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To gate or not to gate: Revisiting drinking water microbial assessment through flow cytometry fingerprinting

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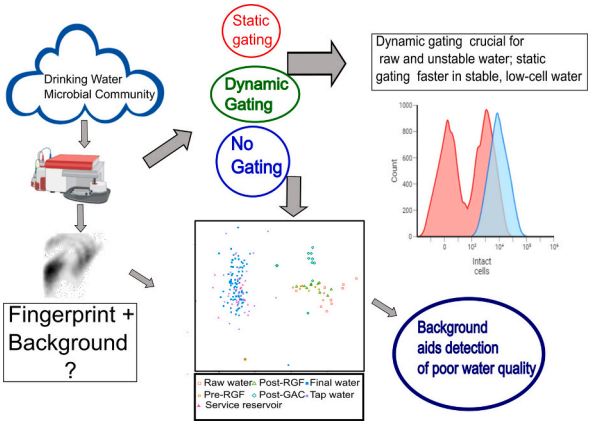
HIGHLIGHTS

- Flow Cytometry Gating strategies (dynamic, static, absent) assessed in drinking water.
- Dynamic gating gave better cell assessment in more variable raw water and distributed waters
- Static gating suited for stable and low cell count final water and is reproducible and rapid.
- Fluorescence fingerprinting cells & background augments traditional metrics for water quality.
- Gate-free strategy including background particles is useful in waters with low residual cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Flow cytometry has been utilized for over a decade as a rapid and reproducible approach to assessing microbial quality of drinking water. However, 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. This work conducts a comprehensive analysis of various gating approaches applied to flow cytometric scatter plots, using a dataset spanning a year. A sensitivity analysis is carried out to examine the impact of different gating strategies on final cell count results. 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. Our conclusions suggest that cell count analysis should be supplemented with fluorescence fingerprinting 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. FCM fingerprinting offers a dynamic approach to quantify treatment processes, enabling options for much better monitoring and control. This study offers new insights into the vagaries of various flow cytometry gating strategies, thereby substantially contributing to best practices in

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the water industry. The findings foster more efficient and reliable water analysis, improving of standardizing methods in microbial water quality assessment using FCM.

1. Introduction

Bacterial 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 (Sartory, 2004; WHO. World Health Organization, 2022). 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 (Hoefel et al., 2003; Cheswick et al., 2019). 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 et al., 2020), tracking bacteria dynamics in DWDS (Gillespie et al., 2014; Besmer et al., 2017; Van Nevel et al., 2017a), 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 et al., 2021).

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 et al., 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 bacterial 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 and 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., 2017b; 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 these approaches are used in the water sector to cope with increased data volumes from high-throughput bench FCM and automated FCM instruments (Suni 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 (Verschoor et al., 2015; Hassard and Whitton, 2019; Staats et al., 2019). 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 et al., 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 fluorescence fingerprinting 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).

Fluorescence fingerprinting using FCM (used with or without gating), is an innovative technique transforming flow cytometric 2D histograms into images, simplifying the comparison and detection of microbial community changes (Koch et al., 2013). It has the potential to reduce gating subjective interpretation as all the fingerprint 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. The Microbial Community Change Detection (MCCD) model offers an advance by monitoring microbial community stability semi-autonomously using an outlier score based on fingerprints and distance-based outlier calculations. This approach could enable early anomaly detection, underpinning proactive responses based on elements of the fingerprint (Sadler et al., 2020). However, most of the water sector is still using offline approaches without fingerprinting 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., 2017b; 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. To address this research gap we undertook a year-long experiment, with each WTW interstage undergoing analysis via FCM circa weekly with final water measured at various frequencies ranging from daily to weekly. The novelty of this work is that resultant flow cytometric scatter graph plots were processed through both static and dynamic gating approaches for comparison (Staats et al., 2019). This study hypothesised that flow cytometric data for cell quantification and fingerprinting analysis could together better inform microbial water quality. It postulated three core hypotheses: (i) the choice between static and dynamic gating significantly impacts FCM results for ICC, TCC, HNA, and LNA; (ii) cell quantification and fingerprinting provide distinct insights into water quality events in WTW; and (iii) a new approach, processing flow cytometric scatter plots without gating prior to fingerprinting, may enable new insights into water quality assessments generally and specifically for WTW and DWDS operations using FCM.

2. Materials and methods

2.1. Monitoring 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 filter (GAC), UV disinfection, and a Chlorine Contact Tank (CCT) (Fig. 1-1). Each treatment process within the WTW underwent weekly monitoring and water quality analysis assessing physical, chemical, microbial, and cytometric parameters, detailed further below. Daily samples were also taken from the final water reservoir outlet and analyzed for the same set of parameters. The studied service reservoir (SR), 16 km downstream from the WTW in the DWDS, had no reported inputs from other supplies throughout the investigation and was sampled weekly. Drinking water obtained from consumer tap samples within this largely single source DWDS were also collected and analyzed circa weekly.

2.2. Physical and chemical parameters

Physical and chemical parameters were measured as follows, with each sample taken in duplicate as per the regulatory procedures stipulated by Standing Committee of Analysts (2010). Initially, sample taps were flushed for three minutes, then flame sterilized for 30 s. After a further 30-s 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 h of sampling, the samples were transported to the laboratory for analysis, kept at a temperature of 4–8 °C. Water constituents including turbidity, pH, and total and free chlorine residual were measured adhering to the Standard Methods for the Examination of Water and Wastewater (APHA-AWWA, 2012), employing the 2130 B nephelometric method for turbidity, 4500 for pH, and 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 min 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.

