

Evaluating flow cytometric metrics for enhancing microbial monitoring in drinking water treatment processes

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ABSTRACT

Flow cytometry (FCM) offers a rapid method for bacterial detection in drinking water but faces challenges in terms of data analysis, particularly gating subjectivity. This study evaluates three metrics derived from the Intact Cell Count (ICC): High/Low Nucleic Acid (HNA/LNA) ratios, Bray–Curtis Dissimilarity Index (BCDI), and FCM fingerprints—to enhance microbial monitoring approaches across different water treatment and distribution stages. ICC provided a direct assessment of microbial load in high cell count scenarios, while HNA/LNA ratios were valuable during low microbial levels. BCDI effectively tracked microbial population changes throughout treatment processes. A lead–lag analysis revealed that ICC changes often precede or coincide with BCDI changes and lead changes in HNA/LNA ratios. FCM fingