

CRANFIELD UNIVERSITY

LEILA CLAVEAU

FROM SOURCE TO TAP:
TRACKING OF DRINKING WATER BACTERIA USING FLOW
CYTOMETRY

SCHOOL OF WATER, ENERGY AND ENVIRONMENT
ENVIRONMENTAL TECHNOLOGY

EngD
Academic Year: 2023 - 2024

Supervisor: Dr Francis Hassard
Associate Supervisor: Prof. Paul Jeffrey
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ABSTRACT

Understanding how water microbial quality changes occur in distributed water is crucial for ensuring water quality, safeguarding public health, optimizing treatment processes, and predicting the impacts of environmental and anthropogenic changes on microbial ecosystems. However, current monitoring tools, such as heterotrophic plate count, have limitations due to their inability to capture the full diversity of microbial communities, low sensitivity to non-culturable microorganisms, and delayed results that hinder real-time decision-making.

Flow Cytometry (FCM) has emerged as a promising alternative offering high-throughput and real-time analysis of microbial cells. Through metrics such as Total Cell Count (TCC), Intact Cell Count (ICC) High Nucleic Acids (HNA), Low Nucleic Acids (LNA), and Bray and Curtis Dissimilarity Index (BCDI) resulting from the CHIC analysis of cell's fluorescence histogram, FCM provides valuable insights into microbial abundance, viability, and metabolic activity. Despite its potential, the application of these metrics lacks standardised guidance, with metrics such as HNA and LNA not fully comprehended leading to risks of incorrect application potentially resulting in misinterpretation of water quality data and suboptimal treatment decisions. Therefore, there is a need for addressing the research question of how to appropriately use these metrics to monitor water microbial quality.

This EngD study aimed to address this gap by identifying the conditions under which the FCM metrics are most valuable and evaluating their potential as early warning indicators for unwanted water microbial quality. A mixed-methods approach was employed, combining the monitoring of 35 Water Treatment Works (WTW)s and 231 associated Service Reservoirs (SR) with in-depth interstage monitoring of one individual WTW. The first case evaluated the relevance of the metrics across different WTW designs, while the second focused on their relevance at different treatment process stages within a single WTW.

The findings revealed that cell counts are most effective in high-cell environments, BCDI is particularly useful in low-cell ones, and HNA/LNA metrics are most relevant in treatment stages involving chemicals. These results highlight the need for a standardised framework to guide FCM users in selecting and interpreting metrics to prevent misinterpretations and support effective water quality management.

Keywords: Flow Cytometry, Drinking water, Longitudinal study, Bray and Curtis Dissimilarity index

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LIST OF ABBREVIATIONS

2D	2 Dimension
A.U	Arbitrary Unit
AOC	Assimilable Organic Carbon
BCDI	Bray-Curtis Dissimilarity Index
BSS	Between Sum Square
CCT	Chlorine Contact Tank
CF	Clarifier
CHIC	Cytometric Histogram Image Comparison
Cl _{free}	Free Chlorine Residual
CQV	Coefficient of Quartile Variation
Ct	Chlorine Contact Time
DAF	Dissolved Air Flotation
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DWDS	Drinking Water Distribution System
DWI	Drinking Water Inspectorate
DWTP	Drinking Water Treatment Plant
E. coli	Escherichia coli
FCM	Flow Cytometry
FIO	Faecal Indicator Organism
FSC	Forward Scatter Size
F-test	Brown-Forsythe test
GAC	Granular Activated Carbon
GW	Groundwater
HNA	High Nucleic Acid
HPC	Heterotrophic Plate Count
ICC	Intact Cell Count
IQR	Interquartile Range
LNA	Low Nucleic Acid
LOD	Limits of Detection
LOQ	Limits of Quantification
LRV	Log Reduction Value

MCCD	Microbial Community Change Detection
MF	Micro Filtration
MPN	Most Probable Number
NMDS	Non-metric Multidimensional Scaling
NTU	Nephelometric Turbidity unit
PCR	Polymerase chain reaction
pH	“Power of hydrogen” negative log of hydrogen ion concentration
PI	Propidium Iodide
r^2	Coefficient of determination
RF	Random Forest
RGF	Rapid Gravity Filter
SD	Standard Deviation
SG	Sybr Green
SGI	Sybr Green I
SR	Service Reservoir
SRO	Service Reservoir Outlet
SSC	Side Scatter Size
SW	Surface Water
SYBR	Sybr Green
TCC	Total Cell Count
TSS	Total Sum Square
UK	United Kingdom
USA	United States of America
U-test	Mann-Whitney U test
UV	Ultraviolet
WHO	World Health Organization
WSS	Within Sum Square
WTW	Water Treatment Work

1 Introduction

1.1 Drivers for the project

Throughout the years, outbreaks of waterborne diseases (T. Koch & Denike, 2009; Mac Kenzie et al., 1994; Olsen et al., 2003), economic losses due to mismanagement of water resources (McIeman et al., 2014; Pauli, 2020; Rodina, 2019), and environmental pollution affecting aquatic ecosystems (Beyer et al., 2016; Phillips et al., 2007) have highlighted the importance of effective water quality management. To this end, since 1949, various organizations and regulations have emerged to ensure proactive risk management. For instance, the World Health Organization (WHO) released the Guidelines for Drinking-Water Quality (WHO, 1984) and introduced the Water Safety Plan (WSP) framework (WHO, 2009) for guiding countries in managing water safety risks. In Europe, the European Commission (EC) established the Drinking Water Directive (European Parliament & Council of the European Union, 1998) and the Water Framework Directive (WFD) (European Commission, 2000) to ensure safe drinking water and promote sustainable water management across member states. Additionally, several countries have developed national organizations tailored to address their unique challenges and enforce compliance with drinking water quality standards (see Table 1-1 for examples of organizations).

Table 1-1: A non-exhaustive list of national organizations ensuring water safety compliance and addressing countries' unique challenges

Country	National organisation	Country's unique challenges
France	<i>Agence Régionale de Santé (ARS)</i> : oversees drinking water quality and public health concerns	<ul style="list-style-type: none">- Impact of agricultural runoff on rural water supplies- Compliance with strict nitrate limits

Germany	<i>Umweltbundesamt</i> (UBA): provides technical guidance and supports federal water regulations	<ul style="list-style-type: none"> - High reliance on groundwater resources - Need for stringent protection of aquifers against industrial pollution
United Kingdom	<i>Drinking Water Inspectorate</i> (DWI): ensures that water companies supply clean and safe drinking water to the public	<ul style="list-style-type: none"> - Aging water infrastructure - Risks of lead contamination in older homes

Sources: Data compiled from

Le Monde, 2024, Drinking water in France threatened with non-compliance due to 'forever chemical'

M. Montginoul et al., 2020, Compliance and enforcement: the Achilles heel of French water policy

To mitigate water safety risks and address challenges, organizations commonly emphasize 3 key elements: ensuring robust water treatment processes, establishing reliable metrics, and implementing a comprehensive sampling strategy (Bjfi, 2023; DEFRA & DWI, 2016; Legifrance, 2024). Table 1-2 illustrates the key elements, their purpose and the commonly recommended practices and metrics

Table 1-2: The 3 key elements commonly set in the water quality management plan of different countries

Key Elements	Purpose	Commonly Recommended Practices and Metrics
Robust water treatment processes	Remove contaminants and ensure water meets health and safety standards	Coagulation, Filtration, Disinfection
Reliable Metrics for Water Quality	Establish measurable indicators to monitor and maintain water safety	Total Viable Count (TVC), coliforms, Disability-Adjusted Life Year (DALY), pH, Turbidity
Comprehensive Sampling Strategy	Ensure representative and frequent testing to identify and mitigate risks	Sampling frequency, sampling points

Sources: Data compiled from Defra, 2016, *The Water Supply (Water Quality) Regulations*
 Bfj, 2023, *Verordnung über die Qualität von Wasser für den menschlichen Gebrauch*
 Legifrance, 2024, *Code de la santé publique*

The application of the recommended practices and metrics have significantly improved water quality management. Notably, in the UK, reports from DWI consistently show compliance levels exceeding 99% for public water supplies, with non-compliance events being rare (DWI & DEFRA, 2023). Non-compliance events refer to instances where drinking water fails to meet established regulatory standards. Despite their rarity, such events still occur, with a relative proportion being microbiologically related often involving the detection of coliforms. Most coliform-related events have been traced to issues at customers’ kitchen taps,

highlighting the importance of maintaining clean tap hygiene. However, a portion of failures remains unexplained (DWI & DEFRA, 2023), underscoring the limitations of current recommended practices, as demonstrated by several studies. One key limitation is that the recommended sampling frequencies may fail to capture critical events, resulting in missed opportunities to identify risks and ensure water safety (Besmer, Hammes, et al., 2017). Another issue lies in the selection of sampling points, which may not adequately help in identifying the root causes of non-compliance events (Doronina et al., 2020; Ellis et al., 2015). Furthermore, the currently recommended metrics face significant challenges. These challenges include evolving threats from climate change, which contribute to extreme weather events and demand faster analytical results (Delpla et al., 2009; Teixeira et al., 2020); the increasing concern of antimicrobial resistance, where pathogens adapt to conventional treatment processes and require more advanced detection (Liguori et al., 2022); and the emergence of unexpected pathogens, such as viruses and protozoa, which are not always addressed by traditional metrics (Hayes et al., 2023; Safford & Bischel, 2019). A summary of the limitations of some of the recommended metrics and practices is provided in Table 1-3.

Table 1-3: A non-exhaustive list the recommended metrics and practices limitations

Recommended metrics			
Metrics	Purpose	Identified limitation	Contributing factor
Total viable Count (TVC)	General Indicator of Microbial Water Quality: Measures the total number of culturable	<ul style="list-style-type: none"> Results take 24–72 hours Lacks specificity Insensitive to viruses/protozoa 	1. Climate Change: Extreme weather events require rapid

	<p>bacteria in a water sample under specific incubation conditions (e.g., 22°C and 37°C)</p>	<p>analytical methods</p> <p>2. Antimicrobial Resistance: TVC fail to detect bacteria entering the viable but non-culturable (VBNC) state</p> <p>3. Emerging Pathogens: TVC cannot differentiate between pathogens and harmless bacteria</p>
<p>Daily Adjusted Life Year (DALY)</p>	<p>Quantifies the overall burden of disease caused for instance by unsafe drinking water, accounting for both the severity and duration of illness</p> <ul style="list-style-type: none"> • Not suitable for real-time monitoring • Complex data requirements • Difficult to attribute health impacts to a single exposure source like water 	<p>1. Climate Change: Extreme weather events require rapid analytical methods</p> <p>2. Antimicrobial Resistance & Emerging Pathogens: DALY models</p>

			require a proper detection of microbial diseases for accurate burden estimation
Log Reduction Values (LRV)	Assess pathogen removal efficiency in water treatment processes to ensure compliance with regulatory standards	<ul style="list-style-type: none"> Indicator organisms (e.g., MS2) may not perfectly mimic real pathogen behaviour Operational constraints due to fluctuating flow rates, disinfectant dosing, etc. Difficulty in detecting very low pathogen concentrations post-treatment 	<p>1. Climate Change: Extreme weather events require rapid analytical methods</p> <p>2. Antimicrobial Resistance & Emerging Pathogens: Pathogens can still be present despite a high LRV</p>
Coliforms	Indicator of Faecal Contamination: Measures the presence of coliform bacteria as an indirect signal of	<ul style="list-style-type: none"> Results take 18–72 hours Poor correlation with pathogens False positives 	1. Climate Change: Extreme weather events require rapid analytical methods

	potential pathogenic microbes (e.g., E. coli, Salmonella).		2. Antimicrobial Resistance & Emerging Pathogens: coliforms can be present without actual faecal contamination and vice versa
Turbidity	Indicator of Water Clarity & Particle Load: Measures the scattering of light due to suspended particles in water, which may indicate microbial or chemical contamination	<ul style="list-style-type: none"> Not a direct microbial risk indicator 	Antimicrobial Resistance & Emerging Pathogens: clear water can still contain viruses and dissolved toxins
pH	Indicator of Water Acidity or Alkalinity: Measures hydrogen ion concentration, affecting corrosion,	<ul style="list-style-type: none"> Not a direct microbial risk indicator 	Antimicrobial Resistance & Emerging Pathogens: water can have normal pH but still be unsafe

disinfection efficiency, and taste.			
Recommended practices			
Strategy	Purpose	Identified limitations	Contributor factor
Sampling frequency	Specifies how often water samples are collected to monitor water quality trends over time, detect contaminations, and ensure regulatory compliance	<ul style="list-style-type: none"> • Risk of missing contamination events 	1. Climate Change: Extreme weather events can cause sudden water quality changes, requiring more frequent sampling 2. Emerging Pathogens & Antimicrobial Resistance may appear sporadically, requiring adaptive, high-frequency monitoring
Sampling points	Specifies the locations where water samples	<ul style="list-style-type: none"> • Limited representativeness 	1. Climate Change: Shifting

are collected to assess quality at critical control points, such as source water, treatment plants, distribution networks, and consumer taps	<ul style="list-style-type: none"> Difficulties to root cause a non-compliance event 	<p>contamination sources (e.g., changing river flows, reservoir levels) affect optimal sampling locations</p> <p>2. Emerging Pathogens & Antimicrobial Resistance may form localized clusters in biofilms, requiring strategic placement of sampling points</p>
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The limitations of the current recommended metrics and practices highlight the need for advanced monitoring technologies that offer more reliable, sensitive, and faster metrics, as well as enhanced sampling strategies that can effectively address emerging risks and help pinpoint the causes of non-compliance events.

Over the two past decade, significant progress has been made in addressing these challenges. Regarding monitoring technologies, innovative solutions providing more sensitive and non-selective metrics and faster have been proposed, allowing for rapid broader detection of microbial contaminants (de Vera & Wert, 2019; Devi et al., 2021; Safford & Bischel, 2019). For sampling strategies, approaches involving more frequent and real-time analyses have been

introduced, improving the ability to capture critical events as they occur (Doronina et al., 2020; Ellis et al., 2015). Furthermore, with the advent of artificial intelligence (AI), many of the suggested tools are observed for their compatibility with AI so they could help build algorithms capable of anticipating failures and identifying potential risks proactively (Doorn, 2021; Kyritsakas et al., 2023). A list of the current solutions and their purposes is presented in Table 1-4.

Table 1-4: Advanced monitoring tools and sampling strategies to improve water safety

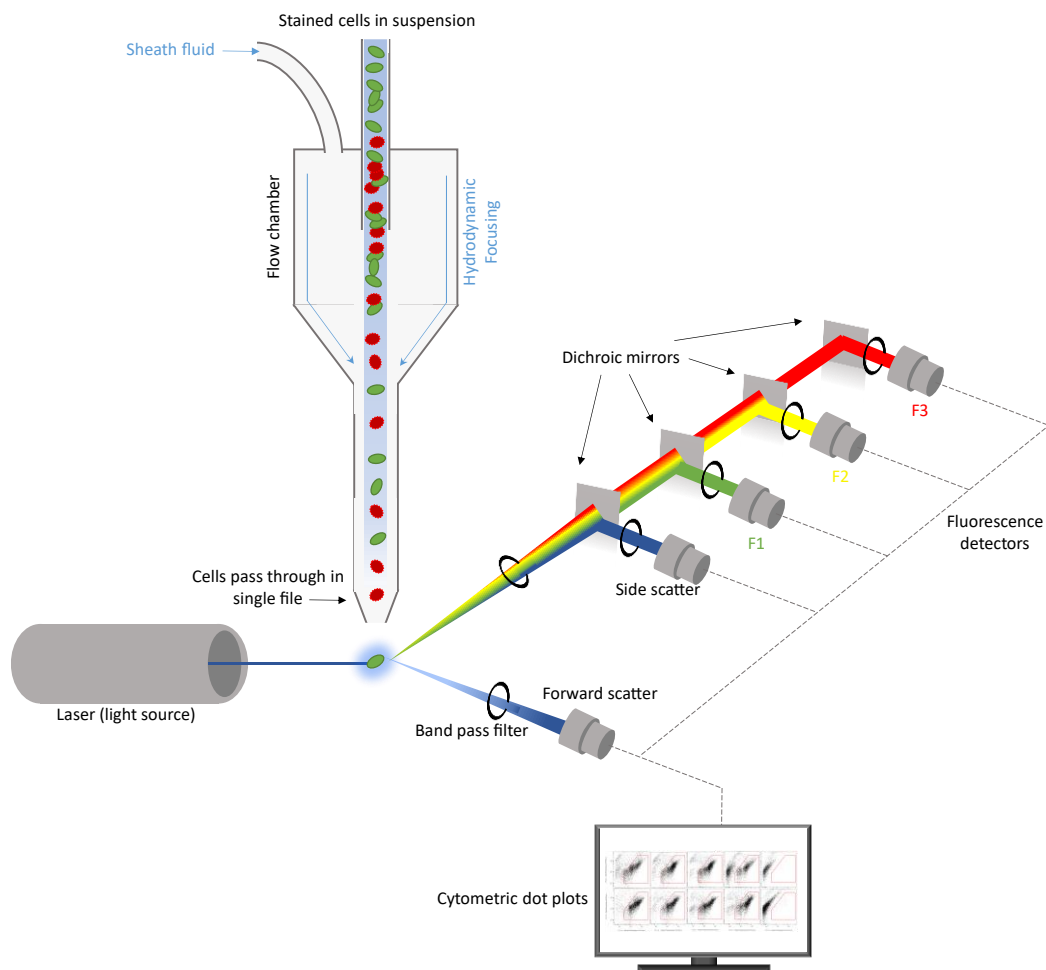
Solutions for reliable metrics			
Potential solution	Purpose	Pros	Limitations
Adenosine Triphosphate (ATP)	Assess microbial activity and total biomass in water systems	<ul style="list-style-type: none"> • Rapid and quantitative assessment • Useful for early detection of microbial contamination 	Does not differentiate between viable and non-viable cells
Quantitative Polymerase Chain Reaction (qPCR)	Detect and quantify specific microbial genes, including pathogens	<ul style="list-style-type: none"> • High specificity and sensitivity • Allows detection of viable but non-culturable (VBNC) organisms • Faster than culture-based methods 	Requires specialized equipment and expertise

MALDI-TOF	Detects potential pathogens, biofilm-forming bacteria, and microbial shifts within treatment and distribution systems	<ul style="list-style-type: none"> • Provides results within minutes to hours • High specificity in bacterial and fungal identification • Requires less sample preparation than molecular methods 	Identification is limited to organisms present in the reference library
Flow Cytometry (FCM)	Assess microbial community composition and dynamics	<ul style="list-style-type: none"> • High-throughput analysis allowing better accuracy when training an AI model • Distinguishes live and dead cells using fluorescent dyes • Enables rapid microbial profiling 	Requires complex data interpretation
Solutions for better sampling strategy			

Potential solution	Purpose	Pros	Limitations
Increase sampling frequency	Increase the likelihood of detecting critical events		<ul style="list-style-type: none"> • Higher costs
Add more sampling point	Enable root cause analysis of critical events	<ul style="list-style-type: none"> • Enhances network-wide water quality assessment • More collected data allows better accuracy when training an AI model 	<ul style="list-style-type: none"> • Increased operational complexity • Potential redundancy if not strategically placed
Real-time monitoring	Enable dynamic response to events as they occur		<ul style="list-style-type: none"> • High initial costs • Sensor calibration and maintenance

Among the emerging tools, flow cytometry (FCM) stands out as a promising approach due to its rapid analysis capabilities, sorting functionality through single-level detection (as illustrated in Figure 1-1), and its flexibility in providing selective or non-selective detection depending on the use of fluorochromes. Most notably, FCM offers real-time analysis showing a potential for AI integration and advanced data processing. Furthermore, FCM provides a variety of metrics that offer valuable insights into the microbial environment of water. These metrics include Total Cell Count (TCC) and Intact Cell Count (ICC) for measuring cell abundance, High Nucleic Acid (HNA) and Low Nucleic Acid (LNA) content for assessing cell viability, and Bray and Curtis Dissimilarity Index (BCDI) resulting

from the CHIC¹ analysis of the fluorescence fingerprints for evaluating cell metabolic activity. However, despite the FCM potential, the application of these metrics is hindered by a lack of standardized guidance (Hassard & Whitton, 2019). Metrics such as HNA and LNA, in particular, are not yet fully understood (Hassard & Whitton, 2019), increasing the risk of incorrect applications, misinterpretation of water quality data, and suboptimal treatment decisions. The following paragraphs discuss each metric giving their purpose and limitations, the additional insights they provide compared to traditional metrics, their current applications, and the gaps that need to be addressed.



¹ Cytometric Histogram Image Comparison

Figure 1-1: Flow cytometer schema showing how cells are processed from the moment they enter the nozzle (flow chamber) to the moment their signal is recorded. Adapted from Safford and Bischel (2019)'s paper and Creative Diagnostics website (<https://www.creative-diagnostics.com/flow-cytometry-guide.htm>)

TCC and ICC are defined as metrics for assessing cell abundance and integrity through cell's membrane. Integrity refers to the structural and functional wholeness of the cell, indicating its viability. These metrics are obtained by combining FCM with the fluorochromes SYBR Green and Propidium Iodide (PI). In brief, the permeant SYBR Green penetrates all cells regardless of their membrane integrity and binds to their nucleic acids, allowing the identification of total cells, while the non-permeant PI only enters cells with damaged membranes, where it also binds to nucleic acids, thereby distinguishing non-intact cells. By combining the two dyes, TCC is derived from the total population of SYBR Green-positive cells, while ICC is determined from the subset of cells stained only with SYBR Green and not PI, indicating intact membranes. Figure 1-2 illustrates the resulting FCM dot plot after running the dyed cells.

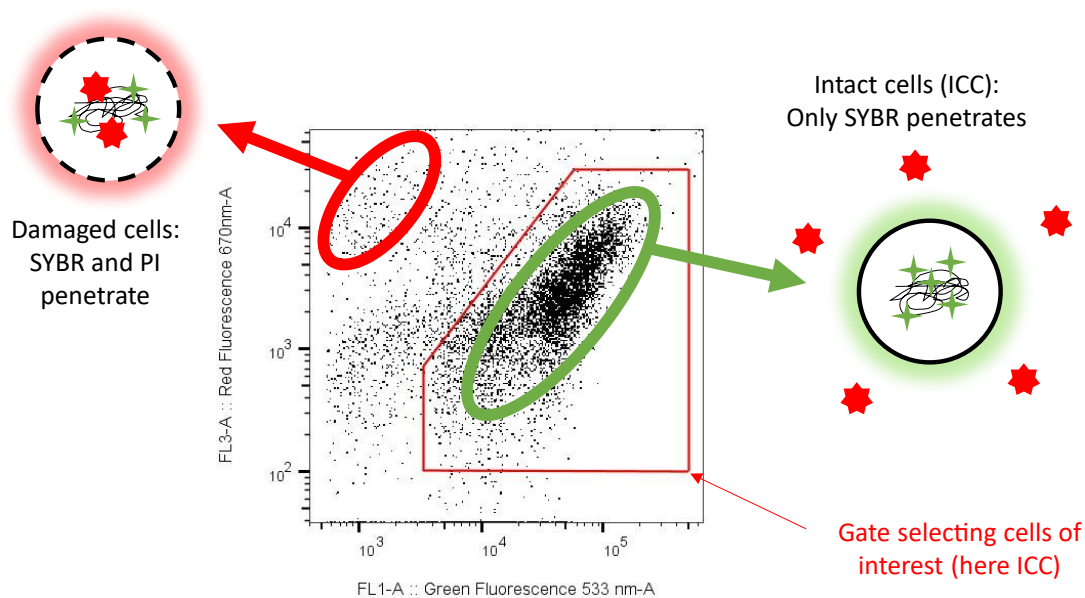


Figure 1-2: Dot plot generated by an Accuri C6 (BD Biosciences) flow cytometer after analysing post-GAC water from a DWDS. Both axes are logarithmic. x-axis represents the Green fluorescence FL1 (Excitation = 497 nm, Emission = 490-650

nm) of SYBR, and the y-axis represents the red fluorescence FL3 (Excitation = 520 nm, Emission = 550-725 nm) of PI

The measurement of TCC and ICC is important because the metrics bring more insight into the microbial water quality compared to traditional culture-based methods like TVC. TCC and ICC enable the detection of 99% of the microbial population thanks to the single-level detection capability and independence from cell culturability of FCM. This allows for the identification of Viable but Not Culturable (VBNC) cells, which are undetectable by culture-based methods. Therefore, with the use of these metrics, studies have demonstrated that the monitoring of the cell population is more accurate and leads to better assessment of WTW performance (Cheswick et al., 2019; Van Nevel, Koetzsch, et al., 2017) and improved treatment optimisation (Cheswick et al., 2022; Gabrielli et al., 2021). Furthermore, the ability to monitor cell integrity (i.e. ICC) through the state of the cell's membrane (damaged or not), enables evaluation of the disinfection efficacy of treatments that target cell membranes, such as chlorine contact tanks (Cheswick et al., 2020, 2022). Ultraviolet (UV) disinfection cannot be optimized through ICC, as UV primarily damages the cell's DNA without affecting the membrane. However, although ICC and TCC are promising metrics, several gaps remain that need to be addressed, particularly the insufficient focus on real-world application issues. For example, users in real-world practices report that the gating process (see Figure 1-2) is subjective and prone to errors (Hassard & Whitton, 2019). Although automated gating methods have been introduced to minimise the subjectivity (Verschoor et al., 2015), there are ongoing questions about whether automated gating should always be used in the data processing workflow, regardless of sample type or cell concentration. Another example is the sensitivity of flow cytometers posing challenges for analysing clean water samples, as their cell abundance often falls below the detection limit (Cheswick et al., 2019). This raises the question of whether values below the detection limit should still be considered, or if alternative methods should be used for analysing low-cell-concentration water. These challenges will be addressed throughout the thesis.

The HNA and LNA are defined as metrics for assessing cell integrity through their genetic material. They are respectively cells with high and low DNA content. The metrics are typically derived from the ICC population, as they focus exclusively on intact, viable cells with undamaged membranes and the potential to grow. The ICC population is divided into 2 subgroups based on their fluorescence intensity in the green fluorescence channel (see Figure 1-2). The subgroup with higher fluorescence intensity is HNA and the subgroup with lower fluorescence intensity is LNA. Figure 1-3 illustrates the resulting FCM plot, showing the distinction between the HNA and LNA subpopulations within the ICC.

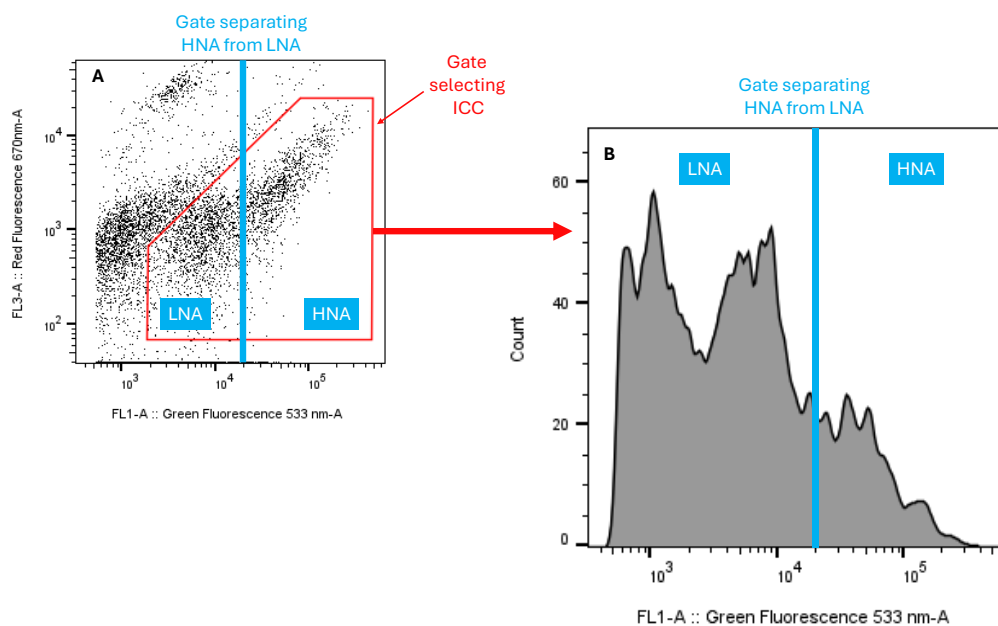


Figure 1-3: Plots generated by an Accuri C6 (BD Biosciences) flow cytometer after analysing a post-GAC water from a DWDS. On plot A, both axes are logarithmic. x-axis represents the Green fluorescence FL1 (Excitation = 497 nm, Emission = 490-650 nm) of SYBR, and the y-axis represents the red fluorescence FL3 (Excitation = 520 nm, Emission = 550-725 nm) of PI. On plot B, x-axis is logarithmic and represents the Green fluorescence, y-axis is linear and represents the cell count.

The use of HNA and LNA is important because the metrics bring more insight into the genetic material of the cell population offering additional information about a sample composition. HNA cells have higher genetic material, which accounts for their stronger fluorescence, while LNA cells have lower genetic material, resulting

in weaker fluorescence (refs). Studies have demonstrated that the use of these metrics enabled observation of how microbial population with varying genetic material respond to environmental factors (refs) and treatment processes (refs). This understanding facilitates selective growth promotion (refs), ultimately leading to better control over process performance (refs). Furthermore, the ability to monitor cell integrity through their genetic material allows for the assessment of treatments targeting DNA, such as UV disinfection, thereby optimizing their performance (refs). However, there are still gaps in knowledge, including a lack of understanding about whether HNA and LNA populations represent distinct microbial groups or are simply different physiological states of the same cells. Another gap is the lack of focus on real-world application issues as, like TCC and ICC, users are concerned about the gating process, wondering whether the gate should be dynamic or static. Additionally, the questioning on whether the fluorescence-based categorization is truly significant in real world-application for understanding cell abundance still remains. While it has been shown that ICC growth accelerates when HNA increases and decelerates when LNA dominates (refs), their significance among other factors in real-world scenarios remains unclear. These gaps will also be addressed throughout the thesis.

The Bray-Curtis Dissimilarity Index (BCDI) is defined as a more advanced metric due to its computational nature, as opposed to the ICC, TCC, HNA, and LNA, which are considered conventional metrics because they do not require computational steps. The BCDI metric is specifically designed for assessing compositional differences between microbial communities in water samples, thereby characterizing changes in microbial populations. This metric is widely used in ecological studies to compare the compositional similarity between two samples (e.g., microbial communities in different environments). In FCM, the BCDI is employed to visualize and statistically evaluate shifts in microbial community composition, for example, before and after water treatment processes (refs). The method to determine BCDI using FCM data was developed by Kosh et al. (2016) and is called Cytometric Histogram Image Comparison (CHIC). In brief, the metric is obtained by comparing pixel values of cytometric histograms generated from FCM data using the Bray-Curtis formula:

Equation 1-1: Formula of the Bay-Curtis Dissimilarity Index

$$BCDI = \frac{\sum |A_i - B_i|}{A_i + B_i}$$

Where A_i and B_i represent pixel intensities in corresponding bins of cytometric histograms from two samples. This process ensures that all features in the histograms are taken into account to calculate compositional dissimilarity.

Figure 1-4 shows the detailed steps of the CHIC method and the resulting plot, which visualizes BCDI values. These plots effectively highlight compositional changes in microbial communities across different conditions.

Image comparison - XOR (Exclusive OR) operation

The operation consist of comparing corresponding pixel values between images

When two pixels are identical, the background is black, when they are different, the background is white

This can be mathematically expressed as:

$$A \text{ XOR } B = (A \cup B) - (A \cap B)$$

Where:

- A XOR is the XOR operation
- (A ∪ B) is the union of the two images
- (A ∩ B) is the intersection of the two images

The result of A XOR B corresponds to the total number of white pixels, which represent the differences between the two images

Images to compare

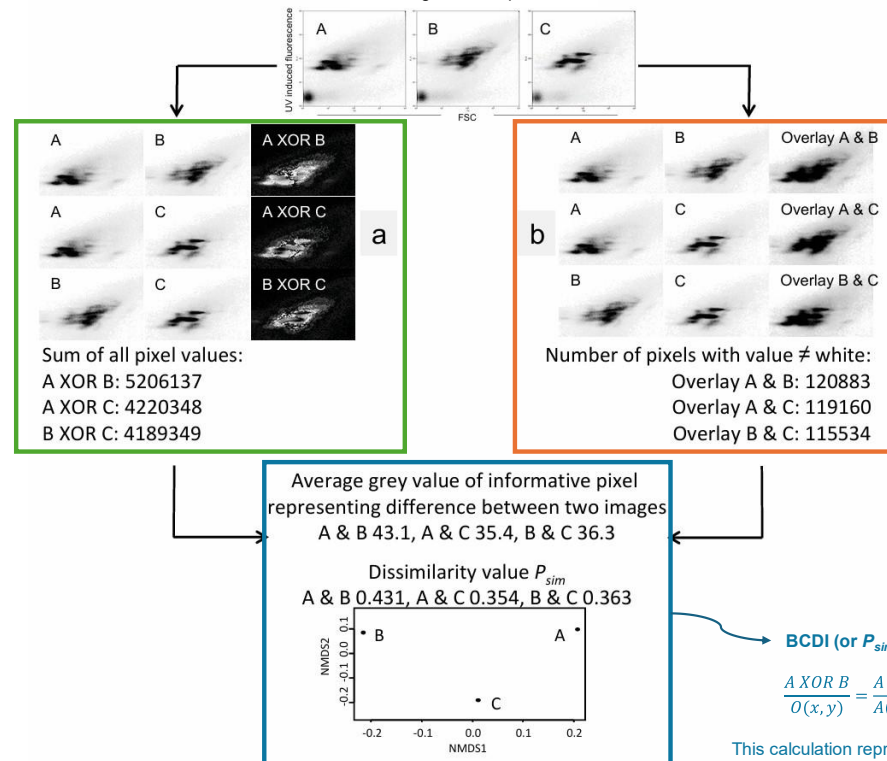


Image superimposing - Bitwise OR operation

The operation highlights all the pixels present in either of the two images

This can be mathematically expressed as:

$$O(x, y) = A(x, y) \cup B(x, y)$$

Where:

- O(x,y) is the resulting image after the Bitwise OR operation
- A(x,y) and B(x,y) are the pixel values of the two input images
- The operator ∪ represents the Bitwise OR operation

The O(x,y) value represents the total number of pixels that are non-zero (different from black)

BCDI (or P_{sim}) calculation

$$\frac{A \text{ XOR } B}{O(x, y)} = \frac{A \cup B - (A \cap B)}{A(x, y) \cup B(x, y)}$$

This calculation represents the difference between two images in terms of their pixel-wise dissimilarity. A higher BCDI value indicates a greater difference between the images

A Non-Metric Dimensional Scaling (NMDS) plot is often used to visualize the distribution of images based on their pairwise differences. In the NMDS plot, the closer the images appear, the lower their calculated dissimilarity

Figure 1-4: Principle of the CHIC analysis and of the determination of the BCDI. Adapted from Kosh et al (2016) 's paper

1 The use of the Bray-Curtis Dissimilarity Index (BCDI) is important because it
2 enables the rapid monitoring of compositional shifts in cell populations within
3 minutes, thanks to its efficient data processing capabilities. This is a significant
4 improvement compared to currently used culture-dependent standardized tools,
5 such as Total Viable Counts (TVC), which require up to 48 hours to yield results.
6 Studies have shown that the use of this metric has allowed for the early detection
7 of microbial community shifts in response to environmental changes (a. favere et
8 al, 2019), water treatment processes (S. Chan et al., 2019), and contamination
9 events (refs). This rapid detection capability supports decision-making
10 processes, enhances monitoring efficiency, and reduces the time required to take
11 corrective actions in water quality management. However, gaps remain in the
12 broader application of BCDI, including its limited use in real-world scenarios.
13 There is a pressing need to apply this metric in longer, longitudinal, real-time
14 studies to validate its applicability under diverse real-world conditions. These
15 conditions include challenges such as extreme weather events, unexpected non-
16 compliance scenarios, and variable environmental factors. Addressing these
17 gaps would enhance the reliability and robustness of BCDI as a practical tool for
18 microbial monitoring in real-world applications.

19 Accordingly, based on the arguments articulate above, it is crucial not only to
20 enhance understanding and interpretability of FCM metrics, but also extend their
21 application to real world scenarios. For this reason, the objectives cited in the
22 following section were addressed.

23 **1.2 Aim and Objectives**

24 The aim of this EngD research is to provide a theoretical framework for the
25 appropriate utilisation in real-world scenarios of both conventional (i.e., ICC,
26 TCC, HNA, LNA) and advanced metrics (i.e., BCDI) offered by flow cytometry. It
27 will contribute to the fundamental understanding of microbial population dynamics
28 and their response to various environmental and treatment conditions and will
29 help to determine the conditions under which the metrics are most valuable and
30 evaluate their potential as early warning indicators for unwanted water microbial
31 quality. This framework will bridge the gap between laboratory-based studies and

practical applications, ultimately enhancing microbial monitoring and improving decision-making processes in water treatment and management.

To achieve the research aim, the following specific objectives have been addressed:

- **Objective 1:** To determine the plus-value of the ICC, TCC, and BCDI metrics by evaluating different WTWs and their associated SRs.
- **Objective 2:** To evaluate the influence of the gating process on the metrics.
- **Objective 3:** To determine the contribution of HNA and LNA to the evaluation of water microbial quality.
- **Objective 4:** To evaluate the complementarity of the metrics and their early warning potential for water microbial quality assessment.

1.3 Thesis structure and format

The EngD thesis consists of six chapters, with four written by publication style. A brief description of each chapter is provided below:

Chapter 1: Provides a general introduction and presents the research context, background, gaps, aim, and objectives.

Chapter 2: Applies the advanced (BCDI) and conventional metrics (cell count: ICC and TCC) to a FCM dataset built over two years by analysing water samples from 35 Water Treatment Works (WTW)s and 231 associated Service Reservoirs (SR), and water samples from interstage treatment processes of one individual WTW. The comparison of the advanced and conventional metrics was performed to determine their ability to detect changes in the microbial population notably the presence of unwanted organisms. The assessment of microbial changes was conducted using the standard Log Reduction calculation for the conventional metrics and the Cytometric Histogram Image Comparison (CHIC) for the advanced metric. (Publication style)

Chapter 3: Gives an understanding of the influence of the gating process on the metrics and their interpretation in regards of the microbial population change. The

previously obtained two-year dataset (Chapter 2) was used for this chapter applying the same metrics but different gating strategies: static, dynamic, and no-gate. (Publication style)

Chapter 4: Elucidates the contribution of the conventional metric HNA/LNA ratio in monitoring water microbial quality. In order to build the dataset, and have a comprehensive understanding of the metric, water samples were collected from interstage treatment processes of one individual WTW and its associated SRs. (Publication style)

Chapter 5: Assesses the complementarity of the conventional and advanced metrics, their ability to be used as early warning indicator, and their conditions for their valuable application. To support this analysis, a two-month dataset was compiled through daily and hourly water analysis at various stages of one individual. (Publication style)

Chapter 6: Offers a comprehensive discussion and summary of the principal findings from each chapter. Additionally, it explains how every chapter contributes to fulfilling the research objectives and the broader implications of the study.

Chapter 7: Highlights the innovative aspects of this research and suggests directions for future studies.

1.4 Publications

At the time of writing this thesis, two papers have been accepted for publication in international peer-reviewed journals as listed below.

Publications included in the thesis

- Claveau, L. et al. (2024) 'Microbial Water Quality Investigation through Flow Cytometry Fingerprinting: From Source to Tap', Sustainable Microbiology, Volume 1, Issue 1. **(Chapter 2)**
- Claveau, L. et al. (2024) 'To gate or not to gate: Revisiting drinking water microbial assessment through flow cytometry fingerprinting', Science of The Total Environment, 912, p. 169138. **(Chapter 3)**

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2 Microbial Water Quality Investigation CHIC analysis: From Source to Tap

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Abstract: Ensuring the quality of treated drinking water is crucial to prevent potential health impacts, regulatory fines and reputation damage. Traditional culture-based microbiology methods often fail to capture the heterogeneity of microbial communities in drinking water. This work investigates water quality in water treatment works (WTW)s and service reservoirs (SR)s using the conventional and advanced metrics offered by flow cytometry (FCM) with the objective of determining their added value in assessing different WTWs and SRs compared to the traditional culture-based methods. A mixed approach was employed combining the monitoring of 35 WTWs and 109 associated SRs with in-depth interstage monitoring of one individual WTW over the period of a year. The first case evaluated the relevance of the metrics across different WTW designs, while the second focused on their relevance at different treatment process stages. The use of Total (TCC) and Intact (ICC) Cell Count metrics confirmed that chlorine concentration contact time (Ct) was pivotal in microbial log reduction across the treatment works. The Bray-Curtis Dissimilarity Index (BCDI) metric exhibited significant deviations during operational events, such as process interruptions, serving as an indicator for identifying poor microbial water quality, but it did not correlate with the presence of microbial indicator organisms in distributed and tap waters. In chlorinated waters with low cell counts, the background signal shows potential to differentiate between different water sources, thereby offering a possibility to characterise breakthrough events in these circumstances which challenge most analytical methods. Interestingly, groundwater from simpler treatment works showed higher occurrence of microbial indicators, while surface water works had lower incidence. These findings underpin the importance of appropriate disinfection even for "low-risk"

source waters and the added value which the statistical interpretation of flow cytometry data can offer for objective decision making.

Keywords: Disinfection; Flow cytometry; Drinking Water; Water Quality; Fluorescent Fingerprint, CHIC Analysis

2.1 Introduction

Ensuring the microbial safety of drinking water is of paramount importance to protect public health. Microbial water safety is influenced by various factors, including source water quality, treatment processes used at water treatment works (WTWs), and the integrity of the drinking water distribution system (DWDS) (G. Liu, Van der Mark, et al., 2013). However, contemporary challenges such as climate change, water scarcity, and polluted water sources complicate the consistent production of microbially 'safe' water, compromising public health protection and increasing costs to utilities and consumers (Semenza & Menne, 2009). Traditional methods of assessing microbial quality, such as culturing microbial indicators, provide only an indicative snapshot of pathways for faecal contamination rather than a comprehensive hygienic assessment (Hassard et al., 2016). These culture-based tests often overlook a significant portion of bacteria (viable but non culturable organisms) and do not easily distinguish pathogenic, infectious organisms from less harmful ones (D. Pinto et al., 2015). Furthermore, culture-based quantification is time-consuming, with results often available too late to inform on practical interventions in treatment, distribution, or use (Van Nevel, Koetzsch, et al., 2017). While advanced techniques, such as quantitative PCR assays and next-generation sequencing (Farkas et al., 2017; Singhal et al., 2015), offer detailed insights and more rapid data, they are not without challenges. These methods can struggle with the specificity and sensitivity necessary for identifying and quantifying viable pathogens or indicator organisms within complex environmental water samples, a crucial aspect of ensuring water safety and public health (H. Liu et al., 2017).

Within this framework, flow cytometry (FCM) is recognised as a transformative approach to assess microbial water quality– a method that has been embraced widely by the water sector. FCM has been applied for its rapidity, reproducibility

and direct assessment concerning microbial populations in water (Hoefel et al., 2003, 2005). Applications range from operational assessments and root cause analysis through to validation of water disinfection (Nocker et al., 2017), contributing significantly to our understanding of microbial dynamics across water treatment systems (Cheswick et al., 2019), and importantly is amenable to automation for rapid user-free diagnostics (Berney et al., 2008). However, the technology is not without its limitations. FCM's wide-spectrum analysis presents challenges in identifying potential risks associated with specific pathogens of concern (Thom et al., 2022), such as viruses (Safford et al., 2023), and differentiating them from the general microbial background, somewhat limiting its deployment for hygienic monitoring.

This study aims to determine the added value of FCM metrics (TCC², ICC³, HNA⁴, LNA⁵, BCDI⁶) in water quality management by extending their application to real-world scenarios, particularly, BCDI which is an innovative application of an established ecological dissimilarity metric, now used in flow cytometry for microbial monitoring. This metric utilizes the FL1 and FL3 fluorescence of cells that allow to monitor their changes and there the changes in microbial community diversity over time. It aims to discern phases of elevated risk across diverse water sources by detecting microbial communities shift that signal potential contamination or microbial regrowth. By correlating other water quality indicators (Coliforms, E. coli, TVC, turbidity, pH) with the FCM conventional (TCC, ICC, HNA, LNA) and advanced (BCDI) metrics, the research aims to enhance FCM's usefulness. Sophisticated techniques, such as Cytometric Histogram Image Comparison (CHIC) and FlowFP, which can elucidate the complexities of microbial communities in water are applied here.

The research navigates these scientific frontiers through a study using a mixed approach to examine over a year firstly data sets across 35 WTWs and 109

² Total Cell Count

³ Intact Cell Count

⁴ High Nucleic Acid

⁵ Low Nucleic Acid

⁶ Bray-Curtis Dissimilarity Index

associated SRs and secondly interstage data sets in one individual WTW, all assets located in southern England. The first case investigates how different treatment processes and chlorine contact times influence microbial water quality and how these factors correlate with the different FCM metrics. The second case explores FCM's latent ability to identify when microbial regulatory compliance issues or public health incidents may occur within a specific WTW and its DWDS. This research presents an opportunity for enhanced water safety management protocols.

2.2 Materials and methods

2.2.1 Sampling strategy

Water quality from 35 WTWs and 109 associated SRs from a supply region in Southern England (UK) was monitored. This provided appropriate spatial resolution within a supply region which had similar operation practise, operating policy (for example, for disinfection) and control setups - permitting direct comparison. These WTWs processed water from various sources: groundwater, surface water, or a blend of both. Distances (hence water age) between the WTWs and their respective SRs varied, with some having immediate post-treatment storage and others being located at a distance from the works (max 16 km). To enable a side-by-side comparison of the WTWs, they were classified into 6 groups based on their treatment processes as illustrated in Table 2-1.

Table 2-1: Description of the monitored WTW designs

WTW No	Flowsheet summary	Raw water source	Disinfection system	Category name	Number of WTW
A	Inline Chlorination	Groundwater	Chlorine gas	Inline Chlorination	9
B	Chlorine Contact Tank	Groundwater	Hypochlorite	CCT	14
C	1. Microfiltration membrane 2. Chlorine Contact Tank	Groundwater	Hypochlorite	MF/CCT	4
D	1. Rapid gravity filtration 2. Granular activated carbon 3. Chlorine Contact Tank	Groundwater	Hypochlorite	RGF/GAC/CCT	3
E	1. Dissolved air flotation 2. Rapid gravity filtration 4. Ozone combined to a Granular activated carbon 5. Chlorine Contact Tank	Surface water (river)	Hypochlorite	DAF/RGF/Ozone-GAC/CCT	2
F	1. Clarifier 2. Rapid gravity filtration	Surface water (river)	Chlorine gas	CF/RGF/Ozone-GAC/UV/CCT	3

-
3. Ozone combined to a
Granular activated carbon
 4. Ultraviolet reactor
 5. Chlorine Contact Tank
-

The sampling frequency was daily for WTWs outlet (final water), and weekly for their interstage processes and source/raw water (when required), and associated SRs (Table 2-2) as this aligned with regulatory monitoring requirements for the water utility. Sampling was conducted in triplicate from April 2019 through to March 2020.

Table 2-2: Summary of the sampling frequency of each treatment

Monitored stage	Sampling frequency
For the 35 WTW and 109 associated SRs	
Raw water (source)	Weekly
Final water (WTW outlet / post-CCT)	Daily
SR outlet	Weekly
For WTW F	
Raw water	Weekly
Post-Clarifier	Weekly
Post-RGF	Weekly
Post-GAC	Weekly
Final water (WTW outlet / post-CCT)	Daily

The raw, final, and SR outlet water samples were collected to ascertain how the WTW process performance and storage conditions influenced total cell counts (TCC) and intact cells counts (ICC), as well as the potential for regrowth across the distribution network. Subpopulations of high nucleic acid content (HNA) and low nucleic acid content (LNA) bacteria were assessed using static gating approaches that have been widely applied for analysis of drinking water when using FCM (Prest et al., 2013). The study indirectly monitored potential regrowth/ingress by quantifying ICC between final water and SR outlet, taking

into consideration the impact of the disinfection residual and temperature. The influence of water transport and storage on cell regrowth was explored by comparing hydraulically linked WTWs and their service reservoirs.

