 and a more detailed report into reactor design was produced for Scottish Water by BHR Group Ltd (Cranfield, UK. Report ref: CR 6714, 1997). A total of 75 WTW had CCT or CWT disinfection stages and of these 60 had a tracer assessment carried out as part of the Ct survey (Table 5.3). In total, 37 of these WTW had hydraulic efficiencies less than the target value of 0.7. Out of 37 WTWs 14 were deemed suitable for additional baffling based on knowledge of the current tank arrangements, while the remaining 23 WTW should theoretically achieve a hydraulic efficiency >0.7 if operated correctly. These 23 WTW have been shortlisted for a follow up tracer test as the evidence shows a hydraulic efficiency less than 0.7 (Table

5.5). At each of these WTW, the technical drawings of the CCT in question indicate appropriate baffling was installed before commission. Therefore, the reason for the poor hydraulic performance may not be directly linked to the reactor design and will require further investigation.

Table 5.3. Hydraulic efficiency (HE) at WTW with CCT and CWT disinfection stages (n = 60). These were classified from poor to excellent as stated by *AWWA (2006)*

Hydraulic Efficiency	Performance classification	Count
>1	Excellent	11
<1>0.7	Excellent	12
<0.7>0.5	Acceptable	8
<0.5>0.2	Compromising	19
<0.2>0.1	Poor	4
<0.1	Poor	6

When first considering the maximum flow conditions and theoretical HRT, 15 of these WTW did not achieve the target value of 20 min HRT (Table 5.4). Calculating the HE based on the tracer tests allowed adjustment of the theoretical HRT at maximum flow considering efficiency, which resulted in an increased number of WTWs below the HRT target (n = 36). This was reflected by the HE adjusted Ct calculation. An assumption has been made here that a constant depth is maintained within the tank and therefore HE is maintained across the flow range, in line with the findings of Chapter 4.

Table 5.4. Hydraulic residence times (HRT) and Ct values at each site calculated from chlorine tracer max flow conditions.

Condition	Flow	Target	Below target (n)
Theoretical HRT	Max	20.0	15
HE Theoretical HRT	Max	20.0	36
Ct	Max	15.0	13
HE Ct	Max	15.0	29
Theoretical HRT	Test	20.0	9
Tracer HRT	Test	20.0	28
Theoretical Ct	Test	15.0	6
Tracer Ct	Test	15.0	19

Using the tracer information, the Ct across the WTWs was calculated based upon the typical chlorine dose applied at the point of disinfection (Figure 5.3). A median Ct of 25.6 mg.min/L was calculated with a minimum and maximum Ct of 1.2 mg.min/L and 758 mg.min/L respectively. Considering the data in (Figure 3.2b), we can see that detections of coliforms in final water samples was correlated with poor Ct.

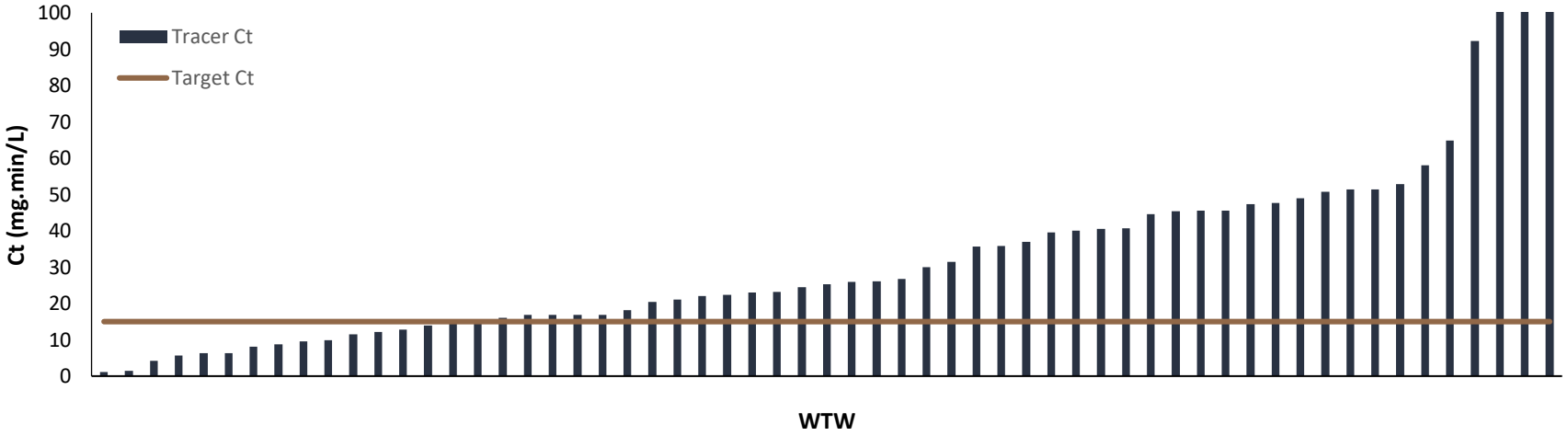

Figure 5.3. Ct values at Scottish Water WTW with CCT or CWT disinfection point. Ct calculated from chlorine dose at the time of the test and tracer derived HRT (n=60), note that the axis has been capped at 100 mg.min/L for clarity.

The solution to Ct issues cannot just be to increase contact time without any consideration for the detrimental effect this could have on water quality. Where Ct values are too low there is a risk of bacteriological issues from undisinfected water entering the distribution. Where the Ct is too high, there is a loss of chlorine residual due to decay which can require secondary dosing downstream in the network which is costly and harder to manage than at the WTW but also there is an increased risk of disinfection-by-product formation. Formation of these unwanted by-products can be rapid, with THM concentrations increasing over two fold with a 15 min increase of contact time with free chlorine from 5 min (Pope *et al.*, 2006). The relationship between Ct and THM concentrations was therefore explored but it was shown that that there was no relationship between these two parameters (data not shown). The total organic carbon (TOC) was instead a stronger driving factor ($r^2 = 0.54$), supporting the previous findings of (Golea *et al*., 2017). This reinforces the point that the primary focus for THM reduction should be on the removal of organics upstream of disinfection, as compromising disinfection and the subsequent failure to disinfect water sufficiently is not a question of if, but when, an individual will become ill from its consumption (Hrudey and Fawell, 2015).

5.3.3 Capable but inefficient CCT

There were 20 WTW where the theoretical HRT at maximum flow was capable of achieving the required HRT of 20 min, yet due to the HE the actual operational HRT was less than this target (Table 5.5). Further investigation of the design drawings for the CCT at these WTW showed that that all 20 of these WTW should have been able to achieve an HE of 0.7. The implication of this is that these chlorine contact tanks are appropriately sized with correct inlet and outlet configurations. Therefore, other factors may have led to the determination of an inaccurate HE for some of these sites. Firstly, these tests rely on the online chlorine monitors being correctly placed at the inlet and outlet of the disinfection contactor. Secondly, there is a possible additive error of 6 min when using online colorimetric chlorine monitors, as each monitor can have a delayed response time of 3 min (White, 2010). Considering this, and assuming the worst-case scenario of 6 min error, this would bring 7 of the 20 CCTs considered here above the 20-min HRT threshold. Based on this information it has been concluded that the certainty around the HE of these contactors was unreliable and requires re-testing. As such these 20 WTW have not been considered further for the calculation of the investments needed to upgrade disinfection contactors in Scottish Water.