2.3. Microbiological parameters

Microbiological assessments were carried out on collected samples following the guidelines outlined by APHA-AWWA (2012). 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 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, 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-h incubation period indicated the presence of *Clostridia perfringens*.

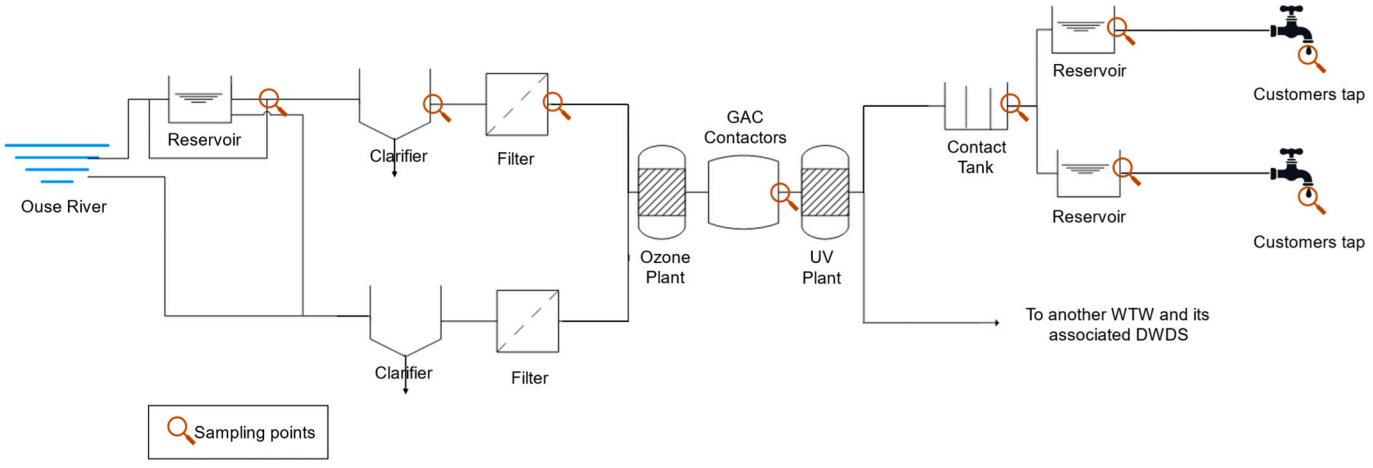

Fig. 1-1. Flowsheet of WTW used in the study. River is located in the South of England with a river abstraction from the Sussex Ouse River (UK). Sample points are indicated in orange. The filter is a Rapid Gravity Filter (RGF).

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 steralised Evian™ water filtered through a 0.2 μm Polyethersulfone (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 0.22 μm (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 (1 mg. 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 min at 35 °C and 400 rpm. Post incubation, 50 lL of each sample was analyzed 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 bacteria 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 0.22 um 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 (Hammes et al., 2008).

2.5. Fluorescence fingerprinting analysis

Flow cytometric scatter plots were delineated using two distinct gating approaches: static and dynamic. 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. Monitoring of the microbial population was two-fold, involving both cell count determination and cellular fluorescence fingerprinting. Cell counts within the gates were automatically quantified using FlowJo, whereas the fluorescence fingerprinting adhered to Koch et al.'s (2013) protocol for Cytometric Histogram Image Comparison (CHIC). This approach enables the interpretation of trends in microbial community structures without a prior definition of gates in a manner that is independent of the user. Briefly, the process of converting FCM scatter plots to bitmap images, extracting features, and comparing images through 'XOR' information retrieval which were conducted using FlowJo (v10) and ImageJ (v1.54d-) software. This process represents a form of 'phenotypic' community analysis relevant on FCM fluorescence data. In this analysis, the FCM data is subjected to transformation, discretisation and is concatenated into a single-dimensional vector which is used as basis for subsequent characterisation. Additional statistical measures, such as Bray-Curtis analysis, and the construction of non-metric multidimensional scaling (nMDS) plots and clustering analysis, were performed using R (2022−02−3). To better characterise contributing factors influencing variability in microbial populations, the operational events noted onsite by WTW staff and metadata including key operational variables such as free, chlorine, temperature, pH etc. were linked to specific measurements. During fluorescence fingerprinting analysis, different evaluation parameters were utilized. Stress measurements 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. As above, *p*-value set at significance of $\alpha = 0.05$ was used to determine the statistical significance of these observations.

2.6. Data analysis

Statistical evaluations of the resulting datasets, each corresponding to a gating method, entailed a series of tests and parameters. Given the non-normal distribution and potential variation in dataset sizes, median values, and Standard Deviation (SD) was 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. The CQV is a measure of relative variability based on the interquartile range (IQR). The IQR, which is the difference between the third quartile (Q3) and the first quartile (Q1), represents the range within which the central 50 % of the data values lie. 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, fluorescence fingerprinting, 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.