To assess the impact of operational events on the microbial population, one WTW with a surface water feed was chosen as a case-study for higher resolution interstage monitoring. This particular WTW (referred to as WTW F) was sampled daily for treated water, and weekly for the raw water and at each stage of the treatment process. Two of its linked SRs (referred to as A and B) were also included as they were exclusively sourced from this WTW during the period of study. The microbial population was assessed by CHIC analysis and enumeration using static gating for cell counts and phenotypic appraisal using FCM. Each sampling event was conducted in duplicate, adhering to the regulatory sampling procedures outlined by Clark (2015). Specifically, sample taps were flushed for three minutes, and flame sterilized before sampling. Subsequent to this, taps were flushed for an additional 30 seconds before the collection of samples into sterile 250 mL sample bottles. These bottles contained a pre-aliquoted dose of sodium thiosulphate to reach 0.1% (w/v) in the water sample. Sodium thiosulphate was added in the form of a few drops of a concentrated solution (18% w/v) to the sample bottle before the water sample was collected - sufficient to quench residual chlorine. Upon collection, samples were transported to the laboratory in the dark for analysis within 24 hours and were stored at temperatures between 4-8°C during transit

2.2.2 Water Chemical, Physical and Microbial Parameters

The following chemical constituents were measured using standard methods: both total and free chlorine residual (4500) (APHA-AWWA-WEF, 2012). Microbiological assessments were carried out on samples collected following standard methods derived from (APHA-AWWA-WEF, 2012). The analysis of *E. coli* and total coliforms was performed using the Colilert® test (IDEXX, UK) based on the most probable number (MPN) enzyme substrate method (9223). This test uses defined substrate technology, wherein bacterial enzymes react with specific nutrients, causing a colour change when the target bacteria - *E. coli* or coliforms

- are present. Heterotrophic plate count (HPC, 9215) at 22 °C was used to determine the total viable counts. *Cryptosporidium* was measured through the method of pathogenic protozoa enumeration (9711), which concentrates water samples, purifies them to isolate protozoa, and uses fluorescent antibodies for their identification under a fluorescence microscope. *Clostridia perfringens* was measured using the membrane filtration method (9260) - the water sample was filtered, the bacteria-retaining filter was placed on a selective medium and incubated at 44°C, and the appearance of black colonies after a 24-hour incubation period indicated the presence of *Clostridia perfringens*. For these sites, log-reduction calculations were performed for TCC and ICC, and results were stratified according to different WTW type including appraisal of the removal across the entire treatment works (i.e., incorporating disinfection processes and upstream process).

2.2.3 Determining process performance

The free and total residual chlorine, reservoir/tank water levels, and flow rates were sourced from a database that received 15-minute interval data from automated meter readings at various locations, including the final water, SR outlet, and final water. Chlorine concentration was ascertained by a number of online probes calibrated on a monthly basis.

In this study, we employed the theoretical Chlorine Contact Time (Ct), a critical parameter in water disinfection representing the product of the concentration of chlorine (C, in mg/L) and the duration the water is exposed to it (t, in minutes) (Rush, 2002). The Ct is important as it quantifies the disinfection strength, informing the likelihood of microbial inactivation. Ct was approximated (Equation 1) using the minimum chlorine contact tank volume and peak flow at each WTW, presenting the lowest potential exposure or a worst-case scenario based on the free chlorine rather than free active chlorine.

Equation 2-1 Formula for the theoretical chlorine contact time calculation

$$Ct = Cl_{min} (mg.L^{-1}) \times \frac{V_{min} (m^3)}{Q_{max} (m^3/min)}$$

Where: Cl_{min} = minimum recorded free chlorine concentration; V_{min} = Minimum volume of water in the contact tank and Q_{max} = Maximum recorded flow rate at CCT.

Chlorine contact tanks are crucial for water disinfection, relying on ideal hydraulic performance and minimal short-circuiting. Effective design, regular flow assessments, and configuration adjustments are essential to prevent dead zones and ensure efficient mixing and flow (Sobsey, 1989). Seasonal temperature changes, affecting water viscosity and flow rates, are also important considerations (Cheswick et al., 2022). This study primarily focused on nominal Ct but also considered the role of temperature and pH on disinfection efficacy (Hua and Reckhow, 2008; Nocker et al., 2017). Lower pH and higher temperatures enhance chlorine disinfection, while alkaline conditions and cooler temperatures have opposite effects. Additionally, these factors influence post-treatment microbial populations. Spatial analysis of WTW outlets and SRs provided insights into chlorine residuals and overall Ct in the water supply zone.

Chlorine contact time was not calculated for WTWA which only uses inline chlorination (Table 2-1).

2.2.4 Fluorescent staining and flow cytometric measurements

Sample preparation commenced with dilution of samples exceeding TCC and ICC concentrations of 1,000,000 and 800,000 cells/mL respectively, using 0.22 μ m and 0.1 μ m Polyethersulfone (PES) filtered sterile Evian™ water. Samples were stained for TCC and ICC detection. The TCC stain involved the use of SGI (10,000x stock. S-7567; Thermo Fisher Scientific, UK) and filtered Dimethyl sulfoxide (DMSO) (0.22 μ m, Z290807, Sigma-Aldrich, UK), diluted to a 100x working concentration. The ICC stain was prepared using a mixture of five parts 100x SGI and one part PI (Thermo Fisher Scientific, UK). Sample staining took place in 96-well plates, which were then incubated using a microplate thermoshaker (Grant Instruments™ PHMP, Thermo Fisher Scientific, UK) at 35 °C and 400 rpm for 15 minutes. Each sample had 50 μ L analysed at a flow rate of 66 μ L.min⁻¹ with a BD Accuri C6 flow cytometer (Becton Dickinson U.K. Ltd., U.K.), employing a 488 nm solid state laser. Green and red fluorescence were

collected at 533 nm (FL1) and 670 nm (FL3) respectively. Samples were assessed for their homogeneity by performing a singlet-doublet analysis. Following the selection of singlets, a static primary gate was applied to delineate cells from background signals and intact or total cells, following Prest et al., (2013). See Figure S2-1 for the illustration of the cells gating process.

Threshold on the green fluorescence (FL1-H) channel was set at 480 arbitrary units (A.U). Microorganism numbers in this gate post-staining with SGI or SGI/PI formed the basis for TCC or ICC per mL calculations using a dynamic gating approach. The high and low nucleic acids content bacteria (HNA and LNA) were determined using a static secondary gate (Gatza et al., 2013; Prest et al., 2013). Log reductions across WTW process steps were calculated post determination of TCC and ICC concentrations, derived from average replicated measurements per sample using Equation 2.

Equation 2-2 Standard formula for the Log Reduction Value calculation

$$\text{Log reduction} = \text{Log}_{10} \left(\frac{\text{Average inlet cells per mL}}{\text{Average outlet cells per mL}} \right)$$

2.2.5 Data analysis

Data were processed using MS Excel, RStudio, and IBM SPSS Statistics 22. Following assessment of independence and normality, Welch's t-test was applied to determine the significance of effects of various treatment technologies on TCC and ICC removal. Linear regression analysis was used to model the relationship between Ct values and the ICC, providing insights into disinfectant impact on the microbial population.

2.2.6 BCDI determination

BCDI was determined via CHIC analysis using FlowJo (v10.7.2), ImageJ (v1.54d), and R (v2022-02-3) software (Koch et al., 2013, 2014). Briefly, FCM FL1 vs FL3 plots were extracted from BD Accuri C6 software as .fcs files and converted to grey-scale images with FlowJo to enable comparison. The samples dyed with SYBR Green and PI for ICC quantification were specifically utilized given ICC significance in assessing viability within chlorinated systems. Prior to

perform the CHIC analysis (see **Error! Reference source not found.** for details), neither inorganic nor organic backgrounds were removed, thus the gating process was not applied. The comparison of the grey-scale images was conducted performing using ImageJ. The resulting XOR and overlap values, representing the pixel differences between the images, were used to calculate the matrix of BCDI values, indicating the dissimilarity between each sample. This matrix was then used to construct a non-metric multidimensional scaling (nMDS) plot, a clustering analysis, and a Pearson's correlation test using R.

The Pearson's correlation test was performed on components within the nMDS. Microbial parameters (total viable count, coliforms, and *E. coli*) were assessed using the R function 'envfit' with the vegan package⁷ (Oksanen et al., 2007). Only parameters with significant effects (p-value < 0.05) and which met the assumptions of the test were represented. Finally, a non-parametric analysis of similarities (ANOSIM) with 9999 permutations (free) was conducted after Clarke (1993), to validate significant differences between the microbial fluorescence at the interstage of WTW F and microbial fluorescence from different SR outlets.

⁷ R package designed for ecological and multivariate statistical analysis

2.3 Results and Discussion

2.3.1 TCC and ICC for assessing microbial removal

The results showed that surface water treatment achieved greater ICC and TCC reductions than groundwater due to higher microbial loads and more intensive treatment. While most WTWs were effective, WTW involving only inline chlorination showed minimal inactivation possibly due to the inefficacy of its chlorine residual (0.4 mg/l). ICC and TCC provided a more comprehensive assessment of microbial removal, revealing variations in chlorine susceptibility and distinguishing physical removal (coagulation) from disinfection (CCT). It was shown that through the strong Ct-ICC correlation, ICC could help optimize disinfection. Finally, Advanced treatments (UV, ozone) showed synergistic effects, further demonstrating the importance of ICC and TCC in evaluating treatment efficiency.

Surface water treatment showed higher ICC and TCC reductions (0.1 to 4.47 logs) than groundwater (0.21 to 3.02 logs), reflecting higher microbial loads and more intensive treatment for surface water (**Error! Reference source not found.**). Microbial counts varied from 45 to 1.3×10^8 TCC/mL and from 20 to 8.2×10^7 ICC/mL in surface water and from 40 to 2.0×10^5 TCC/mL and from 40 to 1.2×10^5 ICC/mL in groundwater, highlighting the importance of thorough treatment and disinfection in improving water quality (Figure 2-1).

While groundwater WTW A had unchanged TCC and ICC counts before (4.0×10^1 - 1.2×10^5 ICC/mL) and after (4.0×10^1 - 1.7×10^4 ICC/mL) inline chlorination (<0.09 log, Figure 2-1A), groundwater WTW B using chlorine contact tank had noticeable change in ICC (1.2 log removal, Figure 2-1B) except TCC that remained almost unchanged (0.3 log removal, Figure 2-1B). The chlorine residual target for WWT A was of 0.44mg/l, while it was of 0.38mg/l for WTW B. With higher chlorine residual and lower cell inactivation for WTW A, this observation suggests that chlorine contact tank is a key treatment for cell inactivation, confirming the importance of sufficient contact time for effective microbial reduction in water treatment.

Despite its noticeable ICC change (1.2 log removal, Figure 2-1), WTW B had the lowest Ct value compared to the other WTWs (Figure 2-1B-F). The latter significantly reduced TCC and ICC (t_{Welch}^8 in all cases $p < 0.05$) attributed to their higher Ct values (t_{Welch} , $p < 0.05$ in all cases) and additional treatment steps. This suggests that effective microbial inactivation and removal depends on achieving an adequate combination of treatment processes, chlorine concentration and contact time, highlighting the critical role of Ct optimization and multi-barrier treatment strategies in ensuring water safety and quality.

While the chlorine residual target for WTW A was 0.44 mg/L, with a median viability of 81% at the inlet and 80% at the outlet of the chlorinated pipe, the residuals for WTWs B, C, D, E, and F were 0.38, 0.44, 0.36, 0.69, and 0.86 mg/L, respectively, with median viability ranging from 62% at WTW B to 70% at WTW E. The fact that WTW A is sourced from groundwater, which is generally considered a low-risk water source (U.S. EPA, 2021) and yet has a higher chlorine residual than WTWs B and D—both also sourced from groundwater—suggests that inline chlorination alone is less effective for microbial inactivation in this context. This could be due to shorter contact time or the protective effect of organic or inorganic matter in groundwater, reducing chlorine efficacy (Safe Drinking Water Foundation, 2017). These findings confirm that a combination of sufficient contact time and additional treatment steps is crucial for effective microbial inactivation and maintaining water safety standards (U.S. EPA, 2007; Nature, 2021).

The results highlight the limitations of inline chlorination, demonstrating the need for adjustments in WTW A to enhance disinfection efficiency. The results also emphasise the need for tailored disinfection strategies that account for variations in source water quality and treatment configurations. Optimizing Ct values and integrating multiple treatment steps can enhance microbial removal efficiency,

⁸ Statistical test used to compare the means of two independent groups when their variances are not assumed to be equal

ensuring compliance with water safety standards and improving overall drinking water quality.

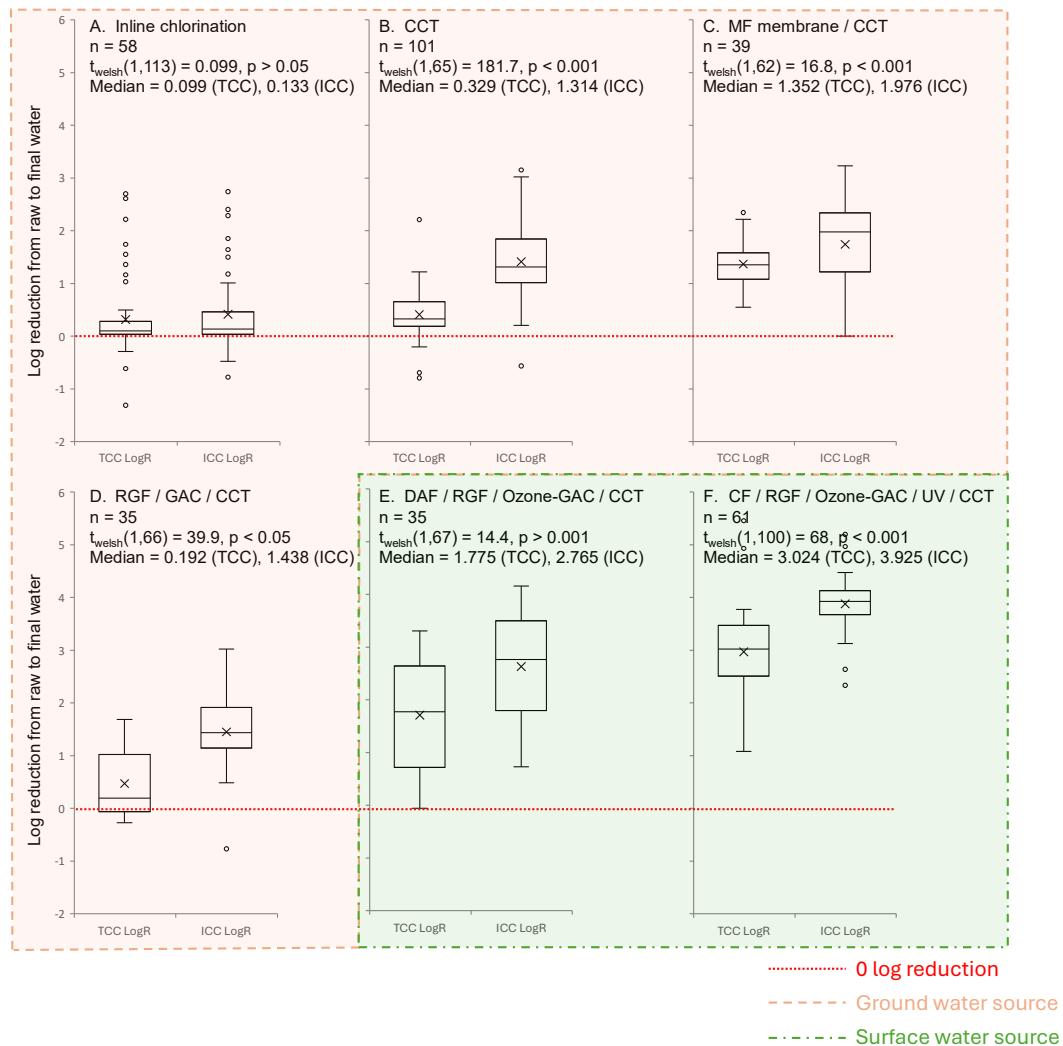


Figure 2-1: Log reduction of total and intact cells across six strata of WTW (raw / Chlorine Contact Tank outlet) between April 2019 and March 2020. n is the number of samples for each WTW. Median initial cell number is presented as $\log_{10}$ of total cell count (TCC) and intact cell count (ICC). Clarification (CF), Chlorine contact tank (CCT); Rapid Gravity Filter (RGF); Granular activated carbon (GAC); Microfiltration membrane (MF); Dissolved air flotation (DAF)

While upstream processes reduced cells for CCT disinfection, a positive Ct-ICC reduction correlation ($R^2 = 0.804$, $p < 0.05$) emerged, albeit subtler than anticipated which could result from varied chlorine susceptibility among drinking water microbial taxa, highlighting FCM's sensitivity in assessing disinfection and

role of upstream processes conditioning water for disinfection (Cheswick et al., 2022).

ICC indicated a 0.191 log cell reduction per 10 mg.min/L increase in Ct (Figure 2-2). CCT and upstream processes affect cell reduction (Cheswick et al., 2019), yet studies show variable susceptibility among isolates to chlorine levels as higher chlorine concentrations for shorter periods were more effective than lower concentrations for longer durations (Mao et al., 2021). One possible explanation for this phenomenon is that at lower chlorine concentrations, microbes may activate stress response pathways that help them survive prolonged exposure, whereas higher chlorine doses overwhelm these defences, leading to rapid inactivation. Additionally, chlorine demand from organic matter in water can reduce the available free chlorine, making prolonged low-dose treatments less effective

However, other factors influence response of cells to different disinfectants. Given the preliminary stresses imposed on microbial populations by initial WTW processes, the disinfection mechanism—oxidative cell damage from free chlorine and/or ozone—becomes more effective (Figure 2-1E). This is attributed to the heightened susceptibility of these pre-stressed cells to protein denaturation, lipid peroxidation, and nucleic acid disruption (Ding et al., 2019a). Thus, while preceding treatments either remove the cells or cause changes to physiology and or permeability of the protective cell envelope, the oxidative action of chlorine and ozone ensures thorough disinfection (Dietrich et al., 2007).

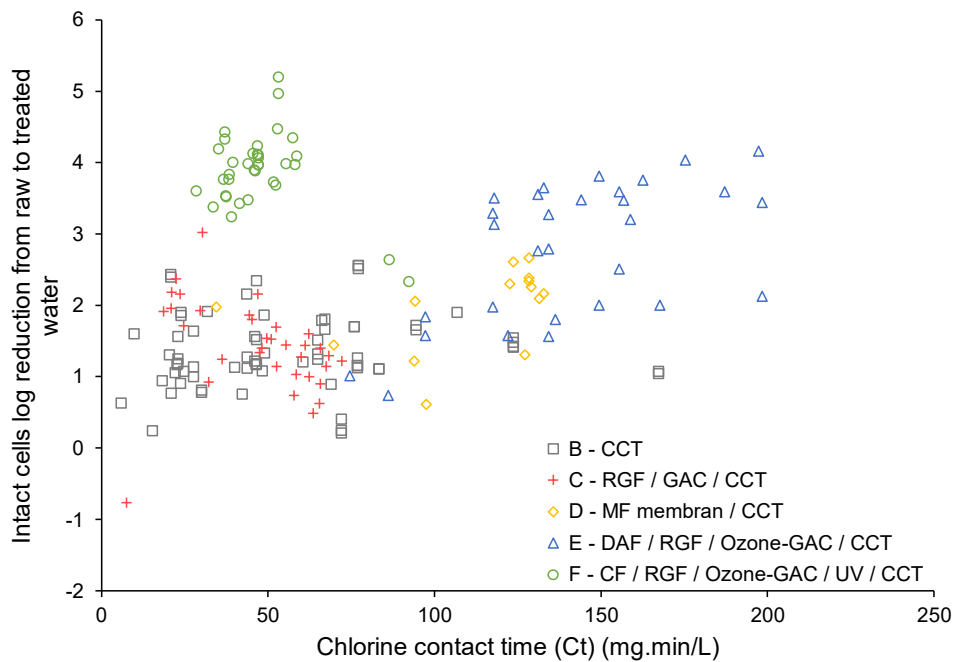


Figure 2-2 Effect of Chlorine Contact Time on Log Reduction of Intact Cells Across Distinct Water Treatment Trains. This table presents the influence of contact time with chlorine on the log reduction of intact cells in various treatment processes

The largest ICC log reduction occurred across the CCT (2.08-3.35), while coagulation/clarification removed most cells, eliminating 5.7×10^6 ICC/mL (Figure 2-1).

The median ICC log reduction across the whole process was 1.5 for MF membrane/CCT WTWs, while clarifier/RGF/ozone-GAC/UV/CCT WTWs showed a total log reduction of 3. The latter, treating surface water, had log reductions which ranged between 3.1 and 5.2, averaging 47.3 mg.min/L Ct, compared to 0.8 - 2.2 mg.min/L for CCT and RGF/GAC/CCT with similar Ct (Figure 2-2).

Figure 2-2 shows the WTW with UV and ozone achieving higher ICC removal than its CT value, likely due to synergistic cell damage across the two processes. While ozone's contribution to log reduction is well-documented (Cho et al., 2010; Ramseier et al., 2011), less is known about UV's role due to its DNA/RNA-targeting disinfection method and the lack of suitable cellular markers for UV damage detectable by standard FCM measurement (Blyth et al., 2021; Farrell et al., 2018; Wang et al., 2010). However, as the dataset only includes raw and final samples, the precise contributions of individual treatment stages cannot be

determined. Variations in factors such as ozone residuals and coagulant doses may also influence observed differences between WTWs, highlighting the need for further research into UV disinfection mechanisms and specifically culture independent approaches for determining its efficacy (Blyth et al., 2021).

The findings highlight the added value of ICC and TCC in assessing microbial water quality by providing a more complete picture of treatment effectiveness. Unlike traditional indicators, ICC and TCC detect both live and non-culturable microbes, revealing inefficiencies, such as WTW A's ineffective chlorination due to its unique inline chlorination.

A strong Ct-ICC reduction correlation ($R^2 = 0.804$, $p < 0.05$) confirms ICC as a quantitative marker for disinfection. ICC also distinguishes between physical removal (e.g., coagulation) and disinfection (e.g., CCT oxidation), showing the highest log reductions (2.08–3.35) in CCT.

Advanced treatments (UV, ozone) showed synergistic effects, reducing ICC beyond Ct predictions. ICC and TCC improve microbial risk assessment, optimizing Ct values and detecting persistent cells missed by culturing.

The findings advance the applicability of ICC and TCC metrics across diverse WTW designs by demonstrating their ability to monitor microbial removal and inactivation in different treatment and disinfection stages, operating differently, unlike traditional culturing methods.

For the water industry, these findings highlight the need to move beyond traditional microbial indicators and integrate ICC and TCC monitoring for better microbial risk assessment. The study demonstrates that chlorine disinfection alone is not always sufficient to treat groundwater as seen in WTW A's failure to inactivate cells effectively. Additionally, the observed synergistic effects of UV and ozone provide a basis for optimizing treatment designs to enhance microbial reduction while minimizing chemical use.

2.3.2 Relating TCC and ICC to water quality indicators

Ensuring microbial compliance, gauged by the absence of faecal indicator organisms (FIOs) in final delivered water, is of paramount importance for water utilities.

The results showed that despite the low cell load of groundwater, they exhibited higher frequency of FIO in final water. FIO was spotted at low (< LOD) and high cell counts, but no correlation between both indicators could be found due to FCM limitations. This highlighted the challenges of using TCC and ICC alone as a microbial risk indicator and emphasizes the need for complementary monitoring approaches to fully assess microbial persistence in treated water

The frequency of FIO-positive samples (detections/sampling effort) in GW with inline chlorination was 0.007%, compared to 0.004% for the surface water RGF/GAC/CCT process and 0.001% for both surface water coagulation-clarifier/RGF/ozone-GAC/UV/CCT (WTW F) and DAF/RGF/Ozone-GAC/CCT (WTW E) processes.

Raw water from surface sources had high FIO detections— with 78% (WTW-E) and 96% (WTW F) detection frequency in these sources. This is 9.75 to 12 times higher than in GW, which had a 0.08% detection rate in their raw water (WTW A). SR samples had negligible FIOs detection (Table 2-1).

Although, final water from groundwater WTWs had low FIO rates (0.007%), these were still higher than those in surface water WTWs (0.001-0.004 despite the greater sampling effort for surface water systems (Table 2-1).

WTWs A and C had more FIOs in final water, despite sourcing from groundwater, and also exhibited higher ICC in treated water. This contrasts with surface water WTWs, where extensive pre-treatment and longer Ct values led to greater microbial reductions. This suggests that while groundwater starts with a lower microbial load than surface water (as reflected in lower raw water ICC and TCC), inadequate disinfection in simpler groundwater treatments can lead to higher ICC persistence in treated water. The strong correlation between high FIO detections

and elevated ICC in final water indicates that incomplete inactivation allows microbial cells to persist, even if FIOs remain low.

These findings challenge the assumption that groundwater is inherently low-risk. Minimal disinfection (as seen in WTW A and C) can leave a residual microbial population, necessitating stronger disinfection to meet compliance and safety standards. The ICC log-reduction for MF-membrane WTW was 0.61-2.66, lower than the 0.9-4.9 range found by Cheswick et al., (2019) likely due to the use of finer ultrafiltration membranes (~0.01 μm) versus the microfiltration membranes (0.1-10 μm) which provide less stringent microbial removal. The smaller pore size of UF membranes allows for greater bacterial retention, contributing to the higher reductions observed in their study.

Additionally, this WTW had low microbial raw water loading (3.34×10^3 - 9.22×10^4 ICC/mL; Figure 2-3A), with peak coliform detections at concentrations below 1000 ICC/mL, challenging the use of FCM (LOD = 200 cells/ml). In such low-biomass conditions, small fluctuations in microbial counts may be difficult to distinguish from background noise, potentially underestimating microbial reductions.

This study advances applicability of TCC and ICC correlation with FIO detection in diver WTW designs. For every WTW, the results show that FIOs do not always correlate with cell counts due to measurement limitations (LOD = 200cells/ml), highlighting FCM's reduced sensitivity at low microbial concentrations, which may mask potential correlations. Additionally, the study challenges the assumption that groundwater treatment is inherently more effective, showing that FIO persistence can occur despite lower initial microbial loads, particularly in systems with minimal disinfection.

The findings suggest that relying solely on cell counts for microbial risk assessment may be insufficient, as FIO levels do not always correlate. Water utilities may benefit from integrating cell count measurements alongside additional testing to gain a more complete picture of microbial persistence.

Table 2-3: One-year summary of *E. coli*/Coliform Positive Sample Detection Across Six Treatment Trains. The table provides an in-depth review of both the quantity and frequency of samples testing positive for *E. coli* and coliforms across six different treatment trains examined in this study

		# of samples with positive detection of total coliforms and/or <i>E. coli</i>			Frequency of samples with positive detection of total coliforms and/or <i>E. coli</i>		
WTW	WTW design	RW ⁹	FW ¹⁰	SR ¹¹	RW	FW	SR
A	Inline chlorination	5	1	0	0.08	0.007	0
B	CCT ¹²	10	1	0	0.09	0.001	0
C	RGF ¹³ / GAC ¹⁴ / CCT	1	2	0	0.03	0.004	0
D	MF ¹⁵ Membrane / CCT	32	0	0	0.42	0	0

⁹ Raw water

¹⁰ Final water

¹¹ Service reservoir

¹² Chlorine Contact Tank

¹³ Rapid Gravity Filter

¹⁴ Granular Activated Carbon

¹⁵ Microfiltration

E	DAF ¹⁶ / RGF / Ozone-GAC / CCT	29	0	0	0.78	0	0
F	Coagulation-clarifier / RGF / ozone-GAC / UV / CCT	52	1	0	0.96	0.001	0

¹⁶ Dissolved Air Flotation

2.3.3 TCC and ICC in full scale DWDS

Service reservoirs (SR)s associated to each monitored WTW were examined for assessing the distribution/storage impacts on water quality. The SRs were divided into sub-groups based on their distance from their respective WTW (Figure 2-5).

The results show that residual chlorine is a key role in controlling microbial regrowth in DWDS, particularly when source of water comes from surface water. Groundwater WTW showed microbial stability in DWDS no matter the distance and the chlorine residual.

Despite variations in free chlorine (0.1–0.35 mg/L) among different supply reservoirs (SRs), similar ICC levels (1.7×10^2 – 1.4×10^3 cells/mL) were observed (Figure 2-2) in SRs fed by groundwater WTWs A, B, and C, no matter the distance, indicating biological stability regardless of distance (hence water age and residence time). In contrast, SRs supplied by surface water WTWs E and F showed a significant correlation between chlorine residuals (0.2–0.4 mg/L) and ICC levels (1.5×10^2 – 1.0×10^5 cells/mL; $p < 0.05$). The higher the distance the SRs and the WTW, the more we could see the trend that lower chlorine residuals corresponded to higher ICC values, suggesting that chlorine plays a key role in controlling microbial regrowth in surface water-fed SRs.

An exception was observed in SRs fed by groundwater WTW D, where ICC levels responded to chlorine depletion. At 0–2 km, ICC remained low (1.2×10^2 cells/mL) with a chlorine residual of 0.2 mg/L, but at 4–6 km, ICC increased significantly (1.5×10^4 cells/mL; $p < 0.05$) despite the same chlorine residual. This suggests that not all groundwater sources behave similarly in terms of microbial stability.

In water distribution systems, maintaining an appropriate free chlorine residual is crucial for controlling microbial regrowth. Studies have shown that lower chlorine residuals can lead to increased microbial activity, as chlorine acts as a disinfectant that inhibits bacterial proliferation. For instance, research indicates that reducing or eliminating chlorine residuals can favour microbial regrowth and alter microbial populations (C. Bertelli et al. 2018).

The observation that SRs fed by surface water WTWs E and F exhibited higher intracellular cell counts (ICC) with lower chlorine residuals aligns with these findings. This suggests that insufficient chlorine residuals may allow for microbial proliferation in the distribution system.

Regarding the exception observed in SRs fed by groundwater WTW D, where ICC increased significantly at greater distances despite similar chlorine residuals, the use of microfiltration (MF) membranes at WTW D may play a role. MF membranes are effective at removing a broad range of microorganisms, including bacteria and protozoa, by physically straining them out of the water. This process can significantly reduce microbial diversity and load, as evidenced by studies showing a decrease in microbial diversity and load by several orders of magnitude after microfiltration and reverse osmosis treatment (A. Ibekwe et al. 2019).

However, the high selectivity of MF membranes might inadvertently favour the growth of certain bacteria that can pass through the membrane or colonize downstream infrastructure. In contrast, other groundwater WTWs employing less selective treatments, such as inline chlorination or granular activated carbon treatment, may maintain a more diverse microbial community, which could suppress the proliferation of specific bacteria through competitive interactions.

These observations highlight the importance of maintaining adequate chlorine residuals throughout the distribution system to control microbial regrowth.

CHIC analysis of the final water originating from surface water and groundwater WTWs (Figure 2-3 B, indicated two distinct populations: (1) final waters from SW, the ones concentrated and not following neither of the nMDS 1 nor 2 axes, and (2) those from GW, with significant inter and intra-population differences (R-value = 0.3181; P-value = 0.0001), and following the nMDS 1 axis driven by ICC and TCC. The fact that SW final waters are more concentrated and do not align with nMDS 1 or 2 axes suggests a more uniform microbial profile, potentially due to extensive pre-treatment and disinfection processes. In contrast, the fact that GW samples exhibit greater variability may suggest that microbial variability in GW-treated water is more influenced by cellular parameters, likely due to differences

in treatment approaches (e.g., inline chlorination vs. membrane filtration) and variations in microbial regrowth potential in distribution.

The findings highlight that treatment history and source water type significantly shape microbial community composition in final treated water.

These findings reinforce the role of ICC and TCC as key microbial indicators in assessing water quality and treatment effectiveness. It shows correlation with chlorine residuals, particularly in surface water-fed systems. It also highlights that MF membranes, while effective in microbial removal, may inadvertently promote regrowth, leading to increased ICC and TCC levels downstream.

On the industry perspective, chlorine residuals could be optimized by monitoring ICC and TCC levels, especially in surface water systems.

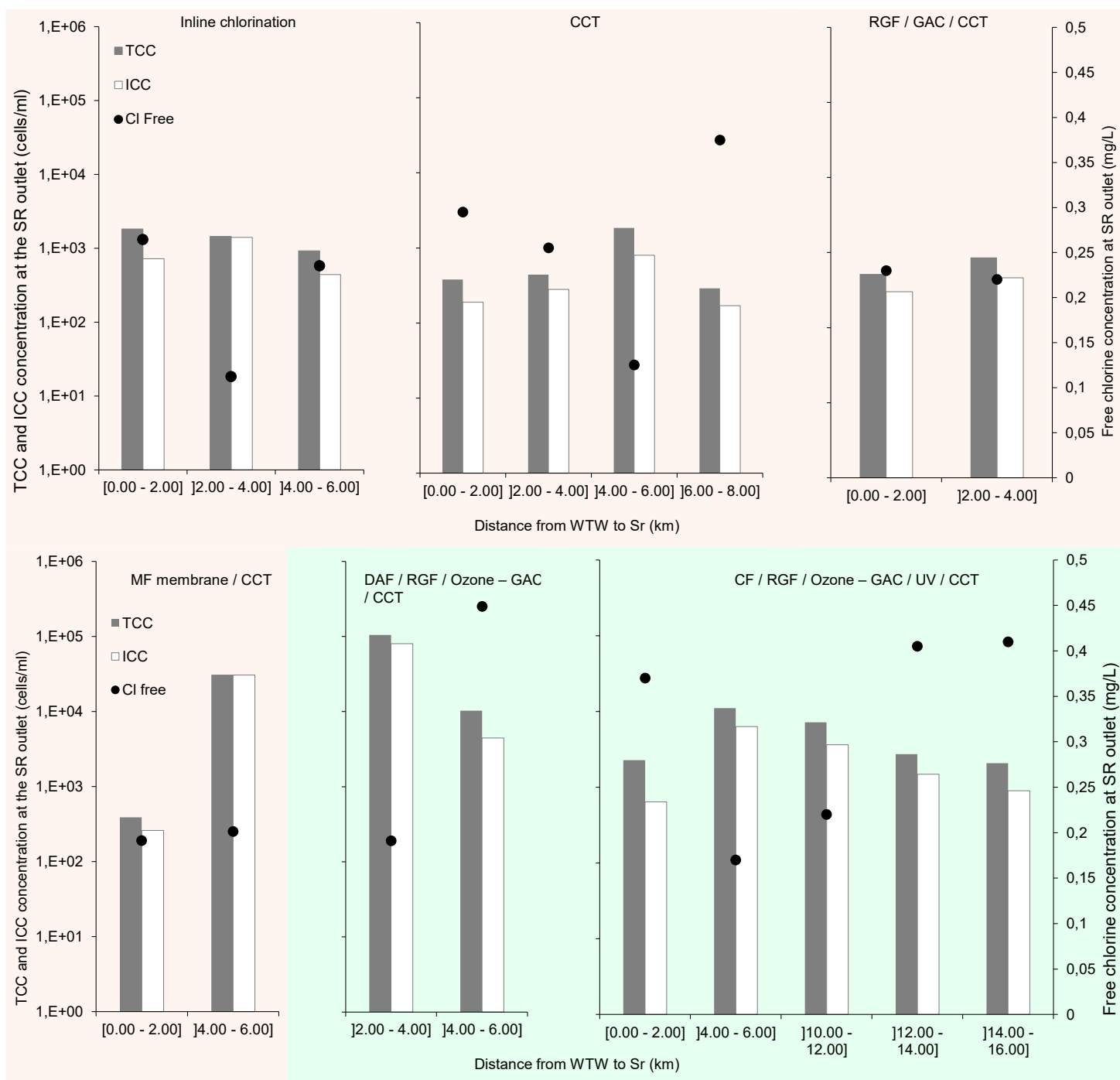


Figure 2-2: Impact of SR Distance on Cell Counts and Residual Chlorine. This table depicts the correlation between the distance of the SR from the WTWs and key variables including TCC, ICC at the SR outlet, and residual chlorine.

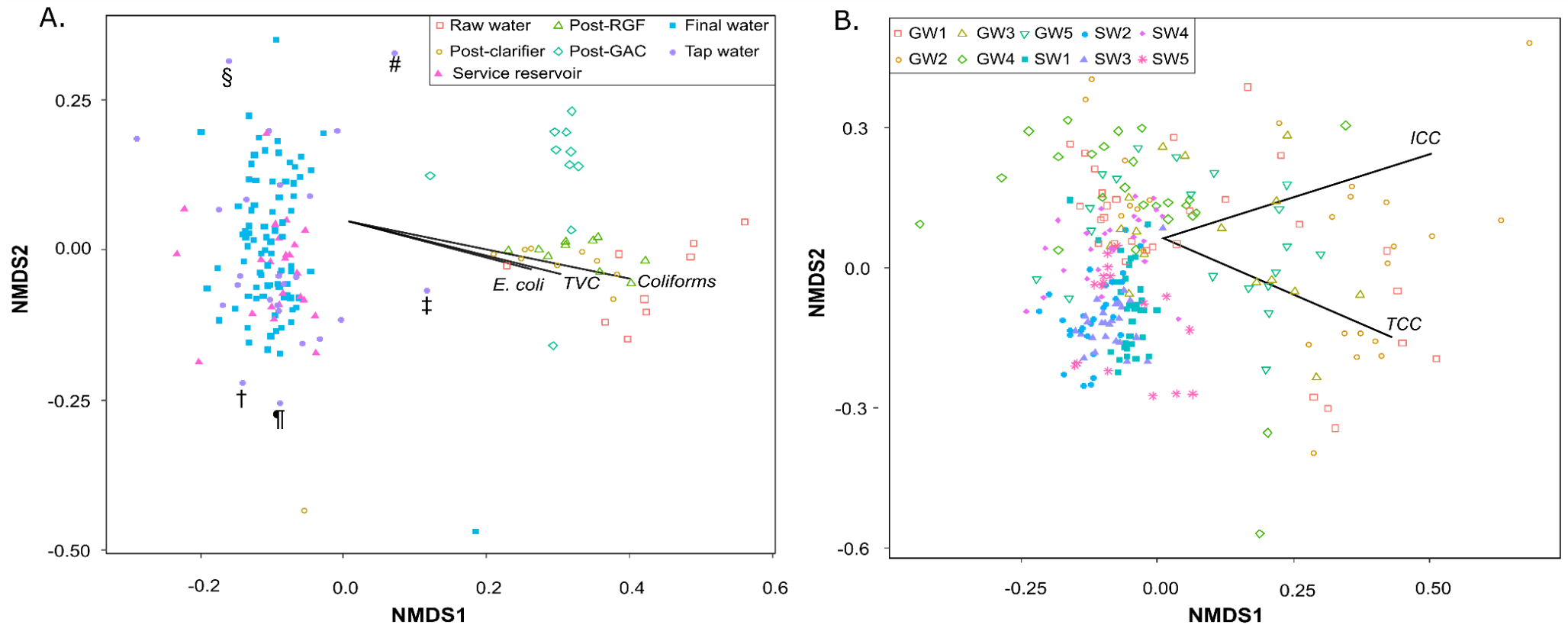


Figure 2-3: Microbial Community Profiling Using NMDS and CHIC Analysis. (A) NMDS ordination plots from CHIC analysis of FL1-FL3 fluorescence histograms, showing clustering of water samples into pre-disinfection, post-GAC, and post-disinfection categories. Correlation vectors indicate relationships between fluorescence histograms and plate count data for heterotrophs, coliforms, and *E. coli*. (B) NMDS ordination plots of FL1-FL3 fluorescence histograms from ten contact tank outlets treating different water sources within the same utility. Clustering is observed for surface water (SW) and groundwater (GW) samples. Correlation vectors depict associations between fluorescence histograms and plate count data for heterotrophs, coliforms, and *E. coli*

2.3.4 TCC and ICC during a microbial and operational event

The results showed that TCC and ICC was able to respond to microbial shift in high-microbial-load water, but CHIC analysis was able to detect events that could only be seen through a thorough analysis, at the pixel-level, of the FL1-FL3 fluorescence plots of samples, emphasizing the early warning potential of cell count and the accuracy of CHIC analysis.

CHIC analysis (Figure 2-3A) of the different stages of WTW F indicated three distinct populations: the first population (1) was composed of final water, SRs, and tap water samples, located on the left-hand side of the plot, and varying along the nMDS 2 axis. The second population (2) was composed of post-GAC samples, located on the right-hand side of the plot, and following the nMDS 1 axis driven by cell count, TVC, *E. coli*, and coliforms. The third population (3) was composed of raw water, post-clarifier, and post-RGF samples, also located on the right-hand side of the plot, and following the the nMDS 1 axis driven by cell count, TVC, *E. coli*, and coliforms.

A similar distinction could be observed with cell counts (Figure 2-4A), where raw water, post-clarifier, post-RGF, and post-GAC grouped as their cell count very between 10^5 and 10^7 cells/ml, while final water varied 10^2 and 10^3 cells/ml, indicating a coherence between cell abundance and microbial community diversity.

The fact that post-GAC samples (population 2) and earlier treatment stages (population 3) cluster together along the nMDS 1 axis, driven by cell count, TVC, *E. coli*, and coliforms, suggests that microbial reductions in these stages are not yet sufficient to differentiate them from raw water and intermediate treatment stages. In contrast, final water, SRs, and tap water (population 1) are distinctly separated along the nMDS 2 axis, indicating that additional treatment processes, particularly disinfection, significantly alter the microbial community, leading to a more stable and biologically controlled final water.

This highlights not only the sensitivity of CHIC analysis as it shows progressive microbial shifts through treatment, with major reductions occurring post-GAC and

disinfection, but also the relevance of cell count. It also emphasizes the importance of GAC in microbial removal, while confirming that effective final disinfection is essential to achieving stable microbial quality in distributed water. The clustering pattern further suggests that biological stability in SRs and tap water is influenced by the extent of microbial removal and inactivation during treatment, reinforcing the need for optimized disinfection strategies.

While GAC contributed to the shift of the microbial population, microbial seeding is usually expected as biofilms can form within the carbon bed, leading to the detachment of bacteria into treated water. However, in this study, GAC filtration was combined with a UV reactor, which is designed to inactivate microorganisms and minimize microbial proliferation. UV treatment is well-documented for its effectiveness in disrupting microbial DNA, preventing regrowth (Hijnen et al., 2006). The microbial shift post-GAC may suggest either a microbial seeding or that UV exposure influenced cells fluorescence by denaturing their genes, highlighting the ability of CHIC analysis to detect this phenomenon.

Within the cluster of chlorinated water samples (final water, SR, and tap water), some tap water samples are more at the extremity of this clustering lowering its compactness. The identification of these samples (Figure 2-4E) allowed to notice that, for some of them, their free chlorine residual ($Cl_f < 0.2$ mg/l) tend to be lower than the other chlorinated samples ($Cl_f > 0.2$ mg/l). When visualising their FL1 FL3 fluorescence plot (Figure 2-4E), we can notice the greater cloud of cells for samples with free chlorine residual ($<0.05 - 0.09$ mg/l) being lower than the WHO (2012) requirements ($0.02 - 0.5$ mg/l).

As for cell counts, they were not able to spot these differences as final water tended to fall below the limit of detection of the FCM (200 cells/ml), highlighting the limitation of cell counts in low-cell-load water.

This indicates that CHIC analysis is able to detect changes in microbial population due to low chlorine residual, highlighting its robustness against the FCM limitations ($LOD = 200$ cells/ml) and also the importance of maintaining adequate chlorine residuals throughout the distribution system to ensure microbial control and compliance with WHO (2012) guidelines.

Within the same cluster (population 1: chlorinated water), another extreme sample was identified with the symbol ¶ (Figure 2-3A). Chlorine residual was not the reason for this shift as it followed the requirement (0.21 mg/l, Figure 2-4E); however, further investigation demonstrated retrospectively that this sample was non-compliant due to the presence of a *Clostridium perfringens* (1 MPN/100mL). When visualising its FL1 FL3 fluorescence plot (Figure 2-4D), we can observe a completely different shape of the microbial population compared to other days preceding and following the event. This highlights the ability of CHIC analysis to identify shift in the microbial population due to microbial contamination.

As for cell counts, on that day, cell concentrations were higher than usual ($> 10^3$ cells/ml, Figure 4-3A), showing its ability to respond to microbial events when the cell load is above the FCM LOD.

This study highlighted the benefits of CHIC analysis, and the early warning potential of cell counts. While cell counts showed ability to warn on microbial shifts in high-microbial-load water, CHIC analysis demonstrated its ability to warn on chlorine decay and microbial non-compliant events even in low-microbial-load water, suppressing the issue with FCM sensitivity.

These findings contribute to knowledge by demonstrating through CHIC analysis the added value and limitations of cell count, and by showing that CHIC analysis is a more sensitive tool than conventional cell counting methods for monitoring microbial fluctuations

On the industry perspective, in addition to cell count, water utilities could use CHIC analysis as an early detection method, allowing for faster responses to microbial instabilities and enhance risk management.

Future studies could assess CHIC analysis in various treatment plants and water sources to validate its effectiveness across different environments and explore the possibility to incorporate artificial intelligence (AI) into CHIC analysis to enhance real-time microbial monitoring and predictive capabilities.

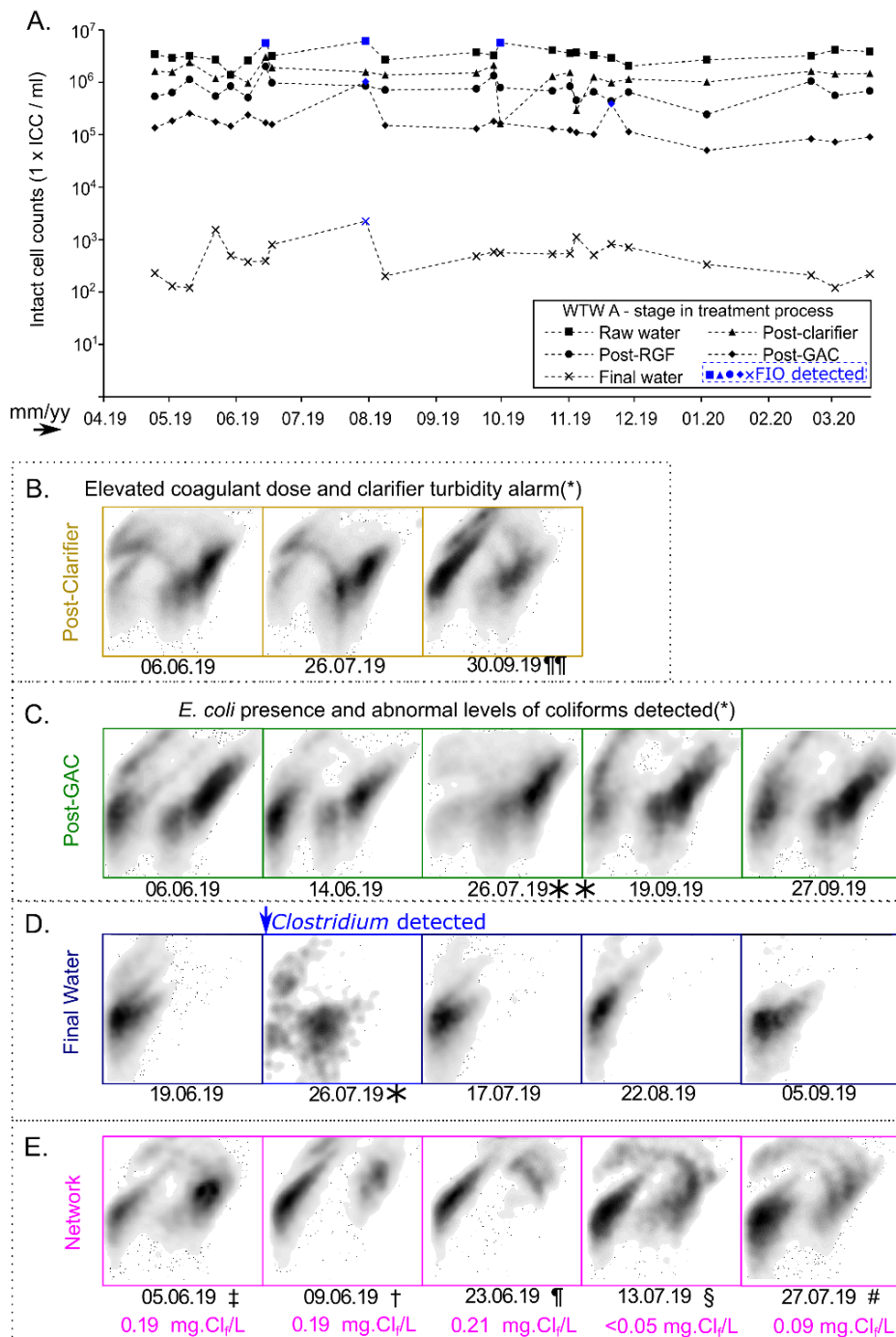


Figure 2-4: A. Intact cells count abundance in the different process stages of the WTW F. Samples were obtained at Raw and post – Clarifier, RGF, Ozonel / GAC, and UV / contact tank. Blue highlighted symbols represent FCM data obtained alongside detection of in process *bacteria faecal* indicator organisms (FIOs) in linked samples. Blue square = *E. coli* > 2420 MPN/100mL, Blue diamond = *E. coli* > 1 MPN/100mL, Blue cross = *Clostridium perfringens* > 1 MPN/100mL. B-E are grey scale FL1-FL3 fluorescence histogram. B. FL1-FL3 fluorescence histogram linked to a process alarm from the pot-clarifier turbidity C. FL1-FL3 fluorescence histogram linked to abnormal detection of faecal indicator bacteria in the post-GAC samples, D. FL1-FL3 fluorescence histogram before, during and post a detection of *Clostridium perfringens*, E. Network samples obtained during periods of normal 0.19 mg/L free chlorine and low <0.09 mg/L free chlorine (footnotes, †, ‡, §, #, ¶ refer to the components within Figure 4A). Rapid gravity filter (RGF), granular activated carbon (GAC), Free chlorine residual (Cl₂), intact cell count (ICC). All FL1-FL3 fluorescence histogram in B-E included background

2.4 Conclusion

This study highlights the value of ICC and TCC as superior microbial indicators, detecting both live and non-culturable microbes to assess treatment effectiveness more comprehensively than traditional methods. It demonstrates their applicability across diverse WTW designs, distinguishing between microbial removal and inactivation. However, the findings emphasize that FIO levels do not always correlate with microbial persistence, suggesting water utilities should integrate ICC/TCC with other monitoring approaches. CHIC analysis proved to bring more sensitivity to microbial shifts than cell counts. It gave more insights into microbial population and detected chlorine decay and non-compliant events, even in low-microbial-load water.