Table 5.5. CCT that are theoretically capable of achieving the required HRT but appear to be inefficient. Sites are anonymised and '-r' denotes retest required.

Site	Treatment	Max flow (ML/d)	Flow during test (ML/d)	Reactor volume (m^3)	Test flow theoretical RT (min)	Test Tracer RT (min)	Hydraulic efficiency	Max flow Theoretical RT (min)	HE adjusted HRT
A-r	Direct filtration	9.50	8.64	420	70	18	0.26	63.6	16.4
B-r	Direct filtration	19.01	9.50	314	48	30	0.63	23.8	15.0
C-r	Fluidised bed filtration	1.81	0.95	450	682	106	0.16	357.1	55.5
D-r	Direct filtration	364.00	198.72	5,686	41	15	0.36	22.5	8.2
E-r	Fluidised bed filtration	1.04	0.61	33	79	35	0.44	46.2	20.4
F-r	Direct filtration	103.68	36.29	1,900	75	35	0.46	26.4	12.3
G-r	Direct filtration	124.42	96.77	1,789	27	5	0.19	20.7	3.9
H-r	Conventional	129.00	90.72	11,100	176	57	0.32	123.9	40.1
I-r	Fluidised bed filtration	0.98	0.60	35	84	37	0.44	52.3	23.1
J-r	Direct filtration	5.18	4.23	184	62	20	0.32	51.0	16.3
K-r	Direct filtration	62.50	32.20	1,563	70	47	0.67	36.0	24.2
L-r	Membrane	16.30	7.40	600	117	33	0.28	53.0	15.0
M-r	Direct filtration	2.40	1.44	90	90	59	0.66	54.0	35.4
N-r	Direct filtration	276.48	259.20	2,363	13	41	3.12	12.3	38.4
O-r	Conventional	32.00	17.00	622	53	36	0.68	28.0	19.1
P-r	Direct filtration	15.98	12.10	304	36	20	0.55	27.4	15.1
Q-r	Conventional	16.00	12.00	473	57	33	0.58	42.5	24.8
R-r	Direct filtration	46.00	33.00	740	32	17	0.53	23.2	12.2
S-r	Conventional	86.40	78.11	3,408	63	37	0.59	56.8	33.4
T-r	Direct filtration	3.36	2.64	116	63	18	0.28	49.8	14.1

5.3.4 Investment required to upgrade short circuiting reactors with sufficient volume

12 of the CCT WTW were identified as suitable for baffling improvements which resulted in a gross cost of £498,558 for a complete baffle upgrade (Table 5.6) which works out at 3.7% of the cost required to replace these CCT. The required amount of baffling was based upon the increase in hydraulic efficiency required to reach the target efficiency of 0.7. The primary goal of baffling is to increase the length to width ratio (L:W) of the reactor. While a minimum ratio of 10:1 can be acceptable, an optimum L:W ratio of 40:1 is recommended, leading to a commensurate reduction in dispersion (Marske and Boyle, 1973). Increasing the L:W not only reduces dispersion but leads to an improvement in HE. (Crozes *et al.*, 1999), a trend that was also observed from the pilot studies undertaken here (Figure 5.4). Based on the relationship between HE and L:W to achieve an HE of 0.7, an L:W of at least 33.1 must be achieved from baffling. As a longitudinal baffle is preferable so that turbulence is reduced (Rauen, 2005), the costs were therefore estimated using the total length of the tank.

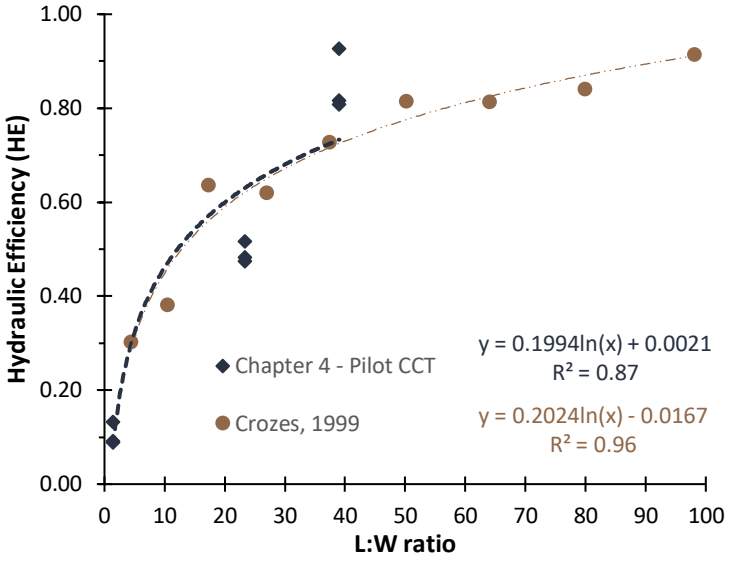

Figure 5.4. Relationship between HE and L:W ratio for rectangular CCT. Equation of line was obtained from pilot studies in chapter 4 and data extracted from Crozes *et al.* (1999) is overlaid for comparison

The most expensive site to baffle is 'G-b' requiring a £ 83,588 investment, whereas the lowest cost site was H-b at almost 6 times a lower cost of £ 14,959 due to the much smaller tank at that site and the overall average cost of the 12 sites included in this assessment was £ 41,547. When considering the costs to replace the current CCT with a new tank the costs for baffling are significantly lower.

What is not specified here but must be considered when installing these baffles is the critically of the arrangement and location within the tank. Increasing the L:W to achieve a given HE does not account for the turbulence regimes within the tank that can occur. CCT should ideally maintain a turbulent flow within each channel of the tank (Re > 2,000). A Reynolds number less than this will result in transitional and eventually laminar flow (Re < 600) that will lead to improper mixing of chlorine with the bulk flow (Chaudhury, 2008).

The pilot studies in chapter 4 only considered a longitudinal baffle arrangement, which is optimal to reduce back mixing and excessive turbulence around each baffle. Lateral baffle arrangements require more baffles than a longitudinal arrangement to achieve the same HE, therefore this point of diminishing returns may occur with fewer baffles. There is therefore an economic trade-off to be made with increasing hydraulic efficiency and the number of baffles introduced to a CCT that must be considered on a site-by-site basis during the planning stage.

Furthermore, in a lateral baffle arrangement the number of baffles introduced to a reactor was shown by computational fluidic dynamics (CFD) to reach a point of diminishing returns when 6 or more baffles were introduced (Wilson and Venayagamoorthy, 2010). The cost of building a replacement tank of the same poor efficiency pales in comparison to baffling (Table 5.7). Replacement of these inefficient tanks with a larger but like-for-like design that is required to achieve the desired HRT is significantly higher in cost (32 times more), and would not be an efficient

Table 5.6. Estimated costing for baffling of WTW with poor hydraulic efficiency but sufficient theoretical contact time. Site names are anonymised and '-b' denotes baffling intervention.

