

CRANFIELD UNIVERSITY

Francesca Palazzo

New insights into drinking water treatment, storage and distribution
systems using Flow Cytometry.

School of Water, Energy and Environment
Master of Science by Research

MSc.
2020 - 2022

Supervisor: Dr Francis Hassard
Associate Supervisor: Prof Peter Jarvis
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ABSTRACT

Excessive microbial regrowth in drinking water distribution systems (DWDS) signifies compromised biostability. In chlorinated DWDS, diminished chlorine residual and substantially elevated water age or transit times can pose risks to water safety. This study delves into microbial community dynamics within DWDS by analysing samples from 119 service reservoirs and 41 water towers across various water sources for six months (March-September 2021). Using Flow Cytometry (FCM) to directly measure microbial populations, surface water exhibited 4-10 times higher microbial loading compared to groundwater and mixed sources. Among these sites, two distinct microbial water quality compliance events (detection of culturable coliform bacteria) were identified through FCM data, each presenting different microbial trends. Factors influencing regrowth in DWDS, notably water age and free chlorine, were scrutinized. Elevated intact cell counts were noted with chlorine levels <0.50 mg/L and water ages surpassing 4 days. Multiple linear regression highlighted temperature as the prime factor affecting cell counts variability in surface and mixed waters. For groundwaters, water age was significant, likely due to decreased disinfectant residuals and minimal treatment of these sources. The Bray-Curtis similarity index, derived from FCM fingerprints, emerged as a potential metric for detecting biological instability in drinking water microbiomes. The findings underscore the necessity of optimally managed DWDS and emphasize the significance of maintaining chlorine levels, especially at higher water ages and temperatures – particularly relevant considering climate change. Through FCM and its fingerprint

analysis, a more detailed view of DWDS dynamics is attainable, promoting possibility for enhanced system control. The implications of this research offering potential for safeguarding public health, ensuring consistent water quality, and pathways for more resilient and sustainable water distribution practices. As a prospective direction for future research, machine learning models could be developed to predict and classify microbial community dynamics in DWDS using the rich dataset provided by FCM fingerprints.

Keywords: Flow cytometry; Drinking water; Service reservoir; Water tower; Water age; Fingerprint analysis.

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LIST OF ABBREVIATIONS

AI	Artificial Intelligence
AOC	Assimilable organic carbon
CFU	Colony-forming units
CHIC	Cytometry histogram image comparison analysis
CRI	Compliance risk index
Ct	Contact concentration time (chlorine)
CV	Coefficient of variation
DBP	Disinfection-by-product
DNA	Deoxyribonucleic acids
DWDS	Drinking water distribution system
DWI	Drinking Water Inspectorate
EPS	Extracellular polymeric substances
FC	Free chlorine
FCM	Flow cytometry
GAC	Granular activated carbon
HDZ	Hydraulic distribution zone
HNA	High nucleic acid content (cells)
HPC	Heterotrophic plate count
ICC	Intact cell counts
IQR	Interquartile range
LNA	Low nucleic acid content (cells)
LOB	Limit of blank
LOD	Limit of detection
LOQ	Limit of quantification
MRA	Multiple linear regression analysis
nMDS	Non-metric multidimensional scaling
NOM	Natural organic matter
NTU	Nephelometric Turbidity Units
PI	Propidium iodide
RNA	Ribonucleic acids
SCA	Standard Committee of Analysts

SD	Standard Deviation
SG	SYBR Green
SSF	Slow sand filter
SR	Service reservoir
TCC	Total cell counts
THM	Trihalomethane
UV	Ultra-Violet radiation
VBNC	Viable but non culturable
VIF	Variance inflation factor
WHO	World Health Organisation
WA	Water age
WT	Water tower
WTW	Water treatment works

INTRODUCTION

Safe drinking water is crucial for public health protection, averting waterborne diseases, and fostering vital agricultural and industrial activities (WHO, 2011). To safeguard this, water treatment processes target specific sources, like surface and groundwater, aiming to neutralize microbial pathogens and hinder their proliferation in drinking water distribution systems (DWDS) up to the point of use (Crittenden et al., 2012). Surface water sources often face contamination challenges from various inputs, encompassing faecal matter from animals and humans, industrial discharges, and land runoff. This confluence can degrade water quality, necessitating multibarrier treatments of varied complexity. Within the UK, a conventional water treatment regimen usually comprises coagulation/flocculation, clarification, filtration, and disinfection stages (Worch, 2019). The bulk of viruses, bacteria, and protozoa are primarily eliminated during the coagulation phase. Nevertheless, disinfection stands out as paramount in protecting public health, through disinfection which is reducing the viability and infectivity of pathogens to acceptable levels defined by the World Health Organisation (WHO). The decline in pathogen counts (evaluated via natural log reductions) between treatment stages offers a tool to gauge disinfection efficacy.

To ensure that disinfection remains potent and effective, there's an imperative to treat water of compounds that might interfere with these processes. For both chemical oxidants, like free chlorine, and physical disinfection methods such as UV, pre-emptively removing turbidity-inducing elements – such as suspended particles and natural organic matter (NOM) – is vital. This step ensures optimal

interaction between pathogens and chemical oxidants, prevents interference with UV processes, and reduces the risk of producing harmful by-products which primarily form through chemical oxidants. Moreover, elements like iron, manganese, ammonia, and hydrogen sulphide can all diminish chlorine's efficiency (Farrell et al., 2018). Consequently, it is advised to manage these elements through apt treatment before chlorine disinfection or UV treatment.

Chlorination is frequently the final step in water treatment works (WTW, Figure 1). In this process, free chlorine (FC) concentrations up to 5 mg/L are introduced to eliminate organisms within a chlorine contact vessel. Before water is distributed via the DWDS (Figure 2), the FC concentration is adjusted to between 0.1 mg /L and 0.5 mg/L. These adjustments serve to i) reduce disinfection by-product (DBP) formation, ii) avert taste and odour issues for consumers, and iii) minimize pipe corrosion within the network. In the DWDS (Figure 2), several processes take place. Natural chlorine decay in water is a decline in the disinfectant's concentration due to various chemical and physical processes, independent of microbial consumption. Chlorine can react with both organic and inorganic substances in water, leading to its reduction. For instance, it forms chlorinated compounds when reacting with NOM and can produce weaker disinfectants like chloramines when combined with inorganic compounds like ammonia. Environmental factors, like exposure to sunlight and increased water temperature, can accelerate chlorine's breakdown. The balance and efficacy of chlorine in water can also be influenced by its pH level, with its potency varying between its different forms. Additionally, chlorine concentration can decrease due

to its reactions with materials lining the pipes and tanks of distribution systems. One reason understanding this decay is crucial is that combined chlorine is less potent as a disinfectant than FC. Secondly, chlorine can combine with biofilm or other DPBs precursors to generate halogenated compounds. This reaction intensifies with prolonged storage or higher temperatures, commonly seen during summers in temperate zones.

During DWDS, water is either pumped or travels by gravity through a pipe network to various storage systems, primarily known as service reservoirs or water towers (Figure 1). As water is distributed, chlorine residual naturally diminishes. Water can also be stored in secondary systems. These structures help balance water supply and demand, offering essential buffering within the network. However, the nature of the distribution can lead to longer distribution durations (often several weeks), resulting in extended water age (WA). This can lead to water contamination due to i) leaks and external contaminants, ii) bacterial regrowth post partial inactivation at the WTW or external contaminants, iii) biofilm development on pipe interiors and service reservoir surfaces, and iv) bacterial proliferation within the DWDS. The DWDS conditions often promote these contamination mechanisms. For instance, many DWDSs in the U.K. and elsewhere are old and require significant asset improvements. This can lead to issues like i) contamination through damaged or corroded pipes and ii) accumulation of organic and inorganic debris due to prolonged asset operation. Low chlorine disinfectant residuals might also arise due to high WA, temperature, and inadequate removal of reactive organics upstream. Therefore, a deeper

understanding of chlorine's functionality and response of microbial populations to this stressor is needed to enhance DWDS asset management.

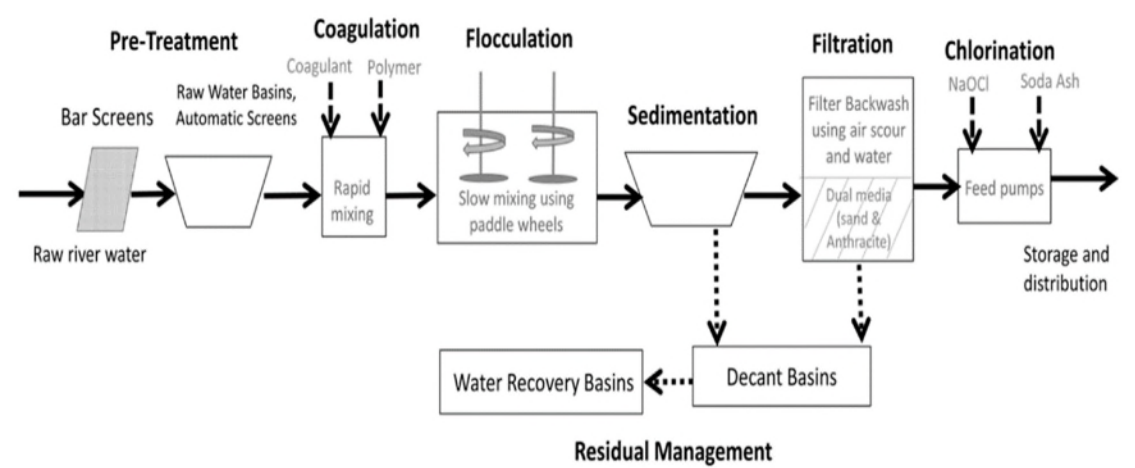


Figure 1 Water treatment process steps commonly applied for UK surface WTW – reproduced from (Bukhary, Batista and Ahmad, 2020).

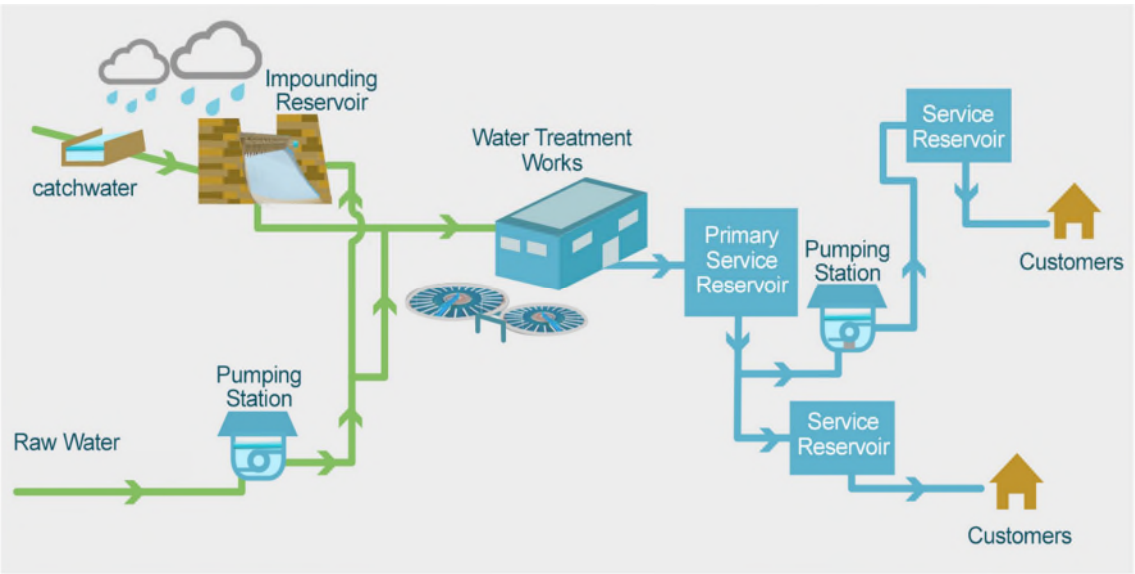


Figure 2 Idealised Drinking Water Distribution System for surface water abstraction (https://www.wsd.gov.hk/en/core-businesses/operation-and-maintenance-of-waterworks/index.html).

1.1 Interaction of chlorine with substances in water

Free chlorine can be introduced into water as either a gas or a liquid. Regardless of the dosing method, once added, it quickly reacts with the water to produce hypochlorous acid (HOCl). This weak acid then partially dissociates into the hypochlorite ion (OCl⁻) and a hydrogen ion (H⁺) based on the subsequent equation:



The conversion between HOCl and OCl⁻ is reversible and influenced by the pH of the water. Given a pKa of 7.6 at 20°C, HOCl is the dominant species at pH values below 7.6, while OCl⁻ becomes more prevalent as the pH rises above this point. While both species possess disinfectant capabilities, HOCl displays markedly superior disinfectant activity. In fact, its reaction kinetics are about a hundred times more potent than OCl⁻ at equivalent molar concentrations, as observed by Crittenden et al., (2012). To ensure optimal disinfection during chlorination, the pH of the water is typically maintained below 7.6. In this environment, HOCl undergoes reactions with various chemicals present in the water, leading to processes such as oxidation, hydrolysis, and deamination, especially with constituents of the NOM pool.

HOCl's reaction with NOM in water leads to the formation of DBPs such as trihalomethanes and haloacetic acids. The diversity and concentration of these byproducts are contingent upon the specific characteristics of the NOM and the applied chlorine dosage. Indeed, the water's origin significantly determines the

nature of this interaction. Upland sources, for example, are richer in humic substances, whereas lowland sources predominantly contain protein-like materials. Several factors, including the pH, temperature, and ionic composition of the source water, modulate the reactivity of HOCl with NOM. Notably, waters with elevated bromide or iodide concentrations may yield more toxic brominated or iodinated DBPs. Additionally, as chlorine engages with NOM, it depletes a fraction that would otherwise contribute to disinfection, thereby influencing the water's chlorine demand. The understanding of source water attributes is imperative for judicious chlorine dosing and curtailing DBP formation through optimised upstream removal of precursors pertaining to their formation. The composition of NOM in water sources markedly dictates the chlorine demand during disinfection. Such NOM predominantly encompasses organic derivatives stemming from plant material decay and microbial activities. Humic and fulvic acids, originating from terrestrial vegetation decomposition, are prominent constituents of NOM and are renowned for their propensity to react with chlorine, subsequently exhausting a substantial portion of the disinfectant.

The primary reactive sites in humic and fulvic acids for chlorine are their functional groups. Carboxylic groups (COOH) within these acids can actively interact with chlorine, especially under specific pH conditions, enhancing the formation of certain by-products. Additionally, phenolic groups in these compounds are particularly reactive. They stand out as significant precursors for the formation of trihalomethanes (THMs), a prevalent group of DBPs. While amino groups are less abundant in these acids, they shouldn't be discounted. When they react with

chlorine, they can contribute to chloramination reactions, adding another layer of complexity to the disinfection process.

Beyond functional groups, the intrinsic aromatic structures of humic and fulvic acids play a pivotal role. These aromatic rings can engage in reactions with chlorine, especially when they carry electron-withdrawing substituents, which can amplify their reactivity. Aliphatic unsaturations, or double bonds present in these molecules, also serve as sites of reactivity. However, compared to the prominent aromatic structures, their significance in chlorine reactions is relatively subdued.

Lastly, the molecular size of these acids plays a discernible role in their reactivity. Larger molecules of humic acid generally present a slower reaction rate with chlorine when compared to the smaller fulvic acid molecules. This reduced reactivity can be attributed to steric hindrance and the decreased accessibility of reactive sites. However, paradoxically, despite their higher reactivity, the smaller fulvic acids might be more instrumental in DBP formation.

Additionally, waters rich in proteins and amino acids can lead to the formation of chloramines when chlorine is added. This is particularly noteworthy as chloramines are a less effective disinfectant compared to free chlorine. Phenolic compounds, another subset of NOM, have their own set of challenges. When they react with chlorine, they can produce compounds that impart undesirable tastes and odors to the treated water. Other components, such as carbohydrates, are less reactive but still play a role in chlorine demand. Tannins, which often stem from plant materials, can also consume chlorine, albeit at a slower rate.

Moreover, in water sources affected by algal blooms, the organic materials released by the algae can significantly interact with chlorine. This can be particularly challenging during bloom events when the organic load increases rapidly precluding adaptive or remedial action at a WTW.

1.2 Mechanisms of chlorine-based disinfection

In aqueous matrices, bacterial concentrations are typically several orders of magnitude less than inorganic particulates or NOM molecules. Given this quantitative disparity, interactions between chlorine and bacterial cells are not anticipated until the breakpoint chlorination threshold is achieved. The breakpoint delineates a pivotal juncture where the majority of reactive organic compounds have been oxidized, paving the way for a subsequent rise in free chlorine (FC) concentrations, as the bulk of the chlorine demand—both organic and inorganic—has been met. However, the intricacies of chlorine's biocidal interactions with bacterial cells have yet to be comprehensively deciphered. While the reactivity of chlorine with inorganic constituents and NOM is well-documented, its multifarious interrelations with bacterial entities warrant further scrutiny. The heterogeneity of bacterial cellular architecture, coupled with diverse metabolic pathways, culminates in a spectrum of responses when confronted with chlorine. Notably, inherent bacterial defense mechanisms, like biofilm formation or inducible stress response pathways triggered by disinfectant presence, augment the complexity of such interactions. Advancements in research methodologies and technologies are crucial to untangle these complexities, ensuring that chlorination protocols maximize disinfection efficacy

without inadvertently fostering bacterial resilience or precipitating undesired sequelae.

Through rigorous research, it's evident that chlorine's interaction with bacteria induces subtle physiological changes, which have detrimental effects on numerous cellular processes. One notable consequence is the modification in the physicochemical properties of the bacterial cell wall and membrane (Venkobachar, Iyengar and Prabhakara Rao, 1977). The complexities of chlorine's interactions with bacterial cells remain somewhat underexplored. Prolonged exposure to chlorine increases the bacterial cell membrane's permeability, eventually leading to cellular breakdown. As highlighted by Haas and Engelbrecht (1980), this increased permeability results in the loss of essential ions such as sodium (Na), potassium (K), and phosphate (P) from the bacterial environment. The exact mechanisms involving bacterial envelopes, including the cell wall and membrane, are less understood. Specific membrane proteins, notably efflux pumps, play a pivotal role in protecting bacterial cells against chemical stressors, inclusive of potent oxidants like free chlorine. These protective barriers restrict chlorine's accessibility to vital intracellular targets, consequently delaying bacterial inactivation. Chlorine's bactericidal action is chiefly grounded in initiating a series of oxidative reactions, disrupting the structural integrity and functionality of essential bacterial enzymes required for replication.