3. Results and discussion

3.1. WTW and DWDS monitoring using FCM

The WTW sample points are presented in Fig. 1-1 and the cell counts, determined using a dynamic gating method, are shown in Fig. 1-2. From the raw to tap water, cell count varies from <LOD to 6.1×10^6 cells/ml. Overall, 41 % of samples obtained from the SR outlet, and 23 % of tap water samples were either at or below LOD. Both ICC and TCC reduced across the WTW treatment processes with the only exception being the UV treatment step, the impact of which could not be detected with FCM. On average, the raw water showed the highest cell count (3.4×10^6 cells/ml) with subsequent treatment steps reducing the cell count; post-clarifier (1.3×10^6 cells/ml), post-RGF (7.7×10^5 cells/ml), post-GAC (1.7×10^5 cells/ml) and final water (666 cells/ml). The cell counts then increased progressively through the DWDS as the average SR cell count was 937 cells/ml and 1.9×10^4 cells/ml in tap water (Fig. 1-1). There was a single drinking water compliance event (operational event) during the sampling regime which was a single colony detection (1 CFU /100 ml) of *Clostridium perfringens* detection in the final water. This is indicated in Fig. 1-1, at the point of detection the TCC was 27,690 cells/ml and the ICC was 18,400 cells/ml. This was compared to counts of

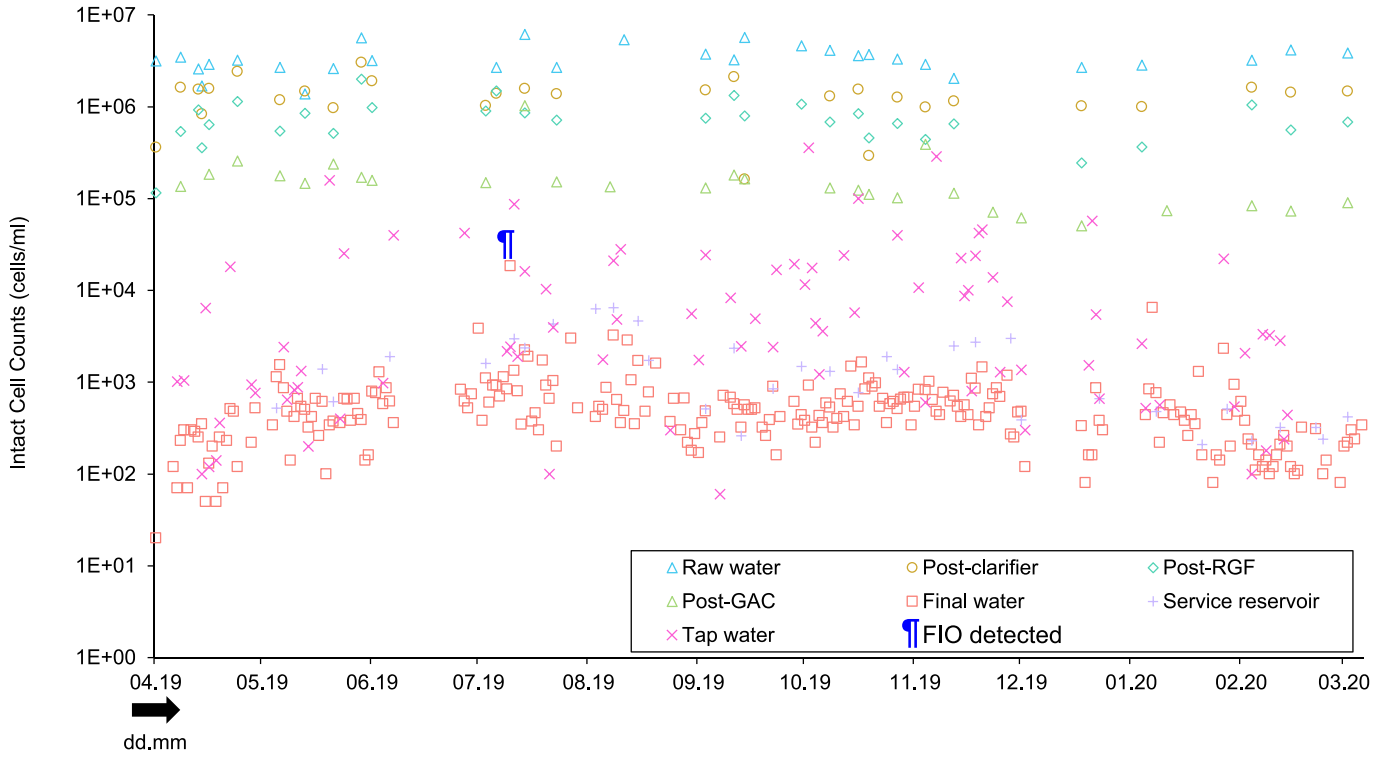

Fig. 1-2. Abundance of Intact Cells Across Various Stages of the Water Treatment Workflow. Sampling was carried out at five distinct stages: Raw, Post-Clarifier, Rapid Gravity Filter (RGF), Ozone/Granular Activated Carbon (GAC), and Ultraviolet (UV)/Contact Tank. The symbols highlighted in blue represent 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.

8400 and 840 cells/ml for TCC and ICC respectfully for the samples taken the day prior to the operational event representing a 3-fold increase in TCC and a 21 fold increase to ICC suggesting inefficient disinfection. Investigation of the FCM data alongside online process datasets determined the likely root cause to be a period of interrupted ozone dosage. The increase in cell count highlights the impact of upstream treatment processes for (i) maintaining efficacious disinfection at WTW and (ii) minimising assimilable nutrients which together act to maintain water which is complaint to standards. There was no evidence indicating any 'enhanced growth' or other signs of compromised water stability. Specifically, there is no observed delayed increase in microbial counts in the SR or tap water. Further work is needed to identify the role of pre-ozonation for providing upstream benefits to disinfection.