Site ID	No of tanks	Length (m)	Width (m)	Depth (m)	Volume (m^3)	HE	Baffles req. for HE = 0.7*	New L:W	Est baffle area (m^2)	Power factor model cost (£)	Lang factor Net cost (£)	Gross cost (£)
A-b	1	20.0	8.3	3.0	496.0	0.34	3	38.6	180	11,564	30,066	66,086
B-b	1	3.4	2.7	1.3	11.5	0.17	5	45.3	21.3	2,763	7,184	15,791
C-b	1	20.0	6.4	3.7	467.2	0.23	3	50.0	219	13,188	34,288	75,366
D-b	2	6.4	4.8	4.8	295.1	0.05	4	33.3	122.9	8,957	23,289	51,189
E-b	1	12.3	2.5	1.7	52.3	0.36	2	44.3	41.8	4,349	11,308	24,855
F-b	1	17.9	17.0	1.8	546.2	0.31	5	37.8	160.7	10,716	27,861	61,238
G-b	1	30.0	12.5	2.8	1065.0	0.23	3	38.4	255.6	14,627	38,029	83,588
H-b	1	4.0	1.0	2.5	9.8	0.09	2	36.0	19.6	2,618	6,806	14,959
I-b	1	7.2	7.0	3.3	165.3	0.48	5	36.7	118	8,713	22,654	49,794
J-b	1	4.0	2.0	1.5	12.0	0.32	4	50.0	24	2,998	7,795	17,132
K-b	1	4.2	2.3	1.8	17.8	0.48	4	45.7	30.9	3,552	9,235	20,298
L-b	1	4.4	3.3	1.5	21.8	0.20	4	33.3	26.4	3,196	8,309	18,262
									TOTAL (£)	87,240	226,824	498,558

**Baffles required calculated from L:W vs HE Figure 5.4*

Table 5.7. Estimated cost of tank replacement to achieve target HRT if baffling was not chosen. Site names are anonymised and '-t' denotes tank construction required.

Site ID	Flow (m^3/d)	Flow (m^3/min)	Required HRT (min)	Volume (m^3)	Calculated RT (min)	New tank min vol required (m^3)	Power factor model cost (£)	Lang factor Net cost (£)	Gross cost(£)
A-t	25000	17.36	20	496.0	28.6	496.0	364,697	948,212	2,084,170
B-t	1296	0.90	20	11.5	12.8	25.7	50,206	130,536	286,919
C-t	25920	18.00	20	467.2	26.0	514.3	373,635	971,452	2,135,251
D-t	4680	3.25	20	295.0	90.8	92.9	118,680	308,568	678,232
E-t	4490	3.12	20	52.3	16.8	89.1	115,430	300,117	659,657
F-t	47520	33.00	20	546.2	16.6	942.9	560,815	1,458,119	3,204,945
G-t	30000	20.83	20	1065.0	51.1	595.2	412,082	1,071,413	2,354,966
H-t	260	0.18	20	9.8	54.3	5.2	17,114	44,496	97,802
I-t	10900	7.57	20	165.3	21.8	216.3	209,116	543,703	1,195,058
J-t	690	0.48	20	12.0	25.0	13.7	32,911	85,569	188,080
K-t	1167	0.81	20	17.8	21.9	23.2	46,800	121,681	267,455
L-t	907.2	0.63	20	21.8	34.6	18.0	39,534	102,789	225,930
						TOTAL (£)	2,341,021	6,086,654	13,378,465

5.3.5 Investment required for reactors with insufficient volume

There were some cases where chlorine contactors could achieve near perfect HE but were not able to achieve the required HRT of 20 min. At some WTW there was no dedicated disinfection stage of treatment (for example, where a CWT is currently used for disinfection credit). In these cases the estimated costs have been calculated for the construction of a new CCT with an estimated HE >0.7. Costs have been estimated using information available from an upgrade of a disinfection contactor currently underway at WTW A (Pers comm C. Murray, Scottish Water, February 2019). It is important to note that this cost does not include instrumentation, dosing equipment, control equipment and overheads and there will also be additional investment required to rectify issues around disinfection pH so that an optimum disinfection pH (<7.5) is always maintained.

Sites were assessed based on both tracer and theoretical HRT at max flow and, where these did not meet the minimum 20 min bulk HRT stated in the SW disinfection policy, a tank replacement cost was calculated (Table 5.8). Tank sizing was based on the maximum design flow of the works, as the bulk HRT should be achieved under the most demanding flow conditions and assumed an operational HE of 0.7. It should be noted that a number of smaller WTW were included in the cost estimations below. The smaller of these sites (A-ci and B-ci) may be more suited to the installation of a chlorine contact pipe (CCP) and so costs are likely to scale differently when assessed in greater detail, although the power factor scaled costs and inclusion of the Lang factor will give a good estimation. The gross investment required for construction of new CCTs across SW was estimated to be £17,508,370. SW have estimated that roughly £ 130 – 240 million of investment will be required to implement the disinfection policy across the business.

This cost includes both the requirement to bring all disinfection control systems, pH dosing arrangements, shutdown procedures and overall Ct up to standard. Therefore, the estimated costs here for tank construction that do not include overheads or other planning and design costs are reasonable.

Without this investment there would continue to be 14 high risk WTW that will be producing non-Ct compliant water in the future, and that have been determined (over a 3-year period), to see 5 times more coliform detections (Figure 2.2b). Based on the findings of Ellis *et al.* (2018) the cost of a bacteriological detection to a water utility is estimated to be £ 4,775 if that detection was to occur at the WTW, therefore by improving the Ct at these sites (reducing failures by 46 Figure 2.2b) an estimated re-sampling and investigation cost saving of £ 267,400 could be made over a 3 year period. There is a further reputational cost associated with these continued failures, particularly in an area of treatment that is non-compliant with the SW policy, that although difficult to financially quantify is of upmost concern.

Table 5.8. Estimated costs for the construction of a new CCT where there is currently insufficient HRT or no dedicated disinfection reactor. Based upon a required HRT of 20 min and HE of 0.7. Each site is anonymised and '-ci' denotes capital investment required.