The Chlorine Concentration-Time (Ct) value holds significant importance in water disinfection, denoting the cumulative impact of residual chlorine concentration

and the duration of exposure to microorganisms in the water. This metric is pivotal for quantifying disinfection efficacy, allowing for informed adjustments to chlorine dosage or exposure time, or both, in order to achieve the desired microbial inactivation. The intricacies of this relationship between Ct values and disinfection efficacy is based on the Chick-Watson Law, which contextualizes the correlation between microbial inactivation and both the disinfectant concentration and exposure time. Exploring in more detail the microbiological mechanics, chlorine's action in the water primarily manifests as a series of oxidative damages to various microbial cellular components. The cell membranes of microorganisms, comprising intricate networks of lipids and proteins, are vulnerable to chlorine-induced oxidative reactions. These reactions can lead to a loss of membrane structural integrity, making it more permeable and often culminating in cell lysis or rupture. Beyond the membrane, chlorine has the capacity to oxidize and denature essential enzymes, disrupting their normal functions and effectively stalling vital metabolic pathways that can culminate in cellular stasis or death. The genetic heart of the cell, its nucleic acids (DNA and RNA), isn't immune to chlorine's effects either. Chlorine can cause mutations or even break genetic sequences, impeding essential biological processes like replication and transcription.

Yet, the efficacy of chlorine disinfection using Ct values isn't just a straightforward calculation. It's influenced by a several key factors. Different microorganisms exhibit distinct resistance patterns to chlorine. For instance, protozoan cysts typically demonstrate higher resistance to chlorine than bacteria, necessitating a

more robust Ct for effective inactivation. The inherent quality of the water also plays a role, with organic and inorganic compounds potentially consuming free chlorine. This can lead to the creation of combined chlorine species, which might not be as potent disinfectants as their free counterparts. This phenomenon, commonly termed "chlorine demand," could require a shift in Ct values to achieve the desired disinfection level. Additionally, water pH and temperature are critical parameters influencing chlorine's efficacy. Depending on the pH, chlorine might exist predominantly as the more potent hypochlorous acid or the less effective hypochlorite ion. Similarly, temperature variations can modulate disinfection rates, with elevated temperatures often amplifying the disinfection process.

At higher Ct levels, chlorine demonstrates an ability to engage and disrupt other cellular components, with nucleic acids emerging as notable targets (Camper & McFeters, 1979). The vulnerability of nucleic acids to chlorine's oxidative actions is especially heightened in prokaryotic cells. Unlike eukaryotes, prokaryotes house their genetic material without the protective confines of a nucleus, leaving it more openly exposed to potential oxidative assaults. Additionally, prokaryotic cells typically wield more rudimentary defence mechanisms against reactive species than their eukaryotic counterparts, further exacerbating their susceptibility to oxidative damage. On the other hand, UV disinfection operates through a different disinfective principle. The primary mode of action for UV disinfection is the absorption of ultraviolet light by microbial DNA and RNA. This absorbed energy results in the formation of pyrimidine dimers, which can prevent replication and transcription, thus rendering the organism inactive. In essence,

UV disinfection's primary objective is to obstruct the genetic machinery of the microorganism, effectively reducing its ability to reproduce and infect. In contrast to chlorination, which attacks multiple cellular components ranging from membranes to enzymes and nucleic acids, UV disinfection more specifically targets the genetic machinery of microorganisms. This focus on DNA and RNA means UV disinfection acts as a more targeted approach, whereas chlorination provides a broader range of microbial disruption. One key attribute of UV disinfection is that it does not provide a residual benefit. Thus, when UV disinfection is used as the primary disinfectant. A secondary disinfectant is normally applied to the water. It is common now in UK surface waters impacted by wastewater discharge or runoff to employ both chlorination and also UV as primary disinfectants.

1.3 Treatment and distribution of water with free chlorine

The effectiveness of chlorine is dependent on several factors including pH (as introduced above) the temperature of the water. Temperature is important for chlorine treatment and distribution as elevated temperatures result in higher movement and collision which is generated among molecules. The effect of this is an increased collision frequency and likelihood of successful reaction between NOM, pathogens or background members of the drinking water microbial community and FC. The net result is an effective doubling of chlorine reactivity with every 10°C increase in temperature. Therefore, seasonal changes at WTW and DWDS require the adjustment on chlorine dosage and concentration (Pizzi, 2010). The latter value together with Ct are considered a key criterion governing

the disinfection of target organisms. The Ct concept has been widely applied in WTW; however, it has been less well studied within DWDS, this is because concentrations of chlorine in DWDS are not considered sufficient to elicit a detectable change in the viability of bacteria. Although theoretically, equivalent Ct values can be created in some extended DWDS despite lower chlorine concentration that presented during drinking water treatment.

$$\text{Ct(mg-min/L)} = \text{Concentration of Free chlorine (mg/L)} \times \text{Residence time (mins)} \quad (1-2)$$

The difference between the initial and the final FC concentration at a specific resident time describes the chlorine demand of different microorganisms. Shang and Blatchle, (2001), demonstrated that organism have a unique chlorine demand even at a similar organic carbon concentration. Other studies have taken this further by investigating disinfection of virus. They showed that inactivation kinetics can differ substantially between strains of the same virus with chlorine e.g., enterovirus (Torii *et al.*, 2022). To compensate for this complexity in organism dose responses alongside variability in the organic dose demand in pre-chlorinated water the effective Ct values are provided in excess to achieve required treatment targets. For this reason, Ct as well as temperature, pH, nutrient concentration are considered external factors whose variation strongly affect the resistance of microorganisms (LeChevallier, Cawthon and Lee, 1988). However microbial community can show a different susceptibility when exposed to chlorine using phenotypic resistance mechanism such as a modification of cell structures with some bacteria capable of producing extracellular polymeric

substances (EPSs) which elicit a chlorine demand and reduce FC. In addition, associating with different surfaces including turbidity particles, macroinvertebrates and algae (Ridgway and Olson, 1982; Cloete, 2003). More resistant organisms require a greater Ct comparison with the sensitive ones to achieve the same log reduction (Helbling and VanBriesen, 2007). It is not practical for most water utilities to monitor specific pathogens of interest as they are i) transient in abundance and distribution, ii) expensive to analyse using molecular biology methods and iii) not representative of risk or water quality assessment. The next section will outline different methods for microbial water quality assessment.

1.4 Microbial Ecology in Drinking Water Systems: Influences and Implications

Drinking water systems present a unique interface for microbial interactions, influenced by a myriad of environmental, infrastructural, and anthropogenic factors. This article aims to dissect the intricacies of microbial ecology in these systems, emphasizing the myriad of factors that sculpt microbial populations from source waters through to distribution networks. Source waters, including rivers, lakes, and aquifers, serve as primary reservoirs of microbial life. Their microbial communities are influenced by environmental determinants such as nutrient levels, temperature, and pH, which are in turn dictated by the catchment area's land use. Urban landscapes and agricultural areas, for instance, contribute distinct microbial and nutrient profiles. An agricultural runoff, enriched with fertilizers, can stimulate eutrophic conditions, potentially amplifying the growth of

specific microbial taxa, including harmful algal blooms. Treatment processes play a pivotal role in dictating the microbial quality of potable water. Conventional treatments, such as coagulation and flocculation, aggregate and remove microbial entities, while disinfection processes, employing agents like chlorine or ozone, exert a selective pressure, impacting microbial viability and community structure. Moreover, biological treatment methods like slow sand filtration (SSF) and granular activated carbon (GAC) filters not only help in the removal of contaminants but also introduce microbial populations. SSF, known for its biofilm-layer or "schmutzdecke," acts as a living filter, capturing pathogens and fostering a microbial community that can potentially seed the drinking water microbiome. Similarly, GAC, with its vast porous structure, supports biofilm growth, and while it's chiefly used for adsorption, it inadvertently hosts microbial communities that can influence the downstream water microbiome. The subsequent distribution of treated water ushers in another layer of microbial dynamics. Biofilm development on pipe surfaces is of particular importance. Embedded within extracellular matrices, these microbial consortia can host a plethora of organisms, ranging from bacteria to fungi, dynamically interacting and potentially impacting the water quality as cells intermittently detach and populate the water column. Loose deposits, a conglomeration of microbes, particulates, and organic matter, lying dormant within distribution systems can become resuspended, introducing microbial pulses into the drinking water. Considering the diversity in drinking water systems, bacterial communities undoubtedly play a central role. However, other organisms such as protozoa, viruses, and fungi also hold significance.

Protozoa, albeit in lower abundances, can act as vectors for diseases. Viruses present a unique challenge due to their resilience and capacity to cause infections. Fungi, often overshadowed in water research, can influence biofilm structures and may pose health risks in certain scenarios. In building plumbing systems, microbial growth is accentuated in zones of water stagnation and thermal stratification. Notably, *Legionella* spp. finds refuge in warmer sections, underscoring the importance of routine management and monitoring of these systems.

Lastly, with the looming specter of climate change, the microbial ecology of drinking water systems faces unprecedented challenges. Rising global temperatures can favor thermophilic microbes in source waters. Altered precipitation patterns could enhance the introduction of allochthonous microbes and organic matter, challenging conventional treatment paradigms. Conclusively, the microbial ecology of drinking water systems is a multifaceted domain, shaped by environmental inputs, infrastructural nuances, and external perturbations. As we navigate the evolving challenges, a thorough understanding of these microbial interactions will be paramount in safeguarding water quality and public health.

In non-chlorinated DWDS, represent complex habitats with distinct ecological dynamics. Within this intricate web of interactions, numerous microbial entities coexist, ranging from bacteria and viruses to a myriad of higher organisms, whose roles are often less explored in the context of DWDS. The microbial landscape of DWDS is undoubtedly influenced by the presence of protozoa, such as *Amoeba*. These unicellular organisms play a dual role: while they actively participate in

microbial predation, they also offer sanctuary to certain bacteria, safeguarding them from adverse environmental conditions and potentially enhancing their survival rates. The presence of minute multicellular organisms, including rotifers and ciliates, showcases the ecological richness of DWDS. By actively consuming bacteria and other microorganisms, they exert selective pressures, shaping the microbial compositions and indirectly influencing the nutrient cycling within these systems.

Nematodes, tiny roundworms, further add to the microbial diversity, and their predatory habits towards bacteria, algae, and other microorganisms are instrumental in governing microbial dynamics. For example free-living species like *Caenorhabditis*, *Rhabditis*, and *Plectus* consume bacteria, algae, and fungi. Bacterial-feeding types include the *Cephalobus* and *Panagrolaimus* genera, while *Aphelenchus* are fungivorous. Additionally, omnivorous nematodes such as *Dorylaimus* and *Mononchus* prey on diverse organisms, from bacteria to smaller nematodes. Notably, in some non-chlorinated systems, crustaceans, predominantly copepods of genera like *Eucyclops* and *Cyclops*, can be observed. These organisms, while unexpected in such settings, bring forth nuances to the microbial interactions, pointing towards intricate food webs and ecological balances. Furthermore, in systems with compromised structural integrity or open storage mechanisms, one might encounter the larval stages of specific insects, such as chironomid midges. Known as bloodworms, these larvae thrive on microbial biofilms, signifying a potential shift in biofilm structures and functionalities due to their feeding habits.

Moreover, photosynthetic entities like green algae (represented by genera like *Chlamydomonas* and *Chlorella*) and diatoms (like *Cyclotella* and *Aulacoseira*) can predominate in DWDS exposed to sunlight, such as open reservoirs. Their presence, while typically non-pathogenic, can influence water aesthetic attributes and introduce additional complexities to the microbial interactions. Exploring the role of granular activated carbon (GAC) filters, it's noteworthy to mention that while they primarily target organic compounds, these filters often become habitats for diverse microbial communities. Chironomid larvae or bloodworms are frequent inhabitants, feasting on the microbial biofilms present. Likewise, even though not insects, oligochaetes find GAC filters conducive, feeding on biofilms and, in turn, potentially influencing their structure and microbial dynamics. In summary, non-chlorinated DWDS provide a mosaic of microbial interactions, governed by bacteria, viruses, protozoa, and an array of higher organisms. Understanding this interplay is crucial, not just from a water quality perspective but also to discern potential ecological shifts, challenges, and considerations in the face of changing environmental scenarios, including the looming spectre of climate change.

1.5 Methods for assessing microbial quality in drinking water

Cultured based microbial methods are widely used and accepted as the gold standard indicators of the bacteriological quality of drinking water (Sartory, 2004). Following European directives (EU, 2020) UK regulations (DWI, 2015) require specific standards determined mainly by the presence/absence of certain pathogens indicating the water quality such as *Escherichia coli*, total coliforms,

Enterococcus faecalis and *Clostridium perfringens* culture on selective media from final and distributed water (0 count CFU/100 ml) where CFU = colony forming units. *E. coli* is a commonly applied microorganism used to detect faecal contamination showing significant changes in water quality caused by ingress or possibly regrowth. As part of the faecal coliform group *E. coli* was thought to be specific to the intestinal tract of warm-blooded animals. Although there is some data to suggest *in situ* regrowth of *E. coli* and presence of some coliforms in cold blooded animals (Verhille, 2013). In addition, these organisms can be more susceptible to FC than some pathogenic organisms, therefore their value for disinfection monitoring has been challenged. The growth of Heterotrophic bacteria provides an overview of the hygienic status of the water highlighting the presence of biofilm or failure in the effectiveness of treatment. The heterotopic plate count (HPC) method is not defined by a clear guideline starting from no limit detection in some countries or no abnormal changes to a maximum of 500 CFU/ml in others showing various approaches across the world (Allen, Edberg and Reasoner, 2004; Chowdhury, 2012). Despite of the fact that all these methods need a low-cost budget it takes at least 18-24 hours for coliforms and *E. coli* detection which means it could be too late to notice any failure in the treatment process.

Faecal indicator organisms are characterized by their presence in waters tainted with faecal material and their absence in pristine, uncontaminated waters. These organisms should also exhibit vulnerability to water treatment processes, notably becoming inactive upon exposure to appropriate chlorine contact times (Mir,

Morató, and Ribas, 1997). Subsequent research has demonstrated that the Ct needed for a 3-log inactivation of *E. coli*, a widely-accepted bacterial indicator of faecal contamination, is shorter than that for pathogens like *Staphylococcus epidermis* and non-clinically concerning environmental bacteria, such as *Mycobacterium aurum*. The WHO guidelines for drinking water safety underscore the importance of rigorous oversight of these control measures. Should faecal contamination arise within the DWDS, an immediate response involves elevating the FC concentration to >0.5 mg/L. This concentration, sustained over 30 minutes, which results in 15 mg min/L, under conditions of a pH less than 8 and turbidity below 1 NTU, stands as the recommended benchmark. In typical operations, a baseline FC concentration of approximately 0.2 mg/L is employed. Yet, this concentration tends to decrease at distal ends of the network, a phenomenon attributed to factors like WA, temperature dynamics, disinfectant efficacy, and interplay with residual natural organic matter and biofilms within the infrastructure (LeChevallier et al., 1996).

Waterborne disease outbreaks in high income countries are rare but do still occur. Mostly, this is due to the aging of water and sewage system as well as black swan events where a hazard can occur due to breakthrough of the multiple protective barriers within drinking water systems (Tzani et al., 2020). For example, detection of compliance organisms in England and Wales occurs infrequently, their status of water quality is of a high standard with The National Compliance Risk Index (CRI). The CRI is a tool used within the UK water industry for assessing drinking water quality. It quantifies deviations from water quality standards, guiding

interventions to maintain or improve water purity. CRI considers the frequency and magnitude of non-compliance events and emphasizes potential health implications. For instance, greater significance might be given to microbial contaminants due to their immediate health risks. By deriving a numerical value for each contaminant or parameter's CRI, water providers can prioritize and allocate resources efficiently. In essence, CRI ensures focus on the most significant public health concerns in water quality management. Specifically, the CRI considers the frequency of non-compliance: how often a particular contaminant or parameter exceeds the set regulatory limits. In addition, the magnitude of non-compliance: The degree to which those limits are exceeded. A slight exceedance might be weighted differently than a significant one. Finally, the health implications and considered depending on the parameter, the potential health risk it poses when it is present above recommended levels might be considered. For instance, microbial contaminants might be weighed heavily due to their direct implications for public health.

Therefore for the sites studied here, a CRI of 2.87 in 2019, an improvement from 3.87 in 2018 and 3.56 in 2017. The CRI is an indication of the occurrence of failures which can contribute to change its value, with the lower numbers constituting the lowest overall risk of compliance events. During the 2019 period in England, a 50% increase in testing occurring compared to 2018. During 2018, there was a significant number of microbial compliance detections for bacterial indicators 5.4% of 12,890 samples for *E. coli* and 12.1% of 11,786 samples for coliforms. The increased testing requirement was due to incentives from the

Drinking Water Inspectorate (DWI) to water utilities to enhance the performance on treatment process based on a report in 2015/2016 (DWI, 2015)

Furthermore, other drawbacks to established methods include a limited sensitivity which may underestimate cell viability and long time consuming labour (Ramírez-Castillo *et al.*, 2015). As discussed by Hammes *et al.*, 2008 less than 1% of bacteria are culturable underneath laboratory conditions and using culture-based method is not possible to enumerate damaged or viable but not culturable bacterial cells (VBNC). Several bacteria enter into this VBNC state induced by the usage of majority of disinfectants (UV, FC) creating a stressful environment (Chen *et al.*, 2018). Using direct microscopy allowed to visualize how HPC method does not determine the difference between active, inactive and dead cells, therefore it was questioned if culturable-dependent methods could actually been replaced or combined with other diagnostic techniques (Hoefel *et al.*, 2005; Vital *et al.*, 2012). This discrepancy to differentiate total from culturable cells lead to the application of culturable-independent methods of microbial water quality assessment (Berney *et al.*, 2008). An alternative approach is the use of flow cytometry (FCM) for directly quantifying the autochthonous bacterial population within drinking water, which will be introduced in the next section.