While the study demonstrated the added value of cell count and CHIC analysis at detecting effectively microbial shifts, their application across more WTW designs, taking into account the geographic regions, require further validation. Furthermore, it is worth noting that the Ct values presented in this study are theoretical. Although the findings provide valuable insights into microbial inactivation, it would be interesting to investigate how the Ct-log reduction trend would compare when using actual measured Ct values in real-world conditions.

The novelty of this research relies on the application of the ICC and TCC in various WTW designs (6), operating differently, to validate its applicability under different scenarios. Another novelty is the introduction of CHIC analysis that, firstly, confirmed the early warning potential of TCC and ICC in high-microbial load water, and secondly, demonstrated its sensitivity to microbial population shift even in low-microbial-load water (cell count < FCM LOD).

This research contributes to knowledge through the expansion of TCC and ICC applicability in different WTW designs and disinfection stages, and by confirming the empirical evidence on Ct-ICC relationships in various WTW designs.

On the industry perspective, water utilities could benefit from integrating ICC and TCC monitoring alongside traditional microbial indicators for better microbial risk management. They could also introduce CHIC analysis to their monitoring

framework as it could serve as an early microbial warning system, allowing utilities to proactively detect microbial instabilities in real time.

Future research could focus on the integration of AI in CHIC Analysis to develop a machine learning model for real-time microbial monitoring and predictive risk assessment. They could also validate ICC, TCC and CHIC analysis applicability in more water sources, treatment technologies, and geographic regions.

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2.6 Appendix

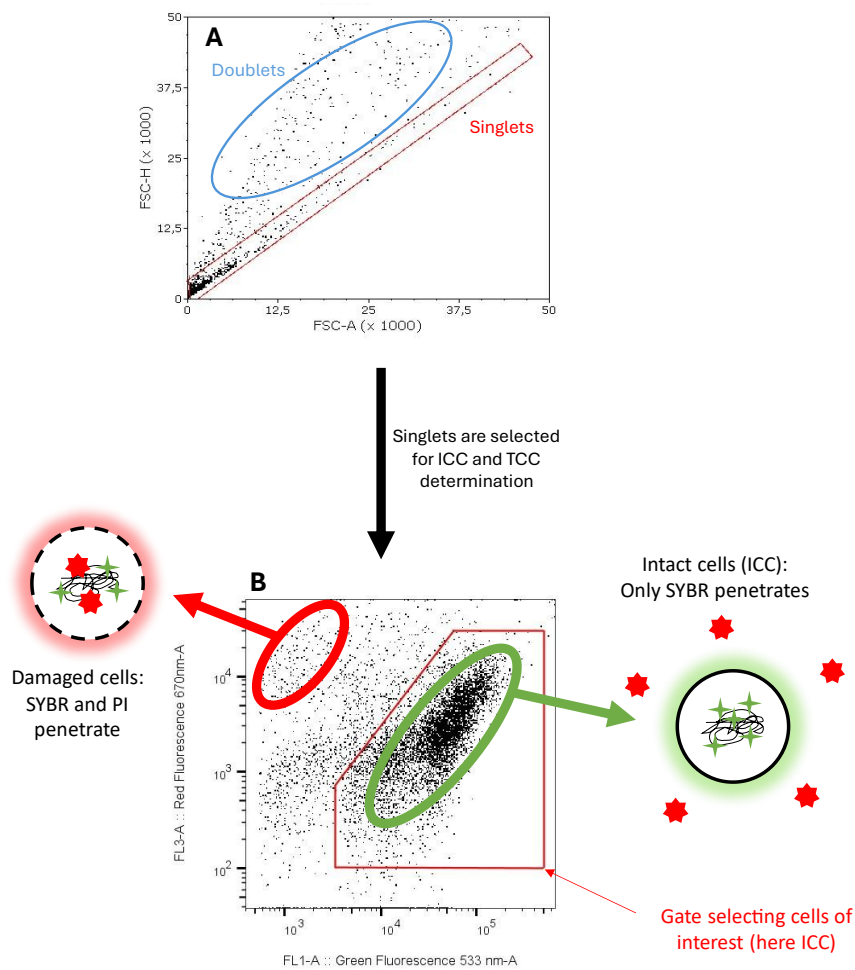


Figure S 2-1: Gating process

Image comparison - XOR (Exclusive OR) operation

The operation consist of comparing corresponding pixel values between images

When two pixels are identical, the background is black, when they are different, the background is white

This can be mathematically expressed as:

$$A \text{ XOR } B = (A \cup B) - (A \cap B)$$

Where:

- A XOR is the XOR operation
- (A∪B) is the union of the two images
- (A∩B) is the intersection of the two images

The result of A XOR B corresponds to the total number of white pixels, which represent the differences between the two images

Images to compare

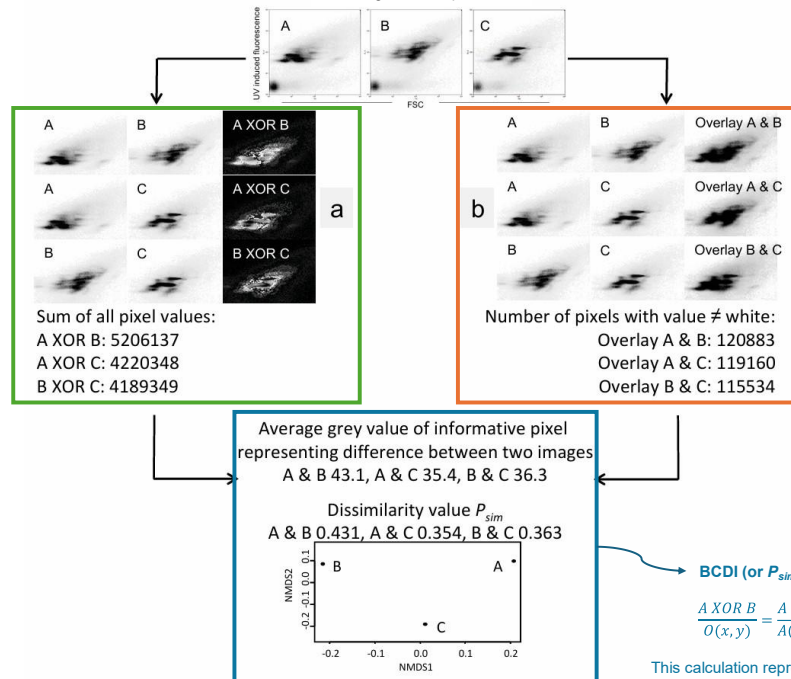


Image superimposing - Bitwise OR operation

The operation highlights all the pixels present in either of the two images

This can be mathematically expressed as:

$$O(x,y) = A(x,y) \cup B(x,y)$$

Where:

- O(x,y) is the resulting image after the Bitwise OR operation
- A(x,y) and B(x,y) are the pixel values of the two input images
- The operator ∪ represents the Bitwise OR operation

The O(x,y) value represents the total number of pixels that are non-zero (different from black)

This calculation represents the difference between two images in terms of their pixel-wise dissimilarity. A higher BCDI value indicates a greater difference between the images

A Non-Metric Dimensional Scaling (NMDS) plot is often used to visualize the distribution of images based on their pairwise differences. In the NMDS plot, the closer the images appear, the lower their calculated dissimilarity

Figure S 1-2: Explanation of the CHIC analysis

3 To Gate or Not to Gate: Revisiting Drinking Water Microbial Assessment Through CHIC analysis

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Abstract: Flow cytometry (FCM) has been utilized for over a decade as a rapid and reproducible approach to assessing microbial quality of drinking water. However, there is an insufficient focus on the practical issues encountered in real world utilization. The need for specialized expertise in gating—a fundamental strategy for distinguishing cell populations—introduces the potential for human error and obstructs the standardization of methods. Furthermore, the sensitivity of FCM (LOD = 234 ICC/ml, 407 TCC/ml) leads to difficulties in analysing clean water samples whose cell concentration tend to fall below the limits. This work's objective is to conduct a comprehensive analysis of the 2 commonly used static and dynamic gating approaches to evaluate their influence on the FCM metrics and explore advanced solutions to monitor clean water. The work was conducted using a dataset built by in-depth interstage monitoring of one individual Water Treatment Work (WTW) over the period of a year. The findings show that dynamic gating, which requires user intervention, is essential for the analysis of highly variable raw waters and distributed water. In contrast, static gating proved suitable for more stable water sources, interstage sample locations, and water presenting a particularly low cell count. Moreover, a “no-gate” approach combined with a CHIC¹⁷ analysis provided a solution to monitor water with cell count below the FCM LOD. Our conclusions suggest that cell count analysis should be supplemented with CHIC analysis to gain a more complete understanding of the variability in microbial populations within drinking water supplies. Establishing dynamic baselines for each water type in FCM monitoring studies is essential for choosing the correct gating strategy. CHIC analysis offers

¹⁷ Cytometric Histogram Image Comparison (Kosh, 2019)

a dynamic approach to assess treatment processes, enabling options for much better monitoring and control. This study offers new insights into the vagaries of 2 common flow cytometry gating strategies, thereby substantially contributing to best practices in the water industry. The findings foster more efficient and reliable water analysis, improving of standardizing methods in microbial water quality assessment using FCM.

Keywords: Flow cytometry; drinking water; cell gating; fluorescence fingerprinting; CHIC analysis; public health

3.1 Introduction

Microbial proliferation in drinking water challenges aesthetic and hygienic standards at drinking water treatment works (WTW), within distribution systems (DWDS), and at the point of use (e.g., tap). Compromised treatment safeguards can result in the distribution of substandard water to consumers, posing public health risks due to the connected elements of the DWDS. This issue is compounded by a lack of dynamic controls in DWDS, deteriorating infrastructure, and climate-linked uncertainties. For over a century, the heterotrophic plate count (HPC) method has assessed drinking water microbiological quality (Payment, Sartory and Reasoner, 2003; WHO. World Health Organization, 2013). However, it is not free from limitations which include underestimating microbial load, displaying taxonomic bias, being time-consuming, and revealing data on trends retrospectively which prevents timely data-led remedial action to counteract poor water quality (Cheswick et al., 2019; Hoefel et al., 2003). Flow cytometry (FCM) has been adopted in several countries' water industries for microbial water quality assessment. FCM has been employed for raw water quality monitoring (Besmer et al., 2014, 2016; Coggins et al., 2020), identifying issues in WTW assets (Phe et al., 2005; Brown et al., 2019; Farhat, Kim and Vrouwenvelder, 2020), tracking bacteria dynamics in DWDS (Gillespie et al., 2014; Besmer et al., 2017; Sam Van Nevel et al., 2017), and other applications including assessing wastewater treatment plant effluents, water reuse systems, and specific asset performance challenges such as membrane integrity (Vital et al., 2010, 2012; Liu et al., 2017; Massicotte et al., 2017; Schleich et al., 2019; Gabrielli, Turolla and Antonelli,

2021). However, despite the multiple applications of FCM in monitoring water quality, there is insufficient guidance to address users' challenges in real-world applications. In particular, there is a lack of guidance on the various gating processes, including the conditions under which they should be chosen to minimize errors. Additionally, there is inadequate guidance on the analysis of clean water, as its cell concentration often falls below the FCM's limit of detection (LOD).

Flow cytometry, when combined with the cellular markers SYBR Green I (SG) and Propidium Iodide (PI), provides a rapid and reproducible characterisation of microbial populations. SYBR Green identifies nucleic acids (DNA and RNA) of all (total) cells, while PI used in conjunction with SG marks cells with compromised membranes (Gatza, Hammes, and Prest, 2013). Integral to the characterisation of drinking water using FCM is the gating step. Gating is used to assess the levels of specific markers of interest on selected particle subsets, without confounding data from other subsets. In this context, some authors may refer to an “analysis region” rather than a “gate”. In drinking water, hierarchical gating is usually applied, consisting of primary gating used to define cell population boundaries, after which the intact cell count (ICC) and total cell count (TCC) are measured. Secondary gating typically distinguishes high (HNA) and low (LNA) nucleic acid content in microbes, although the value of these metrics in practical microbial water quality assessment remains contentious (Prest et al., 2016) as pH and other environmental stressors can change the fluorescence of cells due to changes in how the dyes interact with them (Cheswick et al., 2020). During analysis, singlet gating is sometimes applied to minimise the distortion which aggregates impose on the pulse width and area to distinguish singlets from aggregates (doublets or triplets). Singlet gating is performed using either forward scatter (FSC) or side scatter (SSC) parameters. Further gating levels are rarely applied in drinking water studies and fluorescence compensation is not normally undertaken (Safford and Bischel, 2019). Other dyes exist for evaluating different facets of microbial physiology although their application is currently limited to research applications (Nocker et al., 2011), and rarely applied for operational uses (Wang et al., 2010).

Despite its extensive deployment, the subjectivity of FCM's gating process, which sets cell population boundaries, introduces variability (Chicurel, 2002; Maecker et al., 2005; Bashashati and Brinkman, 2009; Hassard and Whitton, 2019). A biomedical study on intracellular cytokine staining precision found manual gating significantly contributed to this variability, with coefficients of variation between 17–44% on the same samples (Maecker et al., 2005). Moreover, a survey of UK water utilities indicated that most of those applying FCM for drinking water analysis employed a static gating step despite the dynamic processes within WTW and DWDS (Hassard and Whitton, 2019) underscoring the intrinsic biases, human error and variability often associated with the gating process (Van Nevel et al., 2017; Buysschaert et al., 2018; Wang and Brinkman, 2019). In FCM for drinking water analysis, the early approach involved manual gating for cell population selection. As techniques advanced, practitioners adopted static gating, applying consistent criteria across similar water-type samples. This was later complemented by dynamic gating, which adjusts to each sample's unique characteristics through either user-assisted or computer-aided modifications to predefined 'snap' gate (Prest et al., 2013; Maecker et al., 2005). Both of these approaches are used in the water sector to cope with increased data volumes from high-throughput bench FCM and automated FCM instruments (Sun et al., 2014; Bashashati and Brinkman, 2009). When conducting a large study or involving multiple operators or sites, creating a gating template becomes essential. However, despite efforts to lessen subjectivity and improve efficiency, gating variability remains a persistent issue (Hassard, 2019; Staats et al., 2019; Verschoor et al., 2015). Mitigation strategies such as assigning gate configuration to a single expert have limitations, particularly concerning time constraints and the imperatives of other more 'urgent' regulatory analyses in water industry laboratories (Nomura, Maino and Maecker, 2008). Consequently, to improve the reliability and speed of microbial water quality monitoring, novel strategies like machine learning guided assignment of gates, ungated analysis, and CHIC (Cytometric Histogram Image comparison) analysis are being employed, offering new approaches to real-time data interpretation and anomaly detection (Le Meur et al., 2007; Koch et al., 2013; Favere et al., 2020).

Furthermore, another key challenge in drinking water FCM analysis lies in its sensitivity, particularly when dealing with very low microbial concentrations. In clean water, microbial cell counts often fall near or below the limit of detection (LOD) of FCM, making reliable quantification challenging. Distinguishing background noise from true microbial signals can be difficult, particularly when fluorescence intensities of small cells overlap with electronic or optical noise (Buysschaert et al., 2018). The low biomass in treated water further complicates gating strategies, as standard thresholding methods may inadvertently exclude weak fluorescence signals from viable but non-culturable (VBNC) bacteria (Wang and Brinkman, 2019). The presence of residual disinfectants or water matrix effects may also influence fluorescence intensity, leading to potential underestimations of microbial presence (Van Nevel et al., 2017). These challenges highlight the need for refined gating approaches, automated signal discrimination, and alternative strategies such as CHIC analysis to improve microbial detection in low-biomass environments.

CHIC analysis (used with or without gating), is an innovative technique transforming flow cytometric 2D histograms into grey-scale images to allow an image comparison to determine the BCDI (Bray-Curtis Dissimilarity Index) metrics reflecting on the differences between the histograms. This technique simplifies the detection of microbial community changes (Koch et al., 2013). The principle is presented in **Error! Reference source not found.** It has the potential to reduce gating subjective interpretation as the entire sample is described not just subsets of cells. When paired with automated evaluation, it enables quick and precise categorization of water samples through inter-sampling side-by-side comparison (Chan et al., 2018). While traditional offline statistical computation methods offer insights, their retrospective nature makes them inherently reactive. Another innovative technique is the Microbial Community Change Detection (MCCD) model which advances the monitoring of microbial community stability semi-autonomously. It utilizes an outlier score derived from binning the cell population based on fluorescence using a Probability Binning (PB) algorithm, combined with distance-based outlier calculations using the NearestNeighbors model. This approach could enable early anomaly detection, underpinning

proactive responses (Sadler et al., 2020). However, most of the water sector is still using offline approaches without applying innovative techniques and thus new insights into gating effects in drinking water are required. Recently, a new model was developed to investigate bacteriological presence in treated water from WTW using automated FCM. Different machine-learning methods were tested. The best classification accuracy (89.33%) was achieved using a combination of machine learning algorithms increasing options for timely interventions to ensure safe drinking water (Kyritsakas et al., 2023). Despite this advance, there is still a requirement to understand the relative merits of gating as the most commonly applied approach using FCM for drinking water.

The majority of comparative studies have focused on static single-location sampling (Van Nevel et al., 2017; Chan et al., 2018), while longitudinal, multi-location sampling is less well reported (Van Wambeke et al., 2011; Liu et al., 2017; Ling et al., 2018). The latter, increasingly employed in the water industry, evaluates the impact of treatment processes on water quality at various WTW stages by comparing temporally poor but spatially rich sampling between and within works (Li et al., 2017; Bruno et al., 2018).

To the authors' knowledge, no longitudinal drinking water focused study has yet systematically assessed a chosen gating strategy's impact and reliability on determining FCM metrics. To address this research gap, we undertook a year-long experiment by monitoring the interstage processes of one individual Water Treatment Work (WTW). The novelty of this work is that resultant flow cytometric scatter plots were processed through static and dynamic gating and "no-gating" approaches for comparison. This study hypothesised that the conventional flow cytometric metrics (TCC, ICC, HNA, LNA) for cell quantification and the advanced flow cytometric metric (BCDI) for cell community comparison could complement each other to overcome the existing FCM challenges (e.g. FCM sensitivity hindering analysis of low-cell-count water) and better inform microbial water quality. It postulated three core hypotheses: (i) the choice between static and dynamic gating significantly impacts FCM metrics for ICC, TCC, HNA, LNA, BCDI; (ii) cell quantification through the conventional metrics and cell community

state through the advanced metric provide distinct insights into water quality events in WTW; and (iii) a new approach, processing flow cytometric scatter plots without gating prior to executing a CHIC analysis, may enable new insights into water quality assessments generally and specifically for WTW and DWDS operations using FCM.

3.2 Materials and Methods

3.2.1 Monitoring a full-scale WTW and its DWDS

Between April 2019 and March 2020, water samples were collected from various stages of a WTW and its associated DWDS in the South of England. The WTW, which sources water from a river (Sussex Ouse) via a small impounding reservoir, employs treatment processes commonly applied in the UK for surface waters. These include a Clarifier (CF), Rapid Gravity Filter (RGF), Ozone exposure, Granular Activated Carbon contractors (GAC), UV disinfection, and a Chlorine Contact Tank (CCT) (Figure 3-1). Each treatment process within the WTW underwent weekly monitoring of water quality analysis assessing physical, chemical, microbial, and cytometric parameters, detailed further below. Samples were also taken from the final water and service reservoir (SR) outlet to analyse them for the same set of parameters. The final water was sampled daily and the studied SR, 16 kilometres downstream from the WTW in the DWDS, had no reported inputs from other supplies throughout the investigation and was sampled weekly. Drinking water were also obtained from consumers' tap. The household were selected randomly to avoid bias (WHO, 2012) and samples were collected and analysed circa weekly. 250 ml of samples were collected, in duplicate, for each sampling point summarised in Figure 3-1.

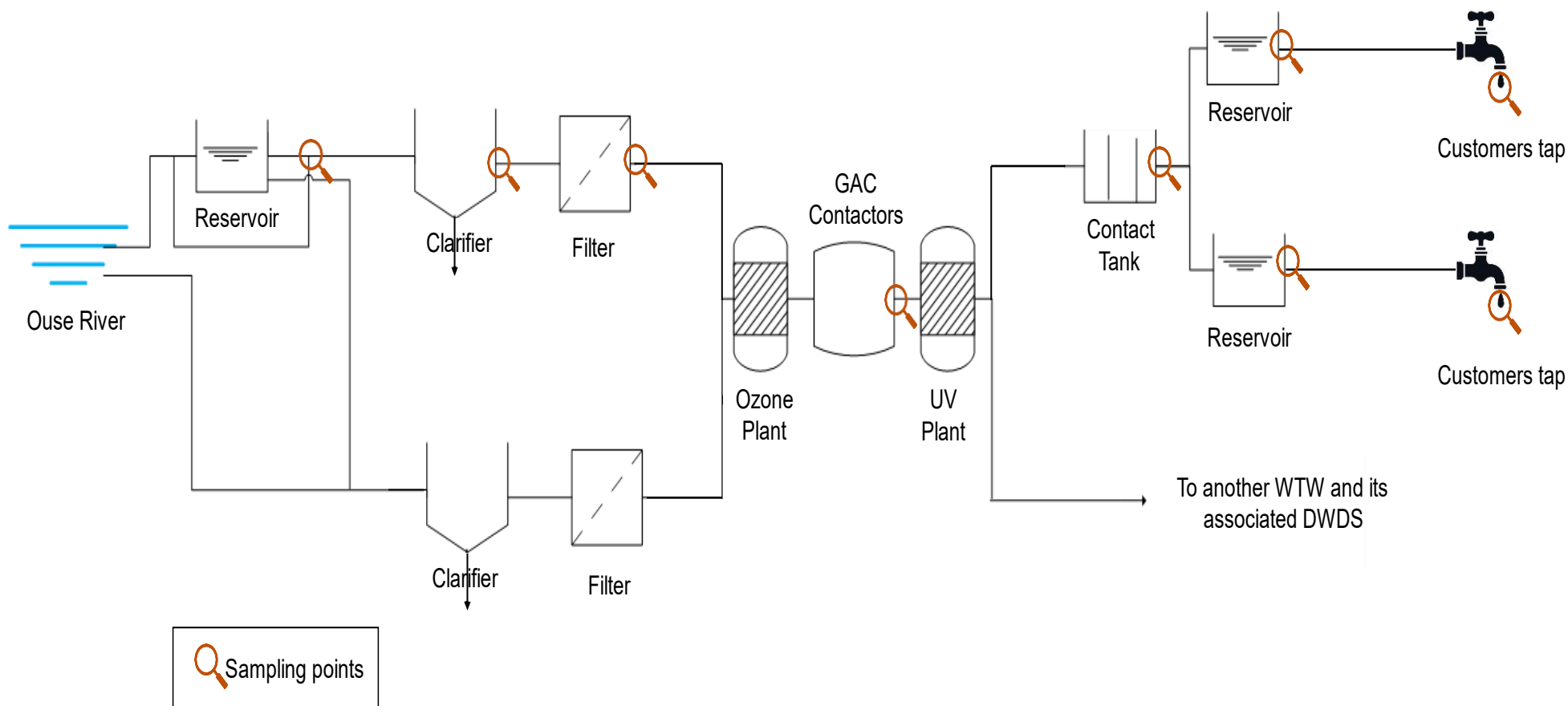


Figure 3-1: Flowsheet of WTW used in the study. River is located in the South of England with a river abstraction from the Sussex Ouse River (UK). Sample points are indicated in orange magnifying glass.

3.2.2 Physical and chemical parameters

Chemical parameters were measured as follows, with each sample taken in duplicate as per the regulatory procedures stipulated by WHO (2012). Initially, sample taps were flushed for three minutes, then flame sterilized for 30 seconds. After a further 30-second flush, samples were collected in sterile 250 mL bottles containing a pre-measured dose of sodium thiosulphate, sufficient to neutralize the chlorine residual/disinfectant. Within 24 hours of sampling, the samples were transported to the laboratory for analysis, kept at a temperature of 4-8°C. Water constituents including total and free chlorine residual were measured adhering to the Standard Methods for the Examination of Water and Wastewater (APHA-AWWA, 2005), employing the 4500-Cl G (DPD colorimetric method) for chlorine residual. In addition to standard methods, we used automated online sensors and automated meter readings (AMR) to measure water quality parameters such as free chlorine, pH, and turbidity for each sample. Chlorine contact time data was obtained from the AMR which records data every 15 minutes from at different points within the WTW. The water utility ensured accuracy by calibrating these online chlorine concentration probes at least once a month against benchtop instruments, using prepared analytical standards.

3.2.3 Microbiological parameters

Microbiological assessments were carried out on every sample (all sampling points included) following the guidelines outlined by APHA-AWWA (2005). The analysis of *Escherichia coli* and total coliforms were performed using the Colilert® test (IDEXX, UK) based on the most probable number (MPN) enzyme substrate method (9223). This test uses defined substrate technology, wherein microbial enzymes react with specific nutrients, causing a colour change when the target bacteria - *E. coli* or coliforms - are present and active. Heterotrophic plate count (HPC, 9215) at 22 °C was used to determine the total viable counts. *Cryptosporidium* was measured through the method of pathogenic protozoa enumeration (9711), which concentrates water samples, magnetically purifies them to isolate protozoa, and uses fluorescent markers for their identification under a fluorescence microscope. *Clostridia perfringens* was measured using the

membrane filtration method (9260) - the water sample was filtered, the bacteria-retaining filter was placed on a selective medium and incubated at 44°C, and the appearance of black colonies after a 24-hour incubation period indicated the presence of *Clostridia perfringens*.

3.2.4 Flow cytometric measurements of drinking water

Cytometric analysis was executed following a specific procedure. If a sample displayed a TCC or an ICC concentration surpassing 1,000,000 and 800,000 cells/mL respectively, it was diluted using sterilised Evian™ water filtered through a 0.2 µm Polyether sulfone (PES) filter. Following dilution, samples were prepared for TCC and ICC detection via fluorescence staining, adopting the protocol detailed by Whitton et al. (2018). For TCC detection, a stain was produced by diluting SG (10,000 × stock. S-7567; Thermo Fisher Scientific, UK) with Dimethyl sulfoxide (DMSO) filtered to 0.22µm (Z290807, Sigma-Aldrich, UK), yielding a working concentration of 100 × SG. ICC detection stain was created by combining five parts of 100 × SGI and one part of PI (1mg.mL⁻¹, corresponding to 1.5 mM; Thermo Fisher Scientific, UK). The samples and stains were combined in a 96-well plate and incubated in a Grant Instruments™ PHMP thermoshaker (Thermo Fisher Scientific, UK) for 15 minutes at 35 °C and 400 rpm. Post incubation, 50 µL of each sample was analysed using a BD Accuri C6 flow cytometer (Becton Dickinson U.K. Ltd., U.K.), equipped with a 488 nm solid-state laser. Green fluorescence was registered in the FL1 channel at 533 nm and red fluorescence in the FL3 channel at 670 nm. To distinguish cells from background signals in the flow cytometric scatter plots, primary and secondary gating was employed (see **Error! Reference source not found.** and **Error! Reference source not found.**, respectively, for more details). Counts of microorganisms within the gate formed the basis for TCC, ICC, HNA, and LNA calculations.

The Limits of Detection (LOD) and Limits of Quantification (LOQ) for FCM were established through serial dilution experiments using a series of high cell count real groundwater samples diluted with 0.22 µm filtered Evian™ water. Specifically, the LOQ values for TCC and ICC were found to be 700 and 1000 cells/ml, respectively. Similarly, the LOD values for TCC and ICC were ascertained to be

200 and 400 cells/ml, respectively, which is broadly in accordance with earlier work (F. Hammes, Berney, Wang, Vital, Köster, et al., 2008). See Figure S 3-3 for details on the determination of LOQ and LOD.

Specifications for the gate configuration

Flow cytometric scatter plots were delineated using two distinct and commonly used gating approaches: static and dynamic (Figure S 3-6). Both were implemented using FlowJo (v10.7.2) software. The static method utilized a fixed gate as outlined by Prest et al. (2013). In contrast, the dynamic method adjusted the gate for each plot to distinctly separate bacteria from background signals. This dynamic approach required human judgment to determine the optimal gate for each sample, relying on the user's expertise. Throughout the study, a single FCM operator was employed to minimise subjectivity related bias in the FCM gating process.

3.2.5 CHIC analysis

The CHIC analysis adhered to Koch et al.'s (2013) protocol for Cytometric Histogram Image Comparison (CHIC). This approach enables the monitoring of shift in the microbial community without a prior definition of gates in a manner that is independent of the user. Briefly, FCM FL1 vs FL3 plots were extracted from BD Accuri C6 software as .fcs files and converted to grey-scale images with the FlowJo¹⁸ (v10.7.2) software to enable comparison. The samples dyed with SYBR Green and PI for ICC quantification were specifically utilized given ICC significance in assessing viability within chlorinated systems. The comparison of the grey-scale images was conducted by using ImageJ (v1.54d). The resulting XOR and overlap values, representing the pixel differences between the images, were used to calculate the BCDI¹⁹ matrix, indicating the dissimilarity between each sample. This matrix was then used to construct a non-metric multidimensional scaling (nMDS) plots allowing the visualisation of the sample

¹⁸ Flow cytometry analysis software used to process, visualize, and interpret flow cytometry data

¹⁹ Bray-Curtis Dissimilarity Index

distribution based on their dissimilarity (BCDI) using R (v2022-02-3). The Figure S 3-4 summarises the steps of the CHIC analysis.

Additional statistical measures, such as clustering analysis, were performed using R (2022-02-3). To better characterise contributing factors influencing dissimilarity in microbial community, the operational events noted onsite by WTW staff and metadata including key operational variables such as free, chlorine, temperature, pH, etc. were linked to the BCDI matrix and add to the nMDS plot. p-value was set at significance of $\alpha = 0.05$ to determine the statistical significance of each factor. During CHIC analysis, different evaluation parameters were utilized. The stress evaluated how well the nMDS plot preserves the original dissimilarity structure of the data and improves interpretation by visualising the data. The Within-Cluster Sum of Squares (WSS) was employed to evaluate cluster compactness, while the Between-Cluster Sum of Squares (BSS) examined the dissimilarity between clusters. The Silhouette measure was used for two distinct assessments: it evaluated how well each data point fit into its assigned cluster and quantified the distinctiveness between clusters. Additionally, an ANOSIM test was applied to determine the significance of the differences between the groups of samples based on the dissimilarity matrix. See Table S 3-1 for a summary of the statistical tests.

3.2.6 Data analysis

Statistical evaluations of the resulting datasets, each corresponding to a gating method, entailed a series of statistical tests. Given the non-normal distribution and potential variation in dataset sizes, median values and the Interquartile Range (IQR) were utilized to capture central tendencies and variations. The Coefficient of Quartile Variation (CQV) was employed to provide insight into the relative variability related to the median. Box and whisker plots were used to visualize data distribution, showing medians, quartiles, and outliers. To compare the dynamic gated and static gated datasets, the Mann-Whitney U test (U-test) and the Brown-Forsythe test (F-test) were applied. The U-test, a non-parametric method, compares medians of two groups, checking for significant differences in central tendency. It assumes independent observations and similar distribution

shapes, which were met. The Brown-Forsythe test assesses variances between groups, determining if data spread is comparable. This robust test also requires independent observations and homogeneity of variances across similar-sized groups, conditions that were fulfilled. These methods were chosen for their suitability in analysing non-normally distributed data and their comprehensive evaluation of both central tendencies and variabilities in the datasets from different gating strategies. Statistical associations between cell count, BCDI (from CHIC analysis), and microbial/chemical indicators were assessed using Spearman's rank correlation coefficient. The variables were ranked, observed a consistent monotonic relationship, and were independent, thus meeting assumptions for this test. These statistical evaluations and calculations were conducted using Python (v3.11) and its libraries, specifically NumPy, Pandas, and SciPy. The tests are summarised in Table S 3-1.

3.3 Results and Discussion

3.3.1 WTW and DWDS monitoring using dynamic gating

The results showed that when analysing chlorinated water, more than 1/3 of the samples fall below the LOD of FCM. Furthermore, the variation of their value could reach 92%, reflecting on the high microbial dynamic of this water, on the sensitivity of FCM, and on the possible inappropriate use of static dynamic.

From the raw to tap water, cell count varies from 1.7×10^6 cells/ml to <LOD. Overall, 96% of samples obtained from the final water, 41% from SR outlet, and 23% from tap water were below LOD.

Typically, the raw water showed the highest cell count (1.7×10^6 cells/ml) with subsequent treatment steps reducing the cell count; post-clarifier (6.8×10^5 cells/ml), post-RGF (3.4×10^5 cells/ml), post-GAC (6.2×10^3 cells/ml) and final water (22 cells/ml). Cell counts then increased progressively until the SR outlet and tap water as cell count was 65 cells/ml and 165 cells/ml, respectively (Table 3-1). While GAC contributed to cell removal, microbial seeding is often expected due to biofilm formation within the carbon bed, which can lead to bacterial detachment into treated water. In this study, GAC filtration was preceded by an

ozone reactor and followed by a UV reactor, both of which play roles in microbial inactivation and growth control. Ozone is known for its strong oxidative properties, capable of lysing bacterial cells and damaging cellular components (Cho et al., 2010; Ramseier et al., 2011), though its precise impact on overall cell removal remains less well-documented in comparison to UV treatment. UV exposure, on the other hand, is widely recognized for disrupting microbial DNA and preventing regrowth (Hijnen et al., 2006). The significant reduction in cell count post-GAC suggests that the combination of ozone pre-treatment and UV post-treatment may have helped mitigate microbial seeding, but further investigation is needed to fully understand the individual contributions of ozone to cell removal in this system.

Variation (CQV) of the data significantly ($p < 0.05$) differed between the stages. While treatment stages recorded a variation ranging from 24 to 38%, chlorinated water (final water, service reservoir, tap water) recorded variation ranging from 42% to 92 % (Table 3-1), suggesting a higher microbial dynamic in the distribution system compared to the treatment process. This is usually attributed to the selective pressure exerted by chlorine, which can lead to microbial fluctuations due to differential resistance among bacteria (Prest et al., 2016). Additionally, factors such as biofilm detachment, residual disinfectant decay, and variations in hydraulic conditions within the DWDS could contribute to these higher fluctuations (Lautenschlager et al., 2014).

The daily monitoring of microbial cell counts provided further insights into water quality fluctuations, particularly in the final treated water. Interestingly, we observed that an instance of higher-than-usual cell counts in the final water was associated with a single colony of *Clostridium perfringens* (1 CFU /100 ml) non-compliance events, suggesting a potential link between elevated microbial counts and faecal contamination risks (Figure 3-2). At the moment of the detection the TCC was of 27,690 cells/ml and the ICC 18,400 cells/ml. The previous day, TCC and ICC were of 8,400 and 840 cells/ml, respectively, representing a 3-fold increase in TCC and a 21-fold increase to ICC. This indicated either a contamination event or a failure in microbial barrier performance.

After investigation, we determined the likely root cause to be a period of interrupted ozone dosage (Figure S 3-5). On the day of the non-compliance event, a negative log removal of ICC was observed (-0.1), indicating regrowth or ingress, along a ozone contact time of 0 mg.min/L, highlighting the critical role of upstream treatment processes in maintaining safe microbial water quality. The absence of ozone exposure may have allowed more resistant microorganisms, such as *Clostridium perfringens* spores, to persist in the final water, contributing to the observed non-compliance.

Ozone is widely recognized as an effective disinfectant that targets a broad range of microorganisms, including bacteria, viruses, and protozoa. However, its effectiveness depends on maintaining adequate contact time and concentration (CT value). In this study, the recorded ozone contact time of 0 mg.min/L suggests a complete failure in disinfection at that stage, allowing microorganisms to persist in the final water. This aligns with previous studies showing that ozone-sensitive cells can rapidly re-establish in treated water when disinfection is disrupted (von Gunten, 2003).

This observation reinforces the potential of FCM as a real-time microbial monitoring tool that can complement traditional culture-based compliance testing by detecting shifts in microbial abundance before regulatory exceedances occur. However, one challenge in interpreting FCM results is the choice of gating strategy, particularly between static and dynamic gating, which can significantly influence data accuracy. In this study, a high microbial variation (CQV 42–92%) was observed in chlorinated water, the use of static gating may have led to an underestimation of microbial fluctuations, potentially causing us to miss the non-compliance event. This further highlights the importance of an appropriate gating strategies.

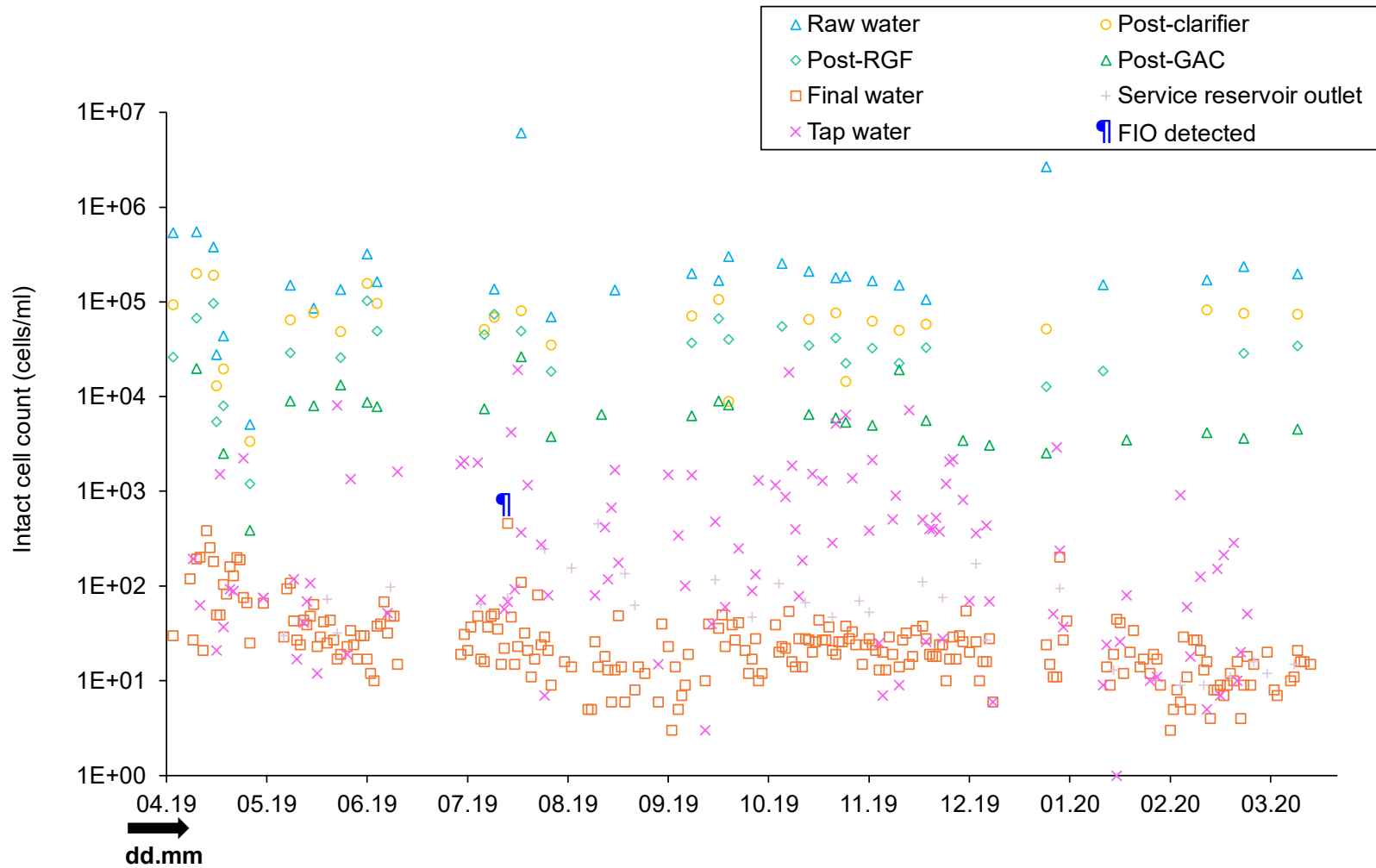


Figure 3-2 Abundance of Intact Cells Across Various Stages of the Water Treatment Workflow. Sampling was carried out at five distinct stages: Raw, Post-Clarifier, Rapid Gravity Filter (RGF), Ozone/Granular Activated Carbon (GAC), and Ultraviolet (UV)/Contact Tank. The symbols highlighted in blue represent Flow Cytometry (FCM) data gathered concurrently with the detection of faecal indicator organisms (FIOs) in the corresponding samples. The symbol ¶ is included as it represented timepoint during detection of faecal indicator in sample

To expand the broader significance of this finding, additional sampling in the SR and customer taps is recommended, as the findings from this study on limited sample size could have some limitations in broader applications. Enhanced monitoring at the SR inlet, within the SR, and in the outlet can help optimize the microbial quality of stored and subsequently distributed water supplies focusing on this key asset for water quality (Doronina et al., 2020; Martel et al., 2002).

Table 3-1 Cell counts during the 1-year period

	DYNAMIC GATE cells/ml (unless stated)	STATIC GATE cells/ml (unless stated)
Raw water (N ²⁰ = 30)	Median = 170,257 IQR ²¹ = (136,050 – 250,470) CQV = 30%	Median = 156,221 IQR = (126,894 – 206,431) CQV = 24%
Post-clarifier (N=28)	Median = 67,563 IQR = (49,850 – 81,132) CQV = 24%	Median = 64,186 IQR = (48,830 – 78,534) CQV = 23%
Post-RGF (N = 28)	Median = 33,767 IQR = (22,564 – 49,249) CQV ²² = 37%	Median = 33,930 IQR = (22,648 – 47,738) CQV = 36%
Post-GAC (N = 28)	Median = 6,159 IQR = (3,762 – 8,299) CQV = 38%	Median = 6,154 IQR = (3,727 – 8,191) CQV = 37%
Final water (N = 239)	Median = 22 IQR = (14 – 34)	Median = 18 IQR = (12 - 30)

²⁰ Number of samples

²¹ Interquartile Range

²² Coefficient Quartile of Variation

	CQV = 42%	CQV = 43%
Service reservoir (N = 31)	Median = 65 IQR = (22 – 102) CQV = 65%	Median = 59 IQR = (20 - 104) CQV = 68%
Tap water (N = 114)	Median = 165 IQR = (43.5 – 1,101) CQV = 92%	Median = 181 IQR = (48 - 805) CQV = 89%

3.3.2 Impact of the gating strategies on drinking water assessment

The results showed that static and dynamic gates give significantly different values when water is less stable, suggesting the use of static gating for stable water, and conversely, dynamic gating for unstable water.

Both gating strategies methods displayed a similar variability in the ICC values across the WTW (Table 3-1), suggesting static and dynamic gating could be both used to monitor the microbial water quality at every stage. However, further investigation showed that in some stages, both gating showed significantly different results ($p < 0.05$, Table 3-2) as they tend to generate outliers (Figure 3-3) or to exclude cell of interest (Figure 3-5), revealing the need to choose the appropriate gating across the WTW stages.

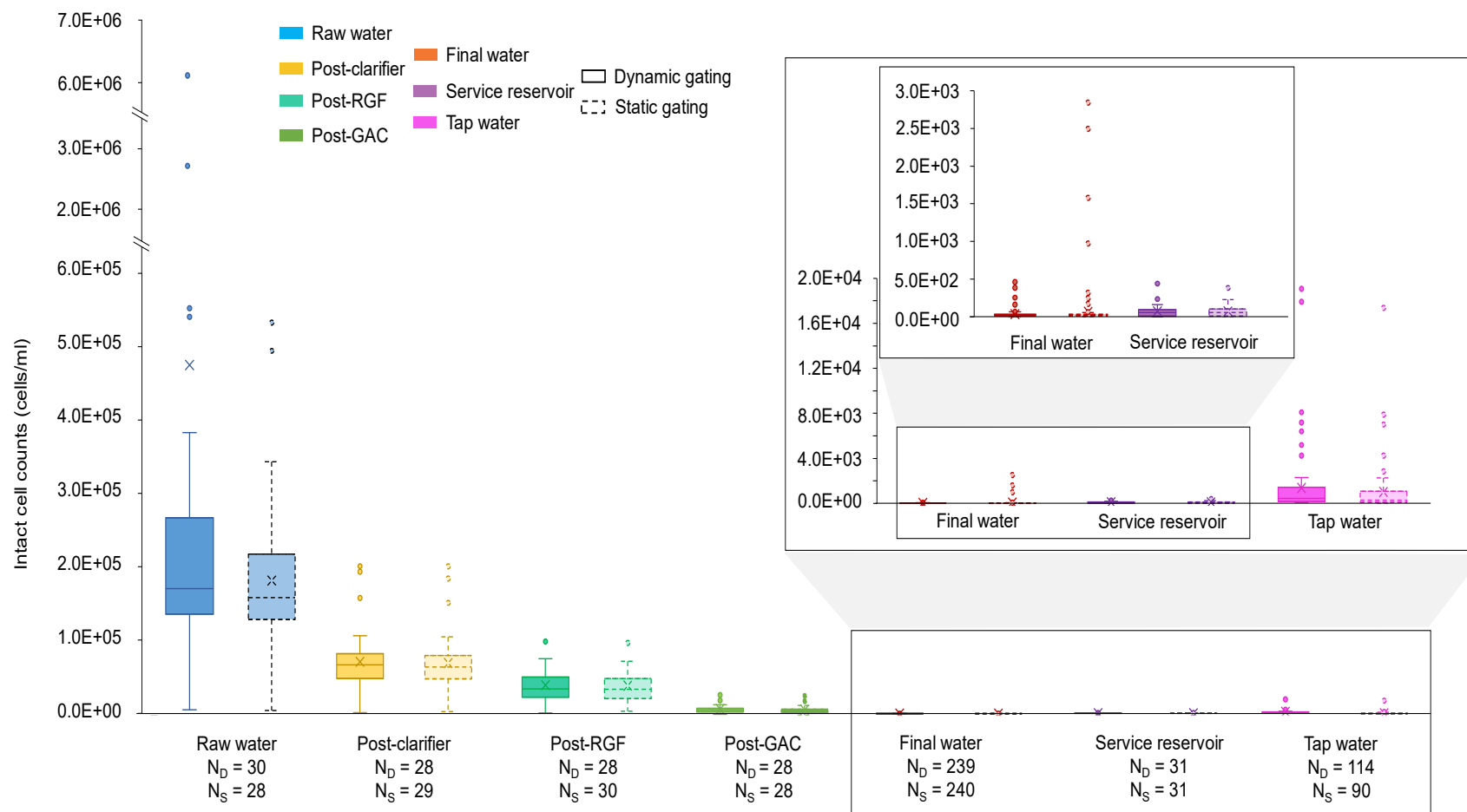


Figure 3-3: Box-and-whisker plots display visualize key patterns and differences in FCM ICC data distribution, spanning stages from source to tap during the water treatment process. The treatment involves chlorination using free chlorine, maintaining residual chlorine in the distribution phase. Disinfected water is demarcated as a cutout. The dynamic gate is presented via a contiguous line, and static gating is presented via a dashed line. The sample size for dynamic and static gate are indicated by N_D and N_S , respectively.