Site	Flow (m^3/d)	Flow (m^3/min)	Volume (m^3)	Calculated RT (min)	Power factor model cost (£)	Lang factor Net cost (£)	Gross cost (£)
WTW A	37,000	25.69	800.0	31.14	*502,355*	1,306,124	2,870,861
WTW B	22,000	15.28	227.0	14.86	216,012	561,632	1,234,466
A-ci	358	0.25	7.1	28.57	21,204	55,130	121,176
B-ci	24	0.02	0.5	28.57	3,468	9,016	19,818
C-ci	776	0.54	15.4	28.57	35,606	92,575	203,480
D-ci	3,888	2.70	77.1	28.57	104,816	272,522	599,004
E-ci	5,184	3.60	102.9	28.57	127,098	330,454	726,338
F-ci	80,000	55.56	1,587.3	28.57	795,028	2,067,074	4,543,428
G-ci	5,760	4.00	114.3	28.57	136,394	354,625	779,465
H-ci	11,232	7.80	222.9	28.57	213,363	554,743	1,219,325
I-ci	576	0.40	11.4	28.57	29,161	75,817	166,647
J-ci	42,000	29.17	833.3	28.57	516,285	1,342,341	2,950,465
K-ci	9,000	6.25	178.6	28.57	183,931	478,221	1,051,129
L-ci	8,640	6.00	171.4	28.57	178,968	465,318	1,022,769
				TOTAL (£)	3,063,689	7,965,592	17,508,370

5.3.6 Ct performance decision tree

Based upon the findings of this chapter a decision tree has been established for use with assessment of CCT performance (Figure 5.5). Adhering to this process will allow for the most appropriate decision to be made for each CCT based upon a simple tracer test and RTD methodology as performed in chapter 4, this process can be applied to both those reactors that have already had a tracer test and for those yet to be tested.

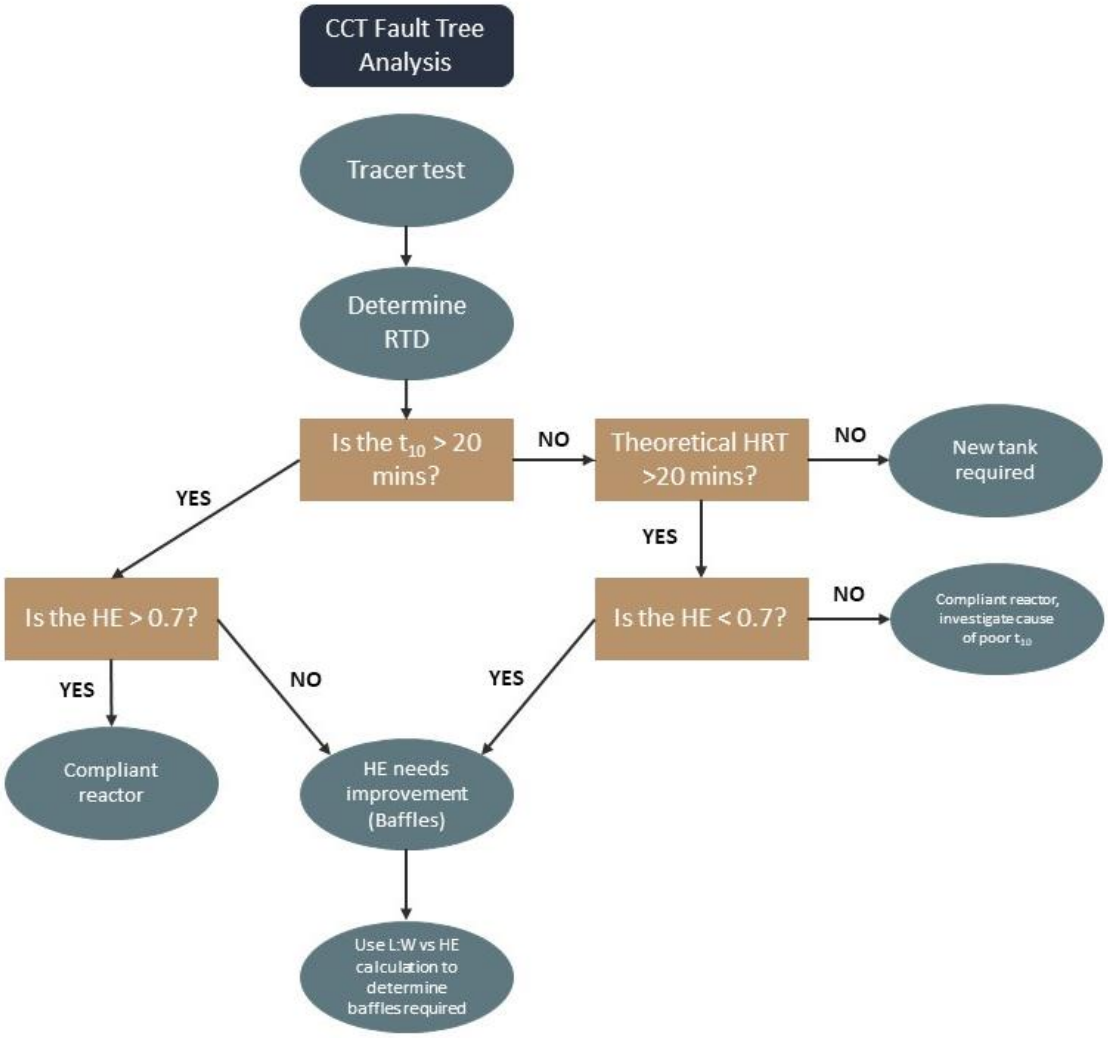

Figure 5.5. Decision tree for investment requirements of CCT across Scottish Water. The L:W vs HE calculation can be found in 6.4 of this chapter.

5.3.7 Alternatives to chlorine disinfection

Chlorination as a disinfection solution for drinking water is not the only option. UV disinfection is an ideal modular solution that can be installed fairly rapidly at a treatment works where applicable. In some cases, UV can be a cheaper solution than installing a new CCT. As an example, a recently constructed UV plant at Scottish Water sized at 33 ML/d cost £1.5 million (RSE, 2018). For comparison, with an equivalent CCT for a similar sized WTW (Table 5.7) it is estimated to cost £2.4 million. This would make UV treatment to meet disinfection requirements an attractive alternative. However, there are a number of factors that need to be taken into consideration. Firstly, regulation within the UK requires all water must have a chlorine residual upon entering distribution and this has to be maintained up to the point of consumption. Therefore, all chlorine dosing ancillary equipment must be maintained and used in conjunction with UV treatment. Secondly, the basic capital costs of UV treatment may seem cheaper than chlorine disinfection but it must be considered that there is less tolerance of the influent water quality (transmissivity of water etc.) which may require investment in upstream processes (pers. comms. G.Moore. Sept 2019). Therefore, each treatment option would require site by site optioneering analysis to determine the most effective installation.

5.4 Conclusions

The data from the Scottish Water Ct survey has proved to be an important starting point for improving the efficacy of chlorine disinfection across the business. The gross investment required to satisfy the requirements of DWQR Information letter 2013/3 addressed by this project equates to £18,006,928. With this investment, all of the high risk WTW (those with CCT) would be compliant with the SW disinfection policy. By bringing Ct across the business up to the same level there will be a concurrent overall reduction in bacteriological failures as the security of this treatment barrier is improved. Large capital investments such as building new tanks will require rigorous design procedures, but this study has identified 12 CCT where a cost-effective solution of baffling can be installed in a much shorter time scale. To assist with the decision process of whether baffling or construction of a new tank is required a simple decision tree process for this evaluation has been described.