1.6 Flow cytometry is an accepted measure of drinking water microbial community

After more than ten years of studies, the benefits of the FCM technique has been demonstrated over conventional HPC and bacterial indicators. In less than 20 minutes (per sample) the total number of bacteria can be assessed (Van Nevel

et al., 2017). Alongside the whole drinking water treatment process, filtration and disinfection, the accuracy of FCM can be <5% coefficient of variation between replicates, compared to ~20% for culture (Safford and Bischel, 2019). FCM is an instrument composed by a fluidic system where a stream of particles is interrogated via a laser through a flow cell. Afterwards these emitted lights are collected by optical mirrors and filters to separate and direct wavelength converted into a voltage by a photomultiplier tube. Finally analogue signal becomes a digital data which can be displayed as histogram or dot plots graphs as shown in Figure 3 (Adan *et al.*, 2017). Passing through this laser beam the light scattered and fluorescence gives information related to size, granularity, nucleic acid content characterizing bacterial cell in a water community. Efforts have been established to standardise the staining of cells and analysis approaches (Hammes *et al.*, 2007).

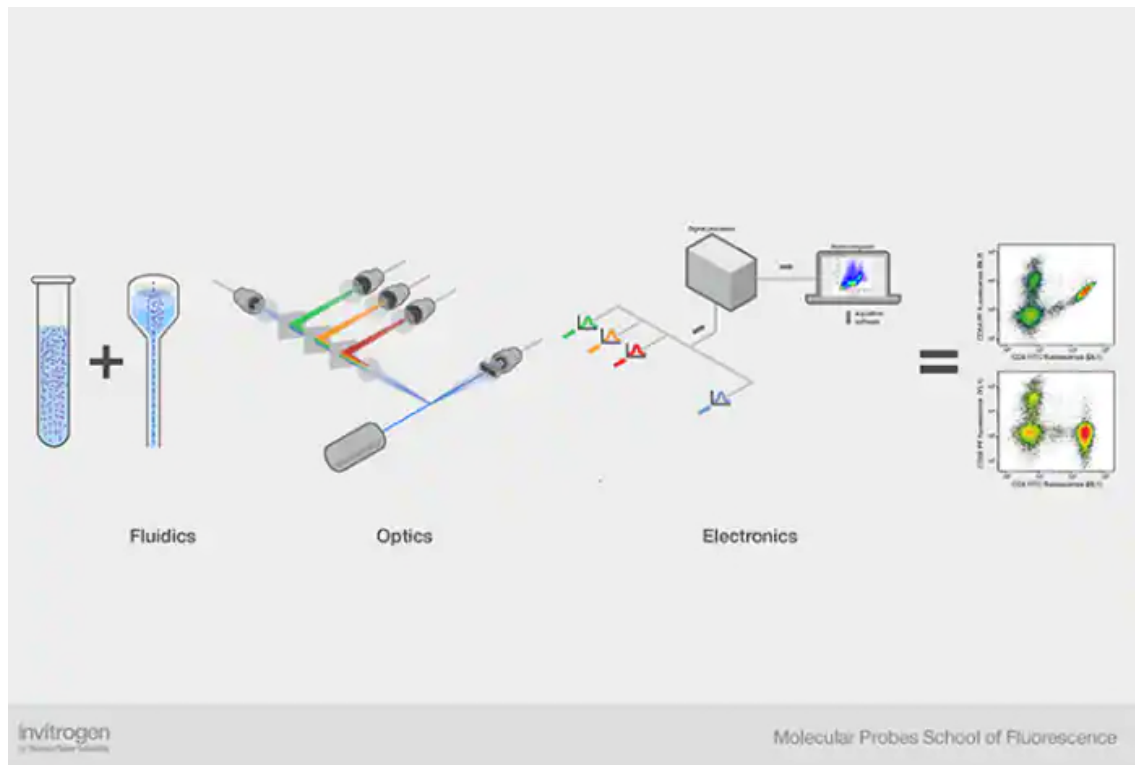


Figure 3 Principle of flow cytometric measurement is based on the passage of cells through a chamber in single file to be detected, counted and sorted by a laser beam. Cell components are fluorescently labelled and then excited by the laser to emit light at varying wavelengths. The light-scatter (forward and side) from the cell and emitted fluorescence from the antibody-fluorophore combination is then collected by optical detectors and is transferred to a digital format to be analyzed on a dot plot or histogram (How a Flow Cytometer Works | Thermo Fisher Scientific - UK).

Using stains like SYBR green (SG) and Propidium Iodide (PI), FCM can quantify total cell count (TCC) and intact cell count (ICC) due to the different membrane permeability of the two dyes. It can be observed the cell integrity by the attachment of the SG, whilst the PI permeability to the membrane reveals whether

the cell wall is intact. As an intact cell membrane is a prerequisite for replication and metabolic activity, cells without an intact cell membrane are considered dead. Consequently, this differential fluorescent staining gives the possibility to discriminate intact from non-intact ones. As stated by Helmi et al (2015), FCM is capable to detect total bacteria cells even if they are present in a VBNC state. A reproducible staining procedure was undertaken by Prest *et al.*, 2013 to demonstrate how FCM method can be repeatable using a combination of cell concentration and the fluorescence fingerprint extremely sensitive to monitor changes to bacterial communities.

1.7 Application of flow cytometry for assessing drinking water treatment, distribution, quality control and biostability.

Generating a FCM fingerprint involves collecting data that culminates in a visual representation, often seen as a dot plot or histogram. In these visualizations, each dot symbolizes an individual cell or particle. The placement of these dots conveys various cellular attributes like size, indicated by forward scatter, and complexity, denoted by side scatter, as well as fluorescence intensity. In drinking water analysis, fluorescence intensity, which mirrors the nucleic acid content within cells, emerges as the pivotal differentiating factor. Consequently, the plot exhibits distinctive clusters or regions, each signifying varied microbial populations. Here, the LNA and HNA microbial groupings command special attention. A closer look at the flow cytometric fingerprint empowers researchers with insights into the structure of the microbial community within the water sample (Lebaron et al. 2001). Any discernible alteration in the fingerprint, be it the emergence of

unfamiliar clusters or the fading of existing ones, can hint at modifications in water quality or the effectiveness of its treatment processes. Furthermore, contrasting these fingerprints across a series of samples can unveil patterns, deviations, or potential episodes of contamination. One of the salient strengths of FCM is its ability to yield swift results, often instantaneously or within a matter of minutes. It paints an intricate portrait of the microbial environment without resorting to traditional culturing methods, which often overlook a vast segment of microbial diversity. Moreover, the process yields quantitative information, facilitating accurate counts of specific microbial populations.

In essence, the fluorescent fingerprinting technique in FCM provides an exhaustive, prompt, and nuanced approach to evaluate the microbial integrity of drinking water. By systematically characterizing and surveilling the microbial entities in water samples, this method plays a pivotal role in upholding the quality and safety standards of potable water. This fingerprint illustrates another discrimination based on the DNA content among bacteria which is the differentiation between high (HNA) and low nucleic acid (LNA) (Wang *et al.*, 2009).

In terms of microbial cell analysis, particularly in the use of FCM, the terms LNA and HNA are frequently encountered. Microbial cells labelled as LNA contain a modest quantity of nucleic acids such as DNA and RNA. When these cells are exposed to fluorescent dyes that bind to nucleic acids, like SYBR Green, the resulting fluorescence intensity is relatively subdued due to the lesser nucleic acid content. Despite their understated presence, LNA bacteria constitute a

substantial segment of the bacterial community in various aquatic environments, including our drinking water. The exact ecological function of LNA cells remains a topic of ongoing research. However, it's acknowledged that this group boasts a rich diversity, encompassing both living and non-living cells.

Conversely, HNA cells are characterized by their abundant nucleic acid content. A hallmark of these cells under FCM is their pronounced fluorescence intensity post staining with nucleic acid-affiliating dyes. This is a direct reflection of their high nucleic acid composition. These HNA bacteria, often more metabolically active than their LNA counterparts, tend to thrive in environments rich in nutrients. They usually signify the more dynamic faction of the bacterial community found in aquatic ecosystems. The technique of FCM, revered for its speed and sensitivity, excels at differentiating and counting microbial cells based on characteristics like nucleic acid content. When exposed to specific staining dyes, a clear distinction between HNA and LNA cells emerges on a cytogram. This demarcation empowers researchers in several ways. They can gauge the relative population of each group within a sample, track variations in these populations which could hint at shifts in water quality or other environmental parameters and evaluate the physiological activity or condition of microbial communities within a specific setting. The interplay and balance between HNA and LNA populations in aquatic systems can shed light on facets of microbial ecology, offer clues about water quality, and even gauge the efficiency of water treatment methodologies.

It is still a matter of debate the interpretation of HNA and LNA to understand whether these clusters represent different types of organisms or organisms which

could exhibit different growth rates. A different response to water physiochemical properties has been demonstrated by the different groups. For example, the LNA tend to be dominant in high conductivity conditions. Whereas the HNA tend to be associated to waters with poor biostability in high turbidity and alkaline environments (Liu *et al.*, 2015).

Drinking water biostability refers to the capacity of treated drinking water to resist changes in its microbial quality during storage and distribution. It emphasizes the water's ability to prevent the growth and multiplication of unwanted microbial populations, ensuring consistent safety and quality from the treatment plant to the consumer's tap. A water supply that is biostable does not support microbial proliferation, primarily because it lacks the essential nutrients these organisms require to thrive. Various factors contribute to water's biostability. These include the concentration of organic and inorganic nutrients, the presence of residual disinfectants, the physical and chemical conditions within the distribution system, and the interaction between microbes and the pipe materials.

Organic matter in the water, particularly biodegradable organic carbon, is a critical factor influencing biostability. High levels of such organic compounds can serve as a food source for microorganisms, enabling them to grow and multiply. Therefore, advanced water treatment processes aim to reduce these organic compounds to minimal levels to enhance the biostability of the finished water. The presence of residual disinfectants, like chlorine or chloramine, in the distribution system plays a crucial role in maintaining biostability. These disinfectants suppress microbial activity, ensuring that treated water remains free

from microbial regrowth during its journey through the vast and complex distribution networks. Physical conditions, like temperature and flow rate within the distribution pipes, also influence biostability. For instance, higher temperatures can promote microbial growth, while stagnant water zones might become hotspots for microbial proliferation due to reduced disinfectant concentrations. Interactions between the microbes and the pipe materials can further impact water's biostability. Some materials might encourage biofilm formation, which are communities of microorganisms adhering to the surfaces. Biofilms can shield microbes from disinfectants, allowing them to flourish and potentially compromise water quality. To ensure drinking water biostability, water utilities and providers often monitor various parameters, including microbial counts, residual disinfectant levels, and organic matter concentrations. By maintaining biostable water, they not only guarantee the consistent delivery of safe drinking water but also extend the lifespan of the distribution infrastructure by reducing bio-corrosion and other microbial-induced damages.

Disinfection impact can influence the response of these two groups with a bigger resistance to chlorine from LNA compared to HNA bacteria (Ramseier *et al.*, 2011). A comparison between *E. coli* strains and natural microbes obtained from a drinking water sample suggested differences in inactivation rates. Inactivation occurred more rapidly within a pure culture of *E. coli*. This is due to the relative chlorine susceptibility of this organism compared to the more heterogeneous response of bacteria in environmental waters to FC. This different susceptibility to chlorination is more representative of waterborne pathogens. In the same

study Cheswick *et al.*, 2020 reveals that changing Ct, prolonging the exposure to chlorine, leads firstly to a damage of ICC. At elevated contact times, a reduction in the TCC occurs as the chlorine starts to break down the inactivated structure of cells resulting in production of more debris located to the bottom left of the fluorescence dotplot. The reason is mainly because as a non-specific oxidant FC is able to degrade key enzymes and cellular DNA alongside increasing membrane permeability (i.e. ICC) without creating major structural damage to the cell surface (i.e. TCC) (Mao *et al.*, 2018). Further experimental data determined by FCM signals show that after disinfection culturability was lost whilst membrane was still not completely damaged (Nocker *et al.*, 2017). Although Ct was considered as a prior factor in determining of coliforms reduction and suppression of intact cells with a value $> 30 \text{ mg min/L}$. Clearly, there has been extensive research demonstrating the use of FCM in treatment and distribution systems. However, there is a knowledge gap understanding whether FCM can be used to optimise the cell loading within different types of DWDS assets.

Accordingly, this research focuses on service reservoirs/water towers, as within these systems there remains a dearth of monitoring data on how the FCM fingerprint changes. This is because FCM is an indirect measure of the bacterial community and as such, taxa cannot be readily identified. However, other works have suggested that the taxonomic composition is reflected within the fluorescent fingerprint. Therefore, in this thesis, it is hypothesised, that additional data inherent within the fingerprint will provide information to elucidate whether process or water quality challenges have impacted microbial water quality. For

example, low chlorine residual has been detected in reservoirs, therefore monitoring using FCM could enable the water industry to manage the risk of bacteriological failures within the network adjusting parameters such as chlorine dosage or investigating the main trigger factor involved. This specific work extends the interest on another pivotal aspect of DWDS management, that is the use of the parameter known as WA. The WA is, the modelled residence time for water to transit from treatment works to reservoir and customers tap. Together with chlorine, the WA can be potentially crucial in determining whether further bacterial growth has occurred. It is acknowledged that there is significant financial investment used for operational activities to investigate bacteriological failure in DWDS. Therefore, FCM data is an opportunity to rapidly cluster sites including all the reservoirs separated according to their specific fingerprint. Consequently, a dynamic baseline can be used to be alerted whenever some variability occurred in the reservoirs based on historical analysis and comparison within upstream DWDS sample locations. Understanding the dynamic interplay between temperature, WA, FC and cell viability represents a novel contribution to the field in an entire DWDS across a regional supply area.

1.8 Aims

The aim is to utilise conventional and advanced fingerprint analysis of FCM data to examine the dynamics of microbial communities under different DWDS. My thesis is to examine factors which contribute to biological stability in DWDS

understanding interdependency between WA, FC and temperature and providing evidence baseline for FCM utilization for microbial water quality assessment.

To achieve the overall aim the following objectives were set:

1. To describe how source water quality impacts DWDS quality using FCM
2. To compare the FCM data with conventional measures e.g., HPC
3. To determine which risk factors contributed to high microbial loading in service reservoirs.
4. To investigate water age, temperature and free chlorine residual as part of an overall DWDS management plan.

To facilitate these objectives this research it was hypothesised that:

1. Variations in water source type (ground, surface and mixed water) and quality impact on the FCM data associated to reservoirs and water towers.
2. No link between coliforms / *E. coli* detections and FCM data. If coliforms failures can not be detected from FCM data, possibly FCM can be considered helpful to monitor and prevent bacteriological events.
3. The increase of Ct in DWDS strongly decreases residual ICC. A defined 'free chlorine' residual operational window will reduce risk of microbial regrowth and high cell loading within network optimizing chlorine dose for each reservoir according to water sources.
4. Water age is an important predictor of ICC in distribution systems highlighting its dynamic interdependency with free chlorine and temperature. This dependency is most apparent within highly loaded

surface water due to poorer biological stability (caused by greater suppressive disinfection effects on the microbial community and increased residual assimilable organics in distributed water).

2 MATERIALS AND METHODS

2.1 Study location and sampling

Over a period of 6 months from March to September 2021 water samples were collected from service reservoirs (SR) and water towers (WT) within the Affinity Water supply area (England) by Affinity Water sampling team. These sampling points were in a broad geographic region located in the South-east of England in the United Kingdom (Figure 4). The supply area is divided for administrative purposes into several zones where different water sources are present such as surface water (lowland, river abstraction), groundwater derived including confined and semi-confined karstic aquifers. The latter consists of porous rock with a shallow aquifer which means that it is strongly impacted by surface run-off during periods of intense rainfall and run-off. In this karstic zone, the WTW received water from a combination of boreholes or rivers, therefore it was referred to it as a zone supplied by mixed water sources where supply is variable depending on the demand and availability of the water.

In this study, 160 sampling points were sampled weekly. Of these sample points, 113 consisted of SR and 40 were from WT. Service reservoirs are often fully- or part-submerged water storage assets, whereas WT represent above ground water storage systems often deployed in very flat areas to provide additional head for supply. These water storage assets are operated within and as part of several DWDS. The majority (56%) of the sample storage assets were fed via groundwater, however, 17% and 27% were fed by surface and mixed supplies

respectfully. Less than 5% of supply was fed from outside the supply area (water derived from other water supply zones operated by other utilities).

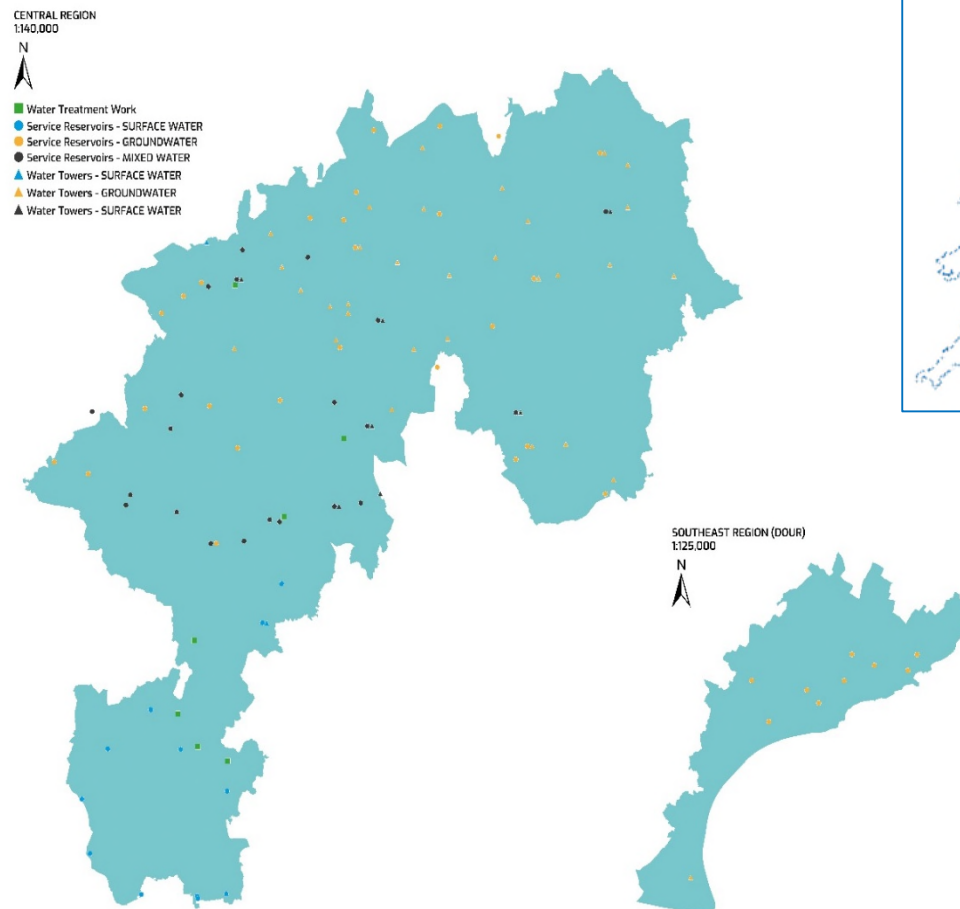


Figure 4 - Water service reservoirs (circles) / water towers (triangles) sampled. Different colours, orange, blue and black define various water sources respectively groundwater, surface and mixed water catchment respectfully. Green rectangles highlight the presence of main water treatment works (Map Produced: 1st August 2018 by GAT, Asset Strategy). On the top right a map of England with blue colour areas representing where AW supply areas are located.