The IQR for raw water was 126,894 – 206,431 cells/ml for static and 136,050 – 250,470 cells/ml for dynamic gating (Table 3-1), constituting a significant difference between both strategies (F-test, 29,27 = 1.69, $p=0.05$, Table 3-2). Similarly, the IQR for final water was 12 – 30 cells/ml for static and 14 – 34 cells/ml for dynamic gating (Table 3-1) which was also shown to be statistically significant (F-test, 238, 239 = 0.035, $p=0$, Table 3-2). The comparison of the data distribution for both strategies revealed that, dynamic gating had the greatest frequency for extreme ICC values (data points beyond 1.5 times the IQR) when applied to raw water, whereas the static gate had greater frequency of extreme values when applied to final water (Figure 3-3), explaining the significant differences between both gating at these stages.

The higher frequency of extreme values observed with dynamic gating in raw water (Figure 3-3) might be due to its high degree of variability in this case i.e., fluvial surface waters without a substantial reservoir buffer, suggesting that dynamic gating would be more effective at capturing microbial outliers, which may include transient microbial populations, aggregates, or high-variability microbial clusters. This aligns with previous studies indicating that dynamic gating can better detect rare or stressed microbial populations in fluctuating environments (Besmer et al., 2014; Van Nevel et al., 2017).

The fact that static gating detected more extreme values in final water might be due to some samples being below the LOD while others were above, suggesting that pre-defined gating thresholds may exaggerate microbial fluctuations in highly stable environments, leading to false positives or misleading trends in compliance assessments.

Despite the low ICC (generally <LOD), the distribution of the final water data shows similar median (18 - 22 cells/ml) and interquartile values (Q25th = 12 - 14 cells/ml, Q75th = 30 - 34) for both dynamic and static gating. However, static gating showed a higher range of cell counts (462 - 2882 cells/ml). The SR and tap water had also similar data distribution for both gating methods (Figure 3-3). This observation shows the practical value of dynamic gate for the quantification of cells in drinking water source waters, sub-potable, and treated supplies.

Table 3-2: Comparison of the ICC measurements from different stages of the monitored WTW using static and dynamic gate strategies. Mann-Whitney U (U) and F-test (F) were performed to assess whether one method produced significantly larger values than the other and whether the variance between the two strategies differed significantly, respectively. The variable 'p' signifies the statistical significance of the observation; a value of $p < 0.05$ indicates a statistically significant observation. All significant observations are emphasized through bold text and underline

DYNAMIC GATE	STATIC GATE						
	Raw water	Post-Clarifier	Post-RGF	Post-GAC	Final water	Service reservoir	Tap water
	Raw water	U(56)=0.903, p=0.367 <u>F(29, 27)=95.54,</u> <u>p<0.05</u>					
	Post-Clarifier	U(55)=0.367, p=0.714 F(27, 28)=1.09, p=0.41					
	Post-RGF		U(56)=-0.0311, p=0.975 F(27, 29)=1.15, p=0.36				
	Post-GAC			U(54)=0.164, p=0.870 F(27, 27)=1.03, p=0.47			
	Final water				<u>U(477)=2.32,</u> p<0.05 <u>F(238, 239)=0.035,</u> p<0.001		
	Service reservoir					U(60)=0.190, p=0.849 F(30, 30)=1.21, p=0.31	
	Tap water						U(202)=0.0920, p=0.927 <u>F(113, 89)=1.48,</u> p<0.05

To demonstrate differences in gating strategies on the ICCs observed, the datasets were compared using a ratio metric: the ICC of the static gate to the ICC of the dynamic gate (Figures 3-4). A gating ratio of 1 means there was no difference between the two strategies. A ratio < 1 indicates a higher count using the dynamic gate. Conversely, a ratio above 1 suggests a higher cell count in the static gate.

The ICC values for raw water, post-clarifier, post-RGF, post-GAC, and service reservoir had the lowest ratios, as shown by a ratio ranging from 0.76 to 1.23. An exception was observed for one of the post-RGF samples, which had a significantly lower ratio of 0.26 (Figure 3-4). The discrepancy was attributed to the high turbidity of this sample, highlighting the influence of the gating when operational events occur. The final water, SR and tap water samples displayed wider ratio ranging from 0.09 to 6.83, from 0.05 to 1.85, and from 0.08 to 1.75, respectively, suggesting greater discrepancies between the two gating methods in chlorinated water samples. The occurrence of greater ratios was more frequent in the final water, SR, and tap water samples, emphasizing firstly, the instability of these samples, and secondly, the impact of the chosen gating strategy on cell count variability. A dynamic gating approach would be more suitable for these samples, as it accounts for variations in fluorescence intensity and shifts in microbial populations, providing a more accurate assessment of microbial dynamics in treated water.

To delve deeper into this observation, the FL1-FL3 fluorescence plots were compared for each stages to visualise how both gating strategies manage to gate the cells for different water origin.

Figure 3-5 provides the FL1-FL3 fluorescence plots for samples from each treatment stage with a green marker indicating samples where the ICC, determined by static gating, was either half (0.5) or twice (2) the ICC determined by dynamic gating. These thresholds were selected to identify which fingerprint elements contributed to the differences in cell counts derived from each gating method deployed.

In the post-RGF, final, and tap water samples, the prevalence of green markers often spans the entire gate. In certain instances, some events even venture outside the gate, implying a challenge to this method in distinguishing between cells and background debris (Figure 3-5). For such samples, it is thus recommended to employ dynamic gating especially as these samples represent water which is intended for human consumption as thus justify analysis and interpretation costs to utilities.

While limited research specifically focuses on gating of microbial cells in drinking water, investigations in other contexts have explored the nuances of gating. For example, Suni et al. (2014) found no significant difference between automated and manual gating when analysing cytokine FCM data due to relative homogeneity of this sample type.

The variability in final water observed in this study arises from the dynamic cytometric background caused by chlorination, compounded by the difficulty in differentiating low fluorescence cell counts (i.e., LNA) from this background (see Figure 3-5).

This variability is partly due to a decrease in fluorescence resulting from oxidative stress (Wert, Dong and Rosario-Ortiz, 2013) but could be a consequence of the inherently binary nature of gating—where a cell is either classified within a gate or outside and thus is inflexible to adapt to inherently dynamic nature of microbial populations in aquatic environments such as drinking water. This also further complicates the setting of arbitrary cell thresholds for assessing microbial water quality.

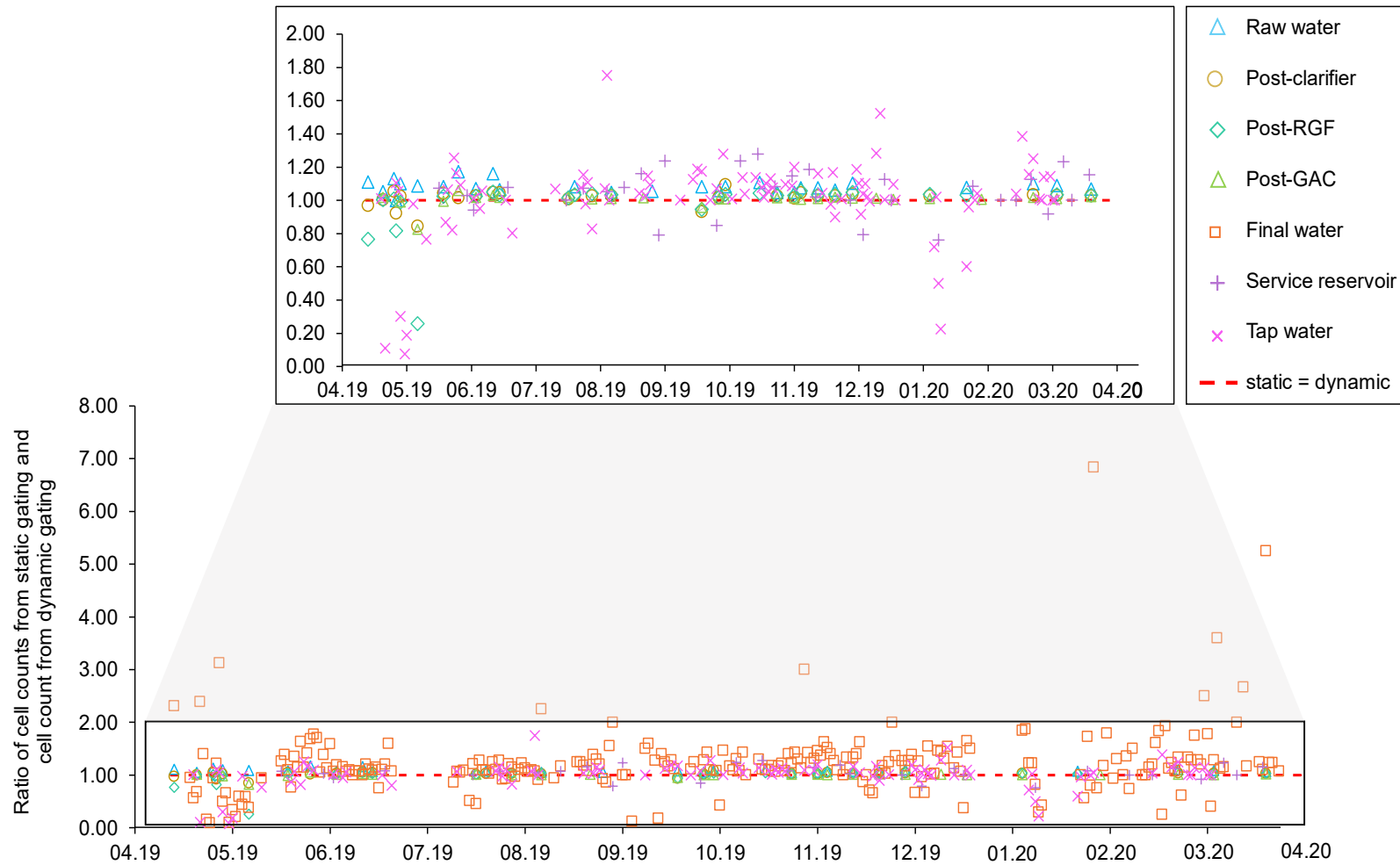


Figure 3-4 Comparative Analysis of ICC values between static and dynamic gating strategies. The data are presented as the ratio of values achieved using static gating to those obtained through dynamic gating. The red line serves as an indicator, illustrating the point at which the values from both gating strategies converge, signifying identical results

Variability in tap water, as illustrated in Figure 3-5, is influenced by events in the DWDS such as ingress (Fox et al., 2015) or regrowth, which can modify microbial populations (i.e., abundance) and distribution (i.e., assembly) (Douterelo, Husband and Boxall, 2014; Lin et al., 2016; Måns, 2021).

Based on the findings by Staats et al. (2019), it has been recommended that manual dynamic gating should be deployed where practical with the gate set above the cell "halo"²³ (i.e. at the boundary of the negative population). This counter increased background, potentially specific to the sample, ensuring better detection of subtle responses.

To translate the implications of these findings to drinking water where cell populations are more heterogenous, it is suggested to use static gates where drinking water is stable such as final waters from a WTW.

In contrast, dynamic gating is essential for monitoring unstable raw water and possibly during DWDS which is often subjected to mixed supplies, ingress and regrowth.

For final water analysis, employing static gating helps minimize user subjectivity, a factor of particular importance when cells count is used in a diagnostic capacity for setting water quality triggers or baselines for operational assessment. However, within these generalisations, disparities did exist between the gating approaches (Figure 1-3, Table 1-3).

When analysing samples with low cell counts in flow cytometry, the differentiation between actual cells and background noise or artifacts becomes challenging. The decision between using dynamic gating versus static gating is crucial in these circumstances. Dynamic gating allows for adaptability and fine-tuning based on the specific characteristics of each sample. This can be particularly beneficial when cell populations are sparse, as it enables a more accurate distinction between target cells and potential interference. On the other hand, static gating,

²³ "Halo" represents low-intensity background fluorescence that can make it harder to distinguish weakly positive cells

with its fixed parameters, may not capture these nuances as effectively, potentially leading to missed cells or inclusion of unwanted signals. As such, during periods of low cell counts, the choice of gating strategy can significantly impact the accuracy and reliability of the analysis. However, unpredictable events, such as the alteration in microbial patterns seen Post-RGF on 10th May 2019 (Figure 1-5), call for adaptive gating strategies.

Operational events such as RGF / GAC backwash or return to service (Vital et al. 2012), pump duty standby (Besmer et al. 2016) or membrane cleaning (Pluym et al., 2023) contribute reproducible differences in microbial populations but are unlikely to cause the significant shifts in abundance and distribution observed at this WTW, as they cause localized and short-term microbial fluctuations. Nomura et al. (2008) emphasised that static gating aids in maintaining consistency of analysis in pre-stained lyophilised human cells. Instrument setup, something not considered here, was noted as being the major source of variability in FCM data generation and its subsequent interpretation. Differences in laser alignment, detector sensitivity, and fluidics stability can lead to shifts in fluorescence intensity, causing misclassification of microbial populations when using static gating. This can be particularly problematic in water quality monitoring, where subtle microbial fluctuations can indicate contamination events. To address this standardized instrument calibration could be introduced to minimize variability and improve reproducibility across different FCM runs. Another approach could be using hybrid gating which combines static and dynamic gating to improve consistency while still adapting to microbial shifts in unstable water condition.

The authors tested a common template with dynamic "snap-to" gates which resulted in least variability between laboratories. Even without use of these dynamic gates, consistent results were obtained using a fixed template checked by an expert operator / analyst reinforcing our work on drinking water cells. The necessity and frequency of review and adjustment are contingent upon the specific water under study.

In this research, the credibility of an emerging method that has been garnering considerable attention: a gate-free approach to FCM. To investigate this,

considering analyses both with and without background, aiming to offer more consistent, unbiased results and circumvent the limitations of traditional gating methods.

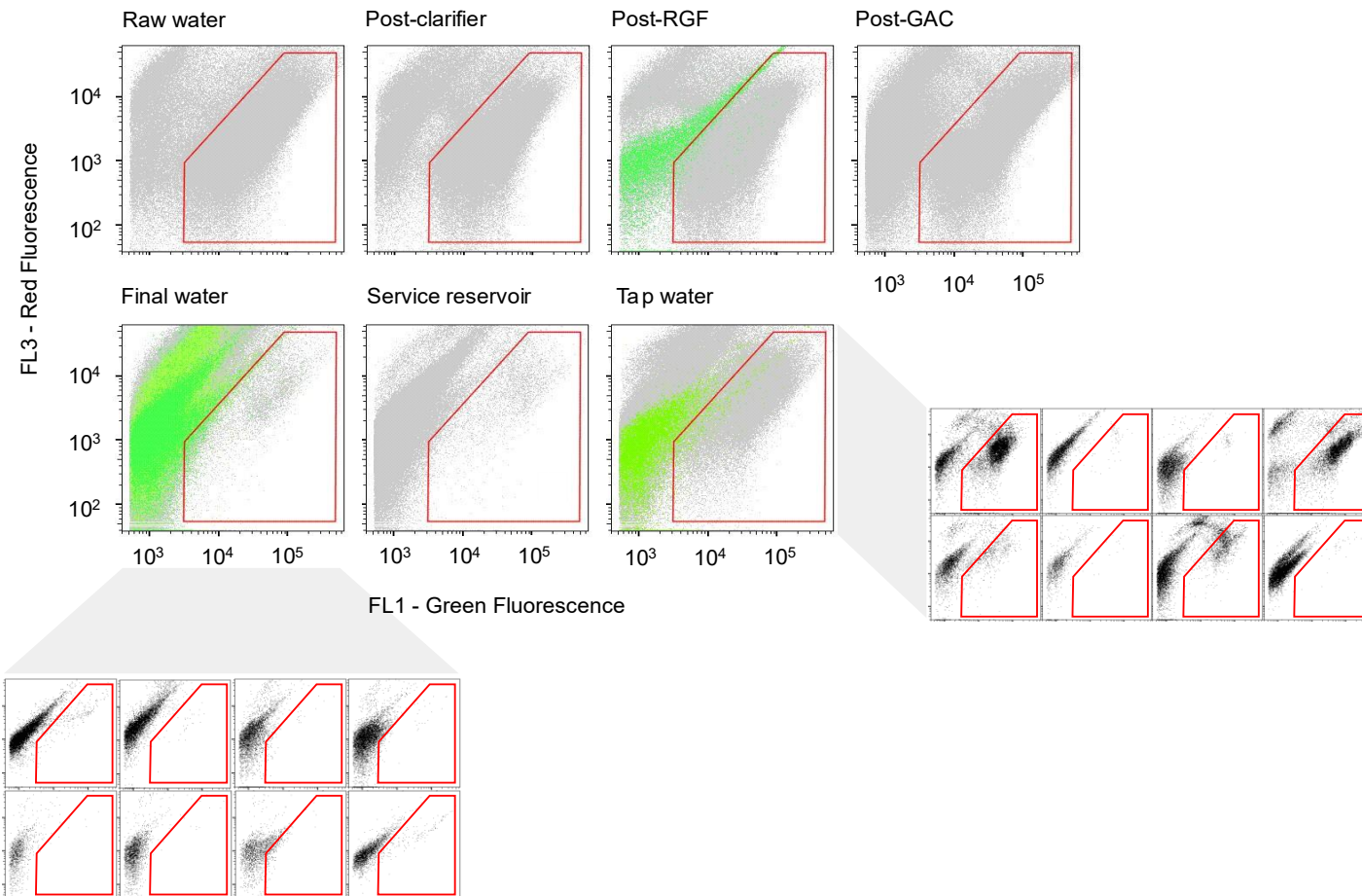


Figure 3-5 Overlapping of the one-year FCM scatter graphs obtained for each stage of the WTW. The X-axis represents the Green fluorescence induced by staining cells with SYBR Green while the Y-axis represents the Red fluorescence induced by using Propidium iodide stain. The red polygon is a gate used for selecting the cell population. The green scatter plot indicate that the cell count determined using a static gate is half or double the one gotten using a dynamic gate. Details of the overlapped scatter plot are provided for Final water and Tap water in order to visualize the dynamic background

3.3.3 Assessing the microbial quality using cell count and CHIC analysis

The results showed that a no-gate strategy using CHIC analysis gives more insight into the microbial population shift as it was observed that non-compliant samples drifted significantly from their usual location.

The 'no-gate' strategy has rarely been applied within the drinking water literature as most studies opt to eliminate background interference through gating before initiating CHIC analysis. In contrast, this work aimed to ascertain the benefits of traditional gated and 'no-gate' strategies using CHIC analysis, examining data from various stages of the WTW. Yet, the CHIC analysis is in broad agreement with Chan et al. (2018) and Cheswick et al. (2019): that is relying solely on cell count is an inadequate metric for assessing microbial water quality. Essentially, similar cell counts in water samples may present different fluorescence on the FLA and FL3 channel of FCM enabling more insights into the processes governing (microbial) water quality. NMDS was used to display differences between distributions of cells.

Figure 3-6A shows the dissimilarity between the samples of each treatment step of the WTW based on their fluorescence on FL1 and FL3 channels using a gated approach. In contrast, Figure 3-6B displays the same information, when background was included in the CHIC analysis. The hypothesis was that greater differentiation between samples can be generated through analysis of all particles in the fingerprint (including cells and background).

The differentiation of the samples coming from the different stages of the WTW (Figure 3-6) was similar for both gating strategies although the location of samples within the NMDS was different. Clean water and raw and treated water were differentiated on the NMDS1 axis for both strategies suggesting that they are equivalent in monitoring changes in cell fluorescence.

When using the static gate strategy clean water samples were more compacted and closer to the centroid of the cluster (silhouette = 0.96, WSS = 0.062) compared to the no-gate strategy ($0.42 < \text{silhouette} < 0.50$, $0.56 < \text{WSS} < 0.74$)

as if the inclusion of the FCM background brought additional insight into the cell population.

It was noticed that the factor driving the difference within the clean water samples was mainly the sample composition in HNA and LNA cells, defined through static gate, suggesting that the background gives additional insight into the metabolic activity of the microbial population in clean water samples. Le Meur et al. (2007) demonstrated that ungated FCM data reveal substantial nonbiological differences in samples.

Pertinent, as this expands FCM's utility as an insightful method for assessing the quality and safety of other aspects of drinking water (e.g., particle penetration). Typically, its primary application is to quantify microbial cell counts, but there's an intriguing aspect that sometimes gets overlooked: the potential value of non-biological content in drinking waters when analysed with FCM. When we discuss non-biological content, we are referring to abiotic particles and substances present in the water that aren't biologically active. These could be minerals, salts, metals, debris or other inorganic or organic materials. In FCM, these elements can generate what is commonly referred to as inorganic "background signals". Under standard conditions, these signals might be dismissed as mere noise or interference, but under specific circumstances, they can provide invaluable insights.

One such circumstance is during situations characterized by a high background, like backwash water events or sloughing from GAC contractors (Hess et al., 2021). In these scenarios, the elevated background can actually be a significant indicator of water quality. The increased non-biological content might signal at operational issues or changes within the treatment processes. Another scenario of interest is during periods of ultra-low cell counts, specifically when these counts fall below the LOD. In such instances, where microbial cells are sparse or potentially undetectable, the background signals offer new insights. Any deviation or change in this background might indirectly indicate shifts which could signal a broader problem like changes to microbial communities, altered growth conditions or even particle associated viruses. For instance, changes in

parameters like pH, turbidity, or nutrient concentrations can provide clues about the underlying biological activity.

Thus, it is essential to view these background signals not as mere static or noise but as potential early warning indicators. Monitoring for sudden shifts or anomalies in these non-biological parameters can pre-emptively highlight potential challenges or changes in water quality. In essence, while the primary focus of FCM in water treatment is understandably on biological content, there's a compelling case to be made for the relevance and utility of non-biological content in certain scenarios.

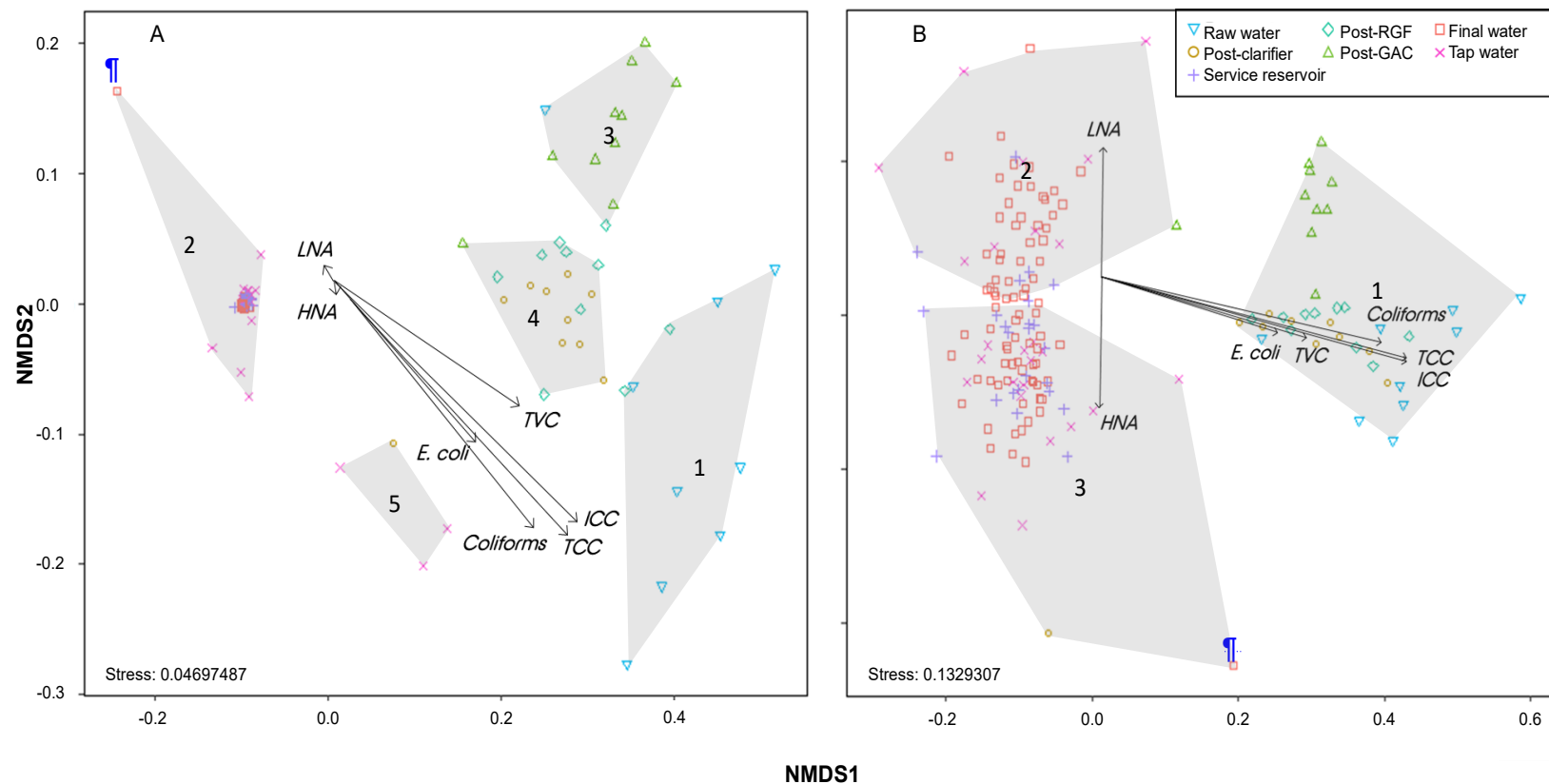


Figure 3-6 A. NMDS ordination plots from CHIC analysis used to produce microbial phenotypic fingerprints, using 'fixed-gate' (Panel A) and 'no-gate' (Panel B) strategies. Utilizing K-means clustering and the gap statistic, optimal clustering was determined, grouping water samples into different clusters based on their similarities. The detailed composition of these clusters can be found in Table 1-3. Correlation vectors are depicted, illustrating linear relationships between cytometric patterns of water samples and plate count data (heterotrophs, coliforms, and *E. coli*). Please note that the *Clostridia* vector was omitted from this analysis due to a single detection event > 0 CFU / 100 ml. This detection event is signified by a (¶), symbol adjacent to the corresponding sample

To discern populations of FCM events that share similar distributions, a k-means clustering approach was employed. The rationale for adopting clustering, particularly the k-means method, is rooted in its ability to group data points based on inherent patterns and similarities. In the context of flow cytometry data, clustering can simplify the complex array of detected events by grouping them into distinct categories or clusters, each representing a specific microbial distribution or pattern. By doing so, this method facilitates a more structured and interpretable visualization of the microbial landscape present in the water samples. This structuring becomes evident when examining the FL1-FL3 fluorescence histogram of clean water, including various stages like final water, SR outlet, post-clarifier, post-RGF, post-GAC, and raw water samples. A distinct difference in their microbial patterns is observable, which is corroborated by the formation of five clusters in Figure 1-6A and three clusters in Figure 3-6B. Such clear delineations underline the effectiveness of the clustering approach in providing a comprehensive overview of microbial diversities and similarities across different water samples.

In the no-gate approach, Cluster 1 represented raw and treated (post-clarifier, post-RGF, post-GAC) water samples with >90% of these samples being co-located. Whereas Clusters 2 and 3 represent clean water samples. The distribution was more variable with (10-45%) of these samples being located in Cluster 2 and 10-80% of samples being located in Cluster 3 (Table 3-3). Cluster 2 was primarily derived from final water and Cluster 3 from DWDS and tap water (Figure 1-6). The separation based on the CHIC analysis was similar to that found using cell counts (Figure 3-2) which shows a clear separation between the clean water samples (final water, and service reservoir outlet) and the treated and raw water samples.

Regarding tap water samples, their cell abundance shows that they were dispersed and sometimes close to the cell abundance of post-GAC water 9 (Figure 3-2) suggesting that tap water microbial quality declines. CHIC analysis (Figure 3-6B) shows that the microbial pattern of tap water is as close as final

water and SR outlet when cell alone is included, but background provides additional bandwidth to quantify differences apparent to water quality.

The sample which also featured a final water Faecal Indicator Organism (FIO) detection was on the edge of Cluster 3 (Figure 3-6B) and provides promise that FCM could be applied in future to signal changes associated with poor water quality, additional datasets or pilot studies would be needed to confirm these observations.

Table 3-3 Overview of the cluster content from Figure 2 for the “fixed-gate” and “no-gate” strategy. The percentage represents the portion of samples within a sample group (e.g., Raw water, post-clarifier, etc.) present in the corresponding cluster. The similarity of the samples within a group, the dissimilarity of the samples between groups, and the compactness of a cluster are respectively represented by the silhouette score, the between-cluster sum of squares (BSS), and the within-cluster sum of squares (WSS)

	STATIC GATE					NO GATE		
	Cluster no 1	Cluster no 2	Cluster no 3	Cluster no 4	Cluster no 5	Cluster no 1	Cluster no 2	Cluster no 3
%Raw water	90	-	10	-	-	100	-	-
%Post-Clarifier	-	-	-	90	10	90	-	10
%Post-RGF	20	-	10	70	-	100	-	-
%Post-GAC	-	-	90	10	-	90	10	-
%Final water	-	100	-	-	-	-	45	55
%Service reservoir	-	100	-	-	-	-	20	80
%Tap water	-	96	-	-	4	-	35	65
Silhouette score	0.554	0.959	0.228	0.4589	0.531	0.651	0.422	0.503
WSS	0.014	0.062	0.121	0.0376	0.0498	0.554	0.563	0.743
BSS			5.679				6.805	

3.3.4 Associations cells count and CHIC analysis with microbial indicators

Figure 3-6B shows that the correlated vectors of the microbial indicators (coliforms, *E. coli*, TVC, ICC, and TCC) point in the direction of the cluster comprised of raw and treated water samples and the vector length indicates a strong correlation.

This observation suggests that the FCM clusters can distinguish between water types and this associates to the presence of microbial indicators (coliforms, $r = 0.5$, $p < 0.001$), and cell counts including ICC ($r = 0.59$, $p < 0.001$), and TCC ($r = 0.51$, $p < 0.001$) (Table 3-4).

During the non-compliant microbial water quality (Figure 3-2; Figure 3-6B), cell count was at its highest during this year ($ICC > 10,000$ cells/ml) and the sample distributed at the extremity of the Cluster 3.

This shows the effectiveness of the CHIC analysis associated with the no-gate strategy to assess water quality. Le Meur et al. (2007) work demonstrated that ungated FCM data can be used to create a systematic and efficient method of data quality assessment. It also raises the prospect of using dynamic models e.g., Sadler et al. (2020) and AI augmented approaches as outlined within on both bacteria and the background populations.

In the literature, HNA and LNA are seen as sensitive to chlorination (Ramseier et al., 2011); therefore, we verified their correlation with chlorine residual and chlorine contact time. The correlation between the FL1-FL3-fluorescence histogram of the clean water samples and the chlorine residual ($p > 0.05$) and contact time ($p > 0.05$) shows that they are not the ones driving the differences between clean water samples along HNA and LNA. However, other studies mentioned a correlation with nutrient availability (Santos et al., 2019; Thyssen et al., 2005; Wang et al., 2009); therefore, this correlation remains to be verified.

Table 3-4 Overview of the Pearson correlation (r) value for each vector shown in Figure 2 for the “fixed-gate” and “no-gate” strategies. The Pearson correlation (r) value for chlorine residual and chlorine contact time vectors are part of other NMDS plots. The plots are not shown because they could not be included in Figure 2 due to their value being only relevant for clean water samples. The p-value represents the significance of the observation. The stress indicates how well the reduced-dimensional space from the NDMS plot fits the original data

	STATIC GATE			NO GATE		
	r	p-value	stress	r	p-value	stress
Coliforms	0.502	<0.001		0.438	0.001	
<i>E. coli</i>	0.228	<0.001		0.186	0.001	
ICC	0.588	<0.001		0.527	0.001	
TCC	0.592	<0.001	0.047	0.528	0.001	0.133
TVC	0.262	<0.001		0.245	0.001	
HNA	0.001	0.927		0.074	0.002	
LNA	0.001	0.926		0.074	0.002	
Chlorine residual	0.07	0.001	0.01	0.022	0.247	0.16
Chlorine contact time	0.037	0.258	9.029 e-05	0.018	0.514	0.123

3.3.5 Future perspectives for the use of FCM and CHIC analysis for microbial quality assessment

Flow cytometry is gaining significance in the realm of microbial counts. However, it is essential to emphasize that FCM should not be viewed as a replacement for the measurement of indicator organisms in compliance monitoring as there is no direct correlation between the total microbial counts ascertained by FCM and traditional indicator organisms. Instead, FCM should be embraced as a supplementary tool. Its strength lies in its ability to provide insights that are both more sensitive compared to culture-based methods and closer to real-time when compared to conventional culture-based methods. Furthermore, while considering microbial culture tests, it is pertinent to note that the majority of bacteria cultured during these tests are non-pathogenic. As a result, they do not pose a significant public health risk, especially for healthy individuals. In the context of drinking water safety, indicator organisms, which suggest potential faecal contamination, play a pivotal role. Fortunately, in water that has been treated appropriately, the presence of these organisms is a rarity and within health-based targets outlined by the WHO.

When investigating deeper into the capabilities of FCM, it becomes evident that the method offers a holistic and reproducible measurement of the microbial community including non-culturable organisms. Such comprehensive insights make FCM superior to the use of surrogate organisms like *E. coli*, known for their high susceptibility to multiple stressors in WTW and DWDS. In addition, it is efficient and ideal for routine measurements, acting as a way of directing more advanced techniques focused on species and strain identification such as Matrix-Assisted Laser Desorption/Ionization - Time Of Flight Mass Spectroscopy, DNA sequencing or qPCR. Furthermore, when direct microbial monitoring is combined with modelling, it can effectively demonstrate the efficacy of innovative WTW and DWDS designs. However, it's worth noting that numerical modelling of WTW and DWDS remains a significant challenge, primarily due to the multifaceted complexities inherent in real-world systems and operation of these assets. In the pursuit of process optimization and investigations, FCM stands out as an

invaluable tool. The emerging trend of online FCM suggests a future where it could be employed for real-time disinfection monitoring leveraging new forms of machine learning to optimise gating to be more dynamic to changing water quality. Additionally, the advent of no-gate CHIC analysis has broadened the horizons, offering more avenues through which FCM can be applied. Lastly, the integration of artificial intelligence and machine learning tools marks a transformative phase in this domain. Such innovations are set to revolutionize the landscape by empowering automated microbial water quality event detection.

3.4 Conclusions

This study highlights the equivalent efficacy of the static and dynamic gating approaches in determining the cell count. Both gating strategies demonstrate value depending on specific circumstances. Static gating showed effectiveness in monitoring water with low cell count, while dynamic gating was ideal for analysing unstable water. In instances of unexpected events, a periodically adjusted static gate was recommended to ensure consistency.

However, the determination of cell count alone was insufficient to holistically interpret water quality. Despite cell count and CHIC analysis yielding similar categorizations for raw, treated, and clean water, the study emphasized the distinct value of CHIC analysis.

This method provided additional insights into the microbial population shift, revealing that two samples with the same cell count might not necessarily present similar microbial risk. Attempts to correlate ICC from clean waters with microbial indicators underlined the challenge of rarely finding contaminated clean water samples, thus inhibiting potential correlation. While CHIC analysis displayed strong correlations with microbial indicators, the correlation between cell counts and these indicators was constrained by the lower limit of quantification of culture-based methods.

The no-gating strategy introduced an innovative perspective into the interpretation of clean water samples by offering additional insight into the microbial population in terms of HNA and LNA. While this approach didn't

significantly impact the interpretation of raw and treated water samples, it provided further understanding of the microbial composition in clean water samples. Though it was determined that this metric does not reflect the state of chlorine residual and chlorine contact time, the study posited it might instead reflect nutrient availability.

In conclusion, this research highlights the importance of choosing appropriate gating strategies for microbial water analysis based on specific conditions and requirements. It emphasizes the necessity of further studies to confirm correlations and causal relationships. It also encourages the exploration of innovative approaches, such as the no-gating strategy, to yield richer insights into water quality analysis.

This research supports the three hypotheses stated in the introduction. It has demonstrated that gating strategy selection significantly impacts FCM metrics, with static gating suited for stable waters and dynamic gating better capturing microbial variability in unstable environments. Additionally, cell quantification (ICC, TCC) and cell fluorescence histogram provided distinct but complementary insights into water quality, reinforcing the need for both conventional and advanced metrics in microbial monitoring. Finally, the no-gating approach introduced a novel perspective on microbial population shift, particularly in clean water samples, though further research is needed to validate its full potential for routine water quality assessment in WTW and DWDS operations.

This research contributes to the field of flow cytometry-based water quality monitoring by introducing the no-gating strategy as a potential tool for characterizing microbial diversity in clean water samples. It also contributes to the validation of the impact of gating strategies on FCM metrics, confirming that static gating is best suited for stable, low-microbial waters, whereas dynamic gating is preferable for fluctuating microbial environment.

The findings have practical implications for water treatment and distribution system operators as it provides an enhanced early detection of microbial events by demonstrating the added value of CHIC, which can improve decision-making in water quality management, and it offers a new perspective on microbial stability.

in treated drinking water through no-gating strategies, which may complement existing regulatory monitoring frameworks.

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3.6 Appendix

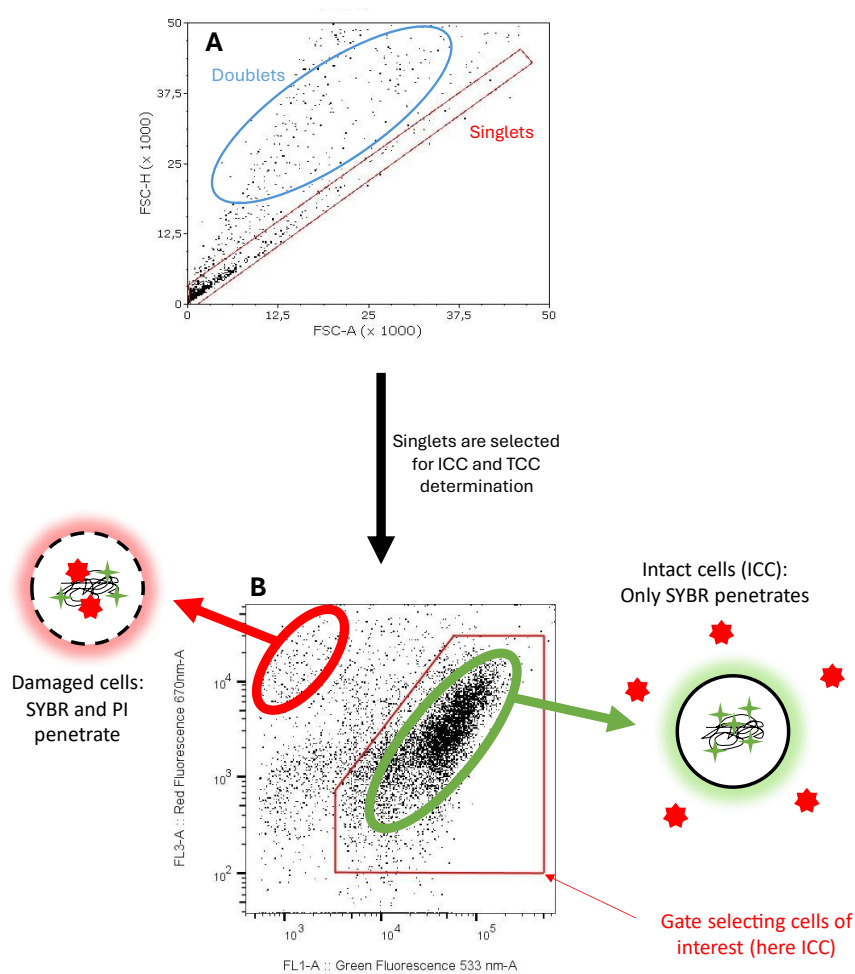


Figure S 3-1: Primary gating

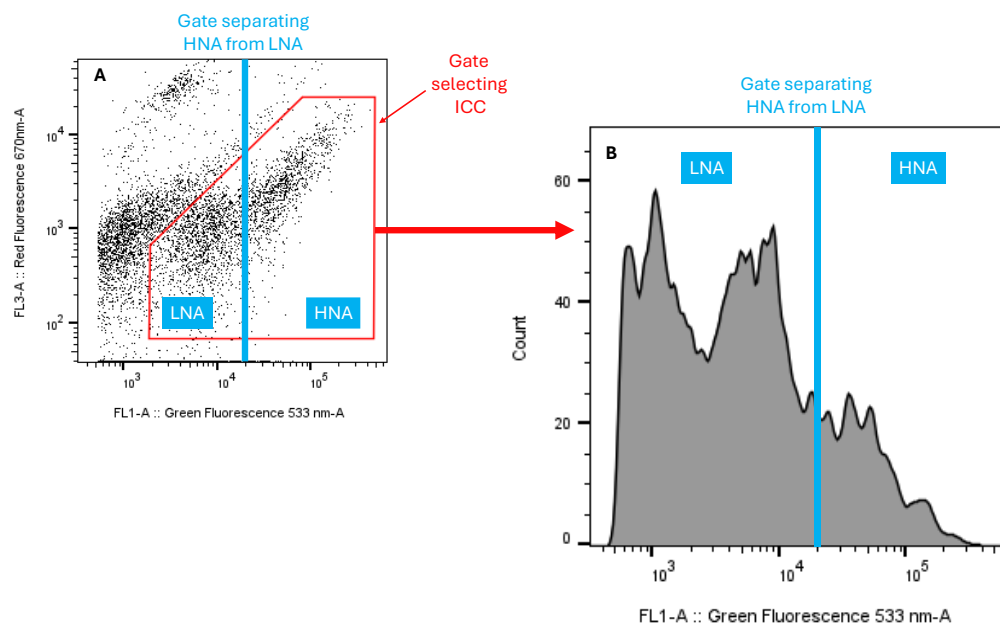


Figure S 3-2: secondary gating

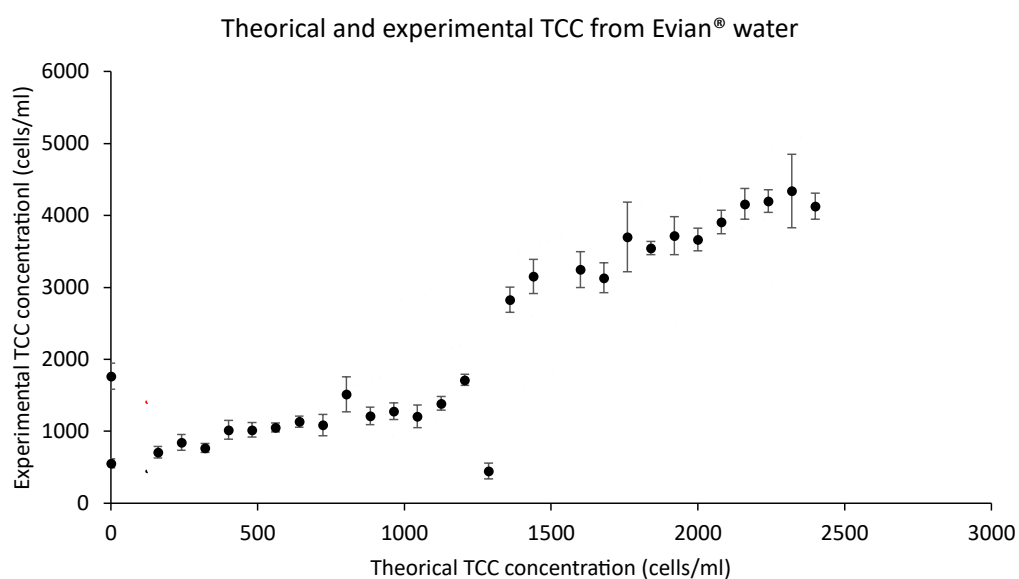
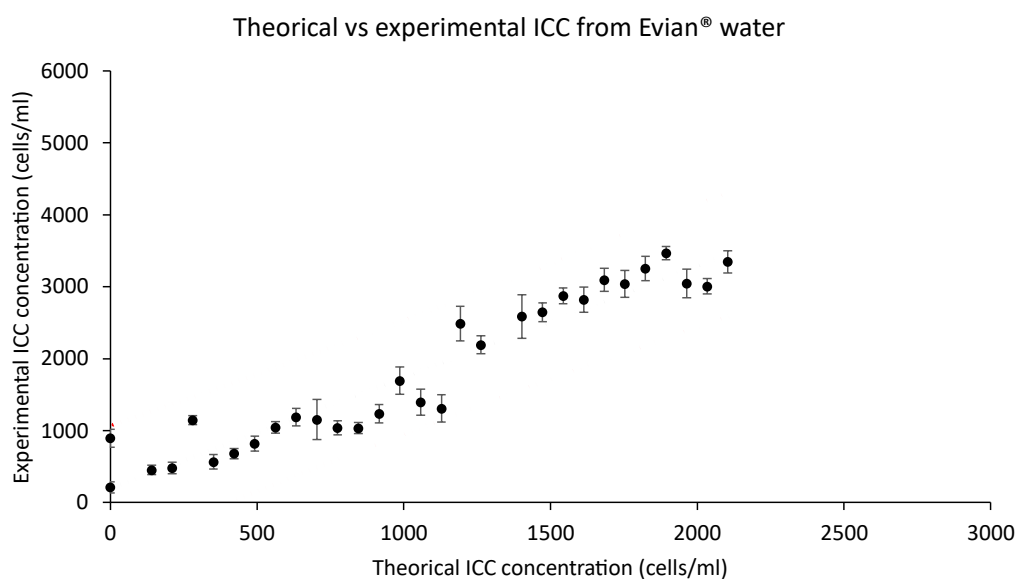


Figure S 3-3: Curve built from analysing a groundwater sample diluting with Evian water in different concentrations to determine the limit of detection (LOD) and quantification (LOQ) of FCM for TCC and ICC

Image comparison - XOR (Exclusive OR) operation

The operation consist of comparing corresponding pixel values between images

When two pixels are identical, the background is black, when they are different, the background is white

This can be mathematically expressed as:

$$A \text{ XOR } B = (A \cup B) - (A \cap B)$$

Where:

- A XOR B is the XOR operation
- $(A \cup B)$ is the union of the two images
- $(A \cap B)$ is the intersection of the two images

The result of A XOR B corresponds to the total number of white pixels, which represent the differences between the two images

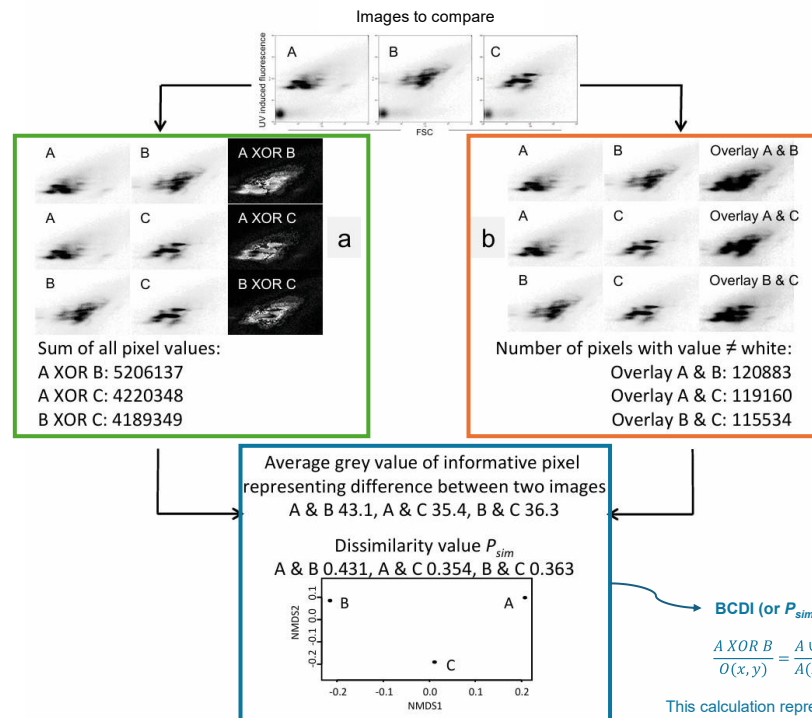


Image superimposing - Bitwise OR operation

The operation highlights all the pixels present in either of the two images

This can be mathematically expressed as:

$$O(x, y) = A(x, y) \cup B(x, y)$$

Where:

- $O(x, y)$ is the resulting image after the Bitwise OR operation
- $A(x, y)$ and $B(x, y)$ are the pixel values of the two input images
- The operator $\cup$ represents the Bitwise OR operation

The $O(x, y)$ value represents the total number of pixels that are non-zero (different from black)

Figure S 3-4: CHIC analysis explanation

Table S 3-1: Statistical tests explanation

Test	Purpose	Value and explanation
Stress (nMDS)	Evaluates how well the nMDS plot preserves the original dissimilarity structure of the data. Lower stress values indicate better representation of the data in reduced dimensions	<p>Value:</p> <p>< 0.1 → Excellent fit</p> <p>0.1–0.2 → Acceptable</p> <p>> 0.2 → Poor fit</p> <p>Interpretation: Ensures that visualized clustering in nMDS accurately represents the actual dissimilarities in microbial communities</p>
Within-Cluster Sum of Squares (WSS)	Measures cluster compactness by evaluating the total variation within each cluster. Lower WSS values indicate tighter clustering	Value: A low WSS suggests that data points within each cluster are closely related, meaning the clustering is well-defined. A high WSS suggests that clusters lack compactness
Between-Cluster Sum of Squares (BSS)	Measures dissimilarity between clusters, assessing how distinct clusters are from one another. Higher BSS values indicate better separation	Value: A high BSS relative to WSS suggests that clusters are well-separated, meaning the clustering method is effective. A low BSS suggests overlapping clusters

Silhouette Measure	Assesses within-cluster cohesion and between-cluster separation, evaluating how well each data point fits within its assigned cluster	<p>Value:</p> <p>Close to 1 → Strong clustering</p> <p>Near 0 → Possible misclassification</p> <p>Negative → Poor clustering</p> <p>Interpretation: Ensures that clusters are distinct and meaningful for microbial community analysis</p>
ANOSIM (Analysis of Similarities)	Tests whether differences between predefined sample groups are statistically significant based on their dissimilarity matrix	<p>Value: Produces an R-value (0 to 1) and a p-value</p> <p>$R \approx 1$: Strong separation between groups.</p> <p>$R \approx 0$: No meaningful difference.</p> <p>$p < 0.05$: Significant difference between groups.</p> <p>Interpretation: Confirms whether microbial community differences between treatment stages are statistically significant</p>
Spearman's Rank	Analyses relationships between HNA/LNA dynamics and other variables, identifying non-parametric correlations	<p>Value:</p> <p>$\rho = 1 \rightarrow$ Perfect positive correlation</p>

Correlation Coefficient		<p>$\rho = -1 \rightarrow$ Perfect negative correlation</p> <p>$\rho = 0 \rightarrow$ No correlation</p> <p>Interpretation: Identifies whether changes in HNA/LNA ratios correlate with microbial population shifts</p>
Random Forest (RF) Algorithm	Identifies key factors predicting microbial growth patterns and their relative importance	<p>Value: RF importance values range from 0 to 1</p> <p>0 $\rightarrow$ No influence</p> <p>1 $\rightarrow$ Strongest influence</p> <p>Interpretation: Determines which environmental or treatment variables most significantly impact microbial growth patterns</p>
Dunn's Test	Conducts pairwise comparisons between groups to identify significant differences	<p>Value:</p> <p>$p < 0.05 \rightarrow$ Significant difference</p> <p>$p > 0.05 \rightarrow$ No significant difference</p> <p>Interpretation: Determines whether differences in microbial communities between treatment stages are statistically meaningful</p>

<p>Mann-Whitney U Test</p>	<p>Compares two independent groups to assess whether one has significantly larger values than the other</p>	<p>Value: Produces a U statistic and a p-value</p> <p>Small U, $p < 0.05 \rightarrow$ Significant difference</p> <p>Large U, $p > 0.05 \rightarrow$ No significant difference</p> <p>Interpretation: Determines whether microbial community characteristics differ significantly between treatment stages</p>
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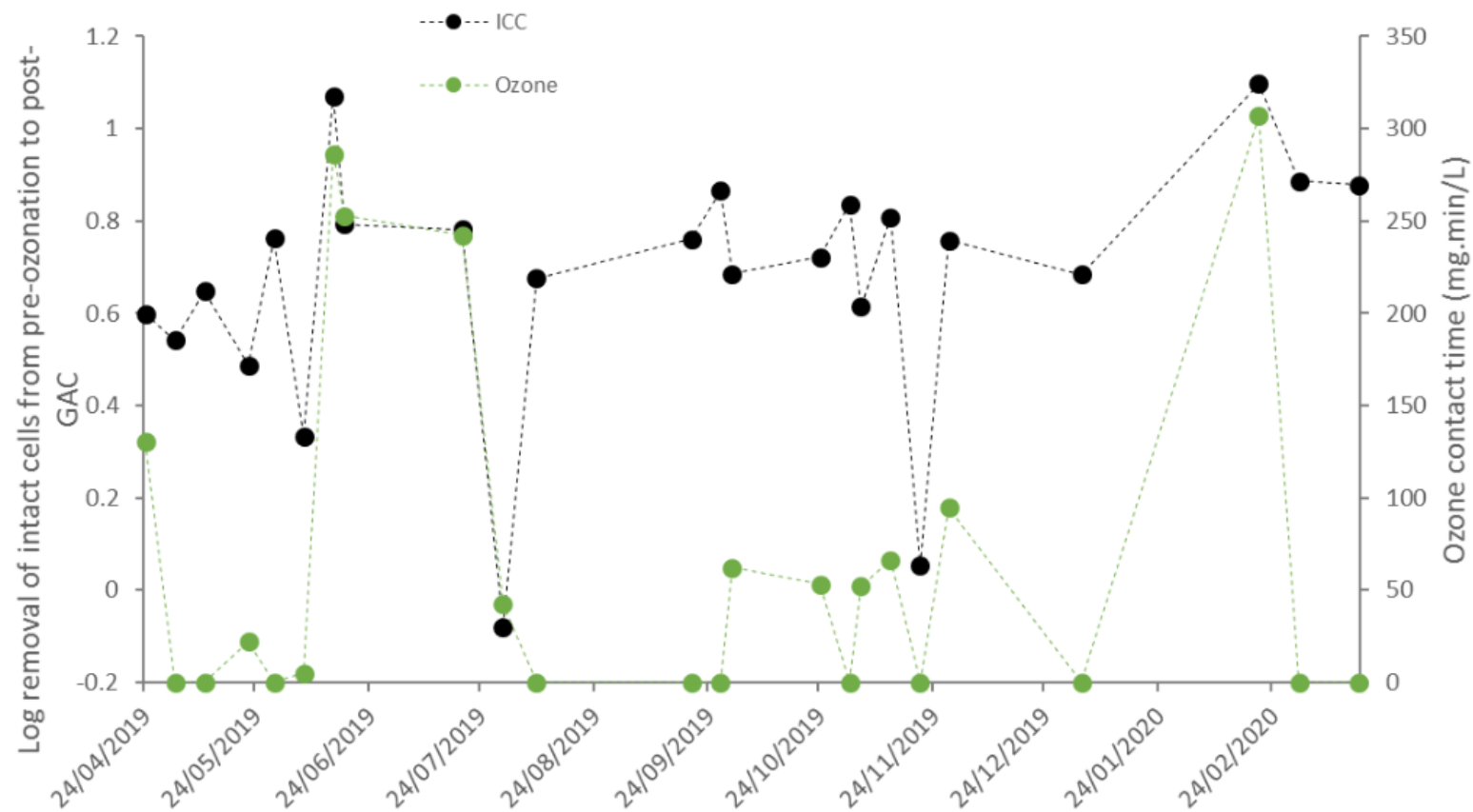


Figure S 3-5: Ozone contact time against log removal of ICC between pre-ozonation and post-GAC.

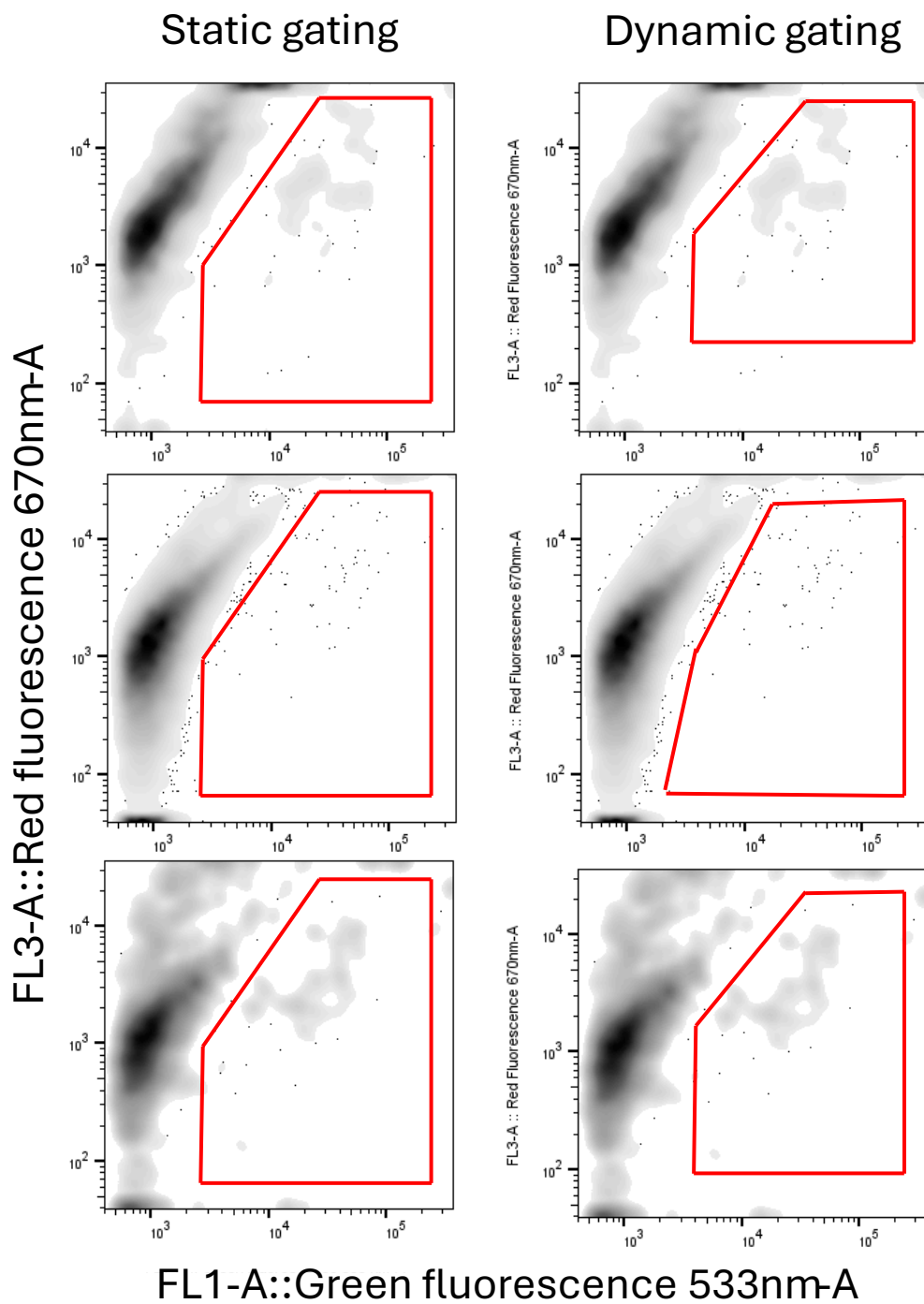


Figure S 3-6: Example of dynamic and static method

4 New insights into regrowth in drinking water treatment and supply using HNA/LNA indicators and CHIC analysis

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Abstract: This study utilizes flow cytometry (FCM) to evaluate the contribution of high (HNA) and low (LNA) nucleic acid cells in assessing water microbial quality in drinking water treatment and supply systems. Our findings indicate that HNA cells, known for their high metabolic rates, rapidly respond to nutrient changes, making them suitable indicators of growth under varying water treatment and supply conditions. However, the study also reveals that HNA and LNA indicators should be used alongside other microbial indicators and environmental condition data, as these factors can significantly influence water microbial quality. Additionally, the research shows that the different microbial communities comprising HNA and LNA cells are dynamic, and their reactivity varies across different water treatment processes. This demonstrates the limitations of solely using HNA and LNA for understanding such complex systems.

The research highlights that CHIC analysis combined with cluster analysis provides a more sensitive evaluation of water quality by capturing a broader range of microbial characteristics compared to using only HNA/LNA ratios. This work advocates for multi-parameter data analysis to advance monitoring techniques for water treatment and supply processes.

4.1 Introduction

Assessing microbial status in drinking water is key to health risk management as active cells can be resistant to disinfection and regrow within drinking water distribution systems (DWDS). The rising use of lower-quality water sources, coupled with the uncertainties of climate change, intensifies pathogen risks (Schijven et al., 2013). This underscores the critical need to understand microbial behaviour in drinking water systems (Ramseier et al., 2011b). Active microbial growth in oligotrophic drinking water indicates poor bio-stability, potential contamination, and possible shifts in water quality. Fluctuating microbial counts may also point to inadequate water treatment (Park et al., 2018). Water of low biological stability has a greater risk of falling below established regulatory safety standards or hygiene norms (Favere et al., 2020a). Early detection of such changes is essential for prompt corrective measures, ensuring water safety and adherence to health regulations (Cheswick et al., 2020). Traditional culture-based methods to quantify these active microbial populations are time-consuming, strain-specific, and economically impractical for routine monitoring (Hassard, Andrews, et al., 2017; Van Nevel, Koetzsch, et al., 2017). Flow cytometry (FCM) has emerged as a powerful tool for rapid and precise measurement of microbial populations including their nucleic acid content - independent of culture-based assessment (Lautenschlager et al., 2013, 2014). The nucleic acid content of bacteria serves as a proxy for evaluating heritage and growth status in aquatic environments, providing an innovative alternative method (Gasol et al., 1999; Longnecker et al., 2005).

In aquatic environments, two distinct microbial populations emerge: high nucleic acid content (HNA) and low nucleic acid content (LNA) bacteria (Hammes et al., 2008). SYBR Green I staining and FCM differentiate microbial groups by sorting cells based on side scatter (SSC) and green fluorescence, indicating nucleic acid content. HNA cells, characterized by high fluorescence intensity and SSC, are larger and thought to possess greater metabolic activity than LNA (Berney et al., 2007; Prest et al., 2013, 2014). LNA bacteria exhibit lower fluorescence intensity and SSC, indicative of a smaller size and possibly reduced specific metabolic

activity (Y. Wang et al., 2009b). HNA are implicated in oligotrophic growth conditions common in more pristine sources such as ground waters whereas LNA are proliferate in eutrophic conditions. Some studies infer LNA represent a different physiological status (Cheung et al., 2015), as LNA bacteria can have lower adenosine triphosphate (ATP) content compared to HNA cells (Zlatanović et al., 2017a). Lower activity in LNA bacteria was determined via sorting and measuring leucine incorporation rates (Lebaron et al., 2001b, 2002), supporting the hypothesis that HNA cells constitute a distinct and diverse group separate from LNA (Chan et al., 2018).

Although FCM is gaining more and more interest in routine monitoring of microbial populations, the direct operational value of nucleic acids metrics in microbial water quality assessment remains contentious (Poitelon et al., 2010). It is not well understood whether HNA and LNA populations represent distinct microbial groups or are different physiological states of the same cells. Additionally, the questioning on whether the fluorescence-based categorization is truly significant in real world-application for understanding variations in water microbial quality still remains. While it has been shown that ICC growth accelerates when HNA increases and decelerates when LNA dominates (refs), their significance among other factors in real-world scenarios remains unclear. This work aims to respond to these questions.

To elucidate the significance of HNA and LNA in real-world scenarios, the in-depth interstage monitoring of one individual Water Treat Work (WTW) and its associated service reservoirs (SR)s was undertaken over the period of a year. Additional factors, such as temperature, distance between WTW and SRs, and chlorine residual were also considered to determine the most significant influences on microbial water quality. To evaluate the contribution of HNA and LNA in understanding variations in water microbial quality, two different experiments were conducted. The first one involved creating experimental samples with varying HNA/LNA ratios using two water samples collected at two of the monitored SRs. These samples were then spiked with nutrients to stimulate microbial growth and subsequently tracked via FCM. The second experiment

consisted in collecting samples from the interstage processes at the monitored WTW. These samples were also spiked with nutrients to stimulate microbial growth and monitored using FCM. The first case evaluated the contribution of HNA and LNA in chlorinated drinking water distribution systems (DWDS), where it is hypothesized that higher HNA concentrations correlate with greater microbial regrowth, while the second focused on their contribution across different treatment processes.

This work postulated three core lines of enquiry: (i) HNA regrowth in DWDS would signal a reduction in chlorine residual; (ii) HNA will regrow quickly during periods of nutrient addition; and (iii) LNA will regrowth slowly after readily biodegradable nutrients are utilised

4.2 Materials and methods

4.2.1 Monitoring full-scale WTW and its DWDS

To address the initial research question, a WTW and its nine service reservoirs (SR)s, underwent weekly monitoring over a year period. The objective was to determine the cell concentration and associated characteristics, including viability and nucleic content, at their respective outlets. Located in Sussex, Southeast England, the site abstracts water from the river Sussex Ouse via a small impounding reservoir and employs treatment processes commonly applied in the UK for surface waters. These include a Clarifier (CF), Rapid Gravity Filter (RGF), Ozone exposure, Granular Activated Carbon contractors (GAC), UV disinfection, and a Chlorine Contact Tank (CCT). An illustration of the site is shown in Figure S 4-1. The SRs were located at distances of 5, 5.5, 6, and 10.5 km from the WTW, each independently connected without water inputs from alternative sources (Figure 4-1). All SR were rectangular design except for SRO-1B which was circular. SRO-1s was ~100 years old and provided water to 6,686 people. SRO-2A, SRO-2B, SRO-C had ages of 89, 89, and 54 years, together serving 6,553 persons. SRO-3 was 68 years old, supplying water to 15,123 individuals. Lastly, SRO-4A, B and C, were 47 years old and provided water to 11,767 individuals. All SRs were reinforced concrete except SRO-2B which was comprised of stainless steel.

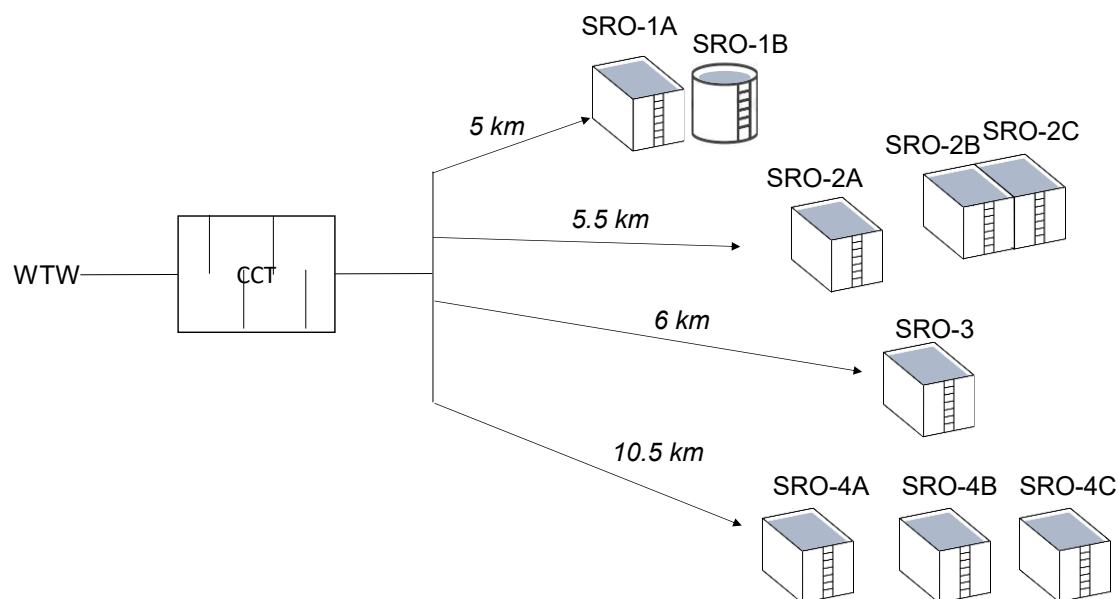


Figure 4-1 illustrates a schematic of a water distribution system, showing the path from a water treatment works (WTW) to various service reservoirs (SROs). The schema is not in scale.

4.2.2 Evaluate HNA and LNA contribution in understanding microbial growth

To evaluate the contribution of HNA and LNA in understanding variations in water microbial quality, two different experiments were conducted.

4.2.2.1 Simulated regrowth of different HNA-LNA ratios

The first experiment involved creating experimental samples with varying HNA/LNA ratios to evaluate the contribution of HNA and LNA in chlorinated drinking water distribution systems (DWDS), where it is hypothesized that higher HNA concentrations correlate with greater microbial regrowth.

2-liter water samples were collected at two of the monitored SRs (SRO-1A and SRO-2A). These SRs were chosen because one has more HNA (80%) than the other (20%), allowing the preparation of samples with a wide range of HNA/LNA ratio. See Figure S 4-2: HNA and LNA gating process for the illustration of the HNA and LNA gating.

The sampled process involved flushing sample taps for three minutes, followed by flame sterilization for 30 seconds. After an additional 30-second flush, samples were collected in sterile 250 mL bottles containing a pre-measured dose of sodium thiosulphate to neutralise the chlorine residual/disinfectant. Upon reaching the lab, the samples were refrigerated at 4-8°C and experimentation began within 24 hours.

A subsample of the water with 20% HNA was filtered (0.22 µm and 0.10 µm) to create a cell-free matrix, confirmed by FCM showing cell count <LOD (refer to Claveau et al., 2024). The matrix was poured in five autoclaved, AOC-free, 250-ml reactors. Aliquots from both water samples, 80 and 20% HNA, were added to each reactor in different volume to achieve varying HNA-LNA ratios (1-0, 0.75-0.25, 0.50-0.50, 0.25-0.75, 0-1). A uniform ICC concentration was maintained across all reactors. Each reactor was then aseptically spiked with 100 µg sodium acetate as an AOC source. The capped reactors were then left in a shaker-incubator at 30°C for 6 days. HNA and LNA growth was measured every 24 hours using a flow cytometer.

To observe microbial regrowth within a short time frame, the incubation temperature and the AOC concentration were balanced. The choice of 30°C accelerates microbial activity while maintaining environmental relevance, and the addition of 10 µg AOC provides sufficient carbon to stimulate growth without overwhelming the system. This combination ensures measurable regrowth dynamics within 6 days while maintaining a realistic representation of drinking water conditions.

4.2.2.2 Simulated regrowth of HNA and LNA originating from different sources

The second experiment also consisted in collecting samples from the interstage processes at the monitored WTW, but this time, to evaluate the contribution of HNA and LNA across different treatment processes.

2-liter water samples were collected at the different stages of the monitored WTW. These stages included the granular activated carbon filter contractors

(post-GAC), the slow sand filter outlet (post-RGF), the chlorine contact tank outlet (post-CCT), and one service reservoir outlet (SRO).

After collection, each sample was stored, filtered for preparing a cell-free matrix, aliquoted with the samples, spiked with AOC, and incubated at 30°C as previously described. The experiment lasted 13 days as each stage may have varying microbial regrowth patterns. HNA and LNA growth was measured every 24 hours using a flow cytometer.

4.2.3 Physical and chemical parameters

For the field study, to evaluate the contribution of HNA and LNA in understating microbial regrowth, their variations were compared with those of physical and chemical parameters. Physical parameters included the monthly average air temperature in Sussex, South East England, sourced from the Met Office (UK). Chemical parameters, such as residual chlorine, were measured according to regulatory procedures outlined by Clark (2015). Each sample was taken in duplicate. Water constituents, including total and free chlorine residual, were measured per the Standard Methods for the Examination of Water and Wastewater (APHA-AWWA, 2005). The methods employed were the 4500-Cl G (DPD colorimetric method) for total and free chlorine residual.

4.2.4 Fluorescent staining and flow cytometric measurements

Cell concentration was determined via FCM alongside viability assessment. If a sample displayed a TCC or an ICC concentration surpassing 1,000,000 and 800,000 cells/mL respectively, it was diluted using filter (0.2 µm polyether sulfone) sterilised water filtered Evian™ mineral water. Following dilution, samples were prepared for TCC and ICC detection via fluorescence staining, adopting standard protocols. For TCC detection, a stain was produced by diluting SG (10,000× stock. S-7567; Thermo Fisher Scientific, UK) with Dimethyl sulfoxide (DMSO) filtered to 0.22µm (Z290807, Sigma-Aldrich, UK), yielding a working concentration of 100×SG. ICC detection stain was created by combining five parts of 100 × SGI and one part of PI (1mg.mL⁻¹, corresponding to 1.5 mM; Thermo Fisher Scientific, UK). The samples and stains were combined in a 96-

well plate and incubated in a Grant Instruments™ PHMP thermoshaker (Thermo Fisher Scientific, UK) for 15 minutes at 35 °C and 400 rpm. Post incubation, 50 µL of each sample was analysed using a BD Accuri C6 flow cytometer (Becton Dickinson U.K. Ltd., U.K.), equipped with a 488 nm solid-state laser. Green fluorescence was registered in the FL1 channel at 533 nm and red fluorescence in the FL3 channel at 670 nm. To distinguish cells from background signals in the flow cytometric scatter plots, primary and secondary gating was employed. The specifications for gate configuration are provided in the next section. Counts of microorganisms within the gate formed the basis for TCC, ICC, HNA, and LNA calculations. The Limits of Detection (LOD) and Limits of Quantification (LOQ) for FCM were established through serial dilution experiments using a series of high cell count real groundwater samples diluted with filtered Evian water and aligned with established limits for FCM using BD Accuri C6 (Hammes et al., 2008). See details in Figure S 4-3.

4.2.5 CHIC analysis

Cell concentration, ICC, TCC, HNA, and LNA data were extracted from .fcs files using FlowJo software (v10.7.2), which facilitated FL1 vs FL3 dot plot extraction.

The CHIC analysis adhered to Koch et al.'s (2013) protocol for Cytometric Histogram Image Comparison (CHIC). This approach enables the monitoring of shift in the microbial community without a prior definition of gates in a manner that is independent of the user. Briefly, FCM FL1 vs FL3 plots were extracted from BD Accuri C6 software as .fcs files and converted to grey-scale images with the FlowJo (v10.7.2) software to enable comparison. The samples dyed with SYBR Green and PI for ICC quantification were specifically utilized given ICC significance in assessing viability within chlorinated systems. The comparison of the grey-scale images was conducted by using ImageJ (v1.54d). The resulting XOR and overlap values, representing the pixel differences between the images, were used to calculate the BCDI²⁴ matrix, indicating the dissimilarity between each sample. This matrix was then used to construct a non-metric

²⁴ Bray-Curtis Dissimilarity Index

multidimensional scaling (nMDS) plots allowing the visualisation of the sample distribution based on their dissimilarity (BCDI) using R (v2022-02-3). The **Error! Reference source not found.** summarises the steps of the CHIC analysis.

Additional statistical measures, such as clustering analysis, were performed using R (2022-02-3). To better characterise contributing factors influencing dissimilarity in microbial community, the metrics HNA, LNA, TCC and ICC were linked to the BCDI matrix and add to the nMDS plot. p-value was set at significance of $\alpha = 0.05$ to determine the statistical significance of each factor. During CHIC analysis, different evaluation parameters were utilized. The stress evaluated how well the nMDS plot preserves the original dissimilarity structure of the data and improves interpretation by visualising the data. The Within-Cluster Sum of Squares (WSS) was employed to evaluate cluster compactness, while the Between-Cluster Sum of Squares (BSS) examined the dissimilarity between clusters. The Silhouette measure was used for two distinct assessments: it evaluated how well each data point fit into its assigned cluster and quantified the distinctiveness between clusters. Additionally, an ANOSIM test was applied to determine the significance of the differences between the groups of samples based on the dissimilarity matrix. Table S 4-1 summarises the statistical tests.

4.2.6 Data analysis

Spearman's rank correlation coefficient analysed the relationships between HNA and LNA dynamics and other variables, revealing non-parametric correlations. A Random Forest (RF) algorithm identified key factors predicting microbial growth patterns. RF importance values usually range from 0 to 1. A value of 0 means that the variable has no/limited influence on the prediction, while a value of 1 indicates it is the most influential, with a comparison of variables considered in the context of these other variables. Dunn's test was used for pairwise comparisons among experimental groups, allowing for an in-depth analysis of the components contributing to the differences in the data. Lower p-values (near 0.05) may suggest significant group differences. Mann Whitney U test was used for dataset comparison to assess whether one group of samples tends to have larger values than the other. The test produces a U statistic to determine whether

there is a statistically significant difference between the two groups being compared. This statistic is associated with a p-value measuring the strength of evidence against the null hypothesis, which states that there is no difference between the two groups of samples being compared. A small p-value (typically less than 0.05) indicates that there is strong evidence to reject the null hypothesis. See Table S 4-1 for summary.

4.3 Results and Discussion

4.3.1 HNA and LNA dynamics in DWDS

The results show that HNA and LNA alone does not account for understanding microbial population dynamism within the DWDS. Other water quality measurements and environmental data were also significant and therefore, are important to be used alongside the metric to model better microbial growth.

The further the SR from the WTW, the higher the ICC and HNA/LNA ratio. Table 4-1 summarises the ICC and HNA/LNA recorded at the CCT and SRs. While the median ICC was of 110 cells/ml and the median HNA/LNA was of 0.47 in the CCT outlet of the WTW, the ICC ranged from 536 to 38,043 cells/ml and HNA/LNA from 0.08 to 39.98 in the closest SRs (5km) and from 7,168 to 11,692 cells/ml and 0.75 to 231.6, respectively, in the furthest (distanced by 10.5km) (Figure 4-3:), suggesting substantial variability in the microbial population and its metabolic activity within this DWDS.

Table 4-1: Summary of the cell counts (ICC) and HNA/LNA ratios recorded during the one-year monitoring of the CCT and the associated SRs. Measurements are recorded at their respective outlet. The median and Interquartile range is given. If the shape or material of the SR is not mentioned, it means that they are rectangular and in concrete.

CCT and Service reservoirs	Median (IQR) Cell counts at the outlet (cells/ml)	Median (IQR) HNA/LNA at the outlet
CCT	110 (40-305)	0.47 (0.04 - 1)

Distance from WTW: 5 km		
SRO-1A	2,470 (370-68,515)	5.0 (3.5-8.8)
SRO-1B (circular)	260 (200-700)	1.75 (0.93-5.2)
Distance from WTW: 5.5 km		
SRO-2A	260 (125-595)	0.83 (0.38-2.0)
SRO-2B (stainless steel)	310 (120-1,090)	0.67 (0.32-1.5)
SRO-2C	350 (225-925)	2.8 (0.70-4.5)
Distance from WTW: 6 km		
SRO-3	260 (100-620)	1 (0.50-2.9)
Distance from WTW: 10.5 km		
SRO-4A	4,460 (720-8,980)	5.1 (2.4-13.6)
SRO-4B	4,270 (695-15,925)	7.5 (4.4-18.6)
SRO-4C	1,485 (415-5,600)	5.9 (2.5-12.8)

SRO-1A had substantially greater ICC (2,470 cells/ml) and HNA/LNA (5,0) compared to SRO-1B (1,104 cells/ml and 1.75, respectively), revealing significant ($p < 0.05$, Mann Whitney U test) differences in microbial abundance and metabolic activity between the two reservoirs. The higher HNA/LNA ratio in SRO explains the high cell counts, as HNA is known for promoting microbial growth (refs). However, both service reservoirs are at equal distance of the CCT, so they would be expected to have similar microbial trend. The significant difference can be explained by either the shape of the reservoirs, as SRO-1A is rectangular, whereas SRO-1B is circular, the chlorine residual, the water age, or the retention time. **Error! Reference source not found.** shows that both SRs had similar chlorine residuals (0.5 mg/L), ruling out chlorine as the primary factor and

suggesting that SR shape, water age, or retention time played a more significant role in the observed microbial differences.

Reservoir shape influences hydrodynamics and water mixing patterns, which can impact disinfectant distribution and microbial growth. Rectangular reservoirs (SRO-1A) may have areas of stagnation where microbial accumulation occurs, whereas circular reservoirs (SRO-1B) generally promote better flow dynamics and reduced sedimentation (LeChevallier & Au, 2004). Water age and retention time also affect microbial stability. Longer retention times can lead to chlorine dissipation, increased biofilm detachment, and greater microbial regrowth (Prest et al., 2016). If SRO-1A had a longer retention time than SRO-1B, it could explain the higher ICC and HNA dominance, as longer water residence promotes microbial proliferation.

The SRO-2s (distanced of 5.5 km) and SRO-3 shared similar cell counts and HNA/LNA ratio (Figure 4-2 C-D), except from SRO-2C who showed a significantly higher HNA/LNA ratio (2.8) ($p < 0.05$, Mann Whitney U test). This suggests that the microbial metabolic activity in SRO-2C is higher, despite similar total cell counts and chlorine residuals (0.5 mg/L) across these reservoirs, eliminating chlorine residual as a primary driver of the observed difference. This elevated HNA/LNA ratio in SRO-2C indicates a shift towards a more metabolically active microbial population, likely due to localized environmental conditions such as hydraulic dynamics, nutrient availability, or biofilm interactions. Retention time may also play a role, as longer residence times can favour HNA cells, which are typically more responsive to available organic carbon and other nutrients (Hammes & Egli, 2005). Additionally, differences in water flow and mixing patterns within the reservoir could influence microbial stratification, potentially leading to higher HNA dominance in certain zones (Prest et al., 2016).

The SRO-4s (distanced of 10.5km) had significantly ($p < 0.05$, Mann Whitney U test) higher cell counts (1,485 – 4,460 cells/ml) and HNA/LNA (5.1 – 7.5) than all the other SRs. It also showed lower chlorine residual (0.3mg/l, Figure 4-2), suggesting that the combination of high HNA and low chlorine residual were the driver for microbial abundance (and hence microbial regrowth). HNA cells are

known to be more metabolically active and can proliferate more effectively under conditions of declining disinfectant levels and increased organic carbon availability (Hammes & Egli, 2005).

The comparison of cell abundance with seasonal temperature fluctuations (Figure 4-2) demonstrated that SRO-4s, along with SRO-1s and the CCT, followed temperature variation trends, while SRO-2s exhibited a delayed response of a few weeks. This suggests that temperature plays a major role in microbial dynamics within these SRs, likely influencing HNA cells metabolism, chlorine decay rates, and overall microbial proliferation (Prest et al., 2016).

The lagged response in SRO-2s may be explained by differences in water retention time, mixing conditions, or localized variations in organic carbon availability, which could delay the impact of temperature fluctuations on microbial regrowth. Longer retention times may buffer temperature changes, resulting in a delayed microbial response compared to SRs with more direct exposure to seasonal variations (Lehtola et al., 2004).

These findings emphasize that HNA/LNA ratios alone do not fully explain microbial regrowth in DWDS, as multiple factors—chlorine residuals, temperature, hydrodynamics, retention time, and biofilm interactions—also play significant roles. The complexity of microbial dynamics in DWDS necessitates expanded monitoring at multiple sampling points, particularly at SR inlets, to better assess microbial changes during water storage and distribution (Dorinda, 2019). Further research is needed to understand how retention time and hydraulic mixing influence microbial responses, especially in mid-distance reservoirs like SRO-2s. These insights are crucial for optimizing seasonal disinfection strategies and ensuring microbial stability in drinking water systems.

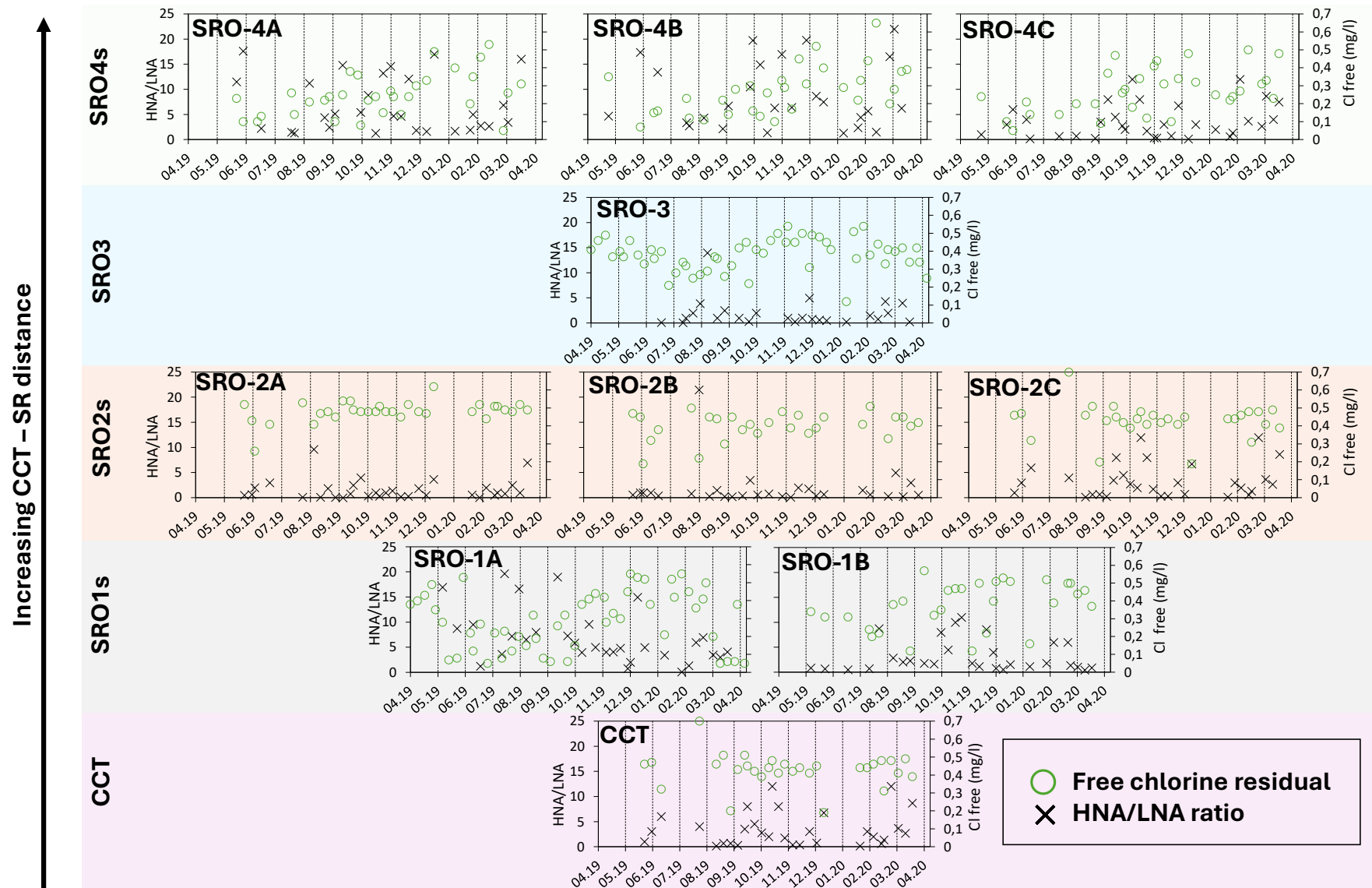


Figure 4-2 Ratio of HNA and LNA of ICC and residual chlorine at the outlet of each SRO and the CCT.

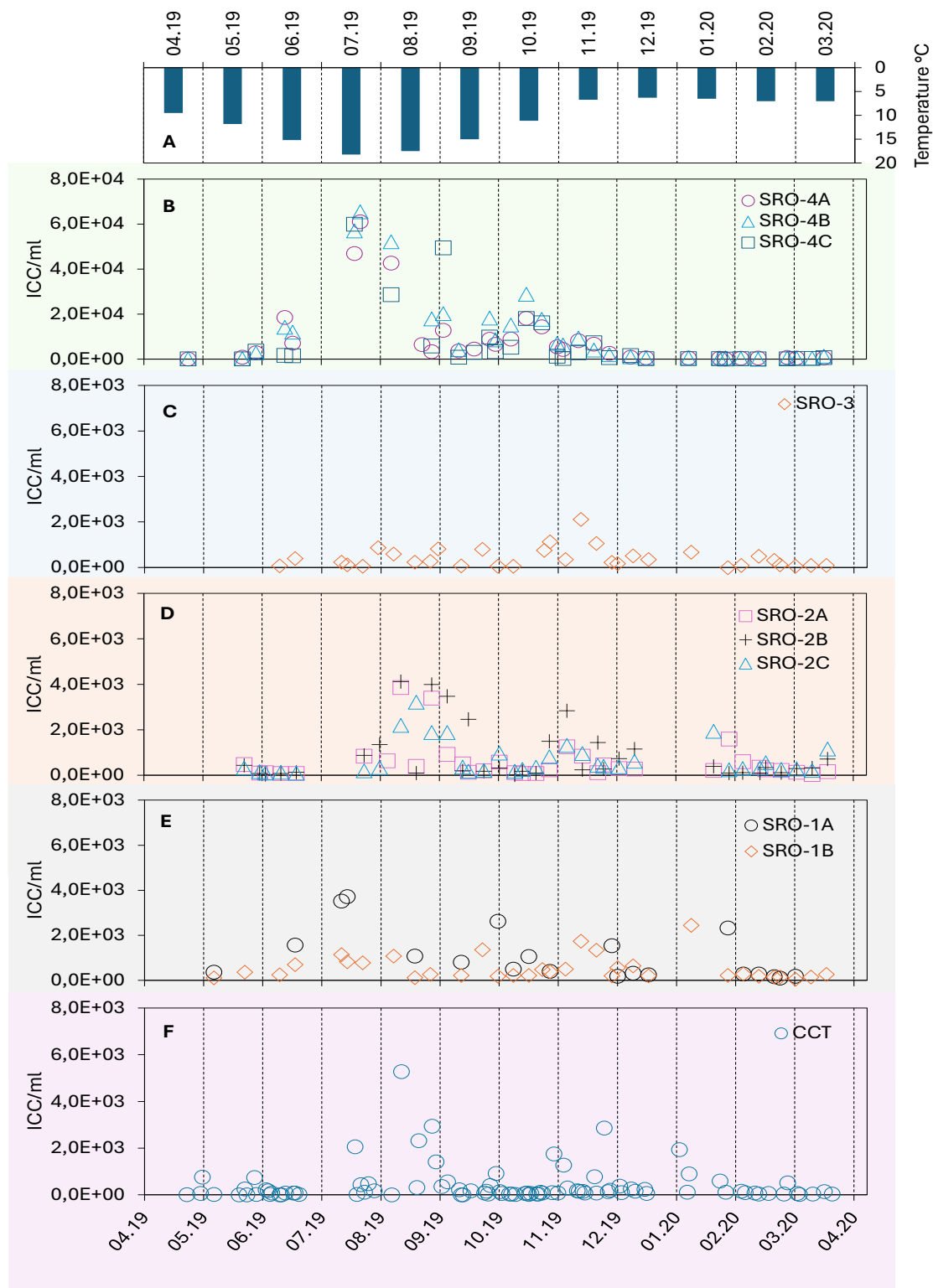


Figure 4-3: The ICC at CCT and Service Reservoir Outlets (SRO). A. The histogram represents the monthly average air temperature in the supply region. **B. C., D., and E.** are ICC for each SROs located at a distance of 5, 5.5, 6, and 10.5 km respectively of the CCT. The graph **F.** depicts the CCT which feeds each of these SR

The RF²⁵ analysis, incorporating the previous factors influencing microbial growth in SRs, revealed distinct trends based on distance from the CCT (Table 4-2). The nearest SRs were primarily influenced by the cell count at the CCT outlet (RF = 0.429 – 0.658, $p < 0.05$), suggesting that microbial dynamics in these reservoirs closely reflect conditions at the treatment plant, with limited regrowth or external influences. Conversely, the furthest SRs were strongly influenced by the seasonal temperature (RF = 0.450 – 0.547, $p < 0.05$), indicating that temperature-driven microbial activity plays a more dominant role in microbial regrowth at greater distances. This aligns with findings that longer retention times allow for greater microbial adaptation to environmental conditions, particularly temperature fluctuations (Prest et al., 2016).

As for SRO-1A, it was primarily influenced by temperature fluctuations (RF = 0.51, $p < 0.05$), providing more insight into the previous observation that reservoir shape (rectangular vs. circular) influences microbial regrowth. The rectangular design of SRO-1A may contribute to thermal stratification and stagnation zones, where localized heating promotes microbial activity, unlike circular reservoirs, which typically have better flow distribution and less temperature variation (LeChevallier & Au, 2004).

Regarding SR-2C, it was primarily influenced by HNA/LNA ratio (RF = 0.450, $p < 0.05$), providing more insight into the previous observations, and suggesting that microbial metabolic activity, rather than total cell count, played a key role in its microbial dynamics. A high HNA/LNA ratio typically indicates a greater proportion of metabolically active bacteria, which may have been promoted by localized variations in nutrient availability, hydraulic conditions, or biofilm interactions (Hammes & Egli, 2005).

In contrast, SRO-2B was equally influenced by cell count at the CCT outlet (RF, $p=$) and the HNA/LNA ratio (RF = 0.734, $p < 0.05$), suggesting a combined effect of initial microbial loading from the treatment plant and localized microbial shifts over time. This may indicate that SRO-2B experiences both external microbial

²⁵ Random Forest

inputs from the incoming water and internal biological processes that drive shifts in microbial composition.

These findings highlight that microbial growth in DWDS is not solely determined by initial cell counts or disinfection but is shaped by a complex interplay of environmental and operational factors. While proximity to the CCT influences microbial loading, temperature, retention time, and microbial metabolic state (HNA/LNA ratios) become increasingly important at greater distances. Future studies should focus on longitudinal monitoring of microbial composition and metabolic activity, integrating real-time temperature and hydraulic data to improve microbial risk assessment and optimize water quality management in DWDS.

The findings partially support the hypothesis stated that HNA regrowth in DWDS would signal a reduction in chlorine residual, but they cannot not universally apply it across all SRs. While SRO-4s (10.5 km from the WTW) had the highest HNA/LNA ratios (5.1–7.5) and also the lowest chlorine residual (0.3 mg/L) and RF analysis showed that distant SRs were influenced more by seasonal temperature than by chlorine residuals, some SRs had high HNA/LNA ratios despite maintaining moderate chlorine residuals and SRO-2B was equally influenced by both initial microbial load (CCT outlet) and HNA/LNA ratio.

Therefore, the findings partially support the hypothesis—since HNA regrowth was observed in SRs with lower chlorine residuals (e.g., SRO-4s)— but it is not the sole determining factor. Other environmental and operational conditions (temperature, retention time, biofilms, and hydraulic dynamics) also contribute to microbial shifts in DWDS.

Table 4-2: Spearman's Rank Correlation Coefficient and Random Forest (RF) to identify relationships influencing variability in ICC at the SR outlet parameters include the ICC from CCT, residual chlorine, temperature, HNA-LNA ratio.

The highlighted cells, shows the parameters contributing the most to the variation of ICC at the SRO

			ICC from CCT	residual chlorine	Temperature	HNA/LNA
SRO-1A	Spearman	ICC from SRO-1A	0.24, p=0.21	-0.32, p=0.10	0.51, p<0.05	0.22, p=0.27
	RF		0.068	0.375	0.475	0.083
SRO-1B	Spearman	ICC from SRO-1B	0.60, p<0.05	-0.46, p<0.05	0.32, p=0.11	0.10, p=0.61
	RF		0.658	0.045	0.109	0.188
SRO-2A	Spearman	ICC from SRO-2A	0.48, p<0.05	-0.21, p=0.30	0.36, p=0.064	-0.26, p=0.19
	RF		0.597	0.131	0.077	0.194
SRO-2B	Spearman	ICC from SRO-2B	0.51, p<0.05	0.11, p=0.59	0.023, p=0.91	-0.36, p<0.05
	RF		0.429	0.033	0.088	0.450
SRO-2C	Spearman	ICC from SRO-2C	0.69, p<0.05	-0.26, p=0.20	0.096, p=0.64	-0.41, p<0.05
	RF		0.079	0.106	0.082	0.734
SRO-3	Spearman	ICC from SRO-3	0.59, p<0.05	-0.58, p<0.05	0.34, p=0.12	0.28, p=0.21
	RF		0.227	0.144	0.515	0.114
SRO-4A	Spearman	ICC from SRO-4A	0.16, p=0.26	-0.36, p<0.05	0.71, p<0.05	0.0074, p=0.96
	RF		0.135	0.121	0.547	0.197
SRO-4B	Spearman	ICC from SRO-4B	0.22, p=0.10	-0.52, p<0.05	0.78, p<0.05	-0.042, p=0.76
	RF		0.055	0.338	0.451	0.156
SRO-4C	Spearman	ICC from SRO-4C	0.20, p=0.15	-0.45, p<0.05	0.65, p<0.05	0.27, p=0.055
	RF		0.062	0.204	0.450	0.285

Distance from CCT



4.3.2 Assessing the contribution of HNA/LNA Ratio on understanding microbial regrowth in DWDS

This subsection is about the findings from the setup 1 at section 4.2.2.1.

The daily monitoring of water samples with increased HNA/LNA ratio (1/0, 0.75/0.25, 0.5/0.5, 0.25/0.75, 0/1) show the contribution of both subpopulations to the microbial growth. The results revealed that none of HNA or LNA account for the increased cell count. However, microbial communities, identified by their fluorescence on the FL1 channel, within both subgroups were found to correlate with increased cell counts at various stages of the growth curve reflecting on the oversimplification of HNA and LNA.

A higher HNA presence does not necessarily correlates with higher microbial growth. On Day 0, the ICC varied from 2033 to 2502 cells/ml, with HNA/LNA ratios between 0.44 and 4.08. By Day 5, these concentrations increased to 48,950 - 4.20×10^6 cells/ml, and HNA/LNA ratios increased to 1.03 - 33.46. The sample with an initial ratio of 1/0 (HNA/LNA) had the lowest growth rate (7,819 cells/day). The sample experienced an 80% decrease in intact cells from Days 0 to 2, followed by a 123% increase from Days 2 to 5 (Figure 4-4B). Notably, this sample did not display an increase in the HNA cells until Day 4, as shown in Figure 4-4A and B. A pairwise comparison using Dunn's test revealed that the dataset from the 1/0 (HNA/LNA) sample was significantly different from the datasets of the other samples ($p < 0.066$, Figure 4-4C, D). This suggests that HNA dominance alone does not drive microbial proliferation and that LNA cells may play a critical role in early-phase microbial regrowth. The initial decline in intact cells followed by a delayed increase in HNA cells indicates that LNA populations may contribute to microbial survival under initial stress conditions, such as chlorine residuals, potentially transitioning into HNA-dominated communities over time. This aligns with previous studies suggesting that LNA cells, though considered less metabolically active, can persist under unfavourable conditions and later shift to an HNA phenotype under improved environmental conditions (Prest et al., 2016).

Contrastingly, samples with higher LNA presence (ratios of 0.75/0.25, 0.5/0.5, 0.25/0.75, and 0/1 of HNA/LNA) exhibited higher growth rates ranging from 1.95×10^5 to 5.26×10^5 cells/day, peaking between 2.05×10^5 - 7.87×10^5 cells/ml on Day 2 (164% increase), before ICC stabilisation. These samples showed rapid microbial growth from Day 1 to Day 2, until the two microbial communities emerged on Day 2, followed by a third community on Day 3 (Figure 4-4A). The microbial communities exhibited different FL1 fluorescence intensities, suggesting physiological or functional differentiation over time. The sample also exhibited more dynamic HNA/LNA ratios with a growth ranging from 3.35×10^3 to 3.35×10^4 % between Day 0 and Day 2. From Day 2 until Day 4, growth rate declined leading to a loss of 94 - 96%. After Day 4, growth seemed to stabilize as increase was of 0.3 - 40% (Figure 4-4A, B). This suggests that LNA-dominated communities exhibit an early advantage in microbial regrowth, likely due to their resilience under initial environmental stress. The initial rapid expansion of LNA populations may contribute to ecosystem establishment, followed by a shift towards HNA dominance as conditions become more favourable for metabolically active cells. The observed community differentiation further supports the idea that LNA and HNA cells play complementary roles in microbial succession, with LNA populations persisting under stress and HNA cells dominating once conditions improve (Prest et al., 2016).

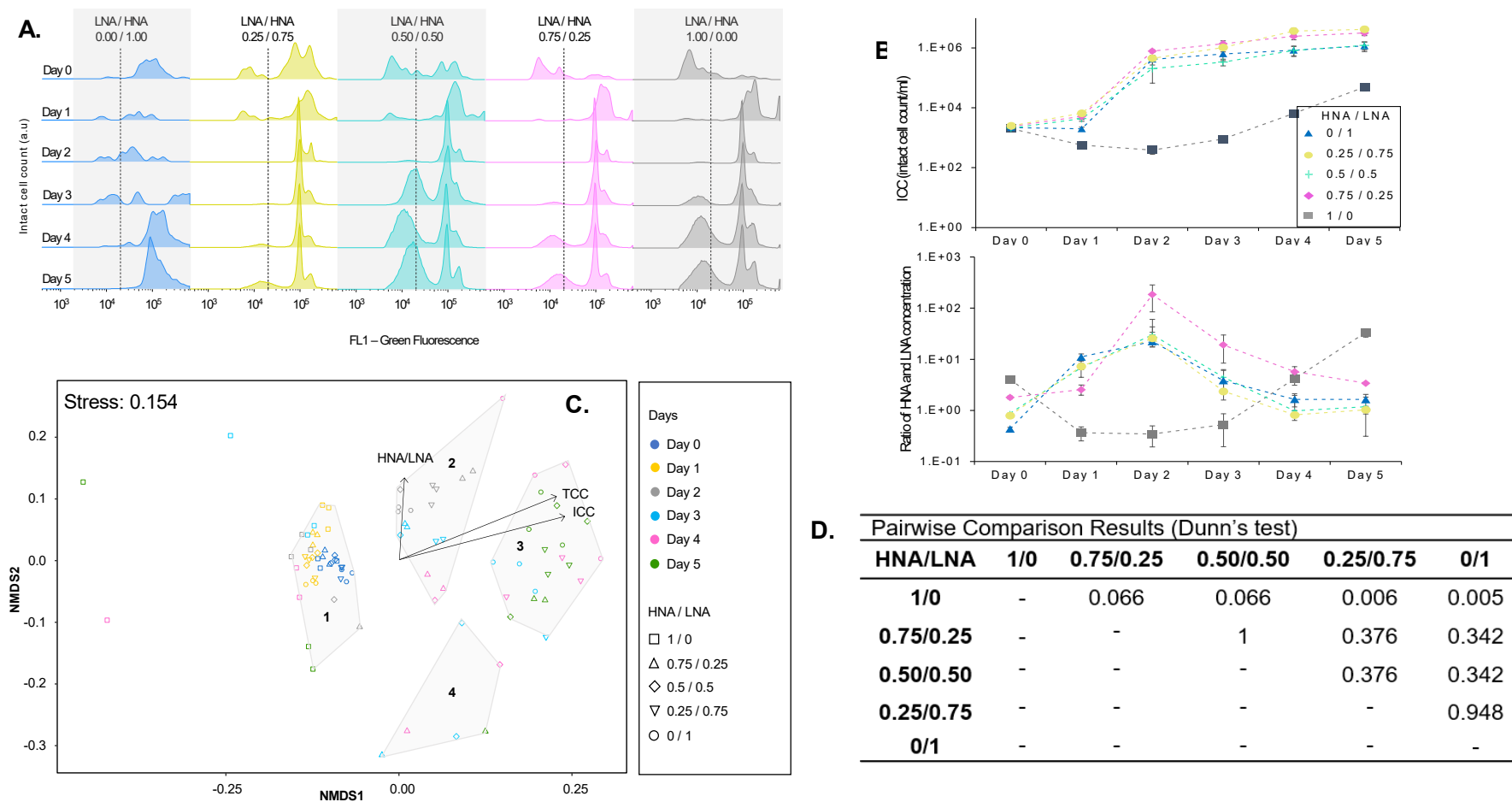


Figure 4-4 Experimental measurements of the daily growth of intact cell count (A) and their corresponding HNA/LNA ratio (B) as a function of their initial HNA/LNA ratio (1-0, 0.75-0.25, 0.5-0.5, 0.25-0.75, 0-1). Error bars show the error on the mean of repeat measurements. Results of Dunn's Test for Pairwise Comparison of the resulting daily intact cell growth for different LNA and HNA ratio. For clarity, a single replicate's fluorescence fingerprint is shown in A, while cell concentration and HNA/LNA ratio data represent mean values of repeats, with error bars indicating mean concentration errors A and mean HNA/LNA ratio errors in B

CHIC analysis followed by a cluster analysis was performed. Cluster analysis grouped the samples' daily measurements into three clusters that shows a positive correlation with the NDMS 1 axis. The ICC and TCC vectors also correlated positively on this axis, suggesting cell concentration as a key differentiator in the observed daily measurements. The HNA/LNA ratio had a negligible impact on the NMDS1 axis but correlated weakly with the NMDS2 axis. See Table 4-3 for the statistical appreciation of the clustering.

The resulting nMDS plot showed that all the daily measurements for the 1/0 (HNA/LNA) sample clustered together, implying no significant change over time. This suggests that the microbial community remained relatively stable, with limited shifts in composition or metabolic activity throughout the experiment. Conversely, for samples with higher LNA presence (ratios of 0.75/0.25, 0.5/0.5, 0.25/0.75, and 0/1 of HNA/LNA) a clear temporal growth pattern could be observed, as their respective measurements progressively shifted along the nMDS1 axis driven by the ICC vector. This indicates that microbial communities in LNA-dominated samples underwent dynamic changes over time, likely influenced by microbial succession, metabolic shifts, or environmental adaptation.

The previously observed microbial communities were correlated with cell abundance (ICC) using a Spearman test. The test revealed a strong monotonic correlation ($r = 0.9927$, $p < 0.001$) between HNA/LNA and ICC for the 1/0 (HNA/LNA) sample, suggesting that in a purely HNA-dominated environment, microbial regrowth is directly proportional to the abundance of HNA cells.

For the other samples (ratios of 0.75/0.25, 0.5/0.5, 0.25/0.75, and 0/1 of HNA/LNA) there was no correlation between HNA/LNA and ICC; however, the Spearman's test revealed significant positive correlations between the HNA/LNA ratio and the three observed microbial communities (Community 1: $r = 0.96 - 0.51$, $p < 0.05$; Community 2: $r = 0.50 - 0.93$, $p < 0.05$; Community 3: $r = 0.86 - 0.93$, $p < 0.05$ (Figure 4-4C). This suggests that in mixed microbial communities, HNA/LNA alone does not explain variations in cell abundance, but rather, the presence and interactions of specific microbial communities drive changes in the

cell count. These correlations imply that microbial growth patterns are more complex, with different microbial groups contributing variably to overall microbial population shift over time, highlighting the oversimplification of HNA/LNA.

Furthermore, the application of a Random Forest analysis (RF) indicated that Community 1 had the most substantial impact on the HNA/LNA ratio (RF importance ranges: 0.37 - 0.55), followed by Community 3 (0.18 - 0.41), and then Community 2 (0.06 - 0.45), suggesting that certain microbial groups play a stronger role in driving shifts in cell abundance.

These findings highlight that cell abundance are not solely dictated by HNA/LNA ratios but are influenced by the composition and metabolic activity of distinct microbial subpopulations. Understanding these dynamics is essential for improving microbial monitoring strategies in DWDS, as changes in community may precede overall shifts in microbial stability and water quality.

4.3.3 Assessing the contribution of HNA/LNA in different treatment stages

This subsection is about the findings from the setup 2 at section 4.2.2.2.

Daily monitoring of water samples from different treatment stages showed HNA/LNA ratio's impact on microbial growth. The results confirmed prior findings from paragraph 4.3.2 stipulating that none of HNA or LNA account for the increased cell count. It also revealed that microbial communities promoting cell growth differ between processes, reflecting the dynamic nature of microbial communities in WTWs. Therefore, due to this dynamism, using HNA/LNA ratios alone oversimplifies the complex microbial phenomena at play.

The starting ICC was different between the different treatment stages (Figure 4-5). Post-GAC had 3,012 cells/ml, post-RGF 21,460 cells/ml, post-CCT water had 159 cells/ml (<LOD), and the SRO 88 cells/ml (<LOD). The HNA/LNA ratio was the highest in post-GAC 6.82, indicating the dominance of HNA cells, whereas post-CCT showed the lowest ratio at 0.19, suggesting LNA cells had greater abundance after chlorine inactivation.

On Day 1 of the experiment, all samples, except for post-GAC, exhibited a significant increase in ICC with growth ranging from 1.51×10^3 to $8.56 \times 10^5\%$ (Figure 4-5B). Post-GAC, however, showed a decrease in ICC by 57.9%. Similarly, the HNA/LNA ratio increased for all samples. SRO showed the largest increase (between 1.46×10^4 and $2.38 \times 10^5\%$), while post-GAC had the smallest increase (34%). These results suggest that microbial regrowth potential varies across treatment stages. While post-CCT and SRO samples, initially low in ICC and higher in LNA, exhibited rapid microbial proliferation, post-GAC, supporting a metabolically active HNA-rich community, exhibited a slow microbial growth. The delayed response in post-GAC may indicate stronger microbial resilience and adaptation mechanisms compared to other treatment stages. Furthermore, when comparing with the findings from the previous paragraph, the % of HNA presence in post-GAC water may reflect a poor environment for which microbial growth is inhibited, such as resource limitations, competitive interactions, or the presence of antimicrobial byproducts. Excessive HNA dominance could lead to increased competition for nutrients, causing a decline in overall microbial proliferation (refs). This suggests that while HNA-rich environments are typically associated with increased microbial abundance (refs), excessive HNA dominance does not always equate to higher growth potential.

On Day 2, post-GAC sample had a significant ($p < 0.05$) increase in ICC and HNA/LNA ratio ($4.37 \times 10^4\%$ and $1.23 \times 10^3\%$, respectively) (Figure 4-5B). By Day 4, the HNA/LNA ratio and the ICC for all samples stabilised (10 and 10^5 cells/ml, respectively) indicating a state where microbial communities reached equilibrium, suggesting that growth rates balanced with resource consumption and environmental constraints.

CHIC analysis (see Table 4-3B for the statistical appreciation of the analysis) revealed that the distinct microbial communities associated with each treatment stage were largely retained over time (Figure 4-5C), suggesting that the microbial community at each stage remained relatively stable which is in accordance with the observed cell abundance and HNA/LNA stabilisation. This shows that microbial succession within the treatment system follows a structured pattern,

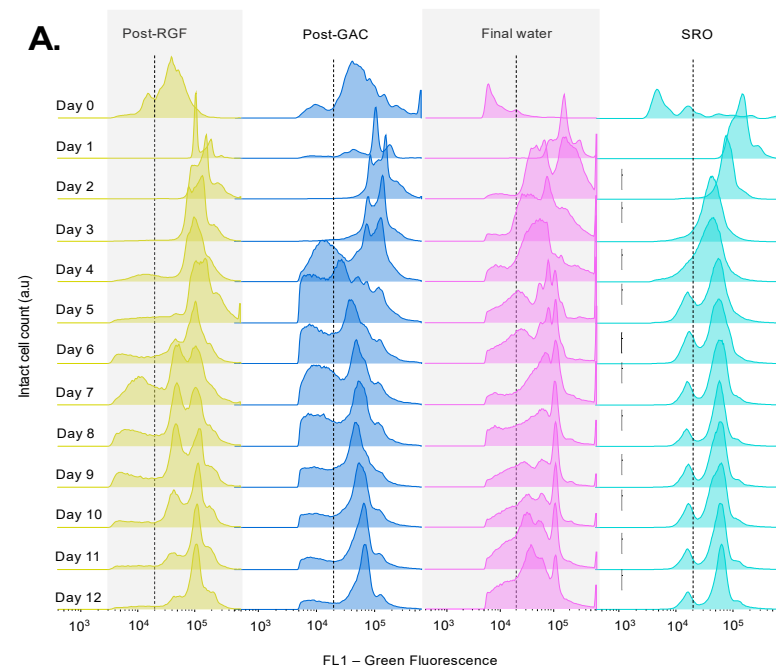
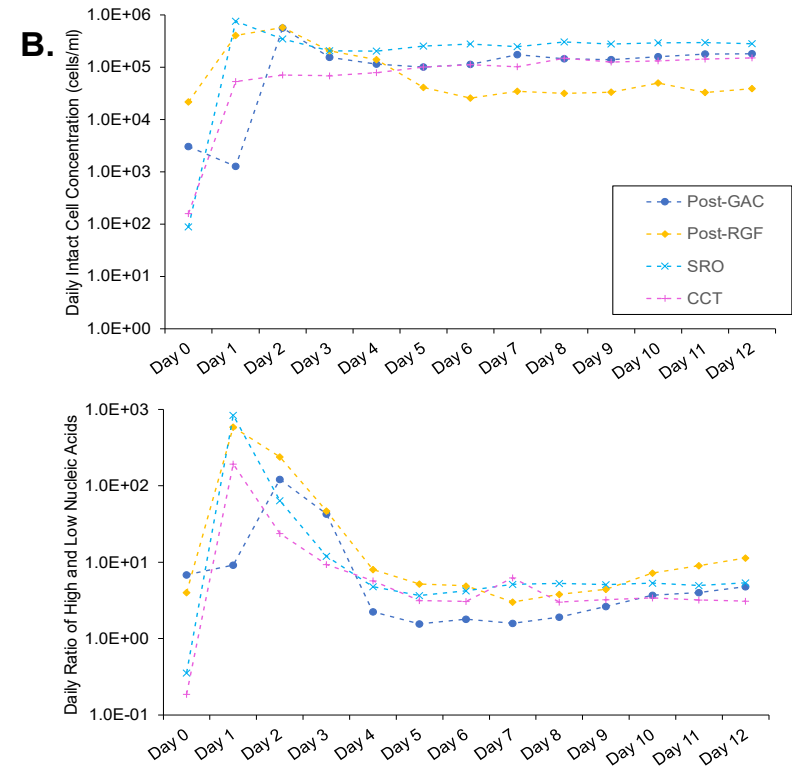
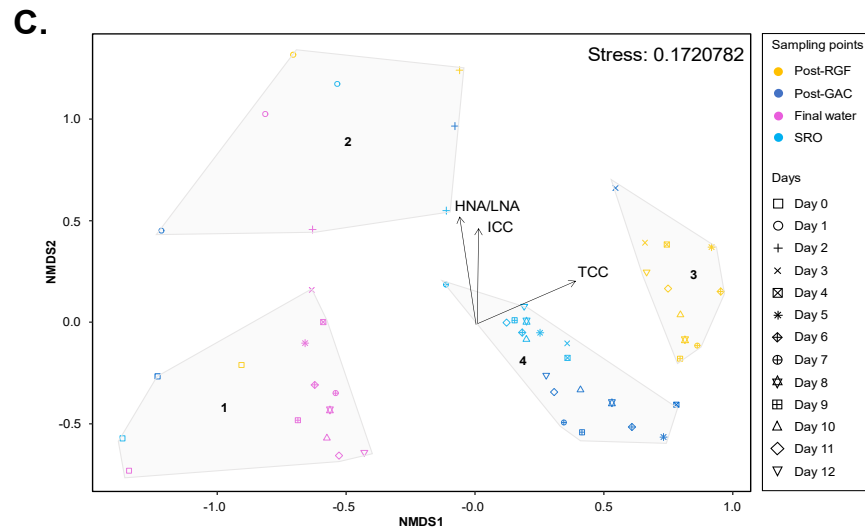
where once established, microbial communities persist. The stability observed indicates that environmental factors such as nutrient availability, disinfection processes, and biofilm interactions play key roles in regulating microbial community composition over time (Q. Li et al, 2017).

There was a significant overlap ($p < 0.05$, Figure 4-5D) between post-RGF and service reservoir as they shared the same cluster (Figure 4-5C), which could indicate similar microbial communities in the populations post-treatment. This suggests a microbial succession from the treatments to the DWDS. The fact that final water is separate from this cluster could be explained by the higher presence of chlorine residual which play a role in the fluorescence of the cells, hence the CHIC analysis.

Through daily monitoring of the FL1 fluorescent profile of each sample (Figure 4-5), five distinct cell communities were identified, labelled as C1, C2, C3, C4, and C5 (Figure 4-6) with their fluorescence intensities spanning ranges from 2.2k-26k for C1, 26k-48k for C2, 48k-88k for C3, 88k-163k for C4, and 163k-612k for C5 (Figure 4-6A). Spearman's test identified significant correlations ($p < 0.05$) between the HNA/LNA ratio and certain cell communities in each sample type. Post-RGF samples correlated with Communities 1, 4, and 5 ($p < 0.05$), Post-GAC with Community 1 ($p < 0.05$), and Final water with Community 5 ($p < 0.05$). In the Random Forest analysis, different drivers were identified for each sample type: Post-RGF was primarily influenced by Communities 1 and 2 (importance scores of 0.27 and 0.28, respectively), Post-GAC by Community 4, Final water by Community 5, and SRO by Communities 1, 4, and 5 (Figure 4-6B). This suggests that distinct microbial communities are associated with specific treatment stages and persist through different stages of water processing and distribution. The observed correlations between cell abundance and microbial communities highlight that cell count fluctuation is influenced by both treatment stage and microbial communities.

The strong influence of Community 5 in final water and SRO samples suggests that this microbial group is highly resilient and may persist despite disinfection efforts, potentially contributing to post-treatment regrowth. Similarly, the

dominance of Community 4 in post-GAC samples implies that this population may be adapted to the specific conditions of granular activated carbon treatment, possibly benefiting from biofilm formation or nutrient retention (Lehtola et al., 2004).



D. Pairwise Comparison Results (Dunn's test)

Process	RGF	GAC	CCT	SRO
RGF	-	0.574	0.826	<0.05
GAC	-	-	0.574	0.082
CCT	-	-	-	<0.05
SRO	-	-	-	-

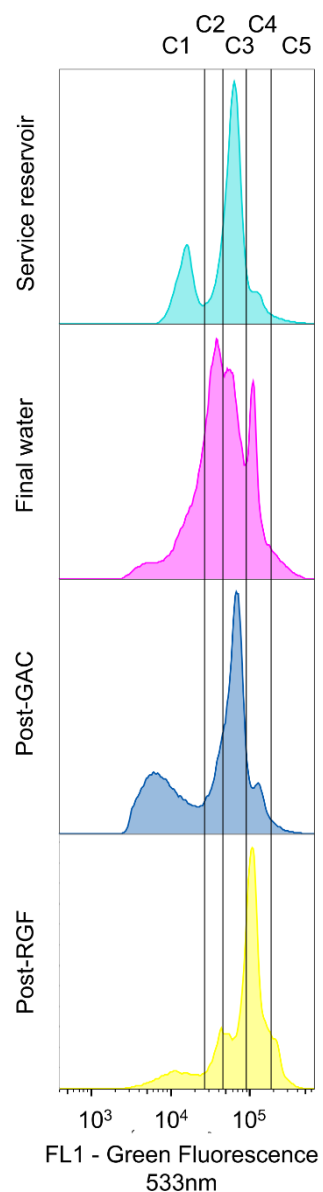
Figure 4-5 Experimental measurements of the daily cell growth of water coming from different stages of a WTW (Post-RGF, Post-GAC, Post-CCT) and a SRO. A. the daily fluorescence fingerprint of each sample. The intact cell count variation on the y-axis scaled for each sample to visualize the microbial communities and their fluorescence. B. the daily variation of the ICC (top) and HNA/LNA ratio (bottom). C. NMDS plot obtained from a CHIC analysis to evaluate the dissimilarities and similarities between and within the samples. The environmental vectors (ICC, TCC, HNA/LNA ratio) were included to determine the factors influencing the dissimilarities, 2D stress is indicated. D. Pairwise comparison results to compare fingerprints between the different source waters after nutrient additional and regrowth experiment. For clarity, a single replicate's fluorescence fingerprint is shown in A, while cell concentration and HNA/LNA ratio data represent mean values of repeats, with error bars indicating mean concentration errors A and mean HNA/LNA ratio errors in B

This data reveals distinct microbial communities across different treatment types, a phenomenon well-documented by Favere et al., (2020) who explored how the source of water influences microbial community structures. Additionally, the influence of treatment on microbial diversity is evident, as demonstrated by Ma et al. (2020) who examined the effects of treatment and distribution processes on microbial diversity in drinking water. Furthermore, the temporal shifts in microbial populations, one of the key findings of this study, align with findings by (Coggins et al., 2020) who investigated the spatiotemporal shifts in pond communities over time. This underscores the complexity and dynamism of microbial ecosystems within water treatment systems, a topic explored in depth by many (Berry et al., 2006; Douterelo et al., 2014, 2019). Moreover, the utility of CHIC analysis for monitoring these variations is highlighted, echoing the conclusion of (Safford & Bischel, 2019a) who reviewed the applications of flow cytometry in water treatment and monitoring. The studies collectively emphasize the intricate interplay of microbial communities in drinking water systems and the importance of advanced monitoring techniques like flow cytometry.

These findings reinforce that microbial shifts in DWDS are not solely dictated by HNA/LNA but are driven by specific microbial groups with different metabolic and survival strategies. Further investigation into the functional roles of these microbial communities is essential to determine their impact on water quality, biofilm formation, and potential risks for microbial stability in distribution networks.

The hypothesis stating that HNAs will regrow quickly during periods of nutrient addition was supported by these experiments, but it was also observed that in the presence of stressors (e.g. chlorine), HNA will not grow directly and will let LNA take the lead until the environment becomes favourable again.

Additionally, the hypothesis stipulating that LNAs will regrow slowly after readily biodegradable nutrients are utilized can also be confirmed as the experiments showed that LNAs started to grow only after a few days of the nutrient spike, probably reflecting when nutrients become less available.



Spearman Rank Correlation Coefficient					
	Community 1	Community 2	Community 3	Community 4	Community 5
Post-RGF	r = -0.835, p < 0.05	r = -0.555, p = 0.049	r = 0.006, p = 0.986	r = 0.824, p < 0.05	r = 0.808, p < 0.05
Post-GAC	r = -0.879, p < 0.05	r = -0.539, p = 0.058	r = 0.187, p = 0.541	r = 0.363, p = 0.223	r = 0.330, p = 0.271
Final water	r = -0.357, p = 0.231	r = -0.159, p = 0.603	r = -0.159, p = 0.603	r = -0.066, p = 0.831	r = 0.912, p = <0.05
SRO	r = -0.484, p = 0.094	r = 0.071, p = 0.817	r = 0.093, p = 0.762	r = 0.385, p = 0.194	r = 0.374, p = 0.209
Random Forest: Score analysis					
Post-RGF	0.27	0.28	0.20	0.13	0.12
Post-GAC	0.15	0.11	0.16	0.32	0.26
Final water	0.24	0.20	0.14	0.04	0.38

SRO	0.23	0.20	0.08	0.24	0.24
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Figure 4-6 A. Fingerprint of each water at the end of the regrowth experiment. C1-C5 represent communities of cells. B. Spearman Rank Correlation Coefficient and Random Forest analysis to determine and evaluate the monotonic relationships between the daily variation of the HNA/LNA ratio and the daily variation of each community composing the HNA and LNA subpopulations of each treatment process

Table 4-3 Silhouette, WSS, and BSS/TSS values evaluating respectively the cohesion of a sample within its cluster, the compactness of the cluster, and the separation of the clusters from each other. These values were calculated for the clustering done in the experiment A: Daily growth of intact cell count and their corresponding HNA/LNA ratio (Figure 4-4C), and the experiment B: Daily cell growth of water coming from different stages of a WTW (Figure 4-5C).

A	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Silhouette	0.55	0.45	0.47	0.44
WSS	0.42	0.13	0.14	0.06
BSS / TSS	75%			
B	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Silhouette	0.54	0.38	0.49	0.45
WSS	2.23	2.10	0.81	1.83
BSS / TSS	80%			

4.3.4 Future perspectives on FCM as a monitoring technique for DWDS

The present study critically examines the contribution of HNA/LNA for the understanding of microbial regrowth in DWDS. Through a systematic approach, the research emphasizes the utility of FCM in monitoring microbial nucleic acid content, differentiating between HNA and LNA microbial populations. FCM's ability to provide rapid and precise measurements renders it an effective complement to traditional culture-based methods, particularly in the context of active bacteria resistant to disinfection and those with propensity to regrow within DWDS during periods of elevated nutrients or temperature or inadequate disinfectant residual. Over a year-long period, cell concentration and viability were monitored weekly across a CCT and SROs. These SROs, despite receiving water treated similarly from the same WTW demonstrated variability in microbial populations, suggesting the influence of environmental factors such as temperature and water age on microbial levels with the ratio of HNA/LNA emerging as a promising indicator of regrowth in DWDS. The study revealed seasonal trends, with a lagged increase in microbial counts in the SROs, implying that elevated water storage times could drive regrowth primarily of HNAs subpopulation.

The study's findings underscore the dynamic nature of microbial communities in DWDS, reflecting the influence of treatment and storage on microbial diversity. This is evidenced by the distinct microbial communities, identified by their distinct FL1 fluorescence, across different treatment processes, which were tracked for temporal shifts, indicating the complexity and dynamism of microbial ecosystems within water treatment systems. The research further highlights the limitations of traditional culture-based methods, which are time-consuming, strain-specific, and economically impractical for routine monitoring. In contrast, FCM provides a more efficient alternative for assessing active microbial populations, aiding in proactive water safety management and adherence to health regulations. Moreover, the study points to the challenges in water quality assessment, noting that microbial

population in drinking water is complex and may not be fully captured by indirect assessments such as nucleic acid content (HNA/LNA).

The novelty of this work lies on the real-world application of the HNA/LNA metrics across different service reservoirs, with different characteristics (material, shape, distance from their WTW). The year-long application of HNA/LNA metrics conducted with a WTW and its DWDS, involving interstage sampling and analysis via FCM, reveals the impact of transient growth conditions on HNA and LNA fractions of planktonic cells for the first time.

CHIC analysis is instrumental for the precise monitoring of microbial regrowth and disinfection efficacy. This technique exploits the unique FL1 and FL3 fluorescence profiles of microbial communities, enabling a detailed analysis of their dynamics. WSS and Silhouette Values provide a robust statistical framework to evaluate the compactness and separation of clusters, essential for discerning various microbial states and interactions. Employing RF algorithm further enriches the analysis, pinpointing the factors driving changes in the microbial landscape. These advanced analytical methods streamline the interpretation process, facilitating both computer-assisted and fully automated analyses, thus enhancing water quality management by allowing for rapid, data-driven decisions.

FCM's capability for quick and practical analysis is a valuable asset for routine water quality monitoring, complementing more intricate methods of species and strain identification. Its direct monitoring potential, when used alongside CFD modelling techniques, strengthens the validation of new designs and operational strategies for DWDS. This synergy between empirical data collection and predictive modelling provides a comprehensive approach to demonstrating the impact and effectiveness of innovative interventions in water treatment and distribution, paving the way for improvements in public health protection and system efficiency.

4.4 Conclusion

This study investigated the contribution of HNA and LNA in understanding microbial regrowth dynamics. To do so, 2 experiments were conducted. The first one consisted in using HNA/LNA to monitor microbial regrowth in a real-world DWDS involving nutrient availability, and environmental conditions. The findings demonstrate that HNA/LNA ratios alone do not fully explain microbial regrowth in DWDS, as multiple factors—chlorine residuals, temperature, hydrodynamics, retention time, and biofilm interactions—also play significant roles and need to be considered.

The second experiment consisted in analysing the microbial regrowth of distinct spiked samples with increased HNA/LNA ratio (1/0, 0.75/0.25, 0.5/0.5, 0.25/0.75, 0/1) and distinct spiked samples coming from different treatment stages of the previously mentioned WTW. The findings demonstrate that, when other environmental or operational factors are not involved, microbial proliferation is not directly driven by HNA/LNA. The strong correlations between cell abundance and specific microbial communities composing the HNA and LNA (Spearman's test, $p < 0.05$) highlight that microbial growth is influenced by complex interactions between microbial communities, resource availability, and treatment processes rather than solely by HNA/LNA. This reflects the oversimplification of the HNA/LNA metric.

Through this study, the three hypotheses stated in the introduction were addressed. Firstly, the study partially supported that HNA regrowth in DWDS would signal a reduction in chlorine residual. That is not always the case as The study then supported that HNAs will regrow quickly during periods of nutrient addition, but it was also observed that in the presence of stressors (e.g. chlorine), HNA will not grow directly and will let LNA take the lead until the environment becomes favourable. Finally, the study supported that LNAs will regrow slowly after readily biodegradable nutrients are utilised as they started to grow only after few days of the nutrient spike, probably reflecting when nutrients become less available.

This study contributes to the better understanding of HNA/LNA contribution to microbial regrowth by applying the metrics in a real-world scenario, across different service reservoirs, with different characteristics (shape, material, distance from WTW), and where other environmental and operational factors are involved.

From an industry perspective, the discovery that certain microbial communities remain stable across different treatment stages suggests that appropriate and well understood design modifications (e.g., retention time adjustments, targeted flushing strategies) can improve microbial quality in DWDS.

Future studies should focus on longitudinal monitoring of the characterised microbial communities (through qPCR) and metabolic activity, integrating real-time temperature, real-time chlorine residual, hydraulic data, and other factors formerly defined, to develop predictive microbial models with the aim to improve microbial risk assessment and optimize water quality management.

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4.6 Appendix

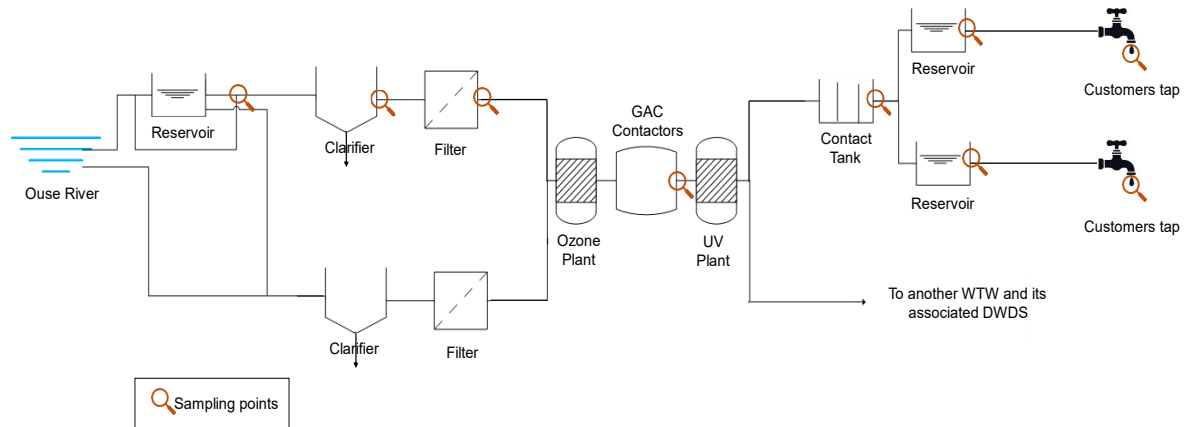


Figure S 4-1: Illustration of the WTW

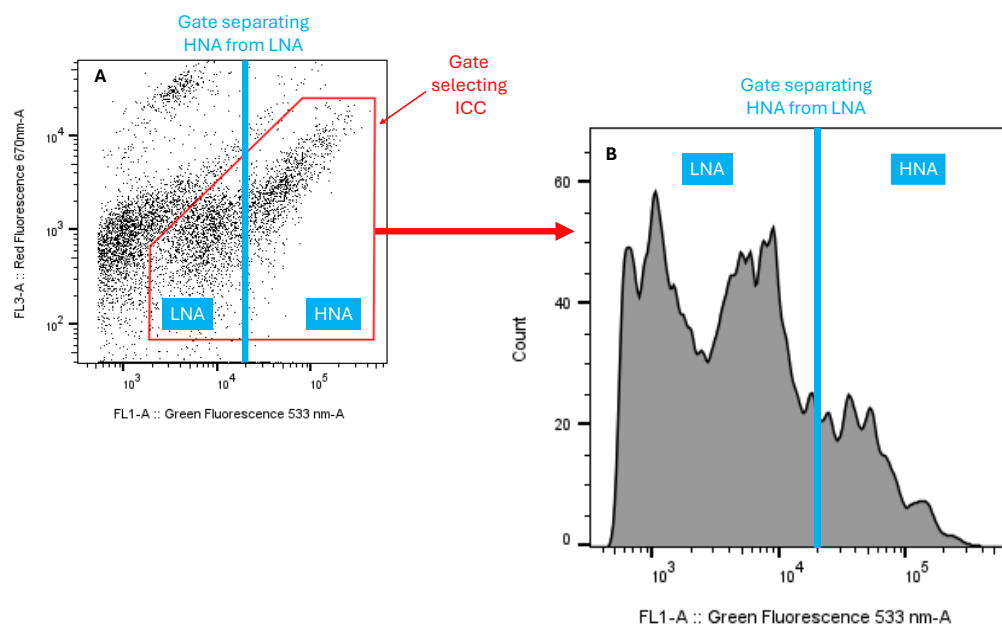


Figure S 4-2: HNA and LNA gating process

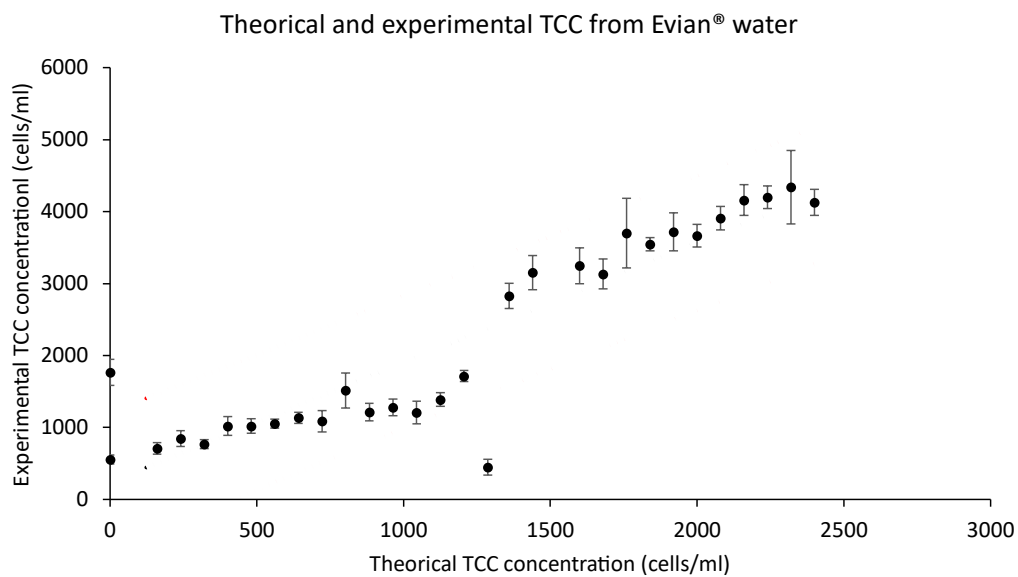
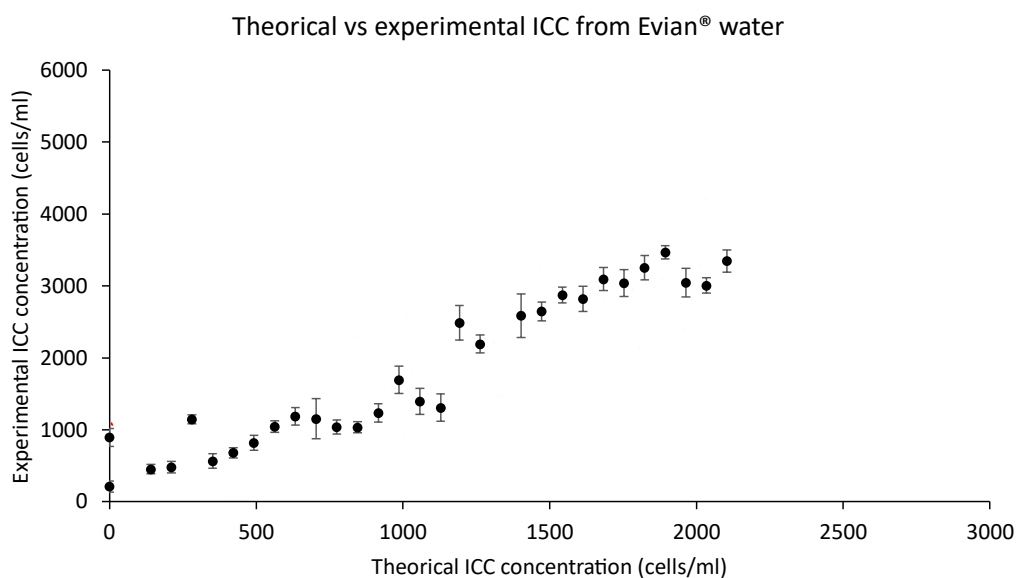


Figure S 4-3: Curve built from analysing a groundwater sample diluting with Evian water in different concentrations to determine the limit of detection (LOD) and quantification (LOQ) of FCM for TCC and ICC

Table S 4-1: Statistical tests explanation

Test	Purpose	Value and explanation
Stress (nMDS)	Evaluates how well the nMDS plot preserves the original dissimilarity structure of the data. Lower stress values indicate better representation of the data in reduced dimensions	<p>Value:</p> <p>< 0.1 → Excellent fit</p> <p>0.1–0.2 → Acceptable</p> <p>> 0.2 → Poor fit</p> <p>Interpretation: Ensures that visualized clustering in nMDS accurately represents the actual dissimilarities in microbial communities</p>
Within-Cluster Sum of Squares (WSS)	Measures cluster compactness by evaluating the total variation within each cluster. Lower WSS values indicate tighter clustering	Value: A low WSS suggests that data points within each cluster are closely related, meaning the clustering is well-defined. A high WSS suggests that clusters lack compactness
Between-Cluster Sum of Squares (BSS)	Measures dissimilarity between clusters, assessing how distinct clusters are from one another. Higher BSS values indicate better separation	Value: A high BSS relative to WSS suggests that clusters are well-separated, meaning the clustering method is effective. A low BSS suggests overlapping clusters

Silhouette Measure	Assesses within-cluster cohesion and between-cluster separation, evaluating how well each data point fits within its assigned cluster	<p>Value:</p> <p>Close to 1 → Strong clustering</p> <p>Near 0 → Possible misclassification</p> <p>Negative → Poor clustering</p> <p>Interpretation: Ensures that clusters are distinct and meaningful for microbial community analysis</p>
ANOSIM (Analysis of Similarities)	Tests whether differences between predefined sample groups are statistically significant based on their dissimilarity matrix	<p>Value: Produces an R-value (0 to 1) and a p-value</p> <p>$R \approx 1$: Strong separation between groups.</p> <p>$R \approx 0$: No meaningful difference.</p> <p>$p < 0.05$: Significant difference between groups.</p> <p>Interpretation: Confirms whether microbial community differences between treatment stages are statistically significant</p>
Spearman's Rank Correlation Coefficient	Analyses relationships between HNA/LNA dynamics and other variables, identifying non-parametric correlations	<p>Value:</p> <p>$\rho = 1$ → Perfect positive correlation</p> <p>$\rho = -1$ → Perfect negative correlation</p>

		$\rho = 0 \rightarrow$ No correlation Interpretation: Identifies whether changes in HNA/LNA ratios correlate with microbial population shifts
Random Forest (RF) Algorithm	Identifies key factors predicting microbial growth patterns and their relative importance	Value: RF importance values range from 0 to 1 $0 \rightarrow$ No influence $1 \rightarrow$ Strongest influence Interpretation: Determines which environmental or treatment variables most significantly impact microbial growth patterns
Dunn's Test	Conducts pairwise comparisons between groups to identify significant differences	Value: $p < 0.05 \rightarrow$ Significant difference $p > 0.05 \rightarrow$ No significant difference Interpretation: Determines whether differences in microbial communities between treatment stages are statistically meaningful

Mann-Whitney U Test	Compares two independent groups to assess whether one has significantly larger values than the other	<p>Value: Produces a U statistic and a p-value</p> <p>Small U, $p < 0.05 \rightarrow$ Significant difference</p> <p>Large U, $p > 0.05 \rightarrow$ No significant difference</p> <p>Interpretation: Determines whether microbial community characteristics differ significantly between treatment stages</p>
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Table S 4-2: Statistical appreciation of the clustering following CHIC analysis

Sample Type	Silhouette Score	WSS Range	BSS/TSS (%)
1/0 (HNA/LNA) sample	0.55	0.42	75
Other Samples with HNA/LNA ratio of: <ul style="list-style-type: none"> - 0.75/0.25, - 0.5/0.5, - 0.25/0.75 - 0/1 	0.44 – 0.47	0.06 – 0.14	75

5 Evaluating Flow Cytometric Analysis Techniques for Microbial Monitoring in Drinking Water Treatment

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Abstract: Flow cytometry (FCM) analysis provides a rapid method for microbial detection in drinking water but faces lack of guidance for using and interpreting its metrics limiting its widespread use. In this study, we concurrently evaluate three FCM metrics, Intact Cell Count (ICC), High/Low Nucleic Acid (HNA/LNA) ratios, and Bray-Curtis Dissimilarity Index (BCDI), for their complementarity and early warning potential. To do so, these metrics were applied across the different stages of a water treatment work (WTW) and associated distribution system. Our findings showed that ICC provided a direct assessment of the microbial loading in high cell count scenarios, while the HNA/LNA ratios were valuable during low microbial loads and treatment stages using chemicals such as after chlorine disinfection. The BCDI metric proved to be versatile across all water treatment stages and in every microbial load scenario. A lead and lag analysis demonstrated that all metrics (ICC, HNA/LNA, and BCDI) have early warning potential, as a shift in one metric consistently led to corresponding shifts in the others. However, the leading metric varies depending on the treatment process, treatment conditions, and environmental factors. The visualisation of all the metrics on a nMDS plot enables the observation of spatial and temporal variations in microbial communities. Combining these FCM metrics improved microbial water quality assessment and supports approaches to optimise water treatment strategies from a microbial perspective.

Keywords: Flow cytometry; Drinking water; Cell gating; Fluorescence fingerprinting; CHIC analysis; Public health; Bray-Curtis Dissimilarity Index.

5.1 Introduction

The integrity of drinking water systems is paramount for public health, economic stability, and societal well-being (World Health Organization, 2008). The microbial safety of drinking water is a critical concern, as microbial proliferation or contamination events can undermine water quality, posing significant health risks and compromising compliance with regulatory standards (Li & Wu, 2019; Newton et al., 2013; Zhang, 2012). Considering water treatment works (WTWs), and drinking water distribution systems (DWDS), this can have direct operational and capital costs and compliance with aesthetic and hygienic standards. Traditional methods like heterotrophic plate count (HPC) and measurement of specific bacteria indicators have served to assess microbial quality, yet they exhibit limitations such as underestimation of microbial load, taxonomic bias, and latency in providing actionable data (Allen et al., 2004; Cheswick et al., 2019; Van Nevel, Koetzsch, et al., 2017). These constraints underscore the necessity for more dynamic, accurate, and real-time methodologies to monitor and ensure water quality, especially in the face of challenges faced by the water sector such as deteriorating infrastructure, climate change, and evolving microbial threats which can negatively impact source waters and treated drinking water (Delpla et al., 2009; Enfrin et al., 2019; Nguyen et al., 2012).

Flow cytometry (FCM) emerges as a robust alternative, offering rapid and reproducible analysis of microbial populations in water (De Roy et al., 2012; Helmi et al., 2014; Robinson, 2022; Van Nevel et al., 2013). It has been increasingly adopted across various sectors of water quality management, from raw water monitoring to assessing treatment efficiencies and tracking microbial dynamics in distribution systems. The adoption of cellular markers like SYBR Green I (SG) and Propidium Iodide (PI) facilitates detailed characterization of microbial communities using FCM, specifically the differentiation of microbial cell viability based on membrane integrity. This serves as a valuable 'direct' measure of cellular disinfection (Berney et al., 2007; Clementi et al., 2014; Hoefel et al., 2003).

Despite its advantages over culture-based methods, FCM's effectiveness is constrained by a lack of guidance challenging the appropriate use of its metrics and their interpretation, limiting its widespread use. However, one advantage of FCM is that it provides a 'multivariate' assessment of each single cell, thus providing numerous additional sources of data that can be derived. These include intact cell count (ICC), total cell count (TCC), high nucleic acid content (HNA)- or low nucleic acid content (LNA)- bacteria, Bray-Curtis Dissimilarity index (BD Accuri, 2013; Chan et al., 2018a; Favere et al., 2020; Hammes & Egli, 2010; Van Wambeke et al., 2011).

The HNA and LNA content and their ratios provide insights into the metabolic status of microbial populations, reflecting the physiological diversity within water samples (J. Liu et al., 2017; Servais et al., 2003; Van Wambeke et al., 2011). The Bray-Curtis Dissimilarity Index (BCDI), a well-established metric in ecological studies, can be employed to quantify the dissimilarity between the microbial population across different water samples, based on FCM data offering a quantitative lens through which microbial "taxa" dynamics can be monitored and understood (Chan et al., 2018b; Koch et al., 2013). CHIC analysis, from which BCDI is derived, allows for an intuitive and comparative analysis of microbial population. This technique, coupled with advanced algorithms, facilitates the detection of microbial population shifts, offering a proactive approach to identifying potential water quality issues (Chan et al., 2018b; Favere et al., 2020; Koch et al., 2013).

For the first time, this study compares three different flow cytometric metrics, ICC, HNA/LNA, and BCDI, using a dedicated sampling of a full-scale WTW to evaluate their complementarity and their early warning potential for assessing drinking water microbial quality. The research framework is set against a backdrop of a 2-month-long monitoring of microbial quality across various WTW stages, employing FCM to gauge the microbial fingerprints in water samples. The study hypothesizes that the appropriate use of the metrics, in the right scenarios, can enhance the microbial monitoring and the decision process in drinking water quality assessment.