Finally, it is important to remember that the primary goal of disinfection is that pathogenic organisms are inactivated before water leaves the WTW, a secondary but important consideration is the formation of DBPs. This study has shown than increased Ct does not lead to increased DBP (THM) formation and that this formation is predominately driven by organic carbon and the free chlorine dose.

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6. Thesis conclusions and implications of the work for the UK water industry:

The current chapter explores the implications of these findings in relation to three questions that have been poised by the industrial sponsors, including progress toward implementation where pertinent followed by the overall thesis conclusions.

6.1. How has FC altered the way we think about disinfection?

Chlorine disinfection remains an industry standard for inactivating pathogens and enabling water companies to meet regulatory standards. Within this thesis the disinfection process has been further explored in relation to the relationship between 'Ct' and microbial inactivation using a non-culture-based technique which has enabled fresh insights to be established. FCM allowed for determination of log inactivation rates both in a laboratory (Chapter 3) and pilot scale environment (Chapter 4), whereby standard HPC measures could not. This enabled direct quantification of the inactivation rates for both laboratory grown and communities within a natural water, showing a significant difference in their respective inactivation profiles (Chapter 3). This granularity of data is critical for driving improvements in disinfection operation and efficacy. This is best illustrated in Chapter 4, where for the first-time changes in hydraulic efficiency and overall Ct have been related to the actual inactivation of organisms. This is data that has only previously been collected in a laboratory study or theoretical modelling of inactivation rates. Extending this further, the results indicate that the use of FC could be applied as an actual Ct validation tool whereby an acceptable 'Log inactivation' could be credited to a disinfection process, allowing for Ct to be adjusted to always meet this requirement and ultimately controlled to resiliently deliver it. This is an application that

is not feasible with culture-based techniques due to both time and practical constraints of the approach. Chapter 3 demonstrated that Ct is intrinsically linked to an increased risk of coliform detections which still remain a critical regulatory driver for water utilities. The findings also indicated that maintaining an ICC/TCC% of ≈1% reduces the number of coliform detections, therefore we can begin to set operational targets linking Ct and ICC/TCC% and hence potentially an early warning of deterioration in the efficacy of disinfection. The current operational approach is to operate within a range of chlorine concentrations (e.g. 0.5 -1.5 mg/L) yet this misses the key point that disinfection should be controlled around the aggregate Ct parameter.

6.2. How do we best describe effective disinfection from an operational standpoint?

As mentioned above, the operational control for disinfection is currently based upon maintaining a chlorine dose within a given 'band'. In reality, each site will require a targeted Ct for the particular water quality at that site. The process should be controlled based upon Ct and the process should be alarmed with a shutdown procedure if the value was to drop below a 'Ct target' for that site. Currently across Scottish Water this can only be done by reference to 'Ct calculation tables', a physical copy of Ct at a given flow rate, dose and pH. We assume that Ct is a constant value, yet there is variability throughout operation of a WTW. This can be as simple as dose changes based upon chemical batching, flow rate changes to deal with demand or backwashes of filters drawing water from CCT. All of which impact the real Ct value. There also then must be consideration of the risk of Ct failure, more specifically 'How long can operation continue without dosing?'

Based on the findings of chapter 4, that HE had a significant impact upon the Ct and subsequent inactivation of microorganisms, what is termed a 'Ct tool' has been developed. In collaboration with Process Instruments Ltd (Burnley, UK) and utilising their CRIUS® controller platform this 'Ct tool' is essentially a controller that allows for a real time display of the live operational Ct. The interface of this tool allows for the operator to input the following:

1) The volume of the tank (m^3), if known
2) Calculated HE based upon L:W of the tank (Chapter 5)
3) Tracer calculated HE based upon theoretical HRT at time of test

There is then a hierarchy of decisions within the software to calculate the actual Ct using the most appropriate residence time calculation. The priority of the system here is to take the value from (3), the known volume of the tank (1) and a flow rate input to the contact tank on site in order to calculate the actual residence time of the tank, in this case the t_{10} as this has an increased safety factor. The chlorine sensor of choice, ideally that located at the outlet of the tank, then uses this true residence time and the chlorine concentration to determine 'Ct' and a signal is sent from the system to the SCADA so that it can be trended alongside other critical water quality parameters.

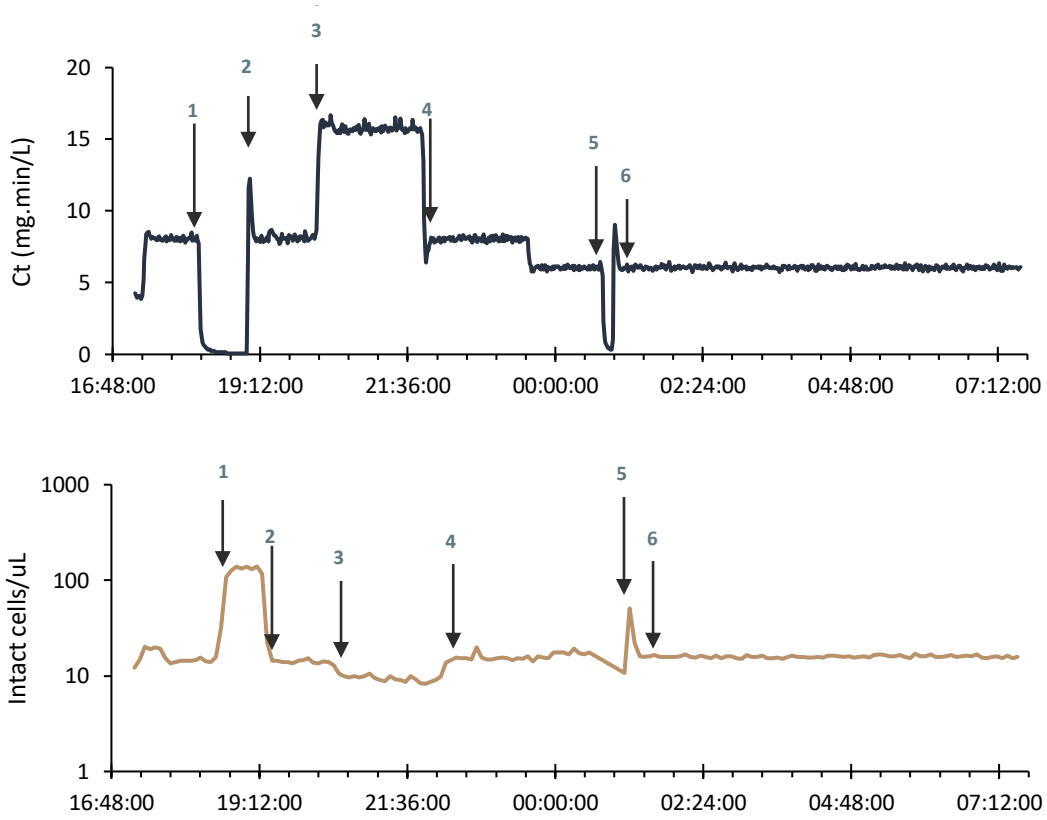

Figure 6.1. Variation of operational Ct over a range of simulated conditions in a pilot scale CCT (a). Response of ICC determined using online flow cytometry located at the outlet of the CCT (b). Descriptions of events: 1: Loss of chlorine dose 45 min, 2: Chlorine dose reinstated, 3: Setpoint of dose increased, 4: Returned to baseline, 5: Loss of chlorine dose 10 min, 6: Steady state operation.