In our initial analysis of the data, we employed a dynamic gating approach. The results indicated that the WTW was effective in both removing and inactivating cells. It is probable that the increased variability in the cell count is linked to increased water ages in distribution (Machell and Boxall, 2014) and variable levels of free chlorine residual (Supplementary Fig. 1, Liu et al., 2013). To validate, the CQV statistic was calculated on each set of samples, which increased from 42 % for final water to 65 and 92 % for SR outlets and tap water samples, respectively (Table 1-1) – highlighting the lower biostability indicative of regrowth, blending of supplies of different quality or, potentially, ingress into the DWDS. The stability in numbers of ICC observed between final water and SR outlet in most samples was indicative of an adequate free chlorine residual (<0.05–1.24 mg/l) supressing the microbial population through inhibiting regrowth (Supplementary Fig. 1). In the absence of a free chlorine residual in most DWDS globally, the microbial water quality deteriorates, leading to bacterial instability as most conventional WTW are not removing assimilable nutrients to levels which inhibit bacterial growth (van der Kooij, 1990; Chen et al., 2001; Hammes and Egli, 2005). Contributing factors to regrowth include DWDS pipe degradation, external contaminants entering the system (i.e., ingress), and the reduction of residual chlorine levels through decay and organics reactive to chlorine (Douterelo et al., 2016; Yan et al., 2022; Wang et al., 2012; Nescerecka et al., 2018). 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

Table 1-1
Cell counts during the 1-year period. SD = standard deviation, CQV = Coefficient Quartile of Variation.

	DYNAMIC GATE cells/ml (unless stated)	STATIC GATE cells/ml (unless stated)
Raw water	Median = 170,257 SD = ± 1.16 × 10^6 CQV = 30 %	Median = 156,221 SD = ± 1.19 × 10^5 CQV = 24 %
Post-clarifier	Median = 67,563 SD = ± 4.81x10^4l CQV = 24 %	Median = 64,186 SD = ± 4.61 × 10^4 CQV = 23 %
Post-RGF	Median = 33,767 SD = ± 2.52 × 10^4 CQV = 37 %	Median = 33,930 SD = ± 2.35 × 10^4 CQV = 36 %
Post-GAC	Median = 6159 SD = ± 5.80 × 10^3 CQV = 38 %	Median = 6154 SD = ± 5.72 × 10^3 CQV = 37 %
Final water	Median = 22 SD = ± 52 CQV = 42 %	Median = 18 SD = ± 2.75 × 10^2 CQV = 43 %
Service reservoir	Median = 65 SD = ± 89 CQV = 65 %	Median = 59 SD = ± 81 CQV = 68 %
Tap water	Median = 165 SD = ± 2.75 × 10^3 CQV = 92 %	Median = 181 SD = ± 2.26 × 10^3 CQV = 89 %

water supplies focusing on this key asset for water quality (Martel et al., 2002; Doronina et al., 2020).

3.2. Impact of gating approach on drinking water assessment using FCM

The ICC was quantified using both dynamic (user defined) and static gating approaches. Regardless of whether dynamic or static gating is applied, both methods display a similar variability in the ICC values across the WTW (Fig. 1-3). Both gating methods provide similar median ICC values. At each sample point the median ICC values for all sample locations were within 22 % of each other, were statistically different in final waters (U test, 477 = 232, $p < 0.05$, Table 1-2), but statistically similar in interstage samples. Therefore, each gating method can be used to detect trends in microbial quality in drinking water but further insights into the vagaries of cell count within different treatment processes was needed (Liu et al., 2013). The raw water had the greatest range in the cell counts from 1.4×10^6–6.1×10^6 and with Q25 and Q75 values of 1.4×10^5 for the static and 2.5×10^5 for the dynamic gate (Fig. 1-3). As for the minimum and maximum, they were 5.1×10^3 and 6.1×10^6, respectively. In contrast, the Q25 and Q75 data were 1.3×10^5 and 2.1×10^5, and the minimum, maximum cells counts were 4.7×10^3 and 5.3×10^5. The Q25 was similar between dynamic and static gates. The Q75 results were generally lower for the static gate compared with the dynamic with the raw water results being most effected by gating strategy (Fig. 1-3).

The SD for raw water was 1.2×10^5 cells/ml for static and 1.2×10^6 cells/ml for dynamic gating (Table 1-1), constituting a statistically significant result (F-test, 29,27 = 1.69, $p = 0.05$, Table 1-2). The SD for final water was 2.8×10^2 cells/ml for static and 52 cells/ml for dynamic gating (Table 1-1) which is also statistically significant (F-test, 238, 239 = 0.035, p = 0, Table 1-2). The frequency of statistically extreme ICC values (data points beyond 1.5 times the IQR) was greatest using dynamic gating applied to raw water, whereas the static gate had greater frequency of extreme values in final water. The ICCs determined using dynamic gating were 0.1–7 % higher than those determined with static gating averaged across all sampling point. Despite the low ICC (generally <LOD), the distribution of the final treated water data shows similar median (18–22 cells/ml) and interquartile values ($Q25^{th}$ = 12–14 cells/ml, $Q75^{th}$ = 30–34) for both dynamic and static gating but with static gating showing a higher range of cell counts (462–2882 cells/ml). The SR and tap water had similar data distribution for both gating methods (Fig. 1-3). This finding shows the practical value of gate adjustment for precise and accurate quantification of bacterial cells in drinking water source waters, sub-potable, and treated supplies. The difference between gating strategies was most apparent in raw water due to its high degree of variability in this case i.e., fluvial surface waters without a substantial reservoir buffer. In addition, the divergence of cell count enumeration between different gating strategies in treated water occurred primarily due to differences between samples near or below derived methodological LOQ and those with higher regrowth potential.