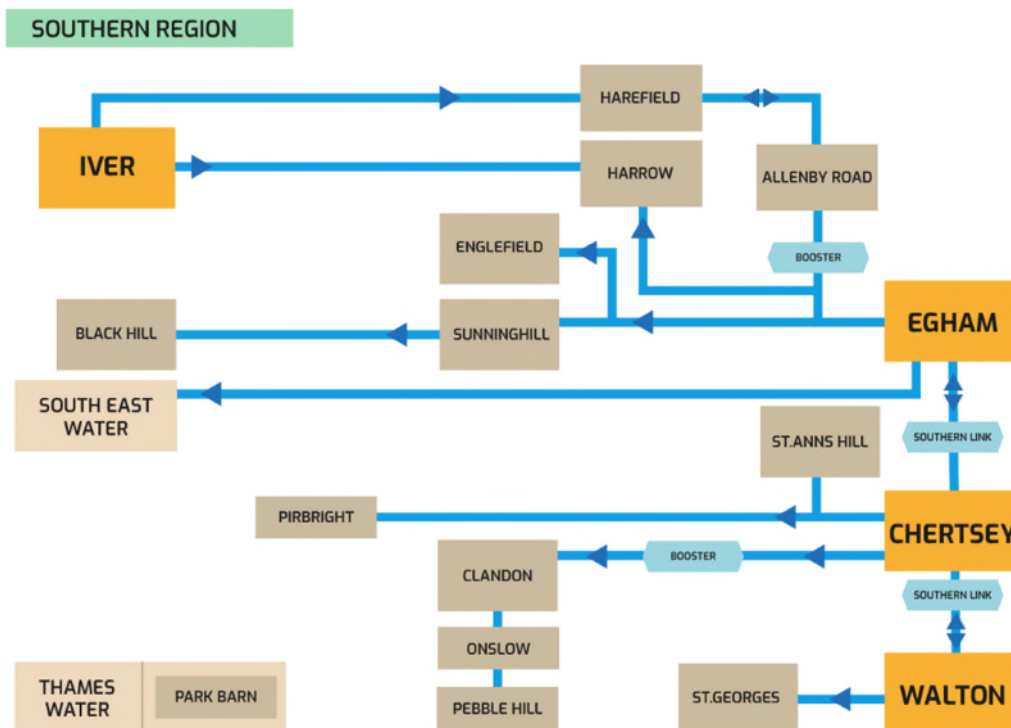


Figure 5 Connections and flow among the service reservoirs in the Southern catchment derived from river Thames water.

The study location was chosen to monitor the whole reservoirs/towers representative of a full-scale groundwater included karstic zone and surface WTW networks reservoirs fed by river Thames (surface water source) in the Southern region (Figure 5).

The water treatment part of the water supply process has three distinct elements: removal of solid contaminants, removal of dissolved contaminants and disinfection. Surface water is typically treated via coagulation, flocculation and sedimentation followed by ozonation and inclusion of adsorption / filtration processes including: granular activated carbon, rapid gravity and slow sand filtration (Figure 1). Compared to other groundwaters, in karstic zones, it is sometimes necessary include an ultra-filtration membrane system due to the less buffering between surface water, rainfall and the aquifer together with the risk of *Cryptosporidium* presence. Groundwaters tend to have lower microbial loading than raw surface waters. This is due to the natural filtering capacity of rock strata, low nutrient conditions and extended WA (often hundreds or thousands of years. Generally, this water presents low turbidity and low biological activity. Therefore, different treatments are designed to reflect these high-level differences between the water sources. Table 1 displays the disinfection process which is applied at the three types of treatment sites:

Table 1 Different disinfection methods used in various type of water sources in Affinity Water Ltd

Type of water sources		Disinfection	Number of sites (SR and WT)
Surface water		Chlorination based disinfection and residual	24 SR and 3 WT
Karstic groundwaters	group	Ultra-filtration (UF) and chlorine residual	37 SR and 7 WT
Groundwater		Chlorination , marginal chlorination and UV	52 SR and 30 WT

Sampling was performed weekly, regulatory sample taps were flushed for at least 3 minutes and the metal tap was disinfected using a flame sterilization technique. Drinking water was sampled in a 350 ml bottle treated with a molar excess of sodium thiosulphate included to remove any chlorine residual. Samples were transported and stored at ~4°C. Analysis for culturable microbial counts within 8 hours and FCM within 24 hours.

2.2 Flow cytometry Measurements

For FCM, water was subsampled from samples obtained from site. Staining and incubation was undertaken as described previously (Cheswick *et al.*, 2019) with full protocol in the Appendix. In short, water sampled were mixed with a

fluorescent stain (SYBR Green I, Life Technologies, USA) for total cell count (TCC) or SYBR Green I and Propidium Iodide (PI, Life Technologies, USA) Intact Cell Count (ICC) at a final concentration 1:10,000. This mixture was incubated for 15 minutes at 37 °C in the dark at 100 rpm. This mixture was then transferred to the flow cytometer for measurement at 200 $\mu\text{L min}^{-1}$. A lower threshold for green fluorescence channel was 90.000. An extended cleaning cycle comprising dilute hypochlorite and detergents was performed between plates comprising 100 samples. Industry standard gating to discriminate TCC (SYBR Green I only) and ICC (SYBR + PI) and the high nucleic acid and low nucleic acid organisms. These fixed gates were applied to separate bacteria from background signals.

2.2.1 Flow cytometry method quality considerations

Limit of quantification (LOQ) and detection (LOD) was examined by experiments where a serial dilution was carried out on the microbial community obtained from bottled mineral water (0.1 μm filtered EVIAN, Evian-les-Bains, France) followed by staining and measurements (Figure A-1). Each dilution was set in triplicates from a range of 0% to 100% (Figure A-2). These values based on the regression approach presented in other studies (Hammes *et al.*, 2007). The LOD was also compared to the cell count from >100 blanks (filter sterilised 0.1 μm) double deionised water. In these samples, both TCC and ICC blanks analysed alongside each batch of samples throughout the experimental plant. The relative means of these samples were in the range of 56-160 cells/ml (Figure A-3). As a result, it was possible to extrapolate the LOD and LOQ value based on blanks which were

respectively for ICC 600 and 1000 and for TCC were 1410 and 2350 similar to previously reported lowest value of 200 cell/ml (Hassard *et al.*, 2019). Another important aspect to consider for the validation of the instrument is to assess the limit of blank (LOB) whose value is determined by the following equation (Forootan *et al.* 2017).

$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645(\text{SD}_{\text{blank}}) \quad (2-1)$$

When replicates of a blank sample are tested it was considered that the LOB is the lowest level analyte concentration which occurs due to the presence of the non-target compounds (e.g. background particles) from water and reagents in the flow cytometer. The LOB is important as it helps to define the lower limits of detection of signal from noise.

A further precise data was determined by repeating several samples in duplicates and triplicates to assess and confirm a reproducibility of the staining method. A pilot study was undertaken involving 20 samples over a 3-week period. To compare the variability between replicates, the Coefficient of variation (CV) value was calculated (standard deviation divided through mean) with values < 5% evident data of a good reproducibility, which was higher than < 2.5% stated by Prest *et al.*, 2013. These 3 weeks trial only covered 2.5% of the total number of samples which was 3390. It is a water industry standard for FCM analysis to analyse singlet samples. This is because of the significant number of samples which preclude method replicates. This is based on the assumptions that error

between measurements is low (<5%) in FCM so value of running method replicates is limited. This assumption was validated through discussions through the UK water industry working group on flow cytometry which recommends using a single aliquot for analysis. The recommendation of repeat measurements of single sample points provides more value to understanding microbial dynamics within DWDS.

The Physiochemical and microbiological analysis methods are presented within (Appendix A).

2.2.2 Hydraulic modelling

With the DWDS network a model simulation was retrieved. The model included the SR and WT volumes and levels with their respective connections. The WA is an important indicator which can be used to manage DWDS. With elevated DWDS WA being indicative of water deterioration caused by disinfection by-products (DBPs). As the bulk water travels through the distribution system, it undergoes several physio-chemical transformations impacting water quality. The behaviour of the water has been represented by mathematical models to estimate hydraulics and calculation of residence time distributions to estimate the WA within the DWDS. For SRs, these models have been applied considering mixing characteristics baffling and flows to estimate the WA. InfoWorks WS software (Innovyze, UK) was used to generate models for each Hydraulic distribution zone (HDZ). This analysis assumed the 'normal' network conditions as per model calibration date, as well as the fact that the reservoirs were completely mixed.

2.3 Statistical data analysis

Statistical processing was executed using MS Excel and SPSS. The analysis involved several statistical tests, each bringing its unique perspective to summarising the results from the data sets. Among these, Pearson's correlation was employed to identify linear relationships between specified variables. This test, which gauges the linear association between two continuous variables, operates under the suppositions of linearity, homogeneity of variances, and a normal distribution of the variables. Its application was particularly aimed at probing the relationships between parameters such as turbidity, FC, TC, Conductivity, and pH in relation to ICC and TCC. Complementing this, the Spearman's correlation was utilized to tease out monotonic relationships among the variables. Unlike Pearson's which focuses on linear relations, Spearman's is geared towards determining how well an ordinal, monotonic function describes the relationship between two variables. Essentially, it was used to assess the consistency and directionality of relationships between parameters. Delving deeper, especially considering the diverse sources of water— groundwater, surface water, and mixed sources, multiple linear regression models were constructed to link these with ICC. Rooted in several foundational assumptions including linearity in relationships, independence of observations, homoscedasticity, normal distribution of residuals, and absence of multicollinearity, this method offered insights into the weight and significance of

various factors, including FC, temperature, and WA distribution, in predicting ICC. Of importance was the adherence to the assumptions intrinsic to each statistical test. Prior to their deployment in this analysis, a thorough validation was conducted to ensure these assumptions held true, thus fortifying the reliability of the results derived.

To investigate this interdependency, multiple linear regression analysis (MRA) was used to compare the impact of FC, temperature and WA on ICC population. Applying a stratification method based on sorting all these data into distinct groups / layers, it was possible to combine them by using analysis data tool (SPSS). To explore this in more detail, a MRA model was developed, and the assumptions of this model are described (Table 2). The model was developed to be able to understand which is the most important factor amongst temperature, WA and FC in governing microbial growth. Ultimately, it could be possible to predict the cell concentration from these variables on a site by site basis. In this study, the multiple regression was used to predict the value of a dependent variable (ICC) based on the three independent variables (WA, temperature, FC).

To be sure that the entire linear regression explains the data analysed it has been checked if these data satisfy each assumption. Table 2 shows assumptions made to model this relationship between a response and a predictor. These assumptions are important to understand through the process including prior to undertaking the model, running the model and interpreting the output of MRA drawing inferences regarding the model estimates or before using the model to make certain predictions.

Table 2 Key assumptions of multiple regression linear model

	Assumptions	Assumption had been met	Result for this study
#1	The relationship between the dependent variable and each of the independent variable is linear	Visually inspecting scatterplots	Linear relation between ICC and WA, temperature and FC
#2	There is no multicollinearity in the data	VIF scores were well below 10, and tolerance scores above 0.2	Predictors are not too highly correlated with one another
#3	The values of observations (residuals) are independent	The Durbin-Watson statistic: the obtained value was close to 2 (Durbin-Watson in range 0.80-1.43)	All the observations, or individual data points, are uncorrelated.
#4	The variance of the residuals is constant, homoscedasticity	Plot of standardised residuals vs standardised predicted values showed no obvious signs of funnelling	The variation in the residuals is similar at each point of the predictor variables across the model
#5	The values of the residuals are normally distributed	The P-P plot created for the model	Normality of the residuals may have been violated
#6	There are no influential cases biasing the model	Cook's Distance values were all under 1	No significant outliers and influential data make the

2.1 Flow cytometry fingerprinting analysis

The fingerprint analysis was performed using cytometry histogram image comparison analysis (CHIC) on FlowJo, ImageJ, and R software according to Koch et al. (2013). In brief, the FCM scatter plots were exported from Attune software as .FCS files and then converted into grey-scale pixel images using FlowJo software. The ICC fingerprint was used due to its importance for viability assessment within chlorinated systems. The inorganic and organic background was not removed prior to fingerprint analysis. Using ImageJ, the cytometric images were compared to one another. The “generate disjunction” function created an XOR image (eXclusive OR) of two cytometric images and a second algorithm produces their overlap, describing the differences in pixels between water samples on a grid. The average grey value per pixel was calculated using the sum of all pixel values from the XOR image and the number of pixels from the overlap image. Statistical analysis was then performed using a dissimilarity algorithm in R. During this procedure, a Bray-Curtis dissimilarity index was calculated and presented as a matrix, and the results were visualized using non-metric multidimensional scaling (nMDS) and correlation vector analysis by superimposing a Pearson’s correlation test on components within the nMDS. The FCM data and conventional microbial parameters (total viable count, coliforms

and *E. coli*) were determined using the R function *envfit* with the *vegan* package and only parameters which had a significant effect (P-value < 0.05) were presented. The author expresses gratitude to Ms Leila Claveau (Cranfield University EngD student for assistance with the FCM fingerprinting analysis.

3 RESULTS AND DISCUSSION

3.1 Microbial water quality in different water sources

FCM was used to assess the variability in cell count values reflecting the differences among surface, mixed and ground water sources. 3,689 samples were analysed for ICC, TCC, HNA and LNA. Overall surface water sources were represented by a cell count which ranged from between 10 and 4 times higher than ground and mixed sources respectively (Table 3). However, microbial abundance in nature including source waters is dynamic varying along a microbial baseline (Besmer and Hammes, 2016) which is likely source water specific. This is reflected by large variability within the water types. In this study, most of the groundwater TCC and ICC were below the LOQ (2000 cell/ml) which reflects in part, the low microbial loading evident in these waters. This has been attributed to the lower nutrients, especially low assimilable organic carbon concentration (Favere *et al.*, 2020) and a degree of protection from intrusion from higher loaded and faecal source contaminated surface waters.

Table 3 Summary of FCM data from different water sources

Water sources	FCM	N	Minimum	Lower quartile	Median	Upper quartile	Maximum	Mean
Mixed	TCC	1016	10	903	3650	16293	320370	12968
	ICC	1016	0	223	1065	6228	131020	6554
	%ICC			25	29	38		51
Surface water	TCC	621	40	1695	18210	48280	1006160	37711
	ICC	621	0	545	8120	34215	687040	26768
	%ICC			32	45	71		71
Ground water	TCC	2052	0	280	750	2608	103380	3362
	ICC	2052	0	60	170	630	74470	1374
	%ICC			21	23	24		41

In this study, the microbial dynamics in DWDS was assessed. Prest *et al.*, 2016 assessed as a key factor the temporal dynamic of microbial community highlighting how seasonal variations can affect bacterial cell concentrations as well as drinking water viability. In Figure 6A there was slight increase in temperature throughout the study period, with a minimum of 12°C in April and peaking at 16.8 °C in July. This trend was not apparent in either groundwater or

mixed supplies. Despite this, there appeared a seasonal trend in all supplies cell counts (ICC) with increases from June to September from 36 to 55 % ICC of TCC suggesting an increase in the number of cells (loading) and the viability of bacteria. Seasonal changes, influencing the drinking water temperature and nutrient availability, have an impact on the biostability of microbial community which can be reflected in FCM fingerprints (De Roy *et al.*, 2012). In drinking water source waters tested here the LNA intact cell count was in a range of 985-1516 / ml in GW, 13393-50223 / ml in SW and 4676-10455 /ml in mixed supplies (Figure 6B,C). For SW the peak occurred in September whereas it was April in GW and July in mixed supplies. The HNA concentration was lower with a range of 11-652 intact cells / ml with more variable loading compared to LNA, reflecting dynamics and variability of this source water (Figure 6D). Other research suggested changes associated in microbial composition revealing a HNA bacteria, rather than LNA, increased mostly in river water specifically after a precipitation events (Besmer *et al.*, 2014) with this trend most apparent in SW as opposed to mixed and groundwaters (Figure 6B,D) with high DNA content (i.e. HNA) thought to originate from eutrophic environments (Prest *et al.*, 2013). And that this population is active despite treated drinking water being considered oligotrophic in terms of growth requirements as the total organic carbon usually is less than 1 mg/L and assimilable fractions being less than 100µg/L (Lebanon *et al.*, 2001; Wang *et al.*, 2009). Elhadidy *et al.*, (2016) explored the dynamics of HNA and LNA populations and found similar proportions of each cell type within colder water conditions suggesting a lower biological activity. In this study, in contrast,

the spring and summer periods could have been a period which had significant HNA growth in catchment. LNA increased with warmer water temperature and higher nutrients with a slower rate compared to HNA bacteria capable to respond more quickly to optimal growth condition. During March and April concentrations of both LNA and HNA bacteria were constant, and particularly the HNA cells showed a large increase during May (Figure 6D) demonstrating a dynamic and seasonal pattern, whereas the concentration of LNA group was more stable over time. These results provided by FCM with a reliable intact cell count data distinguishing between HNA and LNA groups of bacteria, highlighted the different response of these subpopulations to seasonal changes especially in river water due to an increase in nutrients or water temperature. Also, HNA bacteria are more sensitive to changes in environmental conditions than LNA as they were more affected by drop or increase of temperature representing a diverse microbial community (LeChevallier, Cawthon and Lee, 1988).