Essentially with tighter Ct control and operational bands, production would be shut down upon any loss of chlorine dosing. This Ct tool was demonstrated at pilot scale in combination with online intact cell count (ICC) monitoring by FCM (Figure 6.1). Changes in the operational Ct calculated by the Ct tool were directly related to changes in the ICC, both regarding loss of chlorine dosing (Event 1 and 5) and changes in the dose setpoint (Events 2, 3 and 4). The critical result here is that a short interruption to dosing of 45 min causes 10 X the amount of ICC to exit the CCT. If we scale this to the average production volume of a SW WTW (Chapter 5) of 13.6 ML/d this 45 min period would

allow 5.1 x10^{13} ICC to pass through the process un-disinfected, a significant increase in risk.

6.3. Where does the future of FCM application lie?

FCM has been the central tool used for the investigations within this thesis and has without doubt allowed for interpretation of the impact of process variables on bacteriological water quality (Chapter 3 and 4). Academically, there have been numerous demonstrations of the applicability of FCM, yet there are few published demonstrations of its use by the drinking water industry and this thesis attempted to address this (Chapter 2). The insights that FCM can give has gained increasing attention in the UK and globally in the water sector over the past decade. Within the UK, the attention has become widespread and has resulted in the establishment of an industry focused 'Flow Cytometry User Group'. This has provided a platform for water utilities (and universities) to discuss both the appropriate application of FCM and the methodological aspects of this technique. This interest has also been mirrored by the regulatory bodies in the UK, with the Drinking Water Quality Regulator (DWQR) backing Scottish Waters application of the technique and the Drinking Water Inspectorate (DWI) recently commissioning a project to assess how each water company across the UK gathers and uses this new rich data source (DEFRA, 2018). So, this builds to the question of: 'What is the future for FCM?'

6.3.1. As a regulatory monitoring tool

FCM is not a replacement or substitute for the monitoring of indicator organisms (Chapter 2), this is not unexpected as the two approaches are in no way related, but it is important to confirm this lack of relationship as it has been an area of great interest

from the user group. In contrast, it would appear that FCM could be a suitable replacement or addition to HPC monitoring. Across the UK, legislation on acceptable levels of HPC is termed 'No abnormal change', yet an abnormal change is almost impossible to quantify in a dataset that is dominated by blank results (non-detected) (see chapter 2 and 4). FCM on the other hand is data rich, allowing for trending of data and the statistical deviation from the norm can be calculated once a sufficient historic data set is established. FCM also provides more than just a single count from the analysis. Total cells, intact cells and the intact/total percentage are the current standard determinants but the additional HNA/LNA population distinction can also be derived (Chapter 3).

6.3.2. Process monitoring and diagnostics

Monitoring of actual process performance with regards to the microbiology is rarely undertaken as operational practice across the UK water industry. Regulatory compliance is based on samples taken at the WTW, service reservoirs in distribution and customer taps (Scottish Parliament, 2014). The comparative speed of FCM (<15 min/sample) when compared to standard HPC (3-7 days/sample) means there is a potential for use of this method as a near real-time response tool. Revealing the operational dynamics of treatment processes will greatly develop the understanding of operational bands for optimum microbiological water quality, which is a key component for water safety plans (WSP). WSP and the increasing popularity of quantitate microbial risk assessments (QMRA) rely heavily on accurate process removal rates to determine the potential risk to the consumer. As demonstrated in Chapter 2, FCM can provide information regarding the log removal of different treatment processes by inter-stage sampling. These findings

carried out across the SW asset base can allow for comparison of treatment trains (Ho *et al*., 2012) and ranking of individual process efficiencies. In essence, the operation of treatment processes to target an optimal removal will allow for increased confidence in the robustness of that treatment barrier to deal with potential barrier failure at a single point in the treatment train. Given that FCM enables the ability to distinguish between the impact in disinfection of temperature (chapter 3), hydraulic efficiency (chapter 4) and operational robustness (Figure 6.1), this approach allows us to begin to think about how the log removal dynamics of each treatment stage over an hourly, daily and annually cycle are affected. It is posited that this enrichment of available data will be the basis, in the near future, of new understanding in how to manage both individual assets and the overall works to maximise water safety.

6.4. Overall conclusions

The overall aim of this research was to determine how flow cytometric analysis can be applied within the water industry and specifically how can this technology be utilised to assess and aid optimisation of the chlorine disinfection stage within drinking water treatment. In response, the findings of this thesis have demonstrated that key process variables related to Ct need to be correctly controlled to produce optimum bacteriological water quality. In addition, flow cytometry has been demonstrated to be a valuable tool in the assessment of disinfection efficacy by enabling rapid culture independent measurement of disinfection. Objective specific conclusions are included below.

Objective 1 - To critically appraise the most appropriate application of flow cytometry for monitoring bacteriological water quality in drinking water production with a focus on disinfection.

FC was shown to not correlate with coliform detections, although likelihood of coliform detections increased above 10,000 ICC/mL the majority of coliform detections occur when ICC <1,000 cells/mL (Chapter 2)

- Free chlorine alone was not a deterministic factor in the number of WTW coliform detections and the ICC/TCC%. Instead it was demonstrated that this was driven by the Ct (mg.min/L) at the WTW (Chapter 2)
- FC allowed for an evaluation of individual process performance, providing data on both physical and chemical disinfection through the WTW. The key application of this analysis will be in its application to water safety plans (WSP) (Chapter 2).

Objective 2 - Carry out bench scale disinfection experiments to understand how flow cytometric data responds to typical operational parameters that impact disinfection efficacy.

- Disinfectant dose, exposure time and temperature impacted on efficacy of disinfection, the impact of pH contradicted the established chlorine disinfection theory when assessed by FC, whereby enhanced inactivation rates occurred under more alkaline conditions (Chapter 3).
- Ultimately the delivery of an appropriate Ct is crucial for disinfecion, requiring that the dose and contact time are appropriately set and that the chlorine

contact tank has sufficient hydraulic efficiency to deliver the required Ct (Chapter 5).

Objective 3 - To determine the hydraulic efficiency of chlorine contact tanks with different geometries and establish the link between flow behaviour and disinfection efficacy.