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 (Figs. 1-4). A gating ratio of 1 means there was no difference between the two strategies. A ratio nearing 0 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, treated water at various stages (post-clarifier, post-RGF, post-GAC), and service reservoir were closely aligned, as shown by a ratio range of 0.76 to 1.23. An exception was observed for post-RGF samples, which had a significantly lower ratio of 0.26 (Fig. 1-4). In contrast, the final water, SR and tap water samples displayed wider ratio ranges of 0.09 to 6.83. 0.05–1.85, and 0.08 to 1.75, respectively, suggesting greater discrepancies between the two gating methods in the treated and distributed water samples. To delve deeper into this observation, fingerprints were compared from each population of FCM data – to assess which aspects of the fingerprint contributed to differences observed. Fig. 1-5 provides scatter plots for samples from each treatment stage where green markers indicate samples where the ICC, as determined by static gating, was either half (with a ratio of 0.5 or less) or twice (with a ratio of 2 or more) that of the count determined by dynamic gating.

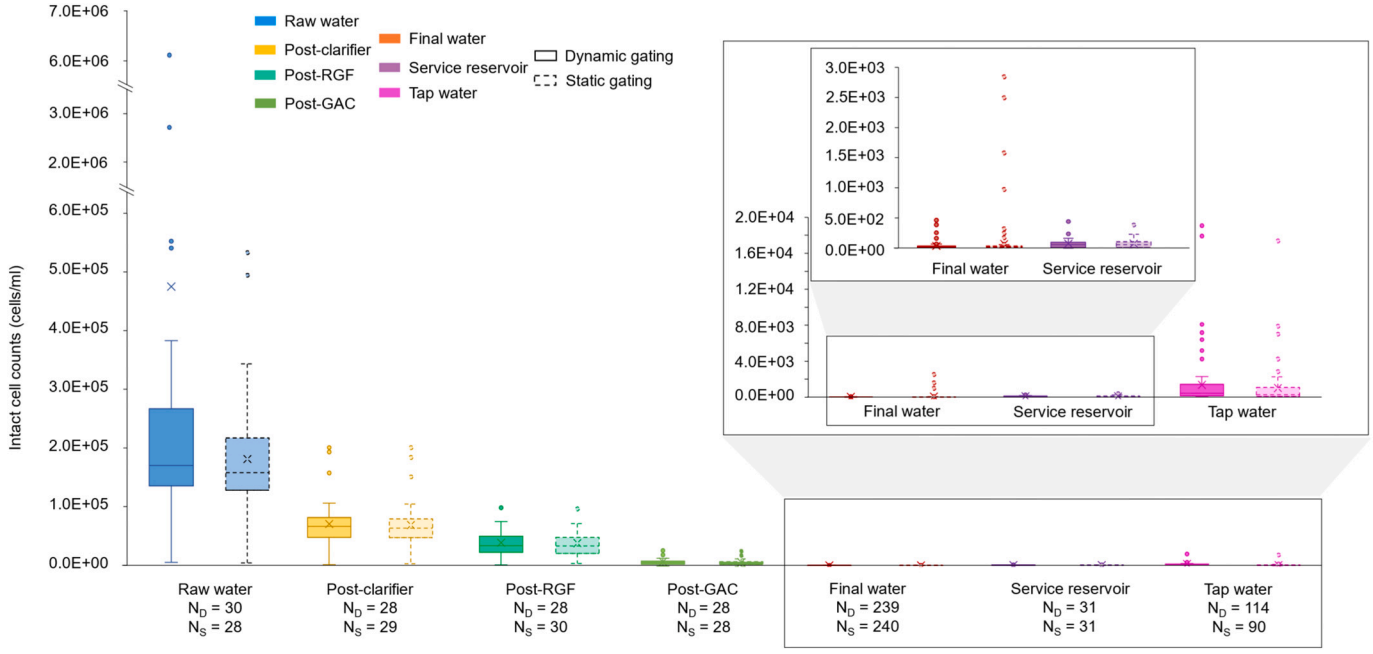

Fig. 1-3. Box-and-whisker plots display visualize key patterns and differences in FCM ICC data distribution, spanning stages from source to tap during the water treatment process. The treatment involves chlorination using free chlorine, maintaining residual chlorine in the distribution phase. Key components represented in the plot include the minimum, first quartile, median, mean (x), third quartile, maximum, interquartile range, whiskers, and extreme values (◯). Disinfected water is demarcated as a cutout. Values below the equipment's Limit of Quantification (LOQ) are included for reference, while those below the Limit of Detection (LOD) have been omitted for clarity. Samples exceeding 40,000 events per second were diluted prior to analysis on FCM. The dynamic gate is presented via a contiguous line and static gating is presented via a dashed line. The sample size for dynamic gate (N_D) and static gate (N_S).

Table 1-2
Comparative Analysis of Intact Cell Count (ICC) Measurements from different points of Water Treatment Works (WTW) process using Fixed-Gate and Adjusted-Gate Strategies. The mean, median, and variability for both strategies were statistically analyzed using the *t*-test, Mann-Whitney *U* test, and F-test respectively. The count of samples included in each comparison is denoted in parentheses. 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.