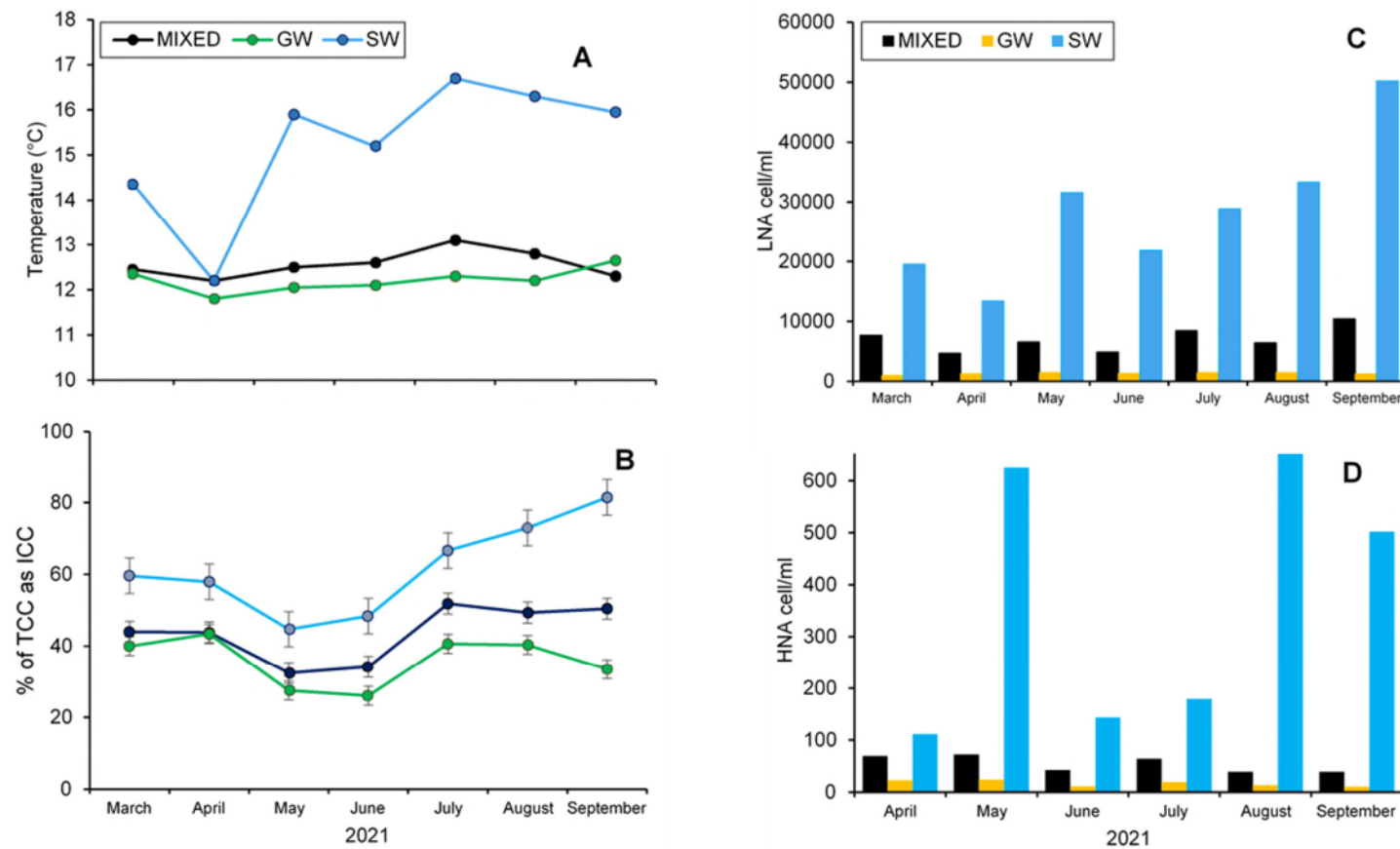


Figure 6 – A average temperature for mixed source water (MIXED), groundwater (GW) and surface water in drinking water storage assets (DWSA), B. percentage of TCC which is ICC in DWSA, C. average LNA abundance in DWSA D. average HNA abundance in DWSA.

As there are site specific factors which govern variability in microbial populations, each site was examined for trends which could indicate changes to raw water quality or efficacy of treatment. One example highlighting the sensitivity of FCM as a direct measure of the microbial population involved a diagnostic investigation of WT- A. This WT had water sourced from surface water (lowland river) from 18 to 29th of March 2021 (period 1), during the study period the source water was gradually adjusted to groundwater in an incremental fashion (13th of April to 3rd of September 2021) which represented period 2. In period 1 the average ICC was 44298 ml which reduced incrementally with each successive sample to 979 /ml below LOQ (1000 cell/ml) during period 2 (Figure 7A). Review of the FCM scatterplots (also known as fingerprints) in Figure 7C suggested that there was a reduction in the bacterial cells as well as the background (events not located within the gate which are indicated by the red events on dotplot). These fingerprints showed the cell population reduction with a change in source water which was evidence in both the density and distribution in events in the FCM scatter plot graph Figure 7C. This microbial water quality change event was similar to a study by Favere *et al.*, (2020) which utilised the Bray Curtis similarity index as a measure of change in water quality during a similar switch from surface water to ground water derived source water. The authors highlighted FCM's value for assessing the loading and biological (in)stability in such systems through use of data embedded within the FCM fingerprints. In contrast, the HPC data did not evidence a significant reduction in cell count suggesting no significant change to the water quality (Figure 7B) Therefore, FCM data characterize directly the

fluctuations of microbial community in source water type and treatments
processes interacting within extended DWDS

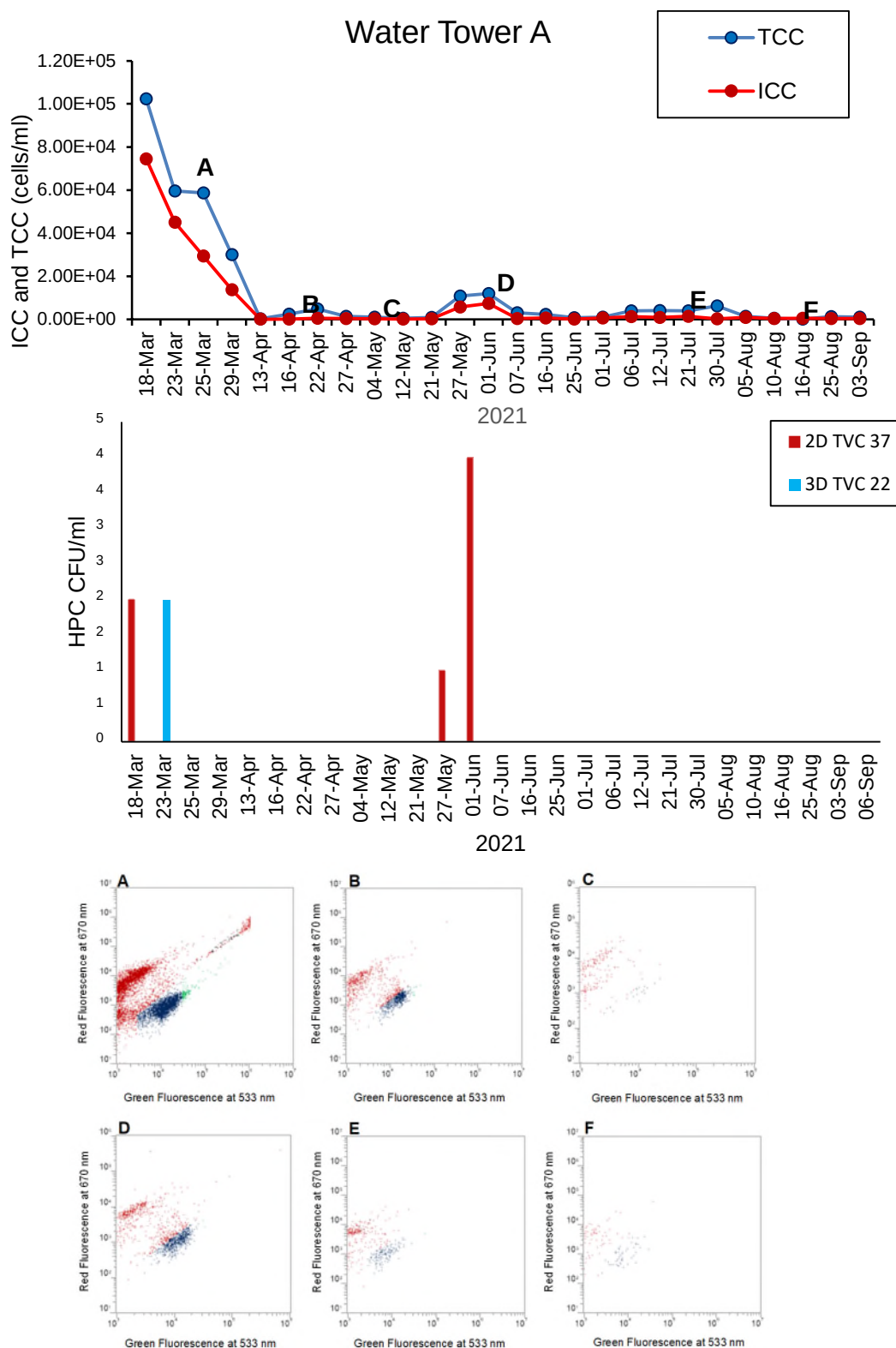


Figure 7 Top. Intact and total cell counts in Water Tower A. **Middle.** Cultured derived data (HPC) including 2 and 3 days **Bottom.** FCM fingerprints correspondent to specific points (A-F) monitor each month the dynamic of ICC population (black dots).

Underestimation by HPC of the absolute cell concentration

Overall, the dataset predicts a specific range of values highlighting natural variation in the water treatment works and reservoirs establishing a better sensitivity than conventional regulatory methods. Comparing HPC count traditional monitoring technique to flow cytometric during the study period in water tower A suggested that HPC largely underestimates the absolute cell concentration with sporadically spread data with many not detected. The whole dataset were analysed for TCC and ICC cell counts have specific trends widely different from HPC cell counts (Figure A-4), all over the three water sources there was a 3-log difference between HPC and FCM. FCM data is direct measurement of the microbial loading / population of a specific reservoir/tower, therefore can detect any changes which could occur from an established and baseline which would ideally be dynamic to season and or water source (under mixed source scenarios). This could allow better asset optimisation through management of source water quality, treatment at WTW and management of key DWDS operational variables such as WA, FC and pH. These aspects will be discussed within the future work section. Below is a discussion delving deeper into the reasons why HPC can underestimate cell concentration.

There are inherent reasons why HPC technique might provide an underestimation of the true bacterial count in water samples. One of the primary reasons revolves around the state in which many bacteria can find themselves: the Viable but Non-Culturable (VBNC) state. In this condition, bacteria are alive

and may even be metabolically active, but they are not amenable to growth on the standard media used in HPC. As a consequence, they remain undetectable to this method. In drinking water treatment and supply systems, bacteria can enter the VBNC state due to various environmental stresses. Disinfectants like chlorine, used to neutralize pathogens, can, at sub-lethal concentrations, push certain bacteria into the VBNC state, making them dormant yet potentially revivable. Temperature variations in the water distribution network can also drive bacteria towards this state, especially prolonged exposure to cold. The nutrient-limited environment of treated drinking water can further compel bacteria to adopt the VBNC state as a survival mechanism. Fluctuating pH and osmotic balances, variable oxygen levels dictated by factors like water flow rates and pipe materials, and lingering disinfectant residues pose additional challenges, sustaining the VBNC condition in some bacterial populations. Given their potential to resurface under specific conditions, understanding and managing the VBNC state is crucial for comprehensive water quality monitoring in drinking water systems. This phenomena has bearing for section 3.3.

The media itself presents another challenge. By nature, the agar used in HPC is selective, designed to support the growth of certain bacterial groups. This means it inherently excludes a range of bacterial species that might be present in water but have different growth requirements. Even among those bacteria that can grow on the agar, competition can arise. Dominant bacterial species might overshadow or even inhibit the growth of others, leading to an imbalanced representation of microbial diversity on the plate. Another overlooked aspect is the potential

aggregation or clumping of bacterial cells. When these aggregates are mistaken for a single colony, it naturally leads to a substantial undercount of the actual bacterial entities. The specificity of HPC to heterotrophic bacteria is another limitation. This method focuses on bacteria that metabolize organic carbon, sidelining other microbial entities like autotrophic bacteria. Water treatment processes further complicate the situation. Procedures like chlorination can induce stress in bacteria, rendering them non-culturable for a period. Though these stressed cells might later recover their ability to grow on media, an immediate HPC assay might miss them. Standard practices in HPC also impose a uniform incubation temperature and duration for all samples. This one-size-fits-all approach doesn't consider bacteria with unique growth requirements, leading to their potential exclusion from the count. Furthermore, while many bacteria require oxygen to grow, there are also anaerobes, which thrive in its absence, and microaerophiles, which need minimal oxygen levels. Traditional HPC might not provide the necessary conditions for these groups. Slow-growing microbes introduce yet another layer of complexity. If an HPC assay is read prematurely, colonies from these slow-growers might not have had enough time to manifest. Finally, the drinking water itself can sometimes work against the HPC method. Residual disinfectants or other chemical agents present in the water might inhibit bacterial growth on the plates, suppressing their actual numbers. In light of these challenges, it's clear why many professionals in the water quality field are exploring or augmenting their toolkit with alternative methods, such as flow

cytometry, which promises a more comprehensive view of the microbial landscape in water samples.

3.2 Diagnostic investigation into two DWDS compliance events

The next component of this study was to see if FCM can provide additional insights in the reasons behind compliance events in DWDS. Compliance events often deemed as 'failures' are defined as deviations to drinking water quality standards which are embedded in UK legislation enforced by the Drinking Water Inspectorate (DWI, 2015). In terms of microbiological consents, there should be no detectable culturable *E. coli* or coliforms (0 units per 100 ml) in final and distributed water until point of use. With units being defined as CFU for HPC and most probable number (MPN) for suspended growth quantification. Within the water supply region, the coliforms and *E. coli* detections during 2015 to 2020 most often occurred in distributed water (i.e. within DWDS) in surface waters with a frequency of 0.08 detections in total compared to 0.02 in groundwater and 0.03 in mixed supplies. Cheswick *et al.*, (2019a) revealed most coliform detections in Scotland were in surface water sources and that the greatest number of coliform detections occurred final waters with low cell counts <10,000 ICC/ml (due to influence of disinfection reducing cell counts). However, as a proportion of the total number of samples, the greatest number of detections occurred in highly loaded waters where the ICC exceed 1 million /ml. In this study, some detection occurred in groundwaters, this could be attributed to the presence of karstic

groundwaters within this supply zone which is more vulnerable to surface water intrusion especially when during periods of rainfall or surface runoff (Figure 8). Consequently contamination is more likely influence fluctuations in microbial community in such groundwaters with strong reaction to aperiodic events increasing the cells concentrations and risk of hygienic issues (Besmer and Hammes, 2016). The next phase of this research was to assess linkages between dynamics of microbial population using direct measurements of FCM alongside quantification of HPC to assess measure of hygienic significance during two water quality events in a retrospective manner.

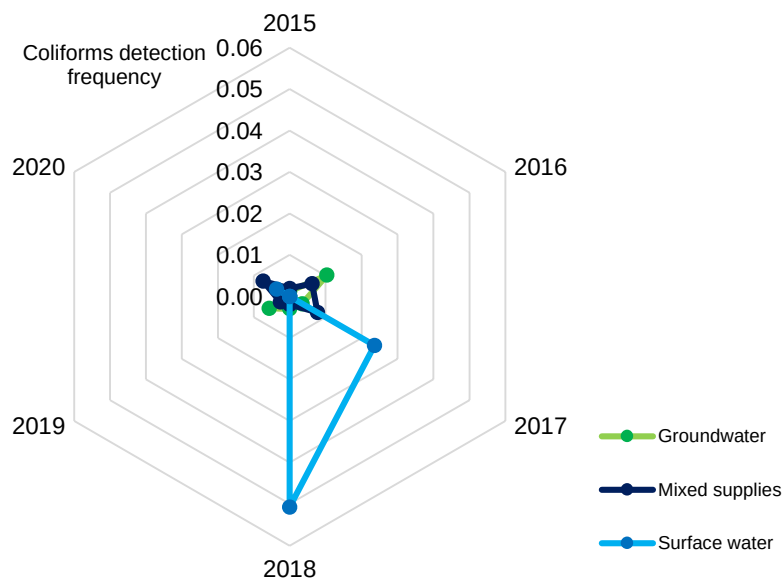


Figure 8 Detection frequencies (amount of detections / number of samples) of coliform and *E. coli* within reservoirs/water tower .

During this study there were two coliforms detections in distributed water. One detection occurred in a service reservoir receiving water from ground source (SR A) and a second supplied by mixed sources (SR B). In the SR A the highest value was 600 ICC /ml observed on 16/04/2021 which was 68 days prior to when the coliform detection which occurred on 21/6/2021 (Figure 9A). Immediately on detection of positive coliform, the SR A was taken out of service, triggering water quality investigation including root cause analysis and SR cleaning procedures according to Affinity Water's standard operating procedures. The consequence was that there was no further data available for this site. This site / failure challenges the utility of FCM to provide a sensitive measure of microbial water quality as there was no visual indication of a contamination events within either HPC (22°C) or FCM datasets. This is, in part attributable to the finding that all samples from this SR were below the LOQ of 1000 cells/ml which was established earlier (see method section 2.2.1). FCM is probably unlikely to provide a sensitive measurement of water quality assessment due to the LOQ> data. Other factors could have contributed to the detection (regrowth, ingress, misconnection, biofilm slough, contaminated sample tap etc), therefore, a set of variables should be considered for each site to determine which ones were involved and this is dependent upon the system developing a good dataset of monitoring results alongside microbial risk assessment and risk management from water safety plans (Lechevallier, Welch and Smith, 1996). In contrast to the above example, the second site with a coliform detection SR B had a gradually increasing trend from baseline of 8320 ICC/ml and 10000 TCC/ml on 15/03/2021

with several peaks resulting on the highest TCC and ICC of 25260 and 17470 cells/ml which occurred directly before and on the same day that the coliform was detected. These specific cases studies were identified by extensive FCM analysis and critically fluorescent fingerprinting with a novel use of their application during water quality events, some of these events also led to compliance failures as it was visualized in Figure 9. Trying to understand if FCM can be used to help prevent water quality events, there were two distinct types of microbial water quality failure. One (SR B) a step change which appears gradual and can be predicted using FCM and another type (SR A) which appears random and because the site is < LOD it is unlikely to be predicted using FCM.