5.2 Materials and Methods

5.2.1 Monitored site

Between August and September 2022, water samples were collected from each stage of a WTW and locations within its downstream DWDS. The WTW treats water from the river Sussex Ouse in the South of England via a small impounding reservoir and employs treatment processes commonly applied in the UK for surface waters. These include a Clarifier (CF), Rapid Gravity Filter (RGF), Ozone exposure, Granular Activated Carbon contractors (GAC), UV disinfection, and a Chlorine Contact Tank (CCT) (Figure 5-1). Each treatment process within the WTW underwent weekly monitoring of water quality assessing microbial state through the cytometric parameters (ICC, HNA/LNA, BCDI) detailed further below and determined using an FCM Accuri C6 (BD, Switzerland). The water post-UV disinfection, final water (contact tank outlet), and distributed water were all monitored daily for the same parameters. In addition to monitoring the treatment processes and distributed water, the pre-contact tank water was analysed every 30 minutes using an inline FCM BactoSense (bNovate Technologies, Switzerland). The pre-contact tank was selected for enhanced monitoring afforded by the BactoSense, to appreciate how each analysis method responds to the influence of upstream treatment processes. The post-contact tank is likely to exhibit low residual cell count which may interfere with trends apparent in each dataset.

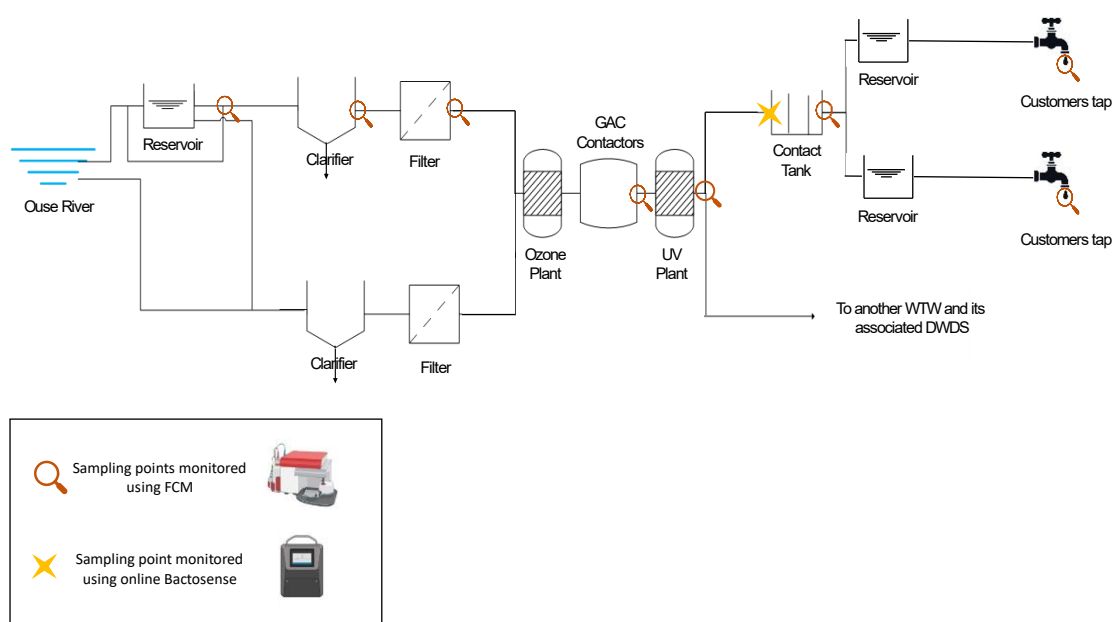


Figure 5-1 Flowsheet of the WTW used in the study. River is located in the South of England with a river abstraction from the Sussex Ouse River (UK). Sample points are indicated in orange magnifying glass and yellow star.

Sampling was conducted in triplicate at the WTW and its associated endpoints and measured 10 times at the pre-contact tank. Each sampling was conducted following the regulatory sampling procedures outlined by Clark (2015). Specifically, the sample taps were flushed for three minutes and flame-sterilized before sampling. Subsequently, taps were flushed for an additional 30 seconds before the collection of samples into sterile 250 mL sample bottles. These bottles contained a pre-aliquoted dose of sodium thiosulfate to reach 0.1% (w/v) in the water sample, which was sufficient to quench the residual chlorine. Upon collection, samples were transported to the laboratory in the dark for analysis within 24 h and were stored at temperatures between 4-8°C during transit.

5.2.2 Offline flow cytometric measurements

Sample preparation commenced with the dilution of samples exceeding TCC and ICC concentrations of 1,000,000 and 800,000 cells/mL respectively, using 0.22 µm and 0.1 µm Polyether sulfone (PES) filtered sterile Evian™ water. The samples were then stained for TCC and ICC. TCC staining involved the use of SYBR Green I (SGI, 10,000 × stock. S-7567; Thermo Fisher Scientific, UK),

filtered with dimethyl sulfoxide (DMSO) (0.22µm, Z290807, Sigma-Aldrich, UK), and diluted to a 100x working concentration. The ICC stain was prepared using a mixture of five parts of 100x SGI and one part of Propidium Iodide (PI) (Thermo Fisher Scientific, UK). The final concentration of SYG was 1× and PI was 3.0 µM. The samples were stained in 96-well plates, which were then incubated using a microplate thermoshaker (Grant Instruments™ PHMP, Thermo Fisher Scientific, UK) at 35°C and 400 rpm for 15 min. Each sample (50 µL) was analysed at a flow rate of 66 µl.min⁻¹ with a BD Accuri C6 flow cytometer (Becton Dickinson U.K. Ltd., U.K.), employing a 488 nm solid-state laser. Green and red fluorescence were measured at 533 nm (FL1) and 670 nm (FL3), respectively. A single static primary gate was applied to delineate bacteria from background signals and intact or total cells, following Prest et al. (2013). The threshold for the green fluorescence (FL1-H) channel was set at 480 arbitrary units (A.U). Microorganism numbers in this gate post-staining with SGI or SGI/PI formed the basis for TCC or ICC per mL calculations using a dynamic gating approach to account for differences in the observed microbial populations, especially following chlorination (Claveau et al., 2023). High and low nucleic acid content bacteria (HNA and LNA, respectively) were determined using a dynamic secondary gate (Gatza et al., 2013; Prest et al., 2013).

5.2.3 Automated inline flow cytometric measurements

The online flow cytometer used in this study was a BactoSense (bNovate, Switzerland), which makes use of the capillary syringe for the alignment of particles. The flow cytometer is equipped with a blue 488 nm laser diode, two fluorescence detectors (FL1: 525/45 nm, FL2: 715 LP), and one side scatter detector (SSC: 488/10 nm). Staining was performed using SYBR Green I in DMSO (0.1 %) and Propidium Iodide in water (0.01%), and cleaning was performed using sterile water with 0.05 % sodium azide, and 0.1 % NaOCl. One sample (90 µL) was taken every 30 minutes using the LDC—Live/Dead Count cartridge (ICC cartridge) to determine the number of ICC.

5.2.4 CHIC analysis and Bray Curtis Dissimilarity Index (BCDI)

Cell concentration, ICC, TCC, HNA, and LNA data were extracted from .fcs files using FlowJo software (v10.7.2), which facilitated FL1 vs FL3 dot plot extraction.

The CHIC analysis adhered to Koch et al.'s (2013) protocol for Cytometric Histogram Image Comparison (CHIC). This approach enables the monitoring of shift in the microbial community without a prior definition of gates in a manner that is independent of the user. Briefly, FCM FL1 vs FL3 plots were extracted from BD Accuri C6 software as .fcs files and converted to grey-scale images with the FlowJo (v10.7.2) software to enable comparison. The samples dyed with SYBR Green and PI for ICC quantification were specifically utilized given ICC significance in assessing viability within chlorinated systems. The comparison of the grey-scale images was conducted by using ImageJ (v1.54d). The resulting XOR and overlap values, representing the pixel differences between the images, were used to calculate the BCDI²⁶ matrix, indicating the dissimilarity between each sample. This matrix was then used to construct a non-metric multidimensional scaling (nMDS) plots allowing the visualisation of the sample distribution based on their dissimilarity (BCDI) using R (v2022-02-3). The Figure S 5-2: Explanation of CHIC analysis summarises the steps of the CHIC analysis.

Additional statistical measures, such as clustering analysis, were performed using R (2022-02-3). To better characterise contributing factors influencing dissimilarity in microbial community, the metrics HNA, LNA, TCC and ICC were linked to the BCDI matrix and add to the nMDS plot. p-value was set at significance of $\alpha = 0.05$ to determine the statistical significance of each factor. During CHIC analysis, different evaluation parameters were utilized. The stress evaluated how well the nMDS plot preserves the original dissimilarity structure of the data and improves interpretation by visualising the data. The Within-Cluster Sum of Squares (WSS) was employed to evaluate cluster compactness, while the Between-Cluster Sum of Squares (BSS) examined the dissimilarity between clusters. The Silhouette measure was used for two distinct assessments: it

²⁶ Bray-Curtis Dissimilarity Index

evaluated how well each data point fit into its assigned cluster and quantified the distinctiveness between clusters. Additionally, an ANOSIM test was applied to determine the significance of the differences between the groups of samples based on the dissimilarity matrix. See Table S 5-1 for details on the interpretation of the tests.

5.2.5 Statistical tests

The Dunn's test was applied for pairwise comparisons among interstage datasets to determine statistical differences in microbial community dynamics. Lower p-values (near 0.05) indicate significant differences between the datasets. Cross-correlation was utilized for determining time lead and lag between the parameters, enabling the identification of temporal relationships and dependencies. The cross-correlation analysis, conducted using Spearman's rank correlation coefficient, is suited for revealing non-parametric correlations. A lower p-value signifies a more significant correlation at a specific time (t).

5.3 Results and Discussion

5.3.1 Comparison of FCM metrics to evaluate their complementarity

The results show that while intact cell count (ICC) is best suited for monitoring upstream treatments with high cell concentrations, HNA and LNA are more appropriate for monitoring downstream treatments, particularly when chemical and physical disinfection is involved. As for BCDI, this metric proved useful for monitoring at every stage of the Water Treatment Works (WTW)s.

5.3.1.1 Cell counts through ICC

The daily ICC exhibited variability across different stages of treatment (Figure 5-2-A). Cell count decreased progressively from raw water (1.9×10^6 to 1.1×10^7 cells/ml) through the treatment processes: post-RGF (6.0×10^5 to 1.7×10^6 cells/ml), post-GAC (1.4×10^5 to 1.0×10^6 cells/ml), post-UV (1.4×10^5 to 8.3×10^5 cells/ml), and pre-CCT (10 to 2.3×10^6 cells/ml). Final water exhibited cell count from 1.4×10^2 to 7.0×10^3 cells/ml) and tap water ranged from 4.0×10^2 to 1.6×10^5 cells/ml which tend to be lower than the FCM's limit of quantification (LOQ), 708

cells/ml (**Error! Reference source not found.**). This highlights the limitations of ICC for monitoring water with low cell count. Figure S 5-3 illustrates the log removal at each stage.

A Dunn's test demonstrated that the dataset for each treatment processes, final water, and tap water were significantly different ($p < 0.05$, Table 5-1), indicating that each treatment stage contributed to a statistically distinct reduction in microbial load. This confirms the effectiveness of the sequential treatment steps in reducing cell concentration and highlights the role of each stage in microbial control.

The significant log removal from post-GAC to pre-CCT is attributed to the location of the pre-CCT sampling point, which was placed immediately after the chlorine dose. This positioning highlights the immediate impact of chlorination on cell counts.

These findings confirm that ICC is effective for evaluating microbial removal efficiency; however, it is most reliable in the early treatment stages, where cell concentrations are higher.

5.3.1.2 HNA/LNA ratio

In contrast to ICC, which showed a progressive decrease across all treatment stages, HNA and LNA ratios exhibited varying trends (Figure 5-2-A). In raw (HNA/LNA = 2.0 ± 0.8), post-RGF (HNA/LNA = 2.0 ± 0.7), post-GAC (HNA/LNA = 1.7 ± 0.4), post-UV (1.5 ± 0.2), HNA cells dominated, indicating a high proportion of metabolically active cells, but HNA gradually declined through the treatments, indicating a slow shift toward metabolically less active cells. At the pre-CCT stage, where hourly measurements were taken using Bactosense© and where chlorine was applied, the HNA/LNA ratio increased sharply by a factor of 2 (HNA/LNA = 4.0 ± 0.1). This suggests that chlorination selectively eliminated LNA cells, which are known to be more sensitive to chlorine due to their thinner and more hydrophobic membranes (refs), rather than directly promoting HNA growth. Additionally, more outliers were observed at this stage, indicating a highly variable microbial response to chlorination. The daily HNA/LNA trend for pre-CCT

stage (Figure 5-2A) showed sporadic events that multiplied the ratio by nearly 10, suggesting that offline measurements may not provide sufficient resolution due to the dynamic nature of microbial shifts in raw water and within the WTW processes. After chlorine disinfection, on average, the HNA/LNA ratio decreased in final water and tap water, but its variation increased (HNA/LNA = 1.9 ± 1.8 for final water, and 2.8 ± 2.0 for tap water), suggesting that while chlorination reduces microbial activity overall, water distribution dynamics introduce additional variability. Possible factors affecting this variability include water age, biofilm formation, chlorine residual decay, and nutrient availability (refs).

The Dunn's test revealed that HNA/LNA datasets for each treatment stage were not significantly different ($p > 0.05$, Table 5-1), except for the pre-CCT stage where the dataset was significantly different from all other treatment stages ($p < 0.001$). This confirms that chlorination is a key turning point in microbial population shifts, favouring HNA cells not by promoting their growth, but by selectively eliminating LNA cells (refs). Additionally, Dunn's test showed a significant difference between post-GAC and the post-UV datasets ($p < 0.001$). This is likely due to UV-induced damage to cellular DNA (refs), which may have altered the fluorescence properties of the cells. Finally significant differences were also observed between final water and tap water datasets ($p < 0.05$), highlighting the influence of distribution network factors such as biofilm formation, residual chlorine decay, water age, and nutrient fluctuations (refs).

These findings contrast with ICC trends, where every stage showed a significant reduction. While ICC effectively tracks microbial removal in early treatment steps, HNA/LNA ratio provides deeper insight into the effects of chemical (chlorination) or physical (UV) disinfection, where selective pressure on microbial populations is more relevant.

5.3.1.3 BCDI

Unlike ICC, which progressively declined across treatment stages, and HNA/LNA, which showed selective shifts in microbial metabolic across disinfection stages, the daily BCDI exhibited broadly similar values across the whole treatment work (Figure 5-2-A). The values of BCDI, for all stages, ranged

from 0.35 to 0.65, reflecting the dynamic nature of microbial communities. Although the BCDI metric reached the same values for every stage, its variation differed. On average, BCDI value for raw water was of 0.49 ± 0.05 , while it was of 0.42 ± 0.04 for post-RGF, 0.50 ± 0.03 for post-GAC, 0.42 ± 0.04 for post-UV, 0.49 ± 0.04 for pre-CCT, 0.40 ± 0.04 for final water, and 0.49 ± 0.05 for tap water. The Dunn's test confirmed the significance of these variations, with a p-value of 0.001 when comparing the dataset of one stage with the following stage (Table 5-1), indicating that each treatment step contributed to a statistically significant shift in microbial community, even though the overall BCDI values remained within a similar range. Final water was the only dataset showing significant differences with all the other stages ($p < 0.001$), likely indicating that the combination of advanced treatment processes (e.g., filtration, UV, and chlorination) led to a fundamental shift in microbial community structure. The significant drop in BCDI suggests that the final disinfection process not only reduced overall microbial abundance but also resulted in a more homogenous and less diverse microbial community. This aligns with previous findings that suggest extensive treatment processes selectively eliminate more sensitive microbial populations, favouring a residual community that is more resistant to treatment conditions (add references here). However, the increased BCDI from final water to tap water ($p < 0.001$) demonstrates potential microbial instability in the distribution network, highlighting the need for improved network maintenance.

These results highlight the importance of BCDI in tracking microbial community throughout the WTW process. While ICC and HNA/LNA provide insights into microbial load and metabolic activity, BCDI captures microbial shifts, making it a valuable complementary metric for assessing treatment efficiency and potentially microbial stability in distributed water

Other studies have also demonstrated the distinct roles that these metrics play in microbial assessment. The cell count served as a crucial metric for detecting system anomalies such as DWDS ingress/leakage (Berney et al., 2008b; F. Hammes, Berney, Wang, Vital, Köster, et al., 2008; Prest et al., 2016), and evaluating the efficacy of different water treatment processes (Van Nevel,

Buysschaert, et al., 2017; Van Nevel et al., 2013; Van Nevel, Koetzsch, et al., 2017c). The HNA/LNA provided insights into the reactivity of microbial populations to oxidative treatment processes (Berry et al., 2006) based on their nucleic acid content (Ramseier et al., 2011). As for the BCDI, it was depicted as helpful for identifying and tracking changes in microbial community over time, without the need to undertake more complex aspects of ordinal scaling but restricted somewhat by its simplification of microbial community (Chan et al., 2018a; Schleich et al., 2019a).

From an industry perspective, these findings suggest that through ICC, log removal of cells should be monitored to assess treatment performance. Through HNA/LNA, disinfection dosing strategies should be optimized to minimize selective microbial shifts that could impact microbial stability. Finally, potential microbial instability in the distribution network should be tracked, identifying the need for improved residual chlorine monitoring and pipeline maintenance.

These findings are not without limitations. While flow cytometry provided high-resolution microbial data, its lack of taxonomic resolution limits the ability to determine which specific microbial species were affected by different treatment stages. Future studies incorporating 16S rRNA sequencing could help link FCM patterns to microbial taxa, providing deeper insights into microbial dynamics in WTWs.

Further research should also investigate the functional implications of the observed microbial shifts by integrating metagenomic analysis. This approach would help determine whether microbial selectivity at each disinfection stage is associated with disinfection-resistant bacteria, which persist through treatment and may pose a risk to water quality, or stressed but viable populations, which might recover in the distribution system and impact long-term microbial stability.

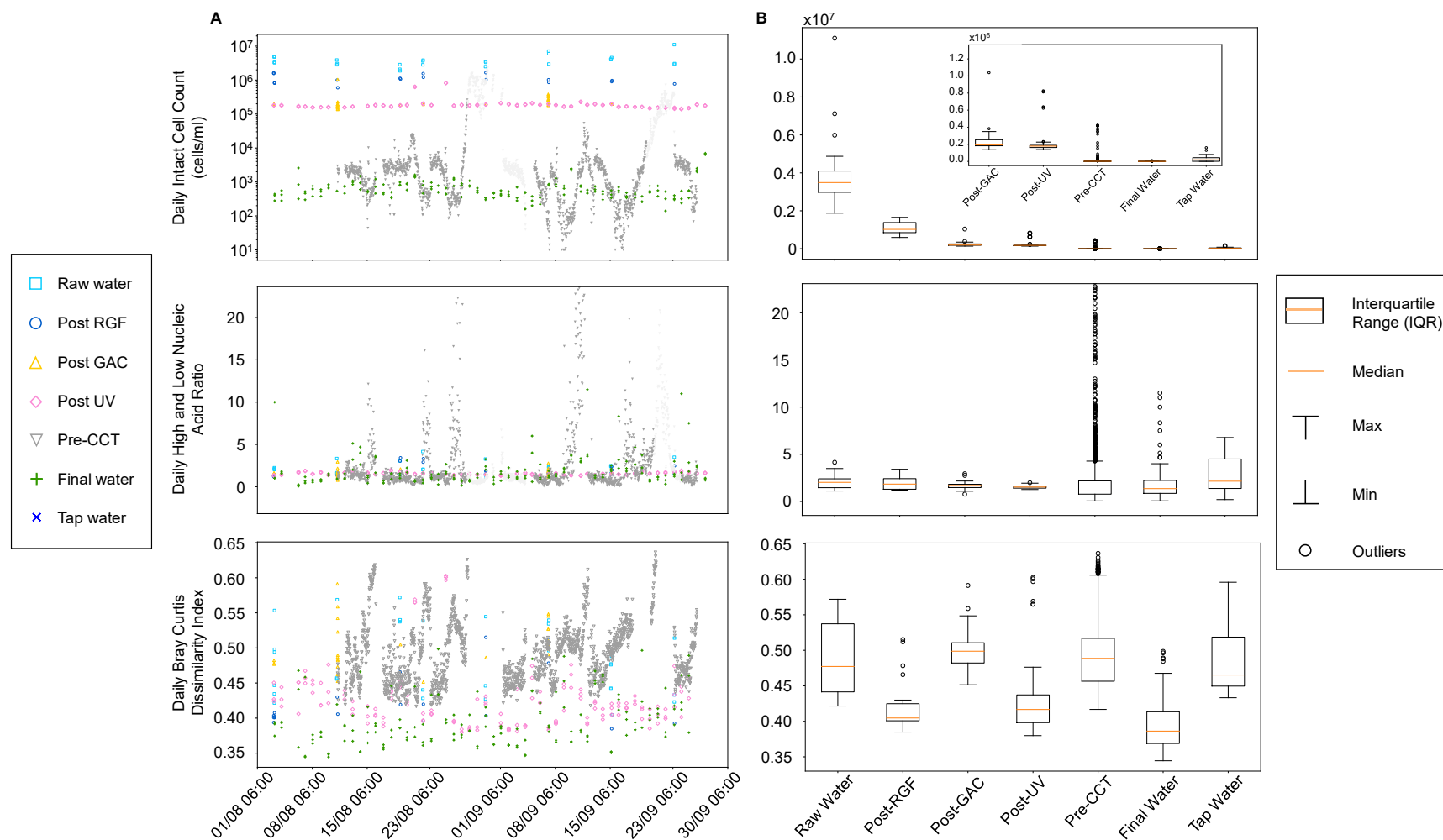


Figure 5-2: A. Daily measurement of cell count, HNA/LNA and BCDI at each stage a WTW. B. Distribution of the measurements at each stage. The middle orange bar represents the median. The outliers represent the data falling outside the main distribution of the measurements. A zoom-in of the daily intact cell count distribution is shown for Post-GAC, Post-UV, Pre-CCT, Final water, and Tap water to see better their distribution.

Table 5-1: Results of the pairwise comparison (Dunn's test) to compare the dataset of each treatment stage between one another and applying different metrics, ICC, HNA/LNA, and BCDI. A number means that the p-value is above 0.05, a start * indicates that p-value is below 0.05, two stars ** indicate a p-value less than 0.01, and 3 stars * indicate a p-value inferior to 0.001.**

ICC							
	Raw water	Post-RGF	Post-GAC	Post-UV	Pre-CCT	Final water	Tap water
Raw water							
Post-RGF	***						
Post-GAC	***	***					
Post-UV	***	***	***				
Pre-CCT	***	***	***	***			
Final water	***	***	***	***	***		
Tap water	***	***	***	***	***	***	
HNA/LNA							
	Raw water	Post-RGF	Post-GAC	Post-UV	Pre-CCT	Final water	Tap water
Raw water							
Post-RGF	0.744						p-value
Post-GAC	0.214	0.337					* <0.05
Post-UV	***	0.191	***				** <0.01
Pre-CCT	***	***	***	***			*** <0.001
Final water	***	*	0.059	*	0.1		
Tap water	0.4	0.251	0.061	***	***	*	
Bray and Curtis							
	Raw water	Post-RGF	Post-GAC	Post-UV	Pre-CCT	Final water	Tap water
Raw water							
Post-RGF	***						
Post-GAC	0.116	***					
Post-UV	***	0.617	***				
Pre-CCT	0.344	***	0.098	***			
Final water	***	***	***	***	***		
Tap water	0.775	***	0.059	***	0.322	***	

5.3.2 Cross-correlation (lead-lag test) to evaluate the early warning potential of the metrics

Due to their large dataset sizes and relative importance on the final microbial water quality, only the stages post-UV (N = 153), pre-CCT (N=1990), and final water (N=163) were used for this subsection.

The results show that when one FCM metric shifts to an unusual state, it often leads to corresponding shifts in the other metrics. However, there was no single dominant metric consistently initiating these shifts, as the treatment conditions influenced which metric responded first. For example, an increase in turbidity due to microbial detachment would first cause a rise in ICC, which in turn could lead to changes in HNA/LNA ratios and BCDI as the microbial population adjusts. Conversely, a sudden increase in chlorine dosing would first alter the HNA/LNA ratio, reflecting the selective impact of oxidative stress on microbial subpopulations, which would then lead to subsequent changes in ICC and BCDI as microbial viability and community shift.

5.3.2.1 Comparison of the metrics' trend

5.3.2.1.1 Post-UV

At the post-UV stage, ICC ranged from 1.4×10^6 to 2.2×10^6 cells/mL and showed a progressive increase on August 11, followed by a gradual decrease in HNA/LNA on August 14, with values varying between 1.3 and 1.9. The increase in ICC, followed by the decrease in HNA/LNA, suggests a shift toward LNA-dominated populations (i.e., less active cells). This trend appears counterintuitive, as HNA cells are typically associated with higher metabolic activity and growth potential (references needed). One possible explanation for this discrepancy is the limited temporal resolution of daily sampling, which may have failed to capture critical change in water microbial quality occurring at shorter timescales. This highlights a key limitation of daily monitoring, as short-term fluctuations in microbial water quality may go undetected. Additionally, during this period, peaks in turbidity (2 NTU, see Figure S 5-4) were recorded at the UV reactor. However, these turbidity spikes were not reflected in ICC or HNA/LNA measurements

(references needed on UV vs. ICC response). This suggests that either the microbial community was unaffected by turbidity changes, or that UV-induced microbial shifts occur independently of bulk cell count variations, or the limitation of daily monitoring. Furthermore, BCDI values (ranging from 0.38 to 0.48) did not follow a similar pattern, suggesting that microbial community remained stable despite changes in ICC and HNA/LNA. This either further supports the idea that UV treatment does not necessarily alter microbial community over daily timescales but may instead inactivate cells proportionally across different microbial subpopulations (refs) or highlight once more the limitation of daily monitoring.

5.3.2.1.2 Pre-CCT

At the pre-CCT stage, ICC, HNA/LNA, BCDI showed significant variations ($p < 0.05$). ICC ranged from 10^2 to 2.3×10^6 cells/ml. HNA/LNA varied between 2 and 40. BCDI fluctuated between 0.4 and 0.65. These variations were linked to the presence of sudden increases observed in each metrics. When ICC abruptly increased from 10^2 to 2.0×10^4 cells/ml on August 18th, this was followed by a rise in HNA/LNA ratios (from 2 to 30) and BCDI (from 0.4 to 0.6), within 24h. A similar pattern occurred on August 21st, where ICC increased (from 10^2 to 7.0×10^3), again followed by an increase in HNA/LNA (2 to 30) and BCDI (0.4 to 0.58). After comparison with the daily turbidity register at the post-UV, stage preceding pre-CCT, these events matched with 2 turbidity events where turbidity was of 0.25 and 2 (Figure S 5-4). These observations suggest that sudden cell count disruptions—possibly due to microbial detachment events—trigger corresponding metabolic and microbial community shifts. The increase in HNA/LNA after ICC peaks could indicate that the newly released microbial populations contain a higher proportion of metabolically active cells, which influence overall community dynamics.

A different trend was observed the following month. On September 7th, a sudden increase in HNA/LNA (from 2 to 40) was followed by a rise in BCDI (0.47 to 0.62) and ICC (10^2 to 5.0×10^5 cells/ml). Similarly, on September 15th, HNA/LNA spike (2 to 20), was followed by an increase in BCDI (0.50 to 0.65) and ICC (10^2 to

2.0x10⁵ cells/ml). Unlike the August events, where ICC shifts occurred first, these September events suggest that metabolic disruptions (HNA/LNA shifts) preceded changes in both microbial community (BCDI) and cell concentrations (ICC). This indicates a reversed causality, where metabolic stress (e.g., oxidative stress, chlorine residual fluctuations, or biofilm detachment) first alters cell physiology, subsequently influencing microbial abundance and community.

These findings demonstrate that the directionality of microbial shifts at the pre-CCT stage is not uniform, and that the different metrics can serve as leading indicators of microbial disturbances depending on the specific treatment conditions.

5.3.2.1.3 Final water

On the other hand, at the final water stage, ICC, HNA/LNA, and BCDI did not follow a similar pattern, but a statistically significant difference was observed among these metrics ($p < 0.05$). ICC daily measurements varied between 500 and 2,500 cells/mL, while HNA/LNA ranged from 0.2 to 5, and BCDI fluctuated between 0.3 and 0.5, which can be attributed to the dynamic nature of microbial population in treated water (references needed). These fluctuations may be influenced by factors such as chlorine contact time, seasonal temperature fluctuations, upstream water quality, and treatment performance, all of which are known to affect microbial stability at this stage (references needed). While these variations confirm that the metrics reflects the microbial water quality dynamism, it could not be determined whether shifts in one metric influence changes in another over time at final water. A cross-correlation analysis was applied to quantify the timing variations between these metrics.

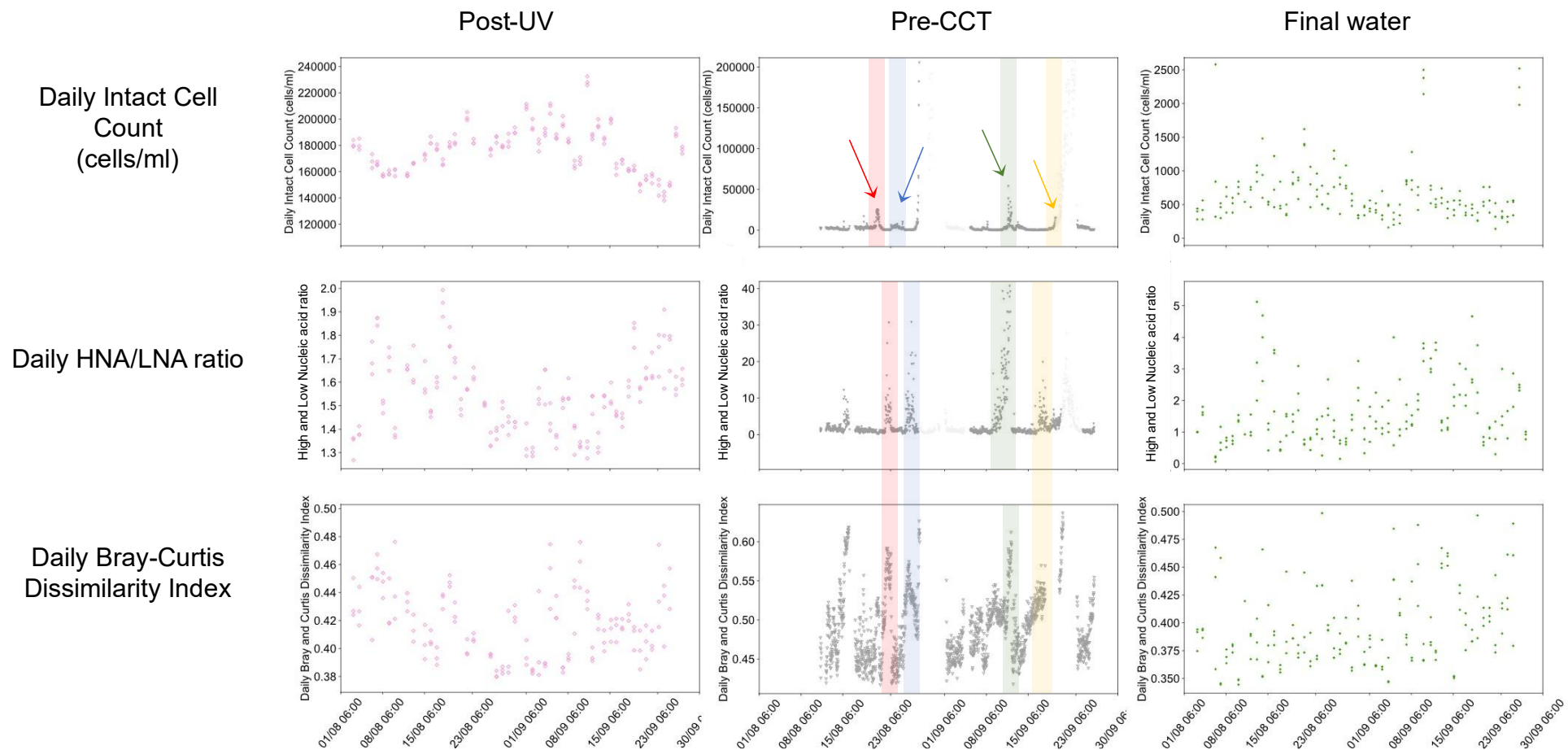


Figure 5-3: Daily results of the water cell count, HNA/LNA ratio, and BCDI at the Post-UV, Pre-CCT, and Final water stage of the WTW. Coloured arrows and background on the pre-CCT graphs represent the periods for which each metrics showed a peak almost simultaneously.

5.3.2.2 Cross-correlation

To delineate the relationships between cell count, HNA/LNA ratio, and BCDI, and evaluate their early warning potential, cross-correlation analysis was used with the help of Spearman's rank correlation. This analysis covers time lags from -10 to 10 days for post-UV and final water, due to their daily analysis, and -48 to 48 hours for pre-CCT, due to its hourly analysis.

5.3.2.2.1 Post-UV

At the post-UV stage, the previous observations showed a negative correlation between ICC and HNA/LNA with a temporal delay for the HNA/LNA ratio to respond to alterations in cell count. The lead-lag analysis confirmed a negative correlation ($\rho = -0.6$, $p < 0.001$) between ICC and HNA/LNA ratio, and a lagged response of the HNA/LNA ratio to ICC fluctuations of 5 days (Figure 5-4). This suggests that ICC serves as an early sign to microbial shifts, with LNA cells responding approximately 5 days later.

This finding contrasts with previous studies, which reported that microbial growth positively correlates with the presence of HNA cells, as HNA-dominated populations are typically associated with higher metabolic activity and proliferation (Bouvier et al., 2007; Lautenschlager et al., 2010; J. Liu et al., 2017; Servais et al., 2003).

The discrepancy could be due to UV treatment's selective effect on microbial populations. Unlike nutrient-driven microbial growth, UV disinfection primarily damages microbial DNA, leading to a delayed metabolic response in surviving cells. This could explain why ICC initially declines, while HNA/LNA only decreases after several days, possibly as damaged HNA-dominant cells undergo repair or transition to a stressed physiological state (references needed). Additionally, the observed delay could reflect differential inactivation rates between HNA and LNA cells, as UV radiation is known to disproportionately affect certain microbial subpopulations based on their cellular repair capacity and DNA content (references needed). Furthermore, external environmental factors, such as organic matter concentration, residual oxidants, or microbial interactions, may

contribute to this delayed response. Future studies incorporating viability staining, metabolic activity assays, and molecular techniques (e.g., qPCR or metagenomics) could help elucidate whether UV-induced shifts in microbial populations are driven by cell damage, metabolic dormancy, or selection for resistant strains.

Still at the post-UV stage, the previous observations could not show an influence between BCDI and the other metrics at the post-UV, but the cross-correlation analysis revealed a positive correlation between the HNA/LNA ratio and BCDI ($\rho=0.4$, $p < 0.001$) with a lagged response of BCDI to HNA/LNA fluctuation of 1 day. Two negative correlations between ICC and BCDI was observed ($\rho=-0.3$, $p < 0.001$ and $\rho=-0.2$, $p < 0.001$) with BCDI responding to ICC fluctuation after 1 and 6 days, respectively. This suggests that changes in microbial metabolic activity (HNA/LNA shifts) influence microbial community (BCDI) within a short time frame, likely due to selective microbial responses to UV treatment. The 1-day lag indicates that as metabolically active (HNA) cells fluctuate, microbial community adjusts shortly after, possibly due to the differential survival and recovery rates of microbial subpopulations exposed to UV stress.

The negative correlations between ICC and BCDI, with BCDI responding at both 1-day and 6-day time lags, suggest that fluctuations in cell count are linked to microbial community shifts, but the effects occur at two distinct timescales. The short-term (1-day) response may reflect immediate reductions in cell count due to UV-induced inactivation, whereas the longer-term (6-day) response could indicate secondary effects, such as microbial regrowth, selection for UV-resistant species, or shifts in communities as surviving cells adapt to post-UV conditions (references needed).

These findings highlight the complex interactions between microbial abundance, metabolic activity, and community in response to UV treatment. While HNA/LNA fluctuations appear to drive short-term shifts in BCDI, ICC reductions contribute to longer-term changes in microbial diversity, possibly due to gradual shifts in microbial competition, recovery, or external environmental influences.

Future research should explore whether these observed relationships persist across different UV doses, water qualities, and seasonal variations. Additionally, incorporating molecular approaches (e.g., 16S rRNA sequencing, metagenomics) could provide deeper insights into which microbial taxa drive these post-UV shifts in community and metabolic activity.

5.3.2.2.2 Pre-CCT

At the pre-CCT stage, previous observations showed a positive correlation between ICC, HNA/LNA, and BCDI, but with varying temporal delays. During the August events, HNA/LNA and BCDI responded with a delay to alterations in ICC, whereas during the September events, ICC and BCDI responded with a delay to fluctuations in HNA/LNA (Figure 5-3).

The lead-lag analysis confirmed the difficulty of visually interpreting these time-dependent relationships, emphasizing the importance of statistical analysis in identifying patterns. The results showed a positive correlation ($\rho = 0.5$, $p < 0.001$) between HNA/LNA and ICC, with a lagged response of ICC to HNA/LNA fluctuations of 48 hours. Additionally, a positive correlation ($\rho = 0.5$, $p < 0.001$) was observed between HNA/LNA and BCDI, with HNA/LNA responding to BCDI fluctuations within 24 hours. Furthermore, both positive and negative correlations were observed between ICC and BCDI. The positive correlation was associated with a 53-hour lag, where ICC responded to BCDI fluctuations, while the negative correlation indicated an immediate response of ICC to BCDI changes (Figure 5-4).

This suggests that microbial shifts at the pre-CCT stage occur in distinct phases, with different leading indicators depending on the treatment conditions. The delayed response of ICC to HNA/LNA fluctuations indicates that metabolic activity changes may precede microbial abundance shifts, possibly due to oxidative stress, nutrient availability, or selective disinfection effects (refs). Conversely, the immediate negative correlation between ICC and BCDI suggests that sudden reductions in microbial abundance (e.g., due to chlorination) lead to a rapid restructuring of microbial community.

The positive correlation between HNA/LNA and BCDI with a short lag (< 24 hours) may indicate that as microbial metabolic activity shifts, microbial community is also affected, possibly due to selective survival of chlorine-resistant microbial subpopulations or community restructuring following stress adaptation (refs).

These findings highlight the complex, time-dependent interactions between microbial abundance, metabolic activity, and community at the pre-CCT stage. The directionality of microbial shifts is not uniform, as HNA/LNA can either lead or follow ICC fluctuations, depending on external stressors.

Future research should explore whether these lead-lag relationships vary under different disinfection regimes or environmental conditions. Additionally, integrating molecular techniques (e.g., metagenomics, qPCR) could help identify specific microbial taxa responsible for these observed shifts.

5.3.2.2.3 Final water

At the final water stage, it was impossible to determine the leading and lagging metrics highlighting either the importance of a high-frequency sampling to undertake lead-lag analysis or the complexity of chlorinated water. Further research would verify whether the lead-lag analysis is possible in chlorinated water.

Our findings show that all FCM metrics (ICC, HNA/LNA, and BCDI) have early warning potential, as a shift in one metric consistently led to corresponding shifts in the others. However, the leading metric varies depending on the treatment process, treatment conditions, and environmental factors. For instance, turbidity fluctuations were found to affect cell count first, which then led to lagged changes in HNA/LNA and BCDI, as the microbial community adapted to the ingress of particles or microbial detachment. In contrast, UV disinfection, under normal conditions, influenced HNA/LNA first, which then triggered delayed changes in cell count and BCDI. Most studies use cell count (ICC) as the primary indicator for determining or predicting microbial population changes (Cheswick et al., 2019a; F. Hammes, Berney, Wang, Vital, Köster, et al., 2008; G. Liu, Van Der Mark, et al., 2013; Van Nevel, Koetzsch, et al., 2017c). However, to our

knowledge, no study has systematically evaluated whether ICC is the most appropriate leading metric across different treatment conditions compared to other FCM parameters.

Furthermore, the complexity of final water prevented us from performing a cross-correlation analysis to determine the leading metrics at this stage. This limitation is likely due to low-resolution (daily) sampling, which is insufficient to capture highly dynamic microbial shifts. This underscores the need for higher-frequency microbial monitoring at the final treatment and distribution stages to better understand microbial stability in chlorinated water.

This analysis advances our understanding of microbial population dynamics in response to treatment processes and supports the development of predictive models for improved water quality management. By identifying time-dependent microbial shifts, these findings provide a framework for optimizing disinfection strategies and enhancing real-time microbial monitoring approaches.

From an industry perspective, the observed bidirectional lead-lag relationships between ICC, HNA/LNA, and BCDI at the pre-CCT stage indicate that chlorination efficacy fluctuates over time. This suggests that real-time microbial monitoring could be integrated into treatment systems to dynamically adjust chlorine dosing strategies, preventing undesirable microbial shifts that could compromise water stability.

While FCM metrics provide high-resolution microbial data, they do not distinguish microbial taxa. Future studies should integrate 16S rRNA sequencing or metagenomics to identify which microbial groups drive ICC, HNA/LNA, and BCDI fluctuations and determine whether specific microbial populations exhibit unique lead-lag relationships. Additionally, factors such as seasonal variations, raw water quality, and operational differences could influence microbial trends. As a result, these findings may not be fully generalizable to all treatment plants, especially those with different source waters and treatment configurations. Further research should explore whether these lead-lag relationships remain consistent under varying environmental and operational conditions.

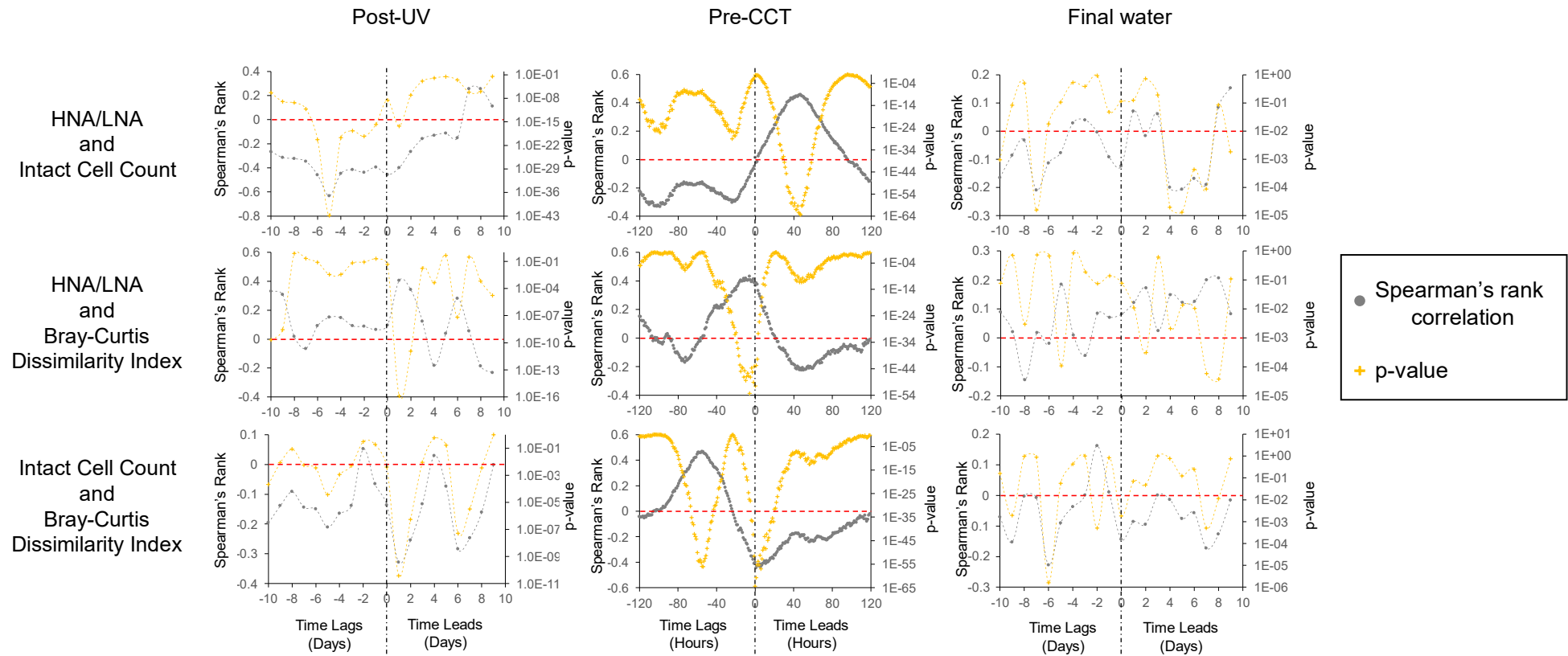


Figure 5-4: Cross-correlation between the different parameters pair wisely at the Post-UV, Pre-CCT, and Final water stage of the WTW. The grey dotted line represents the Spearman's rank correlation at each time lag. The yellow dotted line represents the p-value associated with each Spearman's rank correlation. Note that below 0 the time is seen as a time lag and above zero the time is seen as a lead time.

5.3.3 Multivariate analysis to validate the applicability of nMDS plot to monitor all the metrics together

The results show that ICC enables differentiation between water samples with high cell loads and those with low cell loads. HNA/LNA differentiation was only observed after disinfection, allowing for the distinction between pre-CCT water and post-disinfection water (final water, tap water). BCDI effectively grouped water samples based on treatment type, highlighting its ability to track microbial community shifts across different treatment processes. These findings align with previous observations, further confirming the utility of each metric and demonstrating the applicability of nMDS plots to real-world water treatment conditions.

BCDI values (0.35 to 0.65) highlighted variability across treatment stages. The NMDS plot demonstrated effective clustering (2D stress: 0.139; silhouette score: 0.54; BSS/TSS ratio: 88%), confirming the method's efficacy in distinguishing different microbial water quality profiles (Figure 5-5).

The NMDS analysis, utilizing BCDI values, effectively segregated data into distinct clusters, affirming intra-cluster homogeneity. Cluster 1, with post-UV and select post-GAC points, showed uniformity, while Cluster 2 contained both the raw and post-RGF water with some post-UV and post-GAC samples, indicating similar features. Cluster 3 grouped all pre-CCT samples, and Cluster 4 comprised final and tap water, each showing consistency.

This clustering provides statistical validation of BCDI's utility in discerning microbial water quality across various treatment stages and conditions. Vector display of correlation analysis revealed ICC loading in distinguishing Clusters 3 and 4 (chlorinated, water with low cell load) from Clusters 1 and 2 (unchlorinated, water with high cell load), with a clear orientation towards the latter. This differentiation underscores the sensitivity in the microbial population to chlorine-based disinfection and how its impact can be detected automatically in an FCM user, gating independent way (Claveau et al., 2024).

The vector for the HNA/LNA ratio differentiates chlorinated water clusters, highlighting a higher HNA-content cell prevalence in pre-CCT water (Cluster 3) compared to final and tap water (Cluster 4). This distinction confirms the HNA/LNA ratio's utility in discerning chemical treatment effects and low-cell-count water characteristics. The findings align with Chan et al., (2018) and Favere et al., (2020)'s works which used BCDI associated with NMDS to detect changes in water of the microbial population structure.

Our findings demonstrate the broader comprehension of microbial community shift when considering the HNA/LNA and ICC parameters in addition to BCDI. Integrating cell count, HNA/LNA ratio, and BCDI into a cohesive analytical framework, not only affirms but also expands upon the distinct yet complementary roles each metric plays in evaluating not only changes in microbial population but also in water treatment efficacy. As for NMDS plots and hierarchical clustering, they refine microbial water quality analysis, enabling precise treatment adjustments and ensuring water safety. These tools aid in addressing microbial variability shifts by identifying patterns in microbial community changes, detecting anomalies in treatment performance, and enabling timely interventions. nMDS plots provide a visual representation of microbial similarities and differences across treatment stages, allowing operators to pinpoint shifts in microbial composition. Hierarchical clustering groups water samples with similar microbial characteristics, helping to track the effectiveness of disinfection and filtration processes. By integrating these analytical approaches, treatment plants can proactively adjust processes to maintain stable microbial water quality.

This study provides guidance for the appropriate use of FCM metrics in water treatment monitoring. By understanding which metrics are most relevant under specific treatment conditions, operators can implement more targeted monitoring strategies and optimize treatment processes to maintain safe microbial water quality. For the first time, CHIC analysis has been applied to real-time microbial monitoring, demonstrating its ability to adapt to real-world conditions. This study establishes CHIC as a robust tool for detecting microbial shifts and highlights its potential for continuous water quality surveillance in operational settings.