		STATIC GATE						
		Raw water	Post-Clarifier	Post-RGF	Post-GAC	Final water	Service reservoir	Tap water
DYNAMIC GATE	Raw water	U(56) = 0.903, p = 0.367 **<u>F(29, 27) = 95.54,</u> p < 0.05**						
	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

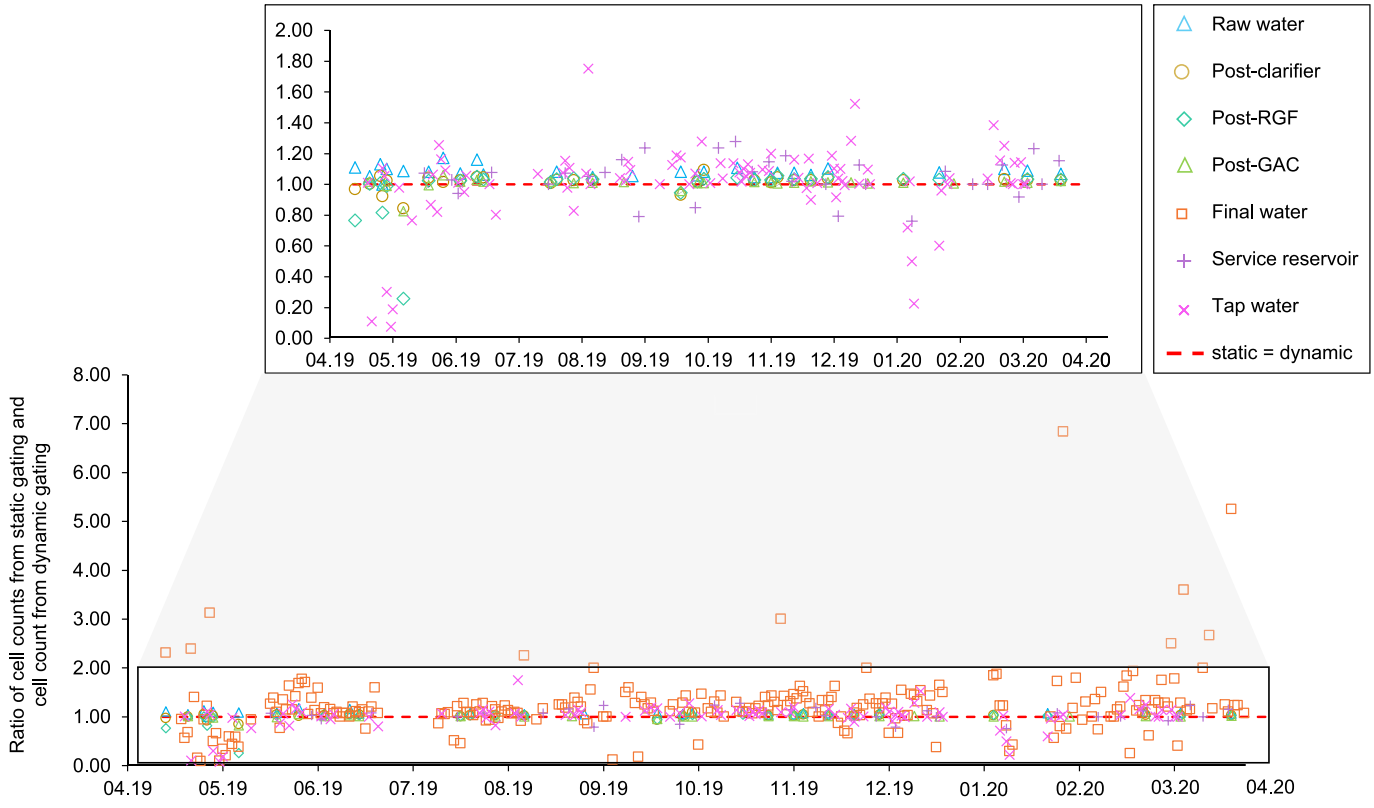

Fig. 1-4. Comparative Analysis of Flow Cytometry (FCM) Intact Cell Count (ICC) values between static and dynamic gating strategies. The data are presented as the ratio of values achieved using static gating to those obtained through dynamic gating. The red line serves as an indicator, illustrating the point at which the values from both gating strategies converge, signifying identical results.