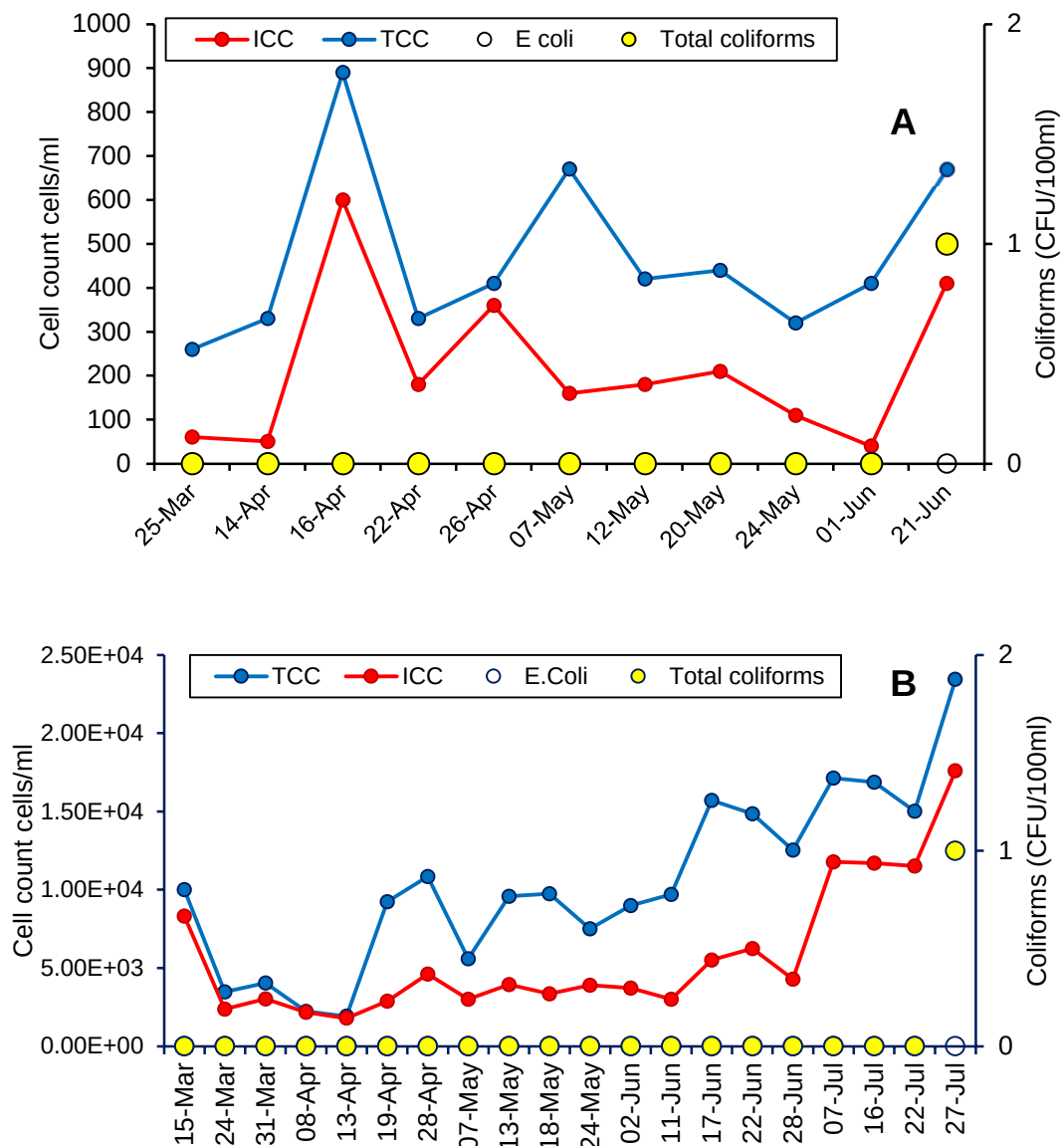


Figure 9 Comparison of ICC and TCC with faecal indicator organisms within
A. SR A groundwater source and B. SR B mixed source water samples.
 Coliforms and *E. coli* detection is displayed on the secondary axis as scatter dots, meanwhile on the primary axes ICC and TCC cells count trends along a certain period of analysis.

To examine the potential correlation between coliform detection and FCM data (specifically ICC and TCC), a Spearman's rank correlation coefficient was determined using data from both reservoirs. The Pearson correlation wasn't appropriate because the data didn't exhibit a linear relationship between the variables. The Spearman's correlation, being better suited for monotonic relationships, was hence chosen. Generally, the ICC cell count showed slightly stronger correlations with values ranging between 0.40 and 0.37, while TCC ranged from 0.35 to 0.37, and the ICC/TCC ratio varied from 0.30 to 0.18 for both reservoirs (as shown in Table 4). However, the p-value is higher than 0.05 indicating the correlation between these two variables is not statistically significant. This means that the correlation is likely due to chance and is not a reliable reflection of this underlying relationship as expected. Notably, SR A presented marginally better correlation values than SR B, though these correlations were relatively weak. This is consistent with findings from other studies, such as Cheswick et al. (2019), which demonstrate that bacteriological failures lack a definitive threshold when it comes to FCM values. These failures can occur at a diverse range of ICC values, spanning both low and high measurements. Therefore, while FCM might not be poised to replace current regulatory indicators, it can complement them by offering a direct quantification of the microbial population for water quality assessment.

Furthermore, microbial cell counts present a straightforward and dependable gauge of the microbial populace. Significant deviations in trends or concentrations could be indicative of compromised water. An abrupt spike in microbial counts can often suggest a recent contamination event, which could be due to breaches in the filtration system, intrusion from external sources, or issues in the distribution network. The sudden introduction of contaminants provides a nutrient source for microbes, leading to rapid growth. In addition to contamination, disruptions in regular treatment processes can play a significant role. For instance, disinfection lapses or changes in source water can lead to inconsistency in microbial removal. As these treatment barriers falter, even temporarily, the microbial population in the water can increase. Another factor to consider is the biostability of the water, which refers to its resistance to microbial growth. If water becomes biologically unstable, often due to an increase in organic matter or a decrease in residual disinfectant levels, it can support extensive microbial proliferation. Waters exhibiting these significant deviations in microbial cell counts have a heightened likelihood of experiencing a microbial compliance event. Recognizing these shifts and understanding their underlying causes is vital for maintaining water quality and ensuring public health safety. Future research could investigate n

The integration of Artificial Intelligence (AI) with flow cytometry data from drinking water holds promising potential for revolutionizing water quality monitoring. Flow cytometry inherently produces extensive data detailing individual cells and their attributes, and manually sifting through this can often miss nuanced patterns

crucial for assessing water quality. AI algorithms excel in rapidly analyzing such vast datasets, pinpointing patterns and anomalies that may indicate the presence of health-threatening microbes. Moreover, these AI models, when trained with sufficient data, can even predict future water quality deteriorations by recognizing early signs of microbial surges or harmful species. This real-time and predictive capability allows for immediate alerts and interventions, safeguarding public health. Additionally, the AI's ability to merge flow cytometry readings with other information sources, like weather patterns or wastewater reports, provides a comprehensive view of water quality influencers. As AI systems continually refine their analytical models, they become increasingly adept at pinpointing threats, ensuring both safer drinking water and more efficient resource utilization.

Table 4 Spearman correlation of ICC, TCC and ICC/TCC% to Total coliforms in Service reservoir A and B.

SR A			SR B		
	Spearman ρ	Significance (P)		Spearman ρ	Significance (P)
	Total coliforms			Total coliforms	
TCC	0.35	0.288	TCC	0.37	0.099
ICC	0.40	0.222	ICC	0.37	0.099
ICC/TCC	0.30	0.370	ICC/TCC	0.18	0.423

3.3 Flow cytometry provides new insights into use of free chlorine for DWDS management.

Free chlorine serves as the primary disinfection residual method at the studied sites to inhibit microbial regrowth. Its effectiveness often appears as a negative correlation between FC concentrations and ICC. Though this trend is observable across mixed, groundwater, and surface water supplies, the necessary FC residuals differ for each. In mixed water supplies, including storage points like WT and SR, an FC concentration of 0.4 mg/L is sufficient to maintain low ICC levels, as depicted in Figure 10A. Surface water storage reservoirs (SRs), however, require a slightly higher FC residual, with a Cl_2 concentration of 0.6 mg/L, likely due to a higher presence of assimilable nutrients (Figure 10B). This observation aligns with Gillespie et al., (2014) who reported similar values in their study of Scotland's surface waters. Groundwater storage, conversely, demonstrated a reduced FC residual requirement of 0.2 mg/L (Figure 10C). Such a low threshold corresponds with findings from other studies; for instance, Nescerecka, Juhna, and Hammes (2018) found bacterial regrowth at chlorine concentrations below 0.3 mg/L and reported a negative relationship with FC residuals. Whitton et al., (2018) conducted a comparative analysis of various chlorine disinfection methods to gauge their efficiency in bacterial cell reduction. Their findings revealed that free chlorine showcased superior efficacy at concentrations exceeding 0.5 mg/L, resulting in lower ICC levels. However, they also pointed out a regrowth threshold below 0.34 mg/L. They emphasized that microbial regrowth is not solely a factor of chlorine concentration but arises from a multifaceted interplay of water's

biological, physical, and chemical attributes, a sentiment echoed by Zhang and DiGiano (2002). Surface water, in this study, appeared to be more vulnerable to bacterial regrowth, a phenomenon also documented by Niquette, Servais, and Savoir (2001). This susceptibility can be attributed to variations arising from the water source and origin. After chlorination, samples with fewer nutrient constraints tend to exhibit higher cell concentrations. Furthermore, diverse microbial compositions, each having distinct chlorine resistance levels, can lead to varying responses, as highlighted by Nescerecka, Juhna, and Hammes (2018).

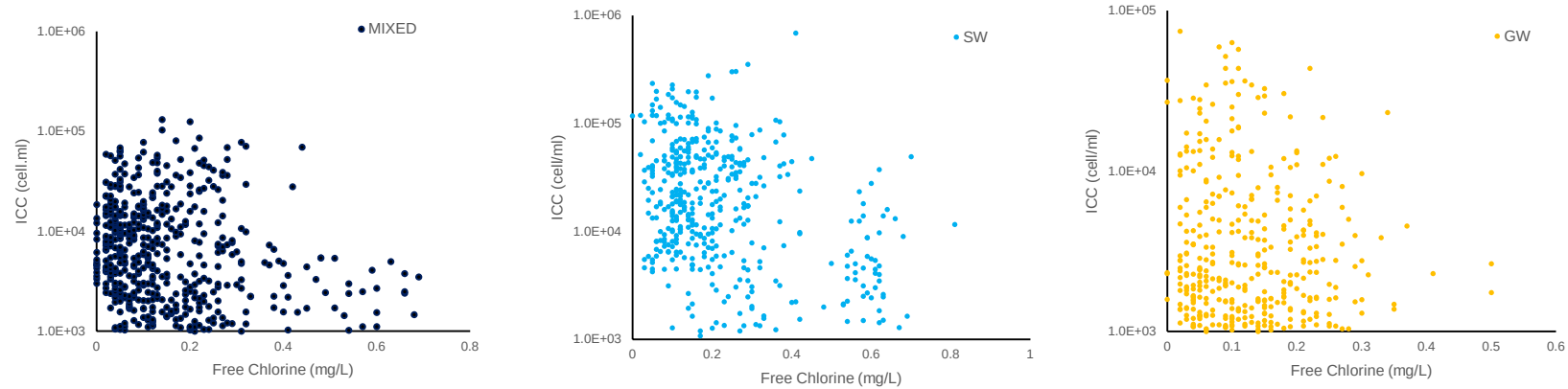


Figure 10 Effect of disinfectant concentrations, Free Chlorine, on the intact cells in service reservoirs and water towers A. Mixed supplies, B. Surface water and C. Groundwater

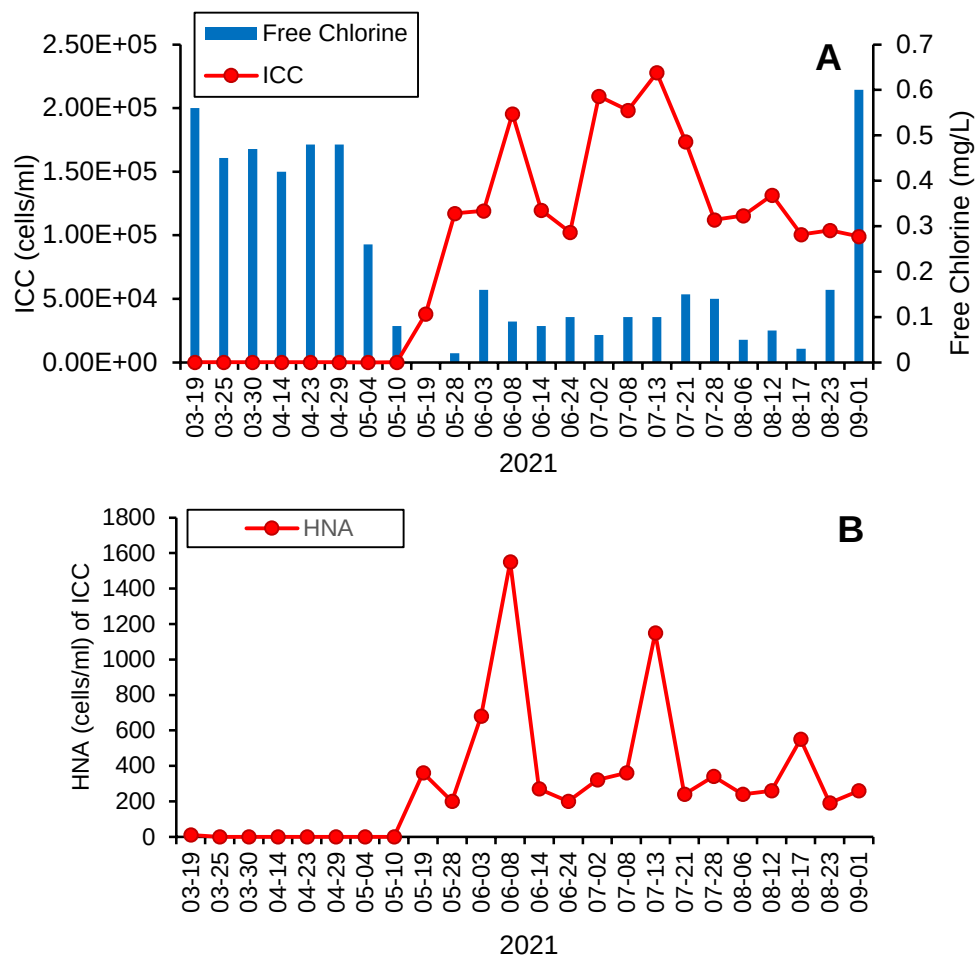


Figure 11 SR C A. ICC population dynamics and Free Chlorine. On the primary axis ICC is displayed as a red line, while FC as a stacked blue bar on secondary axis. B. %HNA of ICC trend line from SR C sample point.

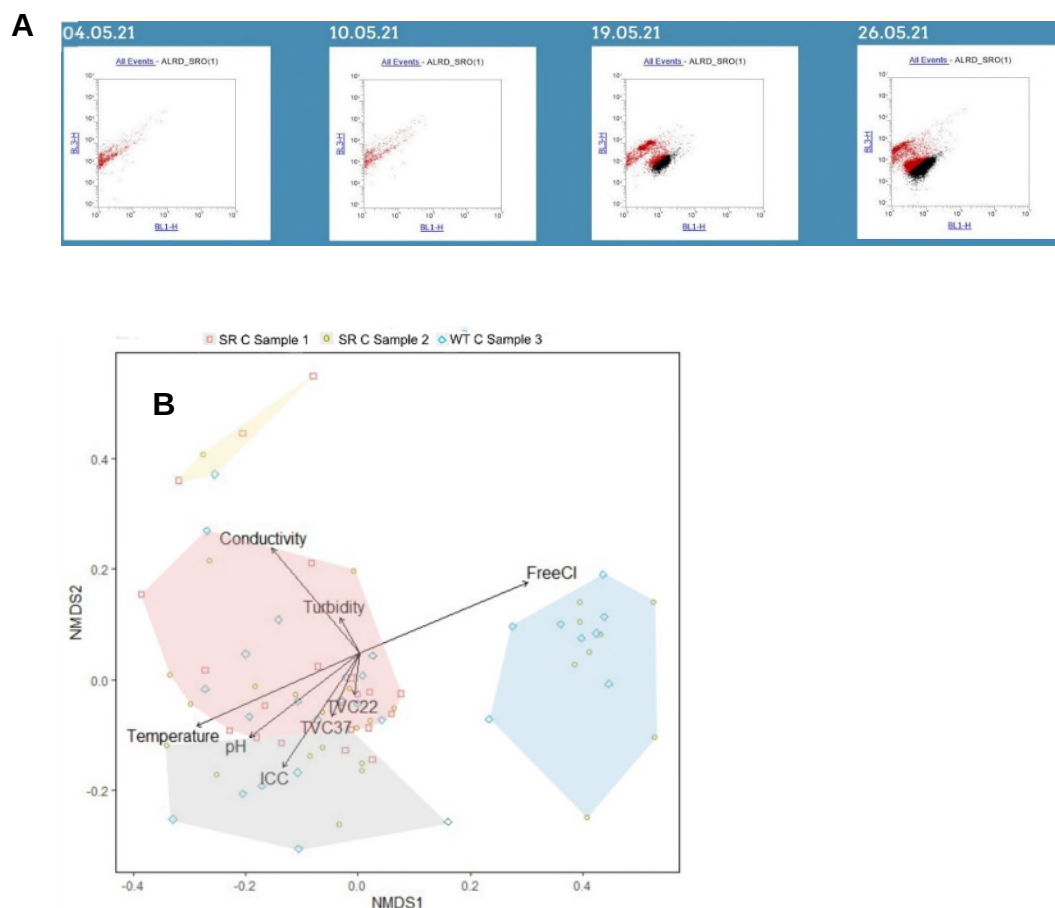


Figure 12 A. FCM fingerprints showing the two events occurred before and after the major FC breakdown. B. Cytometry histogram image comparison analysis (CHIC) with data from SR C and displayed on a non-metric multidimensional scaling plot. Different sample points within the same storage point including both the SR and WT are presented.

In SR C, there was consistently low ICC populations which occurred at sufficient FC concentrations (Figure 11A). From 19/03/21 till 05/04/21, the average of FC was between 0.3 mg/L and 0.6 mg/L which is within the range of values suggested to avoid significant microbial regrowth as stated previously. On the 19.05.21 there was reduction in FC from 0.3 mg/L to <0.1 mg/L, alongside an increase in the bacterial loading. The values for ICC increased 10000-fold

(approx. 4-log). Examination of the FCM ICC data suggested these were primarily due to LNA. Primarily this is due to a decrease in the HNA despite increases to ICC (Figure 11B) which suggested specific regrowth of the LNA organisms alongside a change to the bacterial fingerprint (Figure 12A). There was an increase in the organic and inorganic background during this event as evidenced by the red events on the fingerprint (Figure 12A 19/05/21). Meanwhile the percentage of LNA (composing almost 98% of ICC) showed the same trend as ICC population, the HNA followed a slightly different one (Figure 11A,B). There are several peaks including the highest which occurred in correspondence with the first part of the ICC peak rising on the 6/08/2021. Each peak appeared to be more evident due to a gradual ICC increase in three distinct period (June, July and August) where the FC value was constantly below 0.1 mg/L. As mentioned before HNA group is likely to be more sensitive to changes (i. e. temperature) (Figure 6D).

In this case study, the FCM data were able to detect rapidly changes which occurred to the microbial fingerprint. The concomitant change to the ICC and fingerprint suggested that the fingerprints could be used as a tool to understand the dynamics of cell change from during the process of water storage and distribution. Visual inspection at the fingerprints (Figure 11A) revealed significant cell increases was visualized as black dots. ICC population represented by black dots was barely visible on the 4/05/21 and 10/05/21 samples. There was a substantial increase which occurred on or around the 19.05.2021. The ICC increased from below LOQ to 38,340 / ml. Such events have been tracked by a

continuous inline FCM approach in another study which has a greater sampling frequency. Within this study, they simulated a contamination in drinking water over a short time scale and subsequential disinfection with a shock chlorination (Besmer *et al.*, 2017). In the thesis presented herein, a CHIC was performed to understand statistically the impact of individual cytometric fingerprints of microbial communities, e.g., at certain time points or microenvironmental conditions which is visualised through a non-metric multidimensional scaling (NMDS) plot (Figure 11D). CHIC is an image-based analysis tool without any requirement for an initial manual gating step, so consequently a grey scale images from cytometric raw measurements can be overlapped and differences in the density and distribution of events on the images is to calculate any dissimilarity matrix (Bray-Curtis index) (Koch *et al.*, 2013). On the plot three different sample points from the same storage asset were represented: SRC-1, SRC-2 and WTC. During the distribution and storage process within interconnected assets, the waterflow originated from the same source / WTW. The flow starts at SRC-2 which is fed boosters to WTC based on tower level. The WTC then overflows at night into SRC-1 situated directly underneath the WTC, furthermore SRC-1 and SRC-2 can bypass WTC through connected pipework. There was booster chlorination dosing at the point of SRC-2 whenever a valve to SRC-2 opens. These sample points are strictly interconnected to each other and have the same water feeding them from the same WTW. Despite this, there are significant differences in the microbial fingerprints (Figure 12B). Inspection of the vector analysis (correlation between normalised predictor variables and principal components) revealed a negative

correlation between temperature and FC. These variables appeared to drive the variability in the microbial fingerprint with elevated FC in WTC compared to SR2 which itself does not have booster chlorination. After extended storage times within WTC the fingerprint within SR1 is very different from either SR2 or WTC (Figure 11D). This fingerprinting method to analyse cytometry data proposed in this work demonstrated the importance of good chlorine management within DWDS. Critically, the management of interlinked storage assets with different effective WA, FC and starting cell counts is vital for effective management of these assets and their overall distributed water quality. This will be explored in the subsequent section on WA. Together with CHIC there are different analytical tools (i.e. Dalmatian Plot, CyBar, and FlowFP) which allow retrieving further information of FCM fingerprints giving a wider picture of the microbial system at low costs (Koch *et al.*, 2014). It is recommended that future work could conduct sensitivity analysis to validate how different fingerprints need to present before changes will occur to distribution on NMDS plots. It is considered that fingerprinting will be most valid to compare FCM data from the same source water. As source water effects to microbiome will override smaller, subtle changes which occur to the microbial community during DWDS.

3.4 Water age

The WA is defined as the residence time distribution for water to transit from treatment works to reservoir and customers tap Francisque et al. (2009). It is often modelled and expressed as days that have passed since primary disinfection has been applied (Hrachowitz *et al.*, 2013). There is no convention within the UK water sector for revising WA due to secondary disinfection (i.e., booster chlorination). The WA is an important component of DWDS management, as time is an fundamental component of microbial regrowth (i.e. effective doubling time of heterotrophic bacteria) (Wang *et al.*, 2018) and utilisation of AOC which may be present in drinking water (Pick, Fish and Boxall, 2021), with greater populations of microorganisms occurring during elevated distribution times including evidence of known human pathogens such as *Legionella* spp, *Mycobacteria* spp. and *Pseudomonas aeruginosa* (Wang *et al.*, 2012). Also, chlorine decay and reactivity which is important is dependent on numerous factors such as the water temperature, presence of reactive NOM, DWDS pipe material and presence of biofilm within DWDS. As WA increases residual disinfectant gets used up (FC converted to total chlorine) which can permit for further increases in cell population, as the oxidative stress dose / on viability or suppression of growth response is reduced (Wang *et al.*, 2012). It is well established that the FCM data is important as an indicator of the disinfection efficiency, although should be considered alongside other data (e.g., culture indicators, chlorine residual, colour, taste and odour etc) to judge the hygienic

status of a system. One study looked at the ICC numbers from two different WTWs, and highlighted the reduction in cell counts which occurred after the initial disinfection process (ozone) followed by further increases due to biofilters and subsequent decreases. This is an example of controlling regrowth within WTW and managing biostability into DWDS by minimising assimilable nutrients (Vital et al., 2010). Other key DWDS / environmental variables which are relevant to the microbial population include the water temperature, FC concentration, and rainfall. These factors either directly influence the water quality or indirectly the microbial population, which together are reflected in the ICC change measured using FCM within DWDS. Therefore, increasing the WA could expose the water to some of these factors. During this research this fundamental aspect was explored assessing specifically how WA impacts on absolute number of cells whilst considering / controlling for the impact on other key operational variables (FC and temperature). This dynamic interface/interdependency between WA and FC and its influence on ICC concentrations was assessed within extended DWDS during a 6-month period. It should be noted that the WA is difficult to measure directly and was simulated by network modelling team within Affinity water.

To enable comparison between water types and due to the low temporal resolution of WA data average values were used between different WTW and water types. The WA effect appeared most pronounced in surface waters, possibly due to higher residual organics in these waters. This water could be defined as being less biostable water (than groundwaters) and consequent requirement for higher FC residuals to suppress the regrowth of the microbial

population. Overall elevated WA (5-12 days), there were greater cells counts present and this trend was similar between each of the groups of water sources tested (Figure 13A-C). Therefore, a high value of WA together with a low FC can result in high microbial populations in distributed water. The highest average ICC was reached with a WA average of more than 4 days and FC in the range of 0.03-0.13 mg/L in both groundwaters and mixed sources demonstrating that for these waters a slight decrease of ICC values in the FC range of 0.13-0.23 mg/L is expected. On the contrary, surface waters had a different tendency with the highest peak in ICC within FC range of 0.13-0.23 mg/L. This could be due to a two factors, i) surface waters contain more assimilable organics than most groundwater supplies (as discussed above), ii) the surface supplied DWDS have extended distribution zones and therefore extended WA (evidence by up to 12 day WA) compared to maximum of 7 day and 4 day in mixed and groundwater supplies respectively (Figure 13 A,B). Finally, and most likely is the surface water supplies are influenced by booster chlorination within the DWDS at extended WA (5-12 days). Although the lowest ICC median values < 100 cells / ml in the FC range of 0.23-0.33 mg/L has been detected in mixed and groundwater sources, however, surface water with similar distributed FC range has a lower residual ICC possibly due to elevated log reduction credits achieved at surface water treatment works. The low ICC concentration was reinforced by WA values in a range between 0-1 days together with an elevated FC value. Nevertheless, when FC is above the upper threshold WA becomes important for the regrowth especially in mixed sources. In surface water there is another peak when FC values are higher

than 0.5 mg/L (Figure 13A), but this is not due to WA which seems to be low so therefore some other parameter might be involved for example the variable quality and composition of mixed supplies source water and the role of blending of supplies on ICC. Further work would be needed to investigate this trend in more detail.

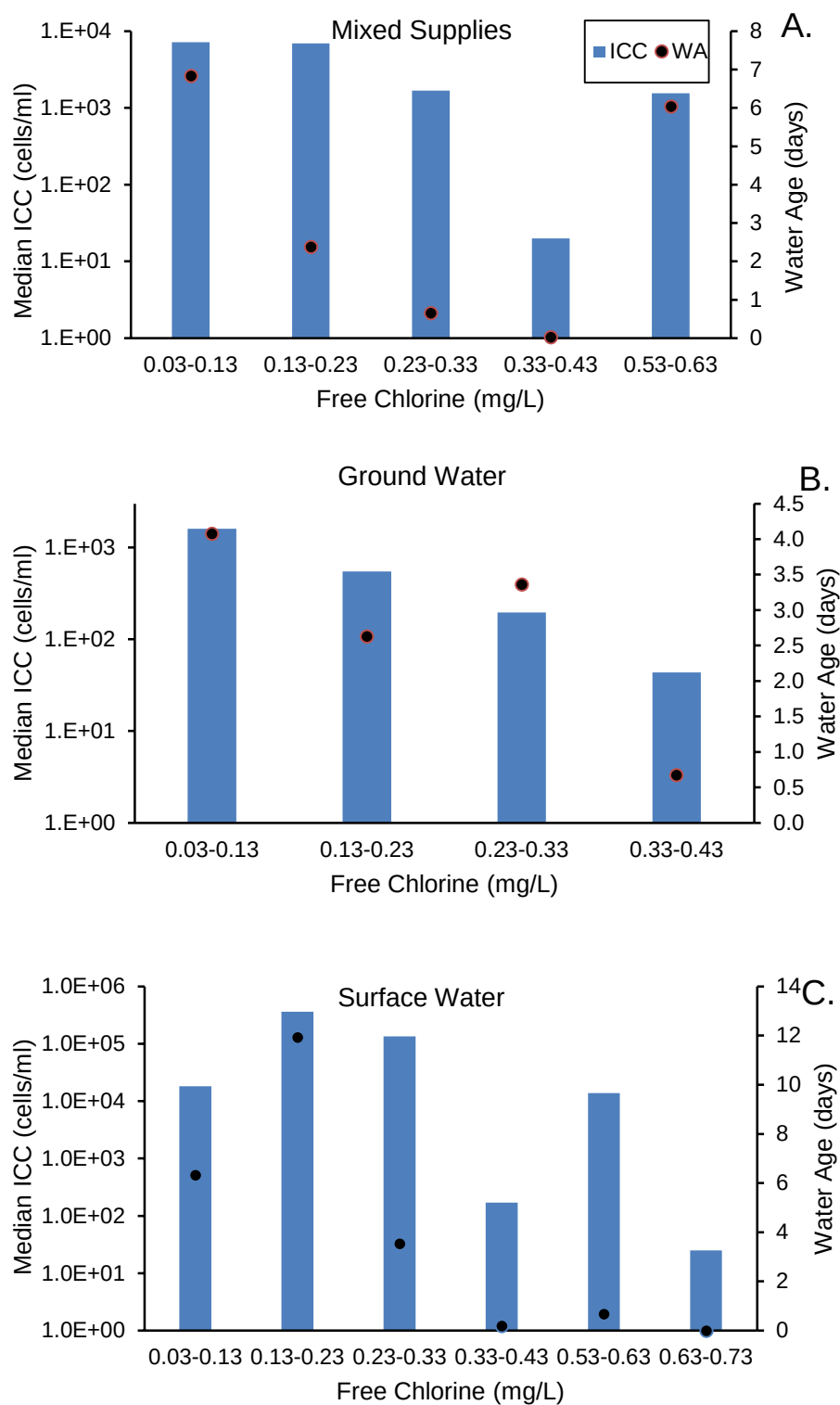


Figure 13 Combined action of Free Chlorine concentration and Water Age on the intact cells count ICC within all the reservoirs/towers analysis along a period of 6 months. A. mixed supplies B. groundwater C. surface water.

Generally, keeping the FC at acceptable levels results in a low regrowth potential irrespective of the WA. However, at extended WA, additional dosing through booster chlorination would be needed (see Figure 11), which raises the prospect of additional disinfection-by-product and taste and odour concerns. Therefore, greater understanding of how cell counts are influenced by FC concentration and WA is important. There is a dynamic interdependency between the two. FC is temperature and time dependent; the absence of FC is related to elevated WA and high temperatures. As described in the methods section, an MRA model was undertaken to gauge the significance of each environmental factor towards bacterial concentrations within DWDS. To build the model it was necessary firstly to be sure no extreme outliers' data were present and then to apply a non-linear transformation to the response variable such as taking the log of the values causing the residuals of the model to become more normally distributed. At this point each assumption was successfully met to proceed with the multilinear regression analysis of all the dataset.

Table 5 Multiple regression linear model results showing ICC influence from three independent variables Temperature, Free chlorine and Water age. Standardise regression coefficients (β) are reported.

	GW	Sig.	SW	Sig.	MIXED	Sig.
Model R square	0.260	<0.001	0.491	<0.001	0.334	<0.001
WA (β)	0.334	<0.001	0.068	0.043	0.202	<0.001
Free Chlorine (β)	-0.177	<0.001	-0.375	<0.001	-0.309	<0.001
Temperature (β)	0.271	<0.001	0.526	<0.001	0.408	<0.001

Despite relatively low R-square values the surface water (SW) derived catchment are well described by the model having a R-square of almost 0.5, $p < 0.001$ (Table 5). This model explained 33% and 26% of the variance for mixed and groundwater (GW) respectively of the ICC ($p < 0.05$). In addition, standardized β coefficients allowed the contribution of all three variables to be directly compared. Examination of these values highlighted the role of temperature (season) in governing variability in ICC in surface and mixed waters with a value respectively of 0.52 and 0.40. In contrast, the WA was crucial in groundwater with 0.33 and then temperature (0.27). In surface and mixed derived catchment temperature contributes substantially to the regression equation, followed in order by FC which has a negative impact (as expected) and the WA with a much lower contribution especially in surface waters. In general, temperature dominated the variability in ICC in surface and mixed water. For all water sources managing the WA is extremely important for understanding and control the regrowth as temperature is not a variable which operators have much control over.

4 Future work and considerations for industry

In this research FCM data helped to create a specific fingerprint for each SR / WT giving a direct assessment of the microbial community and this degree of changes. This is because the fingerprint reflects microbial community structure in the water sample. Shifts or changes in the patterns (like the emergence of new clusters or the disappearance of existing ones) can indicate changes in water

quality or the efficacy of water treatment storage processes. Overall, these FCM data had a significant impact to the water utilities monitoring and leading to several investigations into the DWDS. One of the events occurred was related to Allenby road, a reservoir showing a 10000-fold increase in intact cells count over a very short period suggesting poor water stability or changing quality of fed water. Therefore, a production site inspection reported a FC concentration dramatically dropped below 0.1 mg/l. Outcomes from this action demonstrated how fast and immediate the response was relying on the FCM data for monitoring and diagnosing a specific reservoir asset. Allenby is one of the case studies have been used unveiling how the usage of this instrument gives more information potentially critical to undertake operations in DWDS.

Importantly, FCM has the capability to monitor any changes or confirm the stability in different sites. As a result, alongside the project FCM analysis were required for extra sample points to look around the disinfection process as additional practical / operational benefit on a treatment works. Hence, considering the beneficial use of FCM to identify any changes, one approach to assess consequences for water quality might be a smart sampling which involves sampling specifically when increasing trends of microbial concentration are detected. Elevated microbial ICC and TCC might not be a health risk in themselves, but they suggest that disinfection might not be sufficient. Therefore, it is important to have a baseline range of ICC and TCC depending on site-specific characteristics. In this regards any future measurement can be automatically evaluated against a dynamic baseline that would allow for real-time

quality monitoring with automated alerts and even operational measures. An online system ideally should be developed as an early warning in case of relevant deviations from established (dynamic) baseline conditions. It is then accepted that FCM can be a powerful instrument to minimise the risk of occurrence of failing compliance events throughout the DWDS saving time and costs contributing to the water quality. A key point is to understand the general dynamic of microbial community within distribution network being able to provide fundamental knowledge to define the degree of acceptable change during events of biological instability.

Outcome from this project will be used to elaborate a system throughout the major reservoirs and water towers to create site-specific dynamic baseline based on fingerprints representing FCM data. According to the water source catchment it might be taking in consideration different factors that can cause instability in the distribution system. It has been highlighted from this study that a management plan should be developed to undertake operational actions site and zone specific. As a consequence, together with an early warning system it might be necessary an overall knowledge related to disinfection parameters strictly dependent to other factors as temperature and WA which tend to be vital in accordance to the water sources.