- Hydraulic efficiency was best described by the combination of t_{10} and dispersion index (σ^2)(Chapter 4)
- The tracer mean adjusted Ct, $Ct_{\tilde{x}}$, correlated most strongly with the observed inactivation rate.As such it is proposed that the $Ct_{\tilde{x}}$ is the best indicator of inactivation through a CCT(Chapter 4)
- FC allowed for in-situ evaluation of bacterial inactivation rates, whilst under the same conditions HPC data was not able to provide any insight on process performance (Chapter 4)

Objective 4 - To assess current disinfection practice across Scottish Water and identify a strategy for improvement where required.

- A significant portion of Scottish Water CCT will require investment, to improve hydraulic efficiency or a complete replacement of the contact structure to achieve the Scottish Water disinfection policy specifications (Chapter 5)
- Simple baffle curtain solutions are cost effective (<4% of new tank cost) and can improve hydraulic efficiency (HE) at a minimum of 12 SW WTW (Chapter 5).

A decision tree for evaluating CCT performance and subsequent investment decision making has been provided that is simple to follow and utilises data from this and previous studies to calculate the optimum design (Chapter 5).

6.5. References

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7. Further Work

7.1. Chlorine disinfection mechanism of action and the impact of pH

A critical outcome of Chapter 3 is that there is still an unknown impact from pH on the ICC and TCC data, and further work is required to develop an understanding of this mechanism. Principally, it needs to be established whether: the finding is an artefact of the method dependency on membrane integrity, and why membrane integrity may increase under alkaline conditions. The issue raised here is ideal for the application of a multiparameter viability assay as described by Nocker *et al*. (2011). This approach allows for a systematic evaluation of viability parameters, ranging from membrane permeability (TCC/ICC), membrane potential, enzymatic activity, redox potential, culturability and regrowth lag time (suppression). Applying this methodology over a broader pH range with pure cultures and natural communities (treated water) would lead to a deeper understanding of the critical mechanisms of action building upon the fundamental historic research (Chang, 1944; Friberg, 1956, 1957; Haas and Engelbrecht, 1980).

The pH affect observed within this thesis also emphasis the requirement for a more standardised method for the analysis of water samples by FC. When evaluating data from the literature, many studies do not specify a buffering step for staining of samples. There are some instances where EDTA treatment of cells has been applied, although this is not strictly a 'buffering' step as the EDTA is utilised as an aid to increase dye uptake by the cell. However, EDTA is a pH 8 solution at a standard stock concentration of 0.5 M which is added to samples to give a final concentration of 5 mM, this final concentration

is often enough to alter the pH of the sample. The issue with addition of EDTA is that it alters the uptake of dyes to a point whereby false PI positive cells are observed (Virto *et al.* 2005). Nescerecka *et al.* (2016) developed a pipeline for development FC based staining protocols and found that EDTA decreased the number of intact cells by up to 39% and that more research is required to understand whether the addition EDTA is beneficial to analysis or not. Within this study the staining pH was adjusted to pH 7. It was observed in preliminary studies that FC dot plots and histograms were not consistent if this step was omitted. It would therefore be pertinent for further research to understand what the optimum buffer conditions are and establish what the impacts are of not including this additional step within cytometry workflows.

7.2. Extracting more from FCM analysis

The value in HNA/LNA has been identified as its ability to distinguish two sources that would look similar when only considering cell counts (TCC or ICC). Previous work has demonstrated that changes in the microbial diversity of water can be identified from statistical profiling of HNA/LNA components (Props *et al.*, 2018). However, this does not answer the question of 'do these changes matter operationally?' and returns to the argument of what is abnormal change with regards to the microbiological quality of drinking water? These questions can only be answered with large sources of data and require higher numbers of samples with coliform detections, which can only be obtained over an extended monitoring period. One of the biggest challenges seen from chapter 2 was the limited number of coliform positive samples in final water from three years of analysis (n=92). Therefore, even after 10 years of monitoring, and assuming the same rate of detection, there would only be an estimated 307 detections which is still a rather

insignificant amount. Consequently, it suggested that further work is conducted to enrich this data pool by collaboratively sharing the existing and future datasets generated by drinking water providers. Work is required to ensure that the data is analysed in a comparable way and should include an inter-lab study to ensure consistency.

7.3. FCM is not the only solution

FCM data has been directly linked to HPC and used within a water quality risk assessment. However, the work outlined within the current thesis queries the universal validity of this as no substantive link could be determined between FCM and HPC congruent with previous other studies (Van Nevel *et al*., 2017). However, the uncertainty generated by the differences in findings indicate that it represents an area for further investigations to fully elucidate whether FCM can be used to directly replace HPC and converted into a viable risk tool. The reality may be that FCM alone is not suitable to draw conclusions about the bacteriological water quality and it would be best suited to use in combination with other culture independent techniques. Recent work has demonstrated that Adenosine tri-phosphate (ATP) analysis has value as a rapid method for the evaluation of chlorine damage (Nescerecka *et al*., 2016), this measure of metabolic activity in combination with cell counts can then give a relative activity measure of 'ATP-per-cell' (Lautenschlager *et al.*, 2010).

7.4. Online flow cytometry

To date, there are two commercial options for the online FCM analysis. The first is distributed by Sigrist (Sigrist Photometer AG, Switzerland) known as the 'Bactosense' which has the key selling point that the product is 'Operationally friendly' and does not

require a technical expert to run, maintain or interpret the data from this instrument. Using a cartridge-based system these are pre-loaded with stains and cleaning reagents meaning the operator does not need to make-up these dyes. With a maximum resolution of 30 min this instrument can produce vast quantities of data when compared to traditional regulatory monitoring. The second option is available from OnCyt (OnCyt Microbiology AG, Zurich, Switzerland) who distribute an 'addon' for conventional FCM such as the BD Accuri C6/C6+ instrument (data in Figure 6.1 was collected using this). This product is clearly more suited to a research environment as it requires an experienced operator to maintain, run and interpret data. The benefit of this product is that the options are greatly increased, one can either choose to target more sample points (up to 12 streams) or increase sampling resolution (up to 5 min). A second addon is also available for short term microbial dynamic studies that operates in a continuous measurement function allowing for even greater resolution (<10 seconds). Many of the applications of these products are published and useful reference material for those interested in the application of this technology (Besmer *et al.*, 2014, 2016; Besmer and Hammes, 2016; Page *et al.*, 2017; Props *et al.*, 2018). These initial applications have demonstrated the potential for online flow cytometry in the future, what is required now is further work to demonstrate the true value at full and pilot scale. Specifically, future work should target the assessment of short-term dynamics in treatment processes and the subsequent impact this has on downstream process performance (e.g. loss of coagulation -> filtration removal -> disinfection performance).

7.5. References

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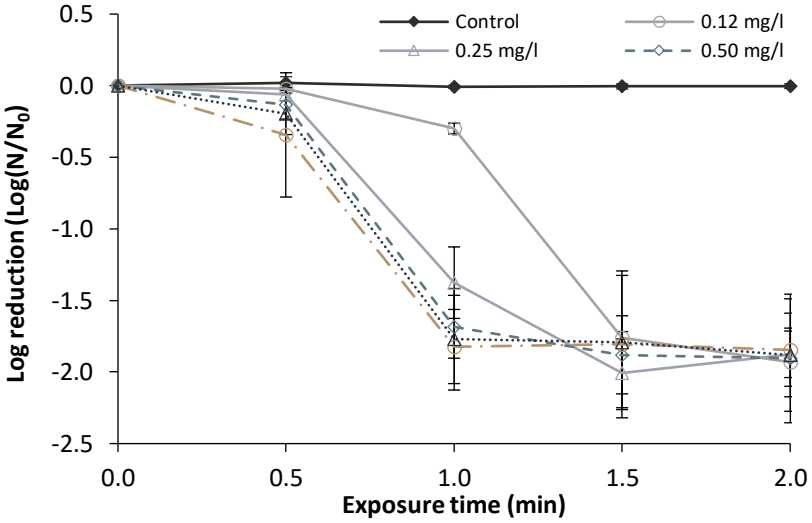
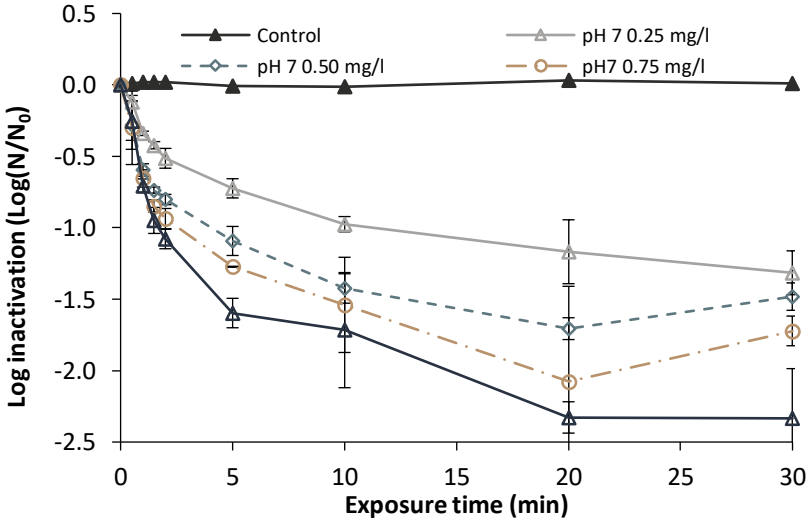
8. Appendices

Appendix A

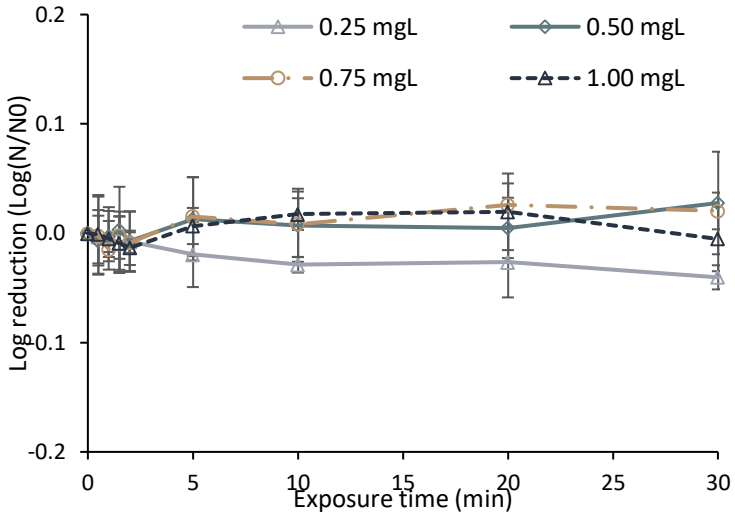
A1. Inactivation curves for E. coli and WTW Filtrate



Appendix A 1. Log reduction of *E. coli* SYBR Green I + Propidium iodide (Intact) cells exposed to chlorine dose of 0.12, 0.25, 0.50, 0.75 and 1.00 mg/L. All tests were carried out in demand free phosphate buffer (pH 7) at room temperature. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats.



Appendix A 2.Log reduction of cells in the WTW Filtrate stained with SYBR Green I + Propidium iodide (Intact cells) exposed to chlorine doses of 0.25, 0.50, 0.75 and 1.00 mg/L. All tests were carried out at room temperature and pH 7. Chlorine was added simultaneously to sampling at T = 0.5. Error bars represent standard deviation of three independent experimental repeats.



Appendix A 3. Log reduction of WTW Filtrate TCC after 30 min exposure to free chlorine doses of 0.25, 0.50, 0.75 and 1.00 mg/L. Chlorine was added at t = 0.5 min. Error bars represent standard deviation of triplicate experimental repeats.

Appendix B

B1. RTD calculations

Firstly, the mean residence time ($\bar{x}$) was calculated from the area under the Concentration (C)/ time (t) curve, Eq. Eq. (8.1):

Eq. (8.1)

$$\bar{x} = \frac{\sum_0^\infty (t \cdot C \cdot \Delta t)}{\sum_0^\infty (C \cdot \Delta t)}$$

Next, this curve was transformed into the E curve, such that the area under the curve was unity. Using the tracer mass (M) and reactor flow rate (Q) Eq. (8.2):

Eq. (8.2)

$$E = C \cdot \frac{Q}{M}$$

The E curve was then translated into an $E(\theta)$ curve from Eq. (8.3):

Eq. (8.3)

$$E(\theta) = \bar{x} \cdot E$$

Finally, the normalised time (θ) was calculated from the measured time (t) and the theoretical retention time (T), Eq.(8.4):

Eq.(8.4)

$$\theta = \frac{t}{T}$$

The RTD variance (σ^2) was calculated by Eq.(8.5):

Eq.(8.5)

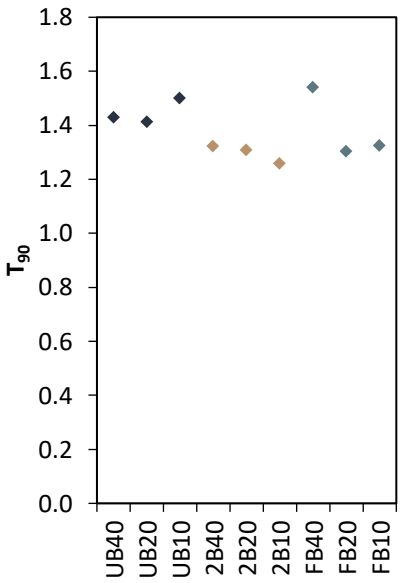
$$\sigma^2 = \frac{\sum t^2 \cdot C \cdot \Delta t}{\sum C \cdot \Delta t} - \theta^2$$

The variance and the mean residence time was then used to calculate the dispersion index of the RTD curve (σ_t^2), Eq. (8.6):

Eq. (8.6)

$$\sigma_t^2 = \frac{\sigma^2}{\bar{x}^2}$$

B2. t_{90}/T



Appendix B 1. t_{90}/T hydraulic efficiency indicator (HEI) for the range of flow and baffling conditions derived in the tracer tests