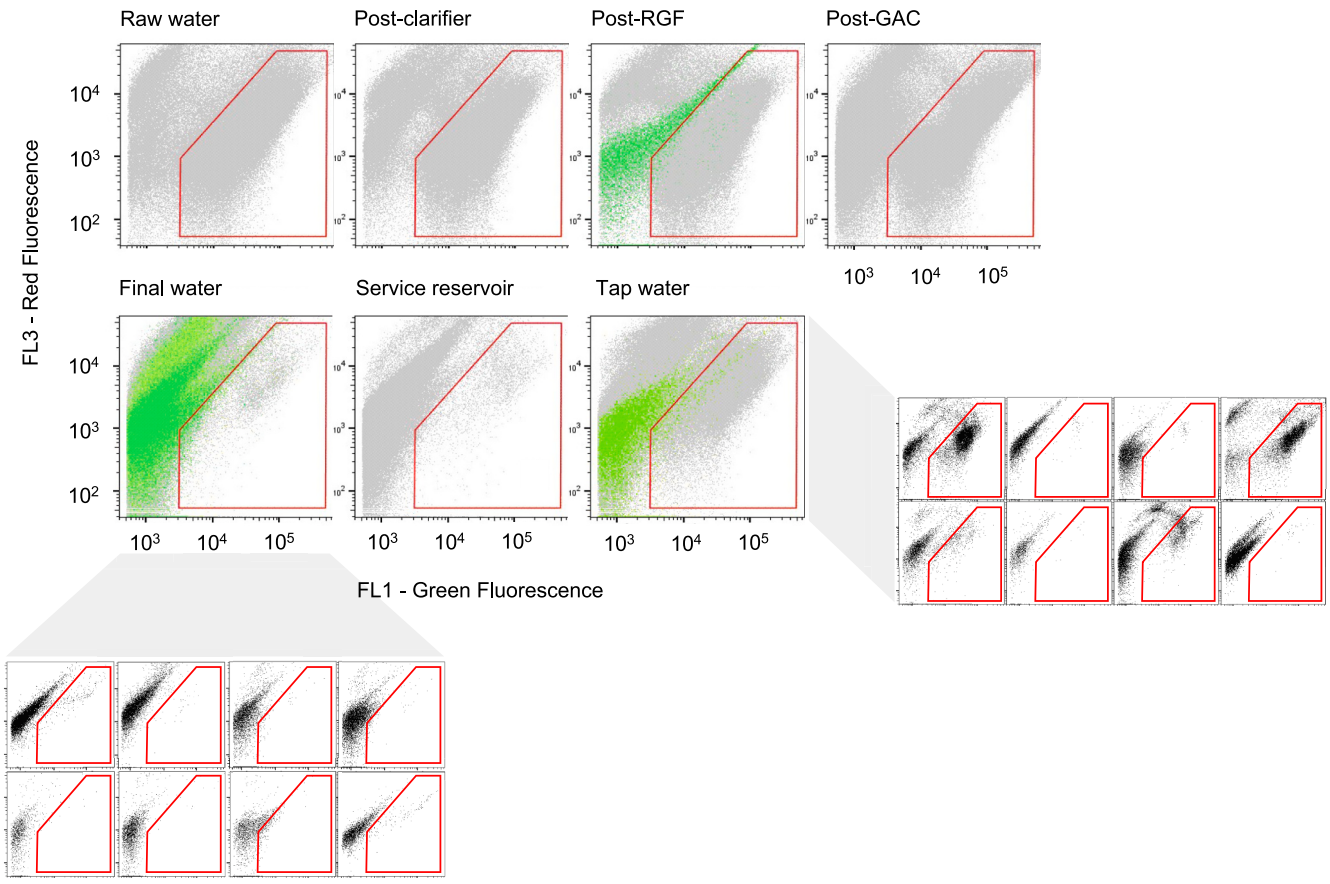

Fig. 1-5. Overlapping of the one-year FCM scatter graphs obtained for each stage of the WTW. The X-axis represents the Green fluorescence induced by staining cells with SYBR Green while the Y-axis represents the Red fluorescence induced by using Propidium iodide stain. The red polygon is a gate used 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 provide for Final water and Tap water in order to visualize the dynamic background.

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 (Fig. 1-5). 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 Fig. 1-5). This variability is partly due to a decrease in fluorescence resulting from oxidative stress (Wert et al., 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.

Variability in tap water, as illustrated in Fig. 1-5, is influenced by events in the DWDS such as ingress (Fox et al., 2016) or regrowth, which can modify microbial populations (i.e., abundance) and distribution (i.e., assembly). 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" at the boundary of the negative population. This counters 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 minimise user subjectivity, a factor of particular importance when cells counts are 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 (Fig. 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, 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

Table 1-3
Overview of the cluster content from Fig. 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	

microbial patterns seen Post-RGF on 10th May 2019 (Fig. 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. Nomura et al. (2008), emphasized 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. 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 this work on drinking water bacteria. 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.

3.3. Assessing the ICC value using cell count and fluorescence fingerprinting of cells

The 'no-gate' methodology has rarely been applied within the drinking water literature as most studies opt to eliminate background interference through gating before initiating fluorescent fingerprinting. In contrast, this work aimed to ascertain the benefits of traditional gated and 'no-gate' strategies using fingerprinting, examining data from various stages of the WTW. Yet, our fluorescence fingerprinting 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