5 Conclusions

The primary objective of the project was to employ advanced fingerprinting techniques using FCM to scrutinize the dynamics of microbial communities within DWDS. The focus was on factors influencing microbial water quality and, more specifically, their interdependencies. The results of the study highlight the importance of considering variables such as WA, FC, and temperature as interconnected elements that significantly impact water quality in distribution systems. FCM data offered new insights into microbial loading and potential regrowth in storage and DWDS. This information is pivotal for assessing and managing risks in various SR. Conclusions tied to specific objectives are presented below:

Objective 1 – To describe how source water quality impacts DWDS quality using FCM

- The type and quality of water sources introduce variations in FCM data within the DWDS. These variations suggest that there's a range of values indicating natural ebbs and flows in microbial concentrations.
- Water quality varies based on its source and specific location. Each reservoir or tower has a distinct microbial fingerprint, showcasing the unique characteristics and quality of the water at each site.
- Observable seasonal patterns influence microbial parameters. These patterns are linked with fluctuations in water temperature and can be influenced by the season.

- FCM fingerprinting is highly sensitive to changes in water source and any significant microbial events. Moreover, this fingerprinting provides supplementary information that is valuable for understanding the broader context of water quality.

Objective 2 - To compare FCM data with conventional measures e.g., HPC

- There's no direct association between the presence of coliforms/*E. coli* and FCM data. High microbial loading doesn't always align with a compliance failure event, suggesting other factors at play. In addition, while changes to the cell count often occur during compliance events. The low frequency of detection and therefore sample size precludes direct statistical linkage.
- No significant correlation was observed between HPC and FCM data. FCM's higher sensitivity provides data that more accurately gauges cell viability, doing so more swiftly and efficiently.
- Two distinct patterns of coliform failures were identified: one that's gradual and predictable, and another that appears randomly with counts below LOD. The latter is difficult to anticipate using FCM alone. It is recommended that other datasets, models and tools be applied to help mitigate these low abundance events.
- While FCM is adept at tracking consistent microbial trends, it's less effective in scenarios where microbial counts suddenly spike but remain below the LOD.

- The project delved into the novel application of FCM data during specific water quality events, exploring whether FCM could be a tool in forestalling water quality issues.

Objective 3 - To determine risk factors contributed to high microbial loading in service reservoirs.

- FC disinfection predominantly determines the reduction of ICC. It's essential to highlight a critical chlorine concentration at 0.42 mg/L. Ensuring that the FC residual remains above this threshold enhances the microbial water quality by inhibiting microbial regrowth and controlling high cell loading.
- It's feasible to establish a specific 'FC residual operational window' tailored to each reservoir or water tower based on their respective water treatment processes. The efficacy of chlorine's influence can vary, generally falling within a range of 0.3 to 0.4 mg/L, contingent upon the water sources.
- Various natural water sources exhibit diverse reactions to FC disinfection. This is largely attributed to the heterogeneity in microbial communities across drinking water reservoirs. The variance in the percentage of HNA within ICC, based on differing water-derived catchment areas, underscores this point. Notably, HNA is particularly reactive to environmental shifts.

Objective 4 - To investigate water age, temperature and free chlorine residual as part of an overall DWDS management plan.

- The study delved into examining the interplay between WA and FC and how they jointly influence ICC concentrations. WA stands out as a pivotal factor, and when paired with FC, it considerably affects microbial growth.
- Water age serves as a useful metric for understanding the microbial population i.e., ICC potable systems. Furthermore, it demonstrates a dynamic relationship with FC and temperature within DWDS which is more nuanced than first considered.
- A multiple regression linear model pinpointed temperature as the primary risk factor for both surface and mixed sources. While ICC values across all water sources were influenced by WA, it was most pronounced in groundwaters.

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Appendix A

5.1 Physio-Chemical standard water analysis

A.1 Turbidity, electrical conductivity and pH

All these physical parameters for potable water were measured following standard practice accepted by SCA. Using a clean cell it was read the turbidity value of the water sample and blank (deionised water) directly from the turbidimeter (Hach 2100N0) digital display. Low and high controls were calculated daily at 20, 200, 1000 and 4000 FTU StabiCal standards (Hach-Lange, UK). Drinking water cannot have turbidity exceeding the PCV of 4.0 NUR for Water Supply Zone or 1.0 NTU for Water Treatment Works. The electric conductivity of each sample was measured by conductivity cells referenced at 20°C and calibrated daily against potassium chloride solution of known strength (0.1M KCl, Merck, UK). Jenway 3540 meter was used to analyse both pH and conductivity pouring the sample in a 100 ml beaker where either a conductivity cell or a pH electrode with temperature probe were placed. pH standard measurements were carried using buffer solution at pH 4.0, 7.0 and 9.22 (Jenway, Stafford, UK). The PCV for conductivity is 2500 $\mu\text{S}/\text{cm}$, whilst the pH range for potable water must be between 6.5 and 10.0 pH units. All controls of these physical parameters were daily randomly performed amongst every 16 samples and run at the beginning and at the end of the batch.

5.1.2 *In situ* measurement

Free and total chlorine measurements were undertaken using the N-diethyl-p-phenyldiamine (DPB) reagents (Palintest, UK) using the colorimetric method with a Hach colorimeter (Hach-Lange, UK).

5.2 Microbiological analysis of drinking water

A.1 Heterotrophic plate count (HPC)

The colony count of heterotrophic bacteria was assessed by carrying out the pour plate method using a solid nutrient medium such as Yeast Extract Agar (YEA, OXOID, UK) to provide a general indication of bacterial population in drinking water under specific test conditions. This method is suitable for the isolation and enumeration of bacteria by testing a volume of 1 ml cultured in 15-20 ml of molten YEA incubated then at 37°C and 22°C respectively for 44±4 and 68±4 hours. A bacteria found growing at lower temperature are representative of the normal waterborne flora, meanwhile at higher temperature the growth is derived from external sources and consequently being significant in terms of human health.

A.2 Total coliforms/ *E. coli* and secondary indicators

Total coliforms and *E. coli* are considered as primary indicators of faecal contamination in the water. The determination of Total coliforms and *E. coli* is based on tests routinely performed by membrane filtration method. A volume of 100 ml of water sample is filtered on a membrane 0.45 µm through a vacuum pump, then placed on a selective media to isolate coliforms colonies. This

medium contains lactose glucuronide (MLGA, OXOID, UK) which let coliforms produce yellow colonies and *E. coli* green colonies due to their specific enzymes respectively β -galactosidase and β -glucuronidase after an incubation period at 30°C for 4 hours followed by 14 hours at 37°C. The total incubation should not exceed 24 hours. Secondary indicators as *Enterococci* and *Clostridium perfringens* were tested by performing a membrane filtration method using selective media to isolate them at different incubation times and temperatures. *Enterococci* grew on a Slanetz and Barley agar (SB, OXOID, UK) at 37°C for 48 hours, meanwhile *Clostridium perfringens* grew on a Perfringens agar base (PAB, OXOID, UK) at 44°C for 24 hours. All these membrane methods determined in accordance of the Standing Committee of Analysts (SCA) were referring to the presumptive presence of bacterial colonies in the water samples. When a microbial growth occurred, there were confirmation steps to follow for each bacteria. To ensure the validation of these analysis several controls, positive and negative, were tested alongside the drinking water samples.

5.1 Flow cytometry fluorescence staining and analysis protocol

In addition of the data obtained by routine analysis it was examined the FCM ones for understanding viable (intact) and total bacterial populations. Together with the usual number of samples some extra are collected in a 300 ml bottle treated with sodium thiosulphate for the FCM analysis. The latter was performed

within 72 hours, which means 3 days a week to organize loading samples already received and stored in a fridge at 4°C. To quantify TCC and ICC population it was followed Ramseier et al 2011 standard protocol combining FCM analysis with staining procedure using two different dyes: SYBR Green and Propidium Iodide (PI). Firstly a SYBR green stock solution was prepared by starting from SYBR green 10000 x (Invitrogen, UK) concentrate diluted in a sterile Eppendorf (1.5 ml) with filtered dimethyl sulfoxide (DMSO, QCL LTD, Sussex, UK), as a result a 100× SYBR Green working solution was produced to be stored at -20°C (10 µl of 10000x SYBR Green in 990 µl of DMSO). To determine total cell it was added this solution to the sample to achieve a 1× final concentration, 5 µl added to a 500 µl of sample volume. For measurement of intact cell concentrations (ICC) a mixture of SYBR green and PI stock solution was made by a mixing of SYBR green and PI in a ratio 5:1 adding 500 µl of 100× SYBR Green to 100 µl of PI (1mg/mL, 1.5 mM, Invitrogen, UK) in a sterile Eppendorf (1.5 ml) stored at 4°C until use within 48 h. In total 6 µl of this working solution was the quantity to add to a 500 µl of sample volume. After staining the sample, it was gently vortexed and then incubated in a heath block shaking incubator (Grant-bio, UK) for 15 min at 250 rpm at 30°C in the dark. To perform FCM analysis for more than one sample it was used a 96-wells microplate filled with 300 µl of each sample where it was added already an aliquote of 3 µl of SG and 3.4 µl of PI respectively in the 1st and 2nd wells. All the samples were transferred using a 6-channels pipette into each wells, then incubated and analysed by the autosampler component of the Flow Cytometer (Thermo Fisher Scientific, UK). Afterwards a dataset was created and processed

using Invitrogen™ Attune™ NxT Software. During these experiments, a 'dot-plots' (FL3 versus FL1) was created characterizing a specific fingerprint based on TCC and ICC for specific reservoirs/water towers.

The instrument Attune™ NxT (Thermo Fisher Scientific, UK). was validated with a setting-up of Standard Operating Procedures and templates required for sample processing. A sample volume of 100 µl was tested with the green fluorescence collected in the FL1 channel at 533 nm and the red one in the FL3 in the channel at 670 nm with a voltage of 350 on FL1 and 465 on FL3 without any compensation. Starting from the template created and kindly provided by Adrian Marsh (Northumbrian Water) it was adjusted the gate which discriminate stained bacteria cell from background, mostly debris made by non-bacterial organic particles (Hammes and Egli, 2005). According to EAWAG method the FL1/FL3 dot plots (or green/red fluorescence density plot) allowed to have this optimal distinction. Therefore, a gate could be set around specific data to represent a certain population following a gating strategy with a fixed template in order to achieve comparable data among samples. In the fixed gate low and high nucleic acid (HNA and LNA) were shown to implement the data around the bacterial population analysed. These typical two clusters bacteria were discriminated in the gating strategy by applying additional gates on the green fluorescence histogram (Wang *et al.*, 2009). Using ultrapure filtered water (0.22 µm PES membrane, Whatman) as a negative control it was possible to adjust the gate to exclude the background noise made by the fluorescent dyes (SY and PI) used for staining the bacterial population. To have a clear density plot without any

background noise it was necessary to filter with Nylon 0.22 μm (Whatman) DMSO used to prepare Sybr green and PI. *Enterococcus faecalis* lenticule stock was used as a positive control having a fixed concentration and viability profile to calibrate the machine. In Appendix section Figure A-1 represented the the lenticule trend which was steady except for the first three weeks where bacterial population was mainly composed by TCC. This behaviour was due to the nature of lenticules which tend to have a lot of dead cells making intact population less clear unless a reactivation occurred. Generally, every week a stock was made by suspend *Enterococcus faecalis* in 10 ml nutrient agar, incubated for 24 h at 37°C then stored at 4°C in a fridge. Of this stock solution 5 μl was resuspended in 10 ml of ringers solution filtered with 0.2 μm PES (Whatman). Afterwards staining procedure followed by FCM analysis was performed.

A.1 Flow cytometric methodology

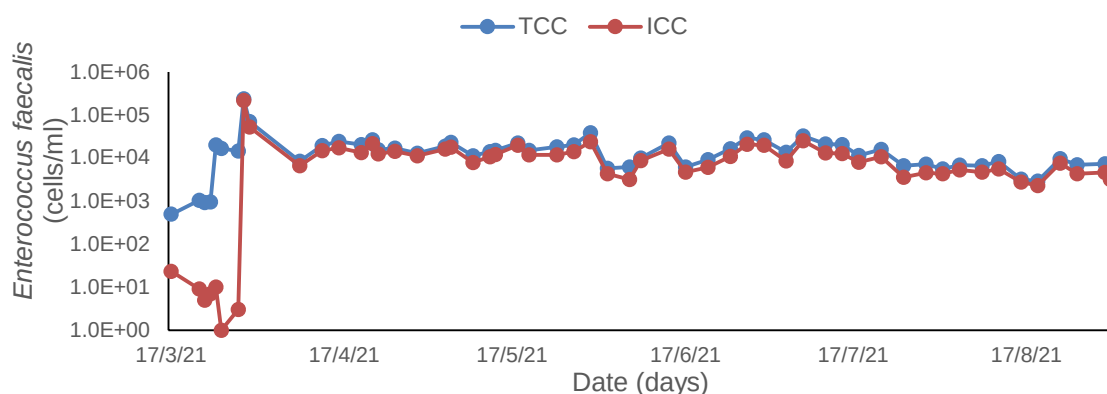
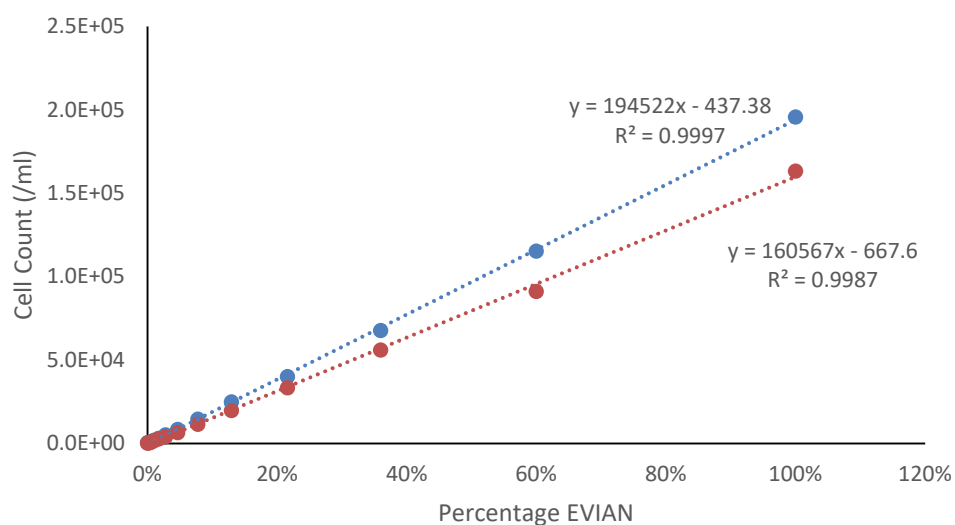


Figure A-1 Dynamic of *Enterococcus faecalis* population used as positive control for validation of the flow cytometer.



Figure_ A-2 Curve of calibration base on different Evian water filtered concentration generating a linear regression equation.

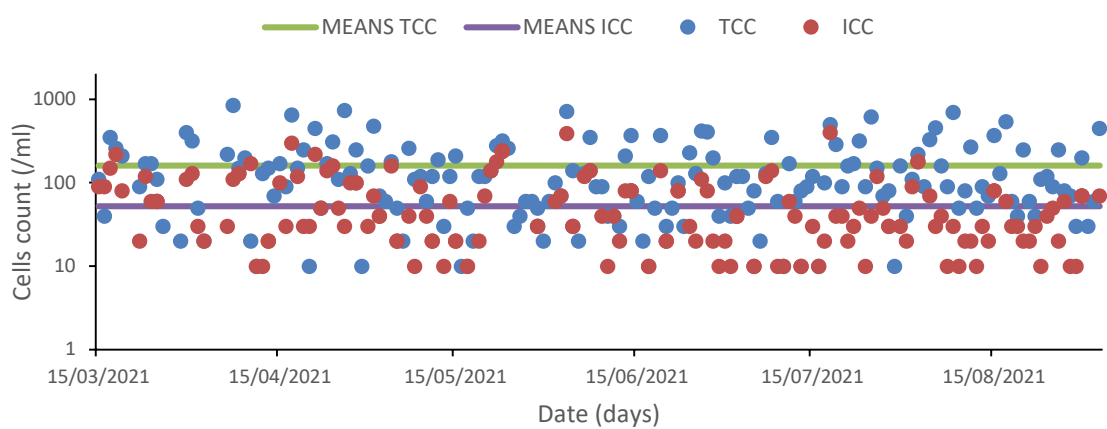


Figure A-3 Dynamic of blanks used as negative control alongside samples analysis throughout 6 months.

A.2 Results

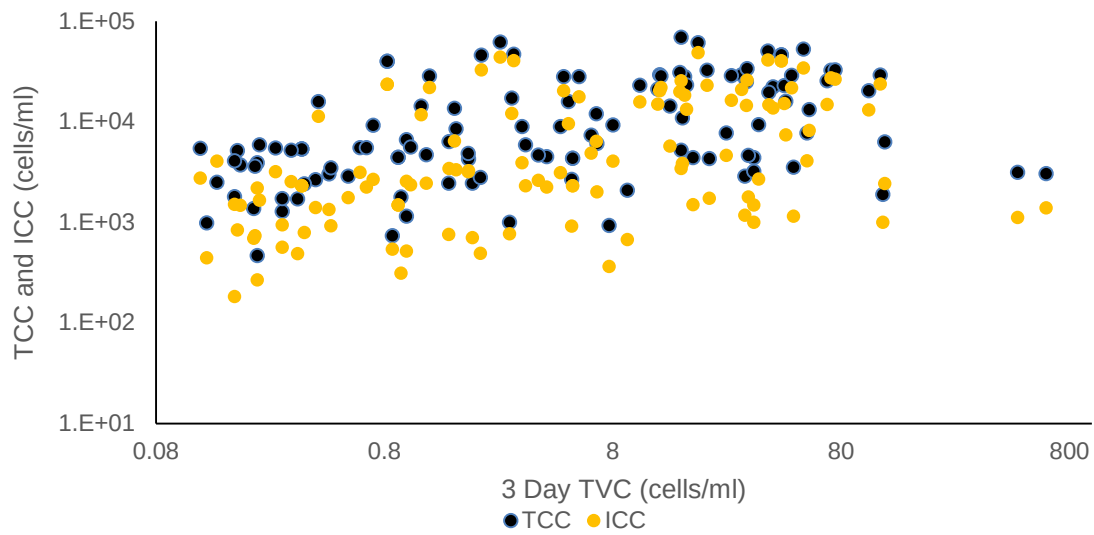


Figure 4 comparison trends between Intact cell counts ICC and Total cell counts TCC to heterotrophic plate counts (HPC) 3 days for each reservoirs/towers