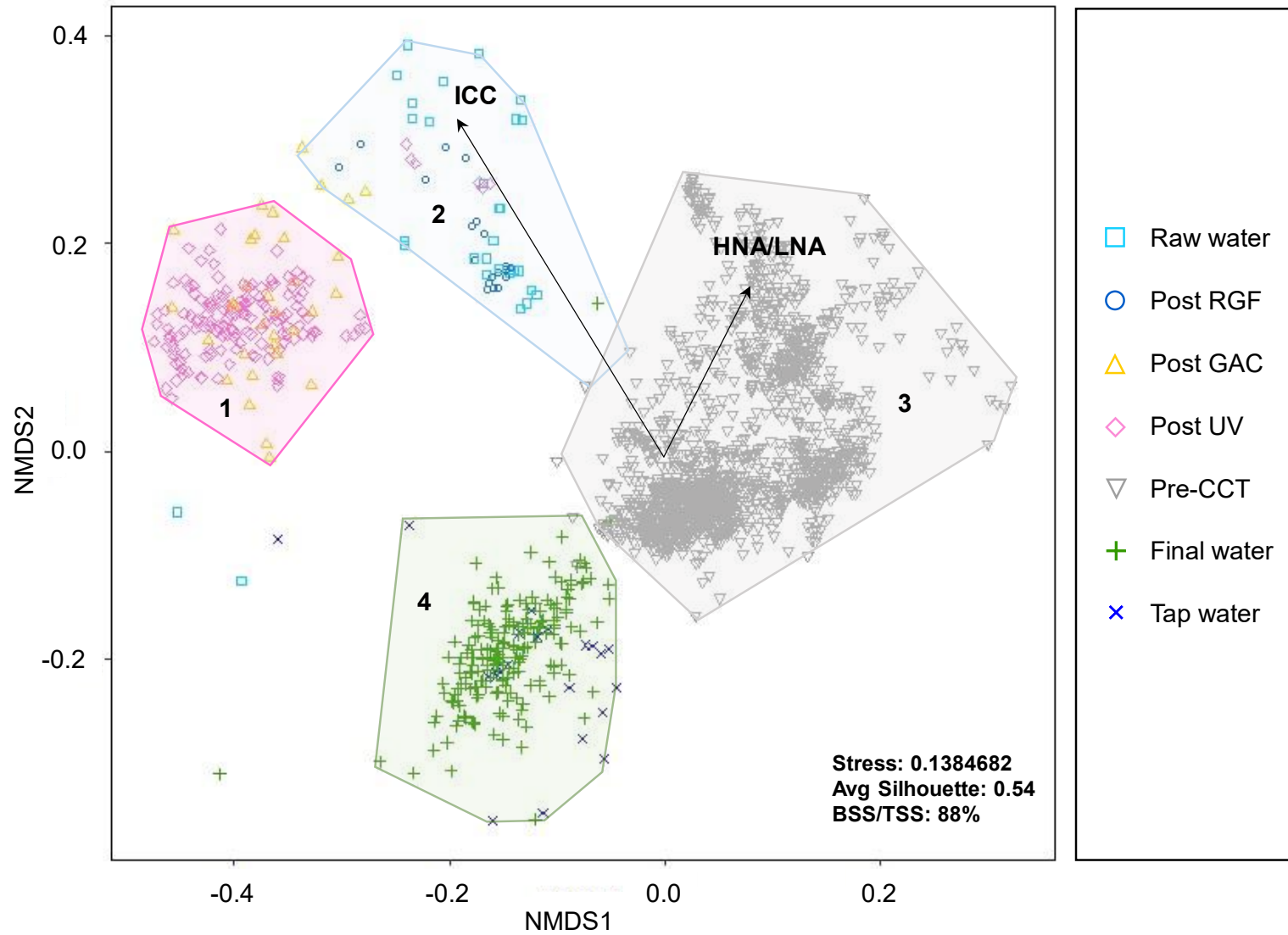


Figure 5-5: Non-Metric Dimensional Scaling (NMDS) plot of daily measurements from different stages of a WTW, including raw water, Post-RGF, Post-GAC, Post-UV, Pre-CCT, Final water, tap water).

5.3.4 Implications for Microbial Water Quality Assessment

This study highlights the utility of flow cytometry metrics—ICC, HNA/LNA ratio, and BCDI—in differentiating microbial populations in chlorinated and unchlorinated water, refining water quality assessment across treatment stages. ICC excels in identifying microbial loads in early treatment, while HNA/LNA ratios offer insights into post-chlorination microbial structures. BCDI's broad applicability aids in comprehensive microbial community shift, enhancing monitoring and treatment strategies. However, limitations include the potential underrepresentation of certain microbes in HNA/LNA analysis and the study's focus on a single facility, which may affect result generality. Future research should expand to multiple sites, explore varied treatment processes, and integrate metagenomic sequencing to deepen microbial community understanding, thereby advancing water treatment efficiency and predictive capabilities.

5.4 Conclusion

In this work, we evaluated the utility of three flow cytometry-based metrics—ICC, HNA/LNA, and BCDI—in monitoring microbial populations across various water treatment stages. Our findings delineate the distinct capabilities of each metric for characterizing microbial water quality dynamics. The ICC effectively discriminates high microbial loads in unchlorinated versus lower counts in chlorinated waters, the HNA/LNA ratio provides valuable insights into microbial community diversity post-chlorination even during low residual ICC, and BCDI enables for detection of microbial community shift across all treatment stages.

The study demonstrates that a combined analysis of these metrics can significantly enhance microbial water quality assessment, allowing more nuance in the management of microbial populations, and thereby potentially improving water treatment outcomes. Through novel application and comparison of these metrics in a real-world water treatment context, this research contributes to the body of knowledge by providing a guidance for selecting appropriate microbial monitoring metrics based on the specific stage of the water treatment process.

and anticipated microbial load. This differentiation based on a multivariate analysis underscores the sensitivity in microbial populations to chlorine-based disinfection and how its impact can be detected automatically in an FCM user, gating independent way.

The integration of real-time monitoring with BactoSense® further enhances microbial surveillance by enabling high-frequency, automated microbial detection, particularly at critical stages such as pre-CCT, where rapid fluctuations in microbial populations can occur. This real-time capability improves the early detection of microbial shifts, offering opportunities for immediate corrective actions in water treatment operations.

The lead-lag analysis enriches our understanding of microbial dynamics, highlighting how specific metrics can serve as early warning indicators for microbial instability, thereby improving water quality management strategies. However, at the final water stage, the complexity of microbial interactions in chlorinated water posed challenges in determining clear lead-lag relationships. This suggests that higher-frequency sampling or more advanced microbial viability assessments may be required to capture rapid microbial shifts in distributed water systems.

Moreover, the implications of this research could help water treatment professionals optimize disinfection strategies, improve treatment efficiency, and enhance public health protection. While the study's findings are insightful, they are based on observations from a single WTW and its corresponding DWDS over a two-month period. This underscores the need for further research across multiple WTWs and over extended durations to validate and expand upon these findings. Additionally, integrating FCM-based methods with other microbial assessment techniques, such as 16S rRNA sequencing or metagenomics, could provide a more comprehensive understanding of microbial dynamics in water treatment processes. Advancing toward more predictive and automated water quality monitoring strategies will support the development of smarter, real-time water management systems, ensuring safer and more resilient drinking water supplies.

5.5 References

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5.6 Appendix

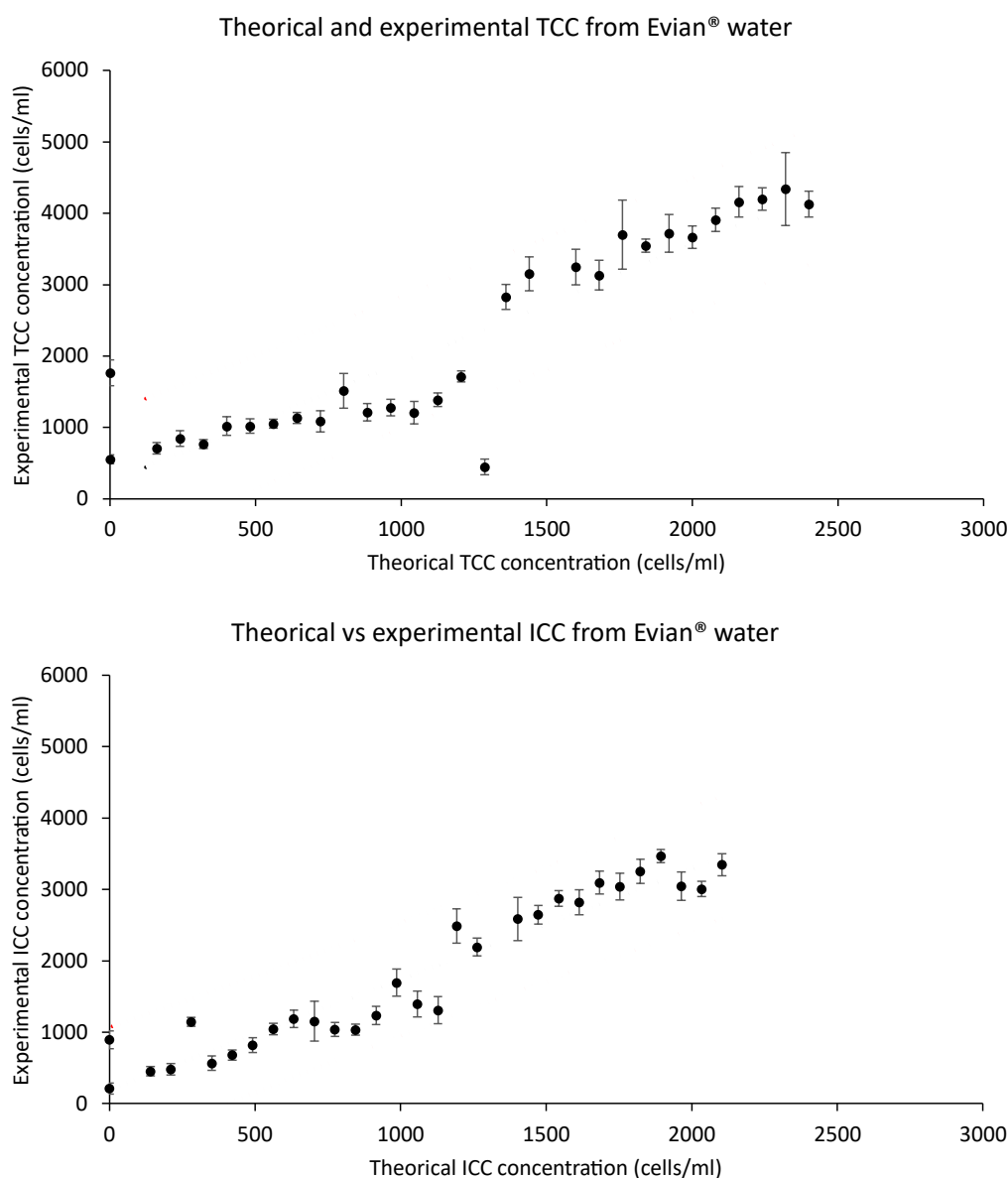


Figure S 5-1: Curve built from analysing a groundwater sample diluting with Evian water in different concentrations to determine the limit of detection (LOD) and quantification (LOQ) of FCM for TCC and ICC

Image comparison - XOR (Exclusive OR) operation

The operation consist of comparing corresponding pixel values between images

When two pixels are identical, the background is black, when they are different, the background is white

This can be mathematically expressed as:

$$A \text{ XOR } B = (A \cup B) - (A \cap B)$$

Where:

- A XOR is the XOR operation
- (A ∪ B) is the union of the two images
- (A ∩ B) is the intersection of the two images

The result of A XOR B corresponds to the total number of white pixels, which represent the differences between the two images

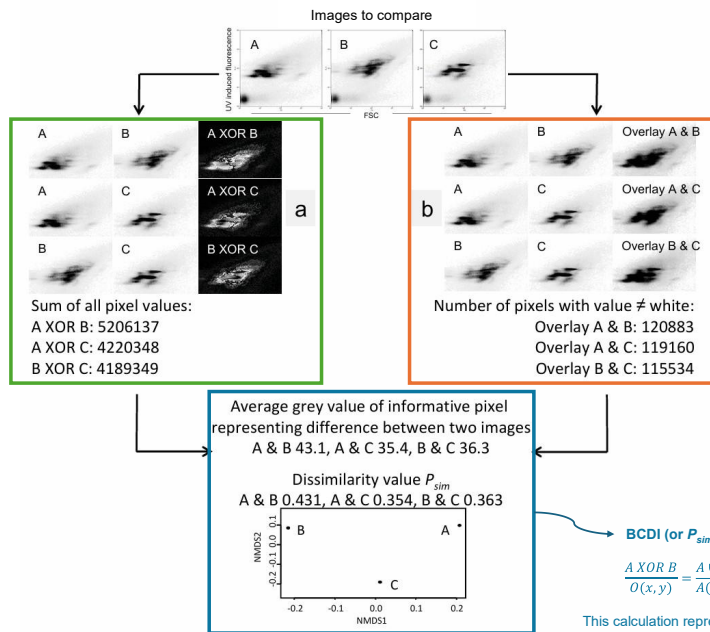


Image superimposing - Bitwise OR operation

The operation highlights all the pixels present in either of the two images

This can be mathematically expressed as:

$$O(x,y) = A(x,y) \cup B(x,y)$$

Where:

- O(x,y) is the resulting image after the Bitwise OR operation
- A(x,y) and B(x,y) are the pixel values of the two input images
- The operator ∪ represents the Bitwise OR operation

The O(x,y) value represents the total number of pixels that are non-zero (different from black)

Figure S 5-2: Explanation of CHIC analysis

Table S 5-1: Statistical tests explanation

Test	Purpose	Value and explanation
Stress (nMDS)	Evaluates how well the nMDS plot preserves the original dissimilarity structure of the data. Lower stress values indicate better representation of the data in reduced dimensions	<p>Value:</p> <p>< 0.1 → Excellent fit</p> <p>0.1–0.2 → Acceptable</p> <p>> 0.2 → Poor fit</p> <p>Interpretation: Ensures that visualized clustering in nMDS accurately represents the actual dissimilarities in microbial communities</p>
Within-Cluster Sum of Squares (WSS)	Measures cluster compactness by evaluating the total variation within each cluster. Lower WSS values indicate tighter clustering	Value: A low WSS suggests that data points within each cluster are closely related, meaning the clustering is well-defined. A high WSS suggests that clusters lack compactness
Between-Cluster Sum of Squares (BSS)	Measures dissimilarity between clusters, assessing how distinct clusters are from one another. Higher BSS values indicate better separation	Value: A high BSS relative to WSS suggests that clusters are well-separated, meaning the clustering method is effective. A low BSS suggests overlapping clusters

Silhouette Measure	Assesses within-cluster cohesion and between-cluster separation, evaluating how well each data point fits within its assigned cluster	<p>Value:</p> <p>Close to 1 → Strong clustering</p> <p>Near 0 → Possible misclassification</p> <p>Negative → Poor clustering</p> <p>Interpretation: Ensures that clusters are distinct and meaningful for microbial community analysis</p>
ANOSIM (Analysis of Similarities)	Tests whether differences between predefined sample groups are statistically significant based on their dissimilarity matrix	<p>Value: Produces an R-value (0 to 1) and a p-value</p> <p>$R \approx 1$: Strong separation between groups</p> <p>$R \approx 0$: No meaningful difference</p> <p>$p < 0.05$: Significant difference between groups</p> <p>Interpretation: Confirms whether microbial community differences between treatment stages are statistically significant</p>
Spearman's Rank	Analyses relationships between HNA/LNA dynamics and other variables, identifying non-parametric correlations	<p>Value:</p> <p>$\rho = 1 \rightarrow$ Perfect positive correlation</p>

Correlation Coefficient		$\rho = -1 \rightarrow$ Perfect negative correlation $\rho = 0 \rightarrow$ No correlation Interpretation: Identifies whether changes in HNA/LNA ratios correlate with microbial population shifts
Random Forest (RF) Algorithm	Identifies key factors predicting microbial growth patterns and their relative importance	Value: RF importance values range from 0 to 1 $0 \rightarrow$ No influence $1 \rightarrow$ Strongest influence Interpretation: Determines which environmental or treatment variables most significantly impact microbial growth patterns
Dunn's Test	Conducts pairwise comparisons between groups to identify significant differences	Value: $p < 0.05 \rightarrow$ Significant difference $p > 0.05 \rightarrow$ No significant difference Interpretation: Determines whether differences in microbial communities between treatment stages are statistically meaningful

<p>Mann-Whitney U Test</p>	<p>Compares two independent groups to assess whether one has significantly larger values than the other</p>	<p>Value: Produces a U statistic and a p-value:</p> <p>Small U, $p < 0.05 \rightarrow$ Significant difference</p> <p>Large U, $p > 0.05 \rightarrow$ No significant difference</p> <p>Interpretation: Determines whether microbial community characteristics differ significantly between treatment stages</p>
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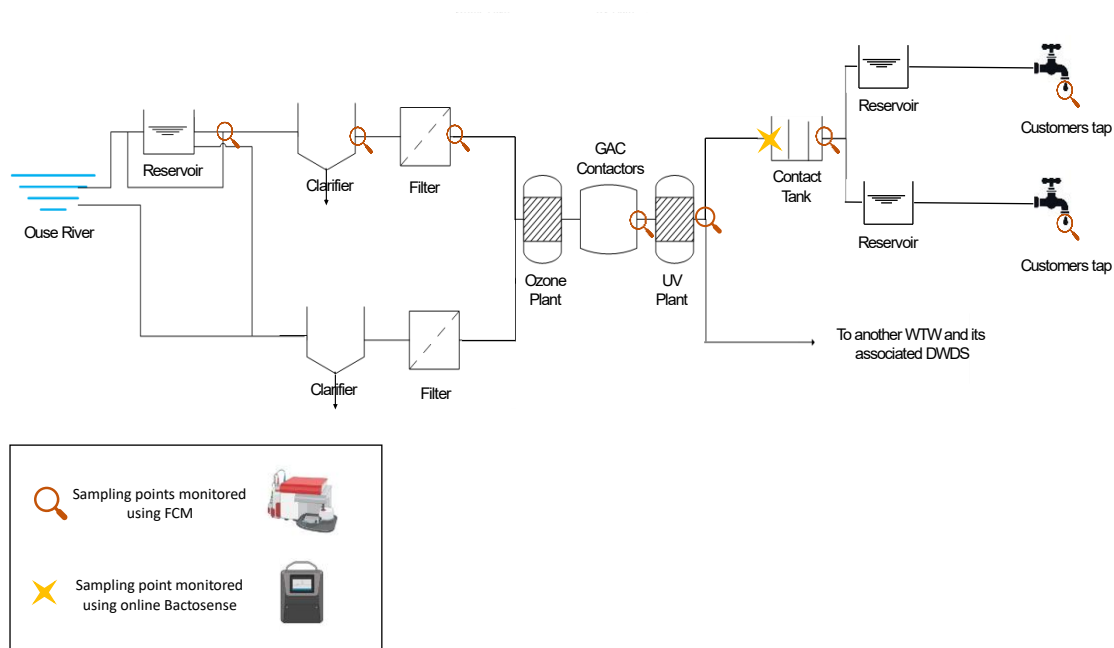


Figure S 5-3: Illustration of the Log removal at each stage of the WTW and log regrowth between final water and tap water

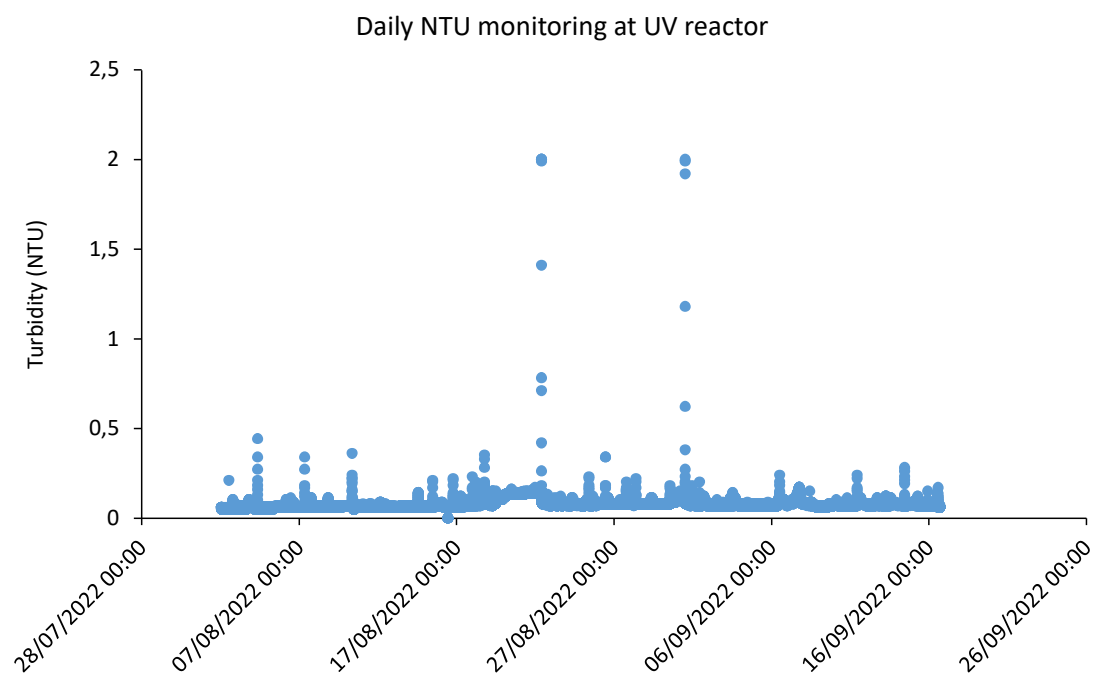


Figure S 5-4: hourly turbidity monitoring at the UV reactor of the monitored WTW

6 Overall discussion and implications of the work

6.1 Introduction

This EngD research addresses a complex issue such as understanding the valuable contribution and the early warning potential of both, conventional (i.e., ICC²⁷, TCC²⁸, HNA²⁹, LNA³⁰) and advanced (i.e., BCDI³¹) FCM metrics, in real-world scenarios, with the aim of providing a practical framework for their appropriate utilisation in decision-making processes in water treatment and management.

To achieve this aim, a mixed approach was employed combining the monitoring of 35 WTWs and 231 associated SRs with in-depth interstage monitoring of one individual WTW over the period of a year. The first case helped to (i) determine the added value of the FCM metrics compared to the traditional metrics (TVC, E. coli, coliforms, Turbidity, pH) to the assessment of water quality across different WTW designs, while the second focused on their added value at different treatment process stages. The second approach also helped to (ii) evaluate the influence of the gating process on the metrics, to (iii) determine the contribution of HNA and LNA to the assessment of the different treatment process stages, and to (iv) evaluate the complementarity of the metrics and their early warning potential for water microbial quality assessment.

This chapter provides a summary of the key findings, and an overview of how the different objectives contributed to achieve the aim of the EngD study.

6.2 Key findings and knowledge gaps filled by this research

As flow cytometry is getting more and more used in the water industry to monitor water microbial quality, bridging the gap between laboratory-based studies and practical applications is of importance to increase our understanding of FCM

²⁷ Intact Cell Count

²⁸ Total Cell Count

²⁹ High Nucleic Acid

³⁰ Low Nucleic Acid

³¹ Bray-Curtis Dissimilarity Index

metrics, particularly their appropriate use in real-world scenarios and their early warning potential to ultimately enhance microbial monitoring and improving decision-making processes.

Chapter 1 identified the research gaps that motivated the aim and objectives of this study. In particular it highlighted the need for: (1) real-world applications of the FCM metrics to bridge the gap between laboratory-based studies and practical applications and understand under which conditions they are the most valuable not only to highlight their contribution to the assessment of water quality but also to develop a practical framework for guidance; (2) addressing the practical issues regarding the FCM sensitivity hindering the accurate analysis of water with cell count below the LOD and the subjectivity of the gating process which can be prone to errors; (3) understanding the contribution of the metrics, particularly HNA and LNA, among other factors to understanding cell growth; (4) long-term, longitudinal, real-time study to demonstrate the benefits of FCM in assessing water quality.

Chapter 2 investigates the real-world applicability of FCM metrics across different water treatment works (WTWs), addressing the lack of cross-site comparisons that confirm their relevance in diverse treatment scenarios. Specifically, it examines whether FCM metrics provide consistent and interpretable insights regardless of water source, treatment design, and disinfection strategies (Ct values).

The study tested the hypothesis that WTW design does not significantly impact system performance evaluations when using FCM metrics, suggesting that these metrics are universally applicable and interpreted consistently across different treatment configurations. Prior studies have demonstrated the effectiveness of FCM metrics in rivers, boreholes, and granular activated carbon (GAC) treatments, supporting their broad applicability.

A mixed-methods approach was used, combining the monitoring of 35 WTWs and 109 associated service reservoirs (SRs) to evaluate the relevance of FCM metrics across different WTW designs, and a year-long interstage monitoring of

a single WTW, assessing how FCM metrics respond to various treatment stages within the same system.

TCC and ICC proved valuable for assessing treatment performance, providing a more sensitive evaluation than traditional microbial indicators (e.g., total viable counts, coliforms). Their ability to detect outliers highlights their strength in identifying inconsistencies in microbial control. BCDI effectively identified treatment failures, offering a proactive risk assessment metric for microbial contamination events.

The study contributed to knowledge through the cross-comparison of different WTW designs which revealed a notable gap in treatment robustness for groundwater WTWs. Although these systems are often considered compliant, they exhibited a higher frequency of non-compliance compared to surface water WTWs. This highlights the need to implement additional treatment barriers in groundwater treatment to enhance microbial safety.

In an industry perspective, the study demonstrates that FCM metrics (TCC, ICC, and BCDI) provide a more comprehensive assessment of treatment performance across different WTW designs. By identifying subtle variations in microbial removal efficiency, FCM can help utilities refine operational strategies. Additionally, the ability of BCDI to detect treatment failures highlights its potential as an early-warning tool for water treatment operators. This allows for proactive intervention, reducing the risk of microbial breakthrough and improving regulatory compliance.

The novelty of this study relies on the first large-scale simultaneous application of FCM metrics across multiple WTW designs over a full year, providing a comprehensive evaluation of their real-world performance and consistency.

The findings support the integration of FCM as a complementary monitoring tool for microbial risk assessment in water treatment, potentially influencing policy frameworks and operational guidelines. Furthermore, they highlight the importance of re-evaluating treatment barriers for groundwater WTWs,

emphasizing the need for enhanced multi-barrier strategies to improve microbial safety

Chapter 3 investigates real-world challenges in FCM monitoring, specifically focusing on the choice of gating strategy and the impact of low-cell concentration monitoring. While flow cytometry is widely recognized for microbial risk assessment, inconsistent gating approaches and failure to account for detection limits (LOD) can compromise the accuracy of microbial metrics (TCC, ICC, HNA, LNA, BCDI), ultimately reducing its effectiveness in water treatment applications

It was hypothesized that inappropriate gating and failure to consider FCM sensitivity limitations will lead to significant inaccuracies in microbial metrics, affecting microbial risk detection. Prior studies have shown that gating selection is subjective, leading to inter-laboratory variability in reported microbial counts. For low-cell-concentration samples, longer run times improve accuracy, yet in operational settings, samples are often analysed in batches, limiting this approach.

An in-depth interstage monitoring of one WTW over one year was conducted to evaluate how gating influences microbial metrics across different treatment process stages. This allowed for a direct assessment of the impact of static vs. dynamic gating strategies under varying microbial loads.

We found that static gating is suitable for high-microbial-load or stable water, ensuring consistent and reproducible results. Dynamic gating is necessary for unstable or low-microbial-load water, improving the accuracy of microbial detection. A no-gate strategy using BCDI (obtained through CHIC analysis) demonstrated value in real-world microbial risk assessment, regardless of microbial load, reinforcing its role in microbial risk detection.

This study contributed to knowledge by bridging the gap between laboratory and real-world application, demonstrating how gating strategies influence microbial monitoring outcomes across different treatment stages. It also contributes to refine the role of BCDI (CHIC analysis) in microbial risk assessment, confirming its applicability regardless of cell concentration.

On an industry perspective, the results support the use of CHIC analysis as a decision-making tool for microbial safety, particularly in monitoring treatment performance. CHIC analysis could aid in optimizing chlorine dosing strategies by providing real-time microbial risk indicators, reducing unnecessary chemical use while maintaining safety.

This study is the first to evaluate the influence of gating strategies on FCM metrics across real-world water sources with different microbial characteristics due to varying treatment steps. It is also the first application of BCDI to long-term WTW monitoring, assessing its performance across multiple treatment stages over a full year.

Regulatory agencies could integrate CHIC analysis into drinking water safety guidelines, enhancing microbial monitoring frameworks. Monitoring through CHIC analysis could improve and accelerate decision-making, enabling water treatment operators to detect microbial risks more efficiently than conventional methods.

Chapter 4 investigates the limited real-world application of HNA and LNA subpopulations in assessing microbial growth, particularly in comparison to other environmental and operational factors such as temperature, chlorine residual, and water age. While prior studies suggest a correlation between HNA presence and microbial regrowth, it remains unclear whether HNA/LNA alone provide sufficient insight into microbial dynamics or if their contribution is overshadowed by other influencing factors.

It is hypothesized that HNA/LNA contribute to microbial growth assessments only when analysed in synergy with other factors. Prior research indicates that HNA cells exhibit higher chlorine resistance due to membrane structure, making them relevant in disinfected systems. However, microbial growth is also significantly influenced by temperature, water age, and ingress events, raising questions about the true significance of HNA/LNA in complex, real-world conditions.

To explore this, a year-long interstage monitoring study of one WTW and nine SRs was conducted, evaluating how HNA/LNA metrics respond under varying

operational and environmental conditions. Additionally, two bench-scale experiments were performed to assess the contribution of HNA/LNA under controlled conditions. The first condition was related to chlorinated DWDS: Water samples from two SRs with different HNA/LNA profiles were spiked with nutrients and tracked using FCM to assess whether higher HNA concentrations correlate with greater microbial regrowth. The second condition was related to WTW stages: Samples collected at different WTW treatment stages were subjected to nutrient spikes to evaluate HNA/LNA contributions to microbial regrowth before entering the distribution network.

We found that HNA/LNA can serve as a rapid indicator of regrowth potential, particularly in chlorinated water, where HNA populations are influenced by chlorine stress. However, HNA/LNA classification oversimplifies microbial community dynamics, as microbial growth is driven by diverse subpopulations within these broad categories. Furthermore, environmental factors such as seasonal temperature shifts, ingress events, and upstream contamination had a more significant impact on microbial regrowth than HNA/LNA alone, demonstrating that their role must be considered within a broader context.

This study contributes to knowledge by improving understanding of HNA/LNA in real-world microbial regrowth assessments, evaluating their response across nine SRs with varying characteristics (shape, material, and distance from WTW) and under diverse environmental conditions. It highlights that HNA/LNA alone are insufficient predictors of microbial growth and should be used alongside other microbial and environmental parameters for robust risk assessment. The conventional approach of dividing microbial populations into two subgroups (HNA/LNA) may not always be relevant, as different microbial communities within each category contribute uniquely to microbial growth.

On an industry perspective, the findings suggest that certain microbial communities remain stable across different treatment stages, indicating that strategic design modifications (e.g., adjusting retention times, targeted flushing strategies) could enhance microbial control in distribution systems. While HNA/LNA provide rapid microbial insights, utilities must integrate them with

temperature, water age, and chlorine residual monitoring for more accurate microbial risk management.

This study's novelty relies on the application of HNA/LNA metrics simultaneously across nine SRs with different characteristics (shape, material, distance to WTW), placing the metrics in varied real-world conditions to assess their reliability in microbial growth monitoring. It was the first long-term monitoring of HNA/LNA response across both WTW treatment stages and DWDS, providing new insights into their role in microbial regrowth potential under operational conditions.

Regulatory agencies could consider integrating HNA/LNA monitoring into drinking water safety guidelines, but with an emphasis on contextualizing results alongside other microbial and environmental factors. Water utilities can enhance microbial risk assessment strategies by recognizing the limitations of HNA/LNA and adopting a multi-factorial approach to microbial monitoring.

Chapter 5 explores the lack of clear guidance on the appropriate use of FCM metrics in real-world water treatment scenarios. While FCM has demonstrated value in microbial monitoring, misapplication or failure to consider operational conditions can lead to inaccurate microbial contamination detection. This study investigates how different FCM metrics should be used under specific conditions to enhance microbial risk assessment.

The hypothesis states that choosing the appropriate FCM metrics based on water conditions significantly improves microbial contamination detection. Prior studies have shown that different microbial metrics (TCC, ICC, HNA, LNA, BCDI) respond differently to treatment processes, disinfection, and microbial dynamics, necessitating a tailored approach for effective monitoring.

An in-depth interstage monitoring study was conducted over one month at a single WTW, utilizing both offline and real-time (inline) FCM monitoring. Offline FCM (daily monitoring) was used to track microbial variations across treatment stages, including RGF, GAC, and UV. Inline FCM (Bactosense), set to record result every 30 min, was deployed at the pre-CCT stages to assess real-time microbial variations and its potential as an early warning system.

We found that cell counts (TCC and ICC) are most effective for monitoring high-microbial-load water, where microbial concentrations exceed the FCM detection limit (LOD). HNA/LNA are more relevant for assessing microbial variations in disinfected water, where subtle microbial shifts are critical. BCDI is universally applicable across all treatment conditions, providing a robust microbial risk indicator in both offline and real-time monitoring settings. The study confirms the complementarity of these metrics, showing that no single metric alone is sufficient for comprehensive microbial monitoring.

This study bridges the gap between laboratory research and real-world applications, particularly by demonstrating the practical value of BCDI in both offline and real-time monitoring. This study was the first to evaluate BCDI's performance across different WTW treatment stages, confirming its ability to provide consistent microbial risk assessment in real-world settings.

On an industry perspective, this study provides practical guidance on selecting appropriate FCM metrics based on water conditions, helping utilities optimize microbial monitoring strategies. The study supports the integration of real-time monitoring (inline FCM) for early warning detection, improving response times to microbial contamination events.

The study's novelty relies on the application of BCDI to long-term, real-world monitoring of multiple WTW treatment stages, demonstrating its real-time microbial risk detection capabilities. Another novelty is the systematic comparison of offline and real-time FCM applications, highlighting their respective strengths and optimal use cases.

Regulatory agencies could integrate BCDI monitoring into drinking water safety guidelines, improving microbial risk detection frameworks. BCDI monitoring could accelerate decision-making in water treatment operations, allowing utilities to proactively address microbial risks before regulatory non-compliance occurs

6.3 Research implications

The implication of the findings of the preceding chapters are explored as follows:

6.3.1 Implications of FCM gating strategies

The thesis provides a substantial contribution to the field of water quality analysis, particularly focusing on the utilization of FCM gating strategies to assess microbial quality in drinking water (Claveau et al., 2024; Gatza et al., 2013). The research critically evaluates different gating approaches—dynamic, static, and gate-free—each offering distinct advantages depending on the water type and its inherent variability (Chapter 3). Dynamic gating emerges as a useful strategy for analysing variable raw and distributed waters, adapting to each sample's unique characteristics to offer a more precise cell count (Chapter 3). This adaptability is particularly valuable in environments where water quality fluctuates, ensuring that microbial assessments are accurate and reflective of the current state (Chapter 2, Chapter 4, and Chapter 5). In contrast, static gating proves advantageous for more stable water sources (Claveau et al., 2024; Staats et al., 2019), especially in the context of final water with low cell counts (Chapter 3) validated using automated inline FCM applied within Chapter 5. This aspect is not directly novel, other researchers have applied automated inline FCM, but no studies have reported comprehensive assessment of data analysis approaches, rather they have undertaken analysis using these metrics without a priori validation (Favere et al., 2020). The study's strength lies in its potential for computer automation and thus reproducibility and speed, enabling consistent and rapid assessments that are vital but sadly missing from most of the routine monitoring undertaken within water utilities to ensure both compliance with water quality standards and protection of public health (Prest et al., 2013c). The introduction of a gate-free strategy (developed in Chapter 3 and applied within Chapters 4 and 5) presents an innovative approach, especially beneficial in waters with low residual cells (Claveau, Hudson, Jarvis, et al., 2024; Claveau, Hudson, Jeffrey, et al., 2024). This method, which includes background particles in the analysis, offers a broader perspective on water quality, revealing nuances that conventional methods of FCM analysis or indeed culture based microbial assessment overlook. CHIC analysis enhances the depth of the analysis and provides opportunity for automated analysis, confirming the findings from earlier work (Sadler et al., 2020). By shedding light on these different strategies and their

applications, the research contributes significantly to the standardization and optimization of microbial quality assessment methods, ultimately supporting efforts to ensure safe and clean drinking water.

6.3.2 Theoretical Implications of FCM Sensitivity

The sensitivity of FCM in detecting minor shifts in microbial viability and community through CHIC analysis and cluster analysis (Chapter 4) highlights the nuanced interaction which can occur due to microbial physiology and disinfection processes. This suggests that pre-treatment processes that induce physiological stress on microbes may enhance the efficacy of subsequent disinfection stages (J. Li et al., 2022; G. Liu et al., 2018; A. J. Pinto et al., 2012; G. Wen et al., 2017). This chapter points to these 'legacy effects' even into downstream DWDS, although further research is recommended to further elucidate this effect. This observation dovetails with theories on microbial resistance mechanisms, positing that stressed microbial cells may exhibit altered membrane permeability, rendering them more susceptible to oxidizing agents like chlorine and ozone. The impact of these treatments however on regrowth within DWDS requires further study. Chapter 4 did examine one aspect of this, which was the increase in HNA/LNA ratio with distance from the WTW due in part to deteriorating free chlorine levels due to decay and subsequent increase in cells. This phenomenon was a facet of the increasing distance (and hence water age) which significantly correlated to the ICC loading. The study suggests that this ratio thus could serve as a useful predictor of regrowth -especially in situations of low residual cell count (Chapter 4 and Chapter 5). Due to the complexity of some chlorination processes within the DWDS, the interplay between chlorine concentration, temperature, assimilable nutrients and starting microbial population requires further targeted research (Gayán et al., 2011; Zlatanović et al., 2017) and this went beyond the aspirations of this thesis. This is particularly relevant considering the transition to greater water storage times and even bulk treated water transfer schemes in the UK and elsewhere.

6.3.3 Bridging Empirical Findings with Theoretical Constructs

The study's integration of CHIC analysis into microbial water quality assessment represents a tangible application of theoretical concepts regarding microbial viability, community dynamics, and disinfection resistance ((Huang et al., 2013; M. Liu et al., 2020; Zhuang et al., 2015)). By linking these empirical findings with existing theories, the research validates appreciation of the complex factors governing microbial behaviour in WTW and DWDS (Chapter 5). This synthesis of theory and empirical evidence highlights the intricate balance between microbial community diversity, treatment technology, and water quality management strategies, setting the stage for future research that can further unravel the complexities of microbial dynamics in water sector but also further afield (such as fluvial, marine and lacustrine systems).

6.3.4 Advanced Methodological Insights

This research has pioneered the use of CHIC analysis to detect minute changes in microbial communities, demonstrating its superiority over conventional microbiological methods (Chapters 2-5). Chapter 5 summarises the critical advancements in the application of FCM for microbial quality assessment in drinking water treatment through a novel statistical framework – developed as part of this thesis incrementally. By integrating ICC, HNA/LNA ratios, and BCDI, the research offers a nuanced approach to detecting microbial dynamics in water treatment processes. Key findings reveal that ICC effectively quantifies high microbial load, while HNA/LNA ratios provide valuable insights during low microbial load conditions, especially post-disinfection (Gillespie et al., 2014; Potgieter et al., 2018). BCDI emerges as a versatile metric across all treatment stages, allowing for a comprehensive detection of microbial community changes (Buysschaert et al., 2019; Favere et al., 2020). By integrating multiple parameters, this approach improves microbial monitoring by providing high-resolution data on cell integrity and disinfection efficacy, enabling more accurate assessments of treatment performance. This, in turn, supports the optimization of treatment processes by allowing real-time adjustments to disinfection strategies, ensuring better microbial control and reducing the risk of under- or

over-dosing disinfectants. Consequently, these methodologies can enhance water quality management and strengthen public health protection in the water industry (Crocker & Bartram, 2014; P. Li & Wu, 2019; Royal Society for Public Health, 2017).

6.3.5 Relevance to the Water sector

The water sector can leverage the insights gained from the application of CHIC analysis in several ways which could enhance drinking water quality assessment and management:

1. **Real-Time Monitoring and Event Detection:** Utilizing CHIC analysis allows water utilities to monitor microbial water quality in real-time. This capability can significantly improve the detection of microbial shift or contamination incidents, enabling prompt responses to mitigate potential risks to public health. Real-time data could inform operational decisions, such as adjusting treatment processes or issuing boil water advisories more swiftly and accurately. Herein represents an opportunity for machine learning and or artificial intelligence to automate some of these aspects especially in remote or unmanned WTW (Rubbens & Props, 2021; Sadler et al., 2020a; Verschoor et al., 2015).
2. **Enhanced Treatment Process Efficiency:** By understanding the microbial dynamics and the effectiveness of different treatment stages, water treatment facilities can optimize their processes. The study's findings on the impact of chlorine concentration, contact time and water ages on microbial dynamics can guide utilities in refining their disinfection strategies, ensuring effective pathogen inactivation while potentially reducing chemical usage. Furthermore, it provides several indicators which could be derived to monitor regrowth within extended DWDS (e.g., HNA/LNA).
3. **Source Water Assessment:** The research highlights the importance of robust treatment regardless of the source water's perceived risk level. This information can drive more comprehensive risk assessments and treatment decisions for various water sources, especially groundwater,

which might be considered lower risk but still shows significant microbial indicators. The relationship between compliance organisms and FCM data is far from clear cut. Additional research is needed in advent of specific and rapid tools for monitoring pathogens cheaply (preferably in real time).

4. **Infrastructure Investment and Planning:** Insights from CHIC analysis can aid in identifying the sections of the water distribution system that are more prone to microbial growth or contamination. This information could be used to prioritize infrastructure investments, maintenance, and replacement to prevent water quality degradation.
5. **Regulatory Compliance and Reporting:** The detailed microbial assessment provided by CHIC analysis can support utilities in meeting regulatory requirements, demonstrating compliance with water quality standards, and providing more accurate reports to regulatory bodies.
6. **Research and Development:** these findings can drive further research into advanced water treatment and monitoring technologies by revealing microbial dynamics that influence disinfection efficacy. A deeper understanding of microbial behavior, such as resistance patterns and survival mechanisms, can lead to innovations in treatment optimization, early-warning detection systems, and automated monitoring using techniques like FCM. By enabling real-time microbial assessments, such approaches can improve process control, enhance water safety, and support the development of adaptive disinfection strategies.

6.4 Limitations of the research

While the research offers significant insights, it is not without limitations. The study's findings are based on data water treatment facilities within one region of England, which may limit the generalizability of the results. Additionally, the research entirely focuses on FCM-based techniques, which, while powerful, may not capture the full spectrum of microbial diversity and dynamics present in water systems. New research into real time detection (such as intrinsic fluorescence) or lab on chip analysis is recommended (see Chapter 7).

6.5 Future research directions

This research has advanced the understanding and application of FCM metrics in real-world water treatment and distribution systems. However, several unresolved questions and emerging challenges require further investigation. Future research should focus on refining the application of FCM, optimizing microbial risk assessment methodologies, and integrating these insights into regulatory and operational frameworks. The following key research directions are proposed:

6.5.1 Standardization and Optimization of FCM Metrics for Water Quality Monitoring

Despite demonstrating the value of TCC, ICC, HNA, LNA, and BCDI, standardized protocols are still needed for their use in different operational contexts. Future research should challenge the gating strategies by using them in additional real-world settings to investigate the robustness of the suggested framework.

6.5.2 Understanding the Role of HNA/LNA in Microbial Growth and Water Safety

The findings suggest that HNA/LNA alone are insufficient for microbial growth modelling but contribute valuable insights when combined with environmental and operational parameters. Future studies should investigate the taxonomic composition of HNA and LNA groups, providing deeper insights into their microbial ecology and role in disinfection resistance. They should also expand HNA/LNA research to different water distribution systems (DWDS) to assess their relevance in microbial regrowth prediction across varied infrastructures. Finally, they should examine more accurately the interactions between HNA/LNA and environmental factors (e.g., temperature, chlorine residual, and nutrient availability) to refine microbial growth models.

6.5.3 Enhancing Real-Time Monitoring and Early Warning Systems

The successful deployment of inline FCM (Bactosense) for real-time microbial monitoring demonstrated its potential as an early warning tool. However, further

advancements are needed to enhance its operational effectiveness. Further study should develop automated data interpretation algorithms to streamline real-time microbial risk assessment and integrate findings with existing supervisory control and data acquisition (SCADA) systems. Furthermore, they should explore the integration of AI and machine learning models to improve anomaly detection and predictive microbial risk forecasting using real-time FCM data.

6.5.4 Regulatory Integration and Industry Adoption of FCM Metrics

While this research demonstrates the practical benefits of FCM metrics in microbial risk assessment, their widespread regulatory and industry adoption require additional work. Future research should collaborate with regulatory bodies to develop guidelines for incorporating BCDI, HNA/LNA, and inline FCM monitoring into drinking water quality regulations. They should also assess the cost-benefit trade-offs of implementing real-time vs. offline FCM monitoring in large-scale water treatment and distribution systems. It would also be interesting to conduct pilot studies with different water utilities to evaluate the feasibility of FCM adoption, identifying operational barriers and optimization opportunities.

6.6 References

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7 Conclusions and Further work

7.1 Overall conclusion

This research has provided a comprehensive assessment of FCM metrics in real-world water treatment and distribution systems, addressing key gaps in their applicability, interpretation, and operational integration. By evaluating FCM metrics across multiple treatment processes, water types, and monitoring conditions, this work offers new insights into their role in microbial risk assessment, early warning detection, and treatment optimization. The findings contribute to both scientific knowledge and industry practice, offering practical guidance for their adoption in water quality management.

This study demonstrated that TCC, ICC, and BCDI provide more precise microbial assessments than traditional culture-based methods. The findings also revealed that groundwater WTWs showed higher non-compliance frequencies than surface water WTWs, despite generally being perceived as microbiologically stable. This highlights the need for additional treatment barriers in groundwater treatment to ensure microbial safety.

Results confirmed that static gating is suitable for high-microbial-load and stable water, while dynamic gating is necessary for low-microbial-load and unstable water. This provides essential guidance on minimizing inaccuracies in microbial assessments, ensuring more reliable FCM-based risk detection.

Findings showed that HNA/LNA classification provides useful but incomplete insights into microbial regrowth potential, as other environmental factors (e.g., temperature, chlorine residual, and water age) often have a greater influence. While HNA cells demonstrated higher chlorine resistance, microbial growth dynamics were better understood when HNA/LNA metrics were considered alongside other operational parameters or when the subgroups composing HNA and LNA were monitored.

A key outcome of this research was the development of guidelines on how to use FCM metrics appropriately under different conditions. We showed that TCC/ICC are best suited for high-microbial-load waters (above detection limits). HNA/LNA

are most relevant for tracking microbial variations in disinfected waters. BCDI is applicable across all scenarios, providing a robust microbial risk indicator. These insights provide essential recommendations for water utilities to optimize their microbial monitoring strategies.

The study highlighted the benefits of inline FCM (Bactosense) for real-time microbial monitoring, showing its early-warning capabilities for detecting microbial variations before regulatory exceedances occur. However, further research is needed to optimize real-time data interpretation and integration with existing monitoring frameworks.

7.2 Contributions to Knowledge and Industry

This research bridges the gap between lab-scale FCM applications and real-world monitoring, offering improved microbial risk assessment strategies for WTWs and distribution systems, a refined understanding of BCDI as a universal microbial risk indicator, supporting its adoption in industry practice, a practical guidelines for choosing appropriate FCM metrics based on water conditions, improving microbial contamination detection accuracy, and finally, evidence supporting regulatory integration of FCM-based microbial monitoring, encouraging its inclusion in drinking water quality frameworks.

7.3 Final Implications and Future Outlook

This research highlights the potential of FCM as a transformative tool in water quality management, offering faster, more accurate microbial assessments than conventional methods. However, its full adoption in the water industry requires further advancements in standardization, automation, and regulatory acceptance. By continuing to refine data interpretation, real-time monitoring capabilities, and integration with conventional indicators, FCM metrics can become an essential component of modern microbial risk management, ultimately ensuring safer drinking water for the future.