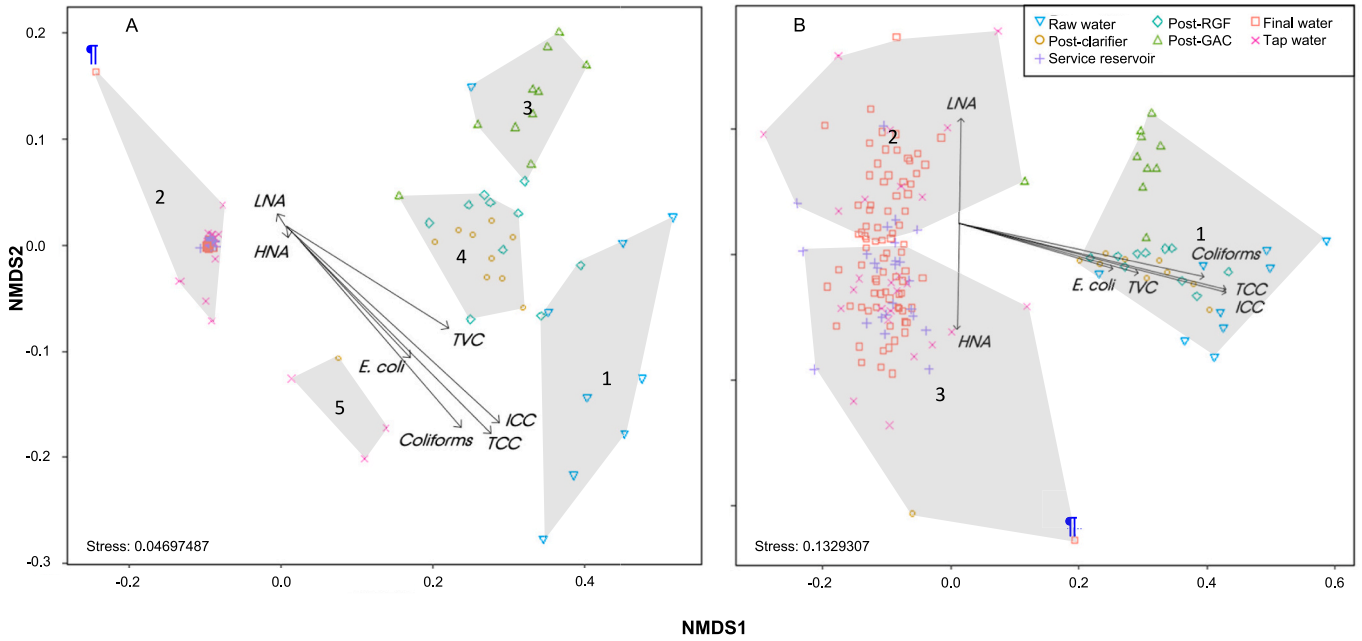

Fig. 1-6. A. Non-metric Multidimensional Scaling (NMDS) ordination plots from Cytometric Histogram Image Comparison (CHIC) analysis used to produce microbial phenotypic fingerprints, using 'fixed-gate' (Panel A) and 'no-gate' (Panel B) strategies. Utilizing K-means clustering and the gap statistic, optimal 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.

Table 1-4
Overview of the Pearson correlation (r) value for each vector shown in Fig. 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 Fig. 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	
E. coli	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

assessing microbial water quality. Essentially, similar cell counts in water samples may present different fluorescence fingerprinting characteristics enabling more insights into the processes governing (microbial) water quality. NMDS was used to display differences between distributions of cells.

Fig. 1-6A shows the dissimilarity between the samples of each treatment step of the WTW based on their microbial fluorescence fingerprinting using a gated approach. In contrast, Fig. 1-6B displays the same information, when background was included in the fluorescent fingerprinting analysis. The hypothesis was that greater differentiation between samples can be generated through analysis of all particles in the fingerprint (including bacteria and background). The differentiation of the samples coming from the different stages of the WTW (Fig. 1-6) 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 fingerprinting. 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 < silhouette <0.50, 0.56 < 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 bacteria suggesting that the background gives additional insight into the size/fluorescence/activity of the microbial population of 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 analyzed 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 produce commonly termed 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 filters (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.

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 fluorescence fingerprinting 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 Fig. 1-6A and three clusters in Fig. 1-6B. 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 1-3. Cluster 2 was primarily derived from final water and Cluster 3 from DWDS and tap water (Fig. 1-6). The separation based on the fluorescence fingerprinting was similar to that found using cell counts (Fig. 1-2) 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 (Fig. 1-2) suggesting that tap water microbial quality declines. Fingerprinting analysis (Fig. 1-6B) shows that the microbial pattern of tap water is as close as final water and SR outlet when cell alone are 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 (Fig. 1-6B) 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 need to confirm these observations.

Associations cells count and cell pattern with microbial indicators.

Fig. 1-6B 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 1-4). During the microbial water quality (Fig. 1-2; Fig. 1-6B), cell count was at its highest during this year (ICC > 10,000 cells/ml) and the fingerprint distributed at the extremity of the Cluster 3. This shows the effectiveness of the fingerprinting 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 fluorescence fingerprinting 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 (Wang et al., 2010); therefore, this correlation remains to be verified.

3.4. Future perspectives for the use of FCM and fingerprinting for microbial quality assessment

Flow cytometry is gaining significance in the realm of bacterial 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 bacterial 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 bacterial 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 modeling, it can effectively demonstrate the efficacy of innovative WTW and DWDS designs. However, it's worth noting that numerical modeling 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 optimize gating to be more dynamic to changing water quality. Additionally, the advent of no-gate FCM fingerprinting 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.

4. Conclusions

This study underscored the equivalent efficacy of the static and dynamic gating approaches in determining the ICC value and their association with microbial indicators. Both gating strategies demonstrate value depending on specific circumstances. Static gating showed effectiveness in monitoring low-cell-count water, while dynamic gating was ideal for analysing microbiologically unstable water. In instances of unexpected events, a periodically adjusted static gate was recommended to ensure consistency. However, the determination of ICC value alone was insufficient to holistically interpret water quality. Despite cell count and fluorescence fingerprinting yielding similar categorizations for raw, treated, and clean water, the study emphasized the distinct value of fluorescence fingerprinting. This method provided additional insights into the cellular composition, 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 both cell count and fluorescence fingerprinting methods displayed strong correlations with microbial indicators, the correlation between cell count 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 methods and strategies for 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.

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CRediT authorship contribution statement

L. Claveau: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **N. Hudson:** Funding acquisition, Investigation, Methodology, Supervision. **P. Jeffrey:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. **F. Hassard:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper. The decision to publish the research rested solely with the authors and the funder did not influence the decision to publish the research.

Data availability

Data associated with this manuscript is available at: 10.17862/cranfield.rd.23735649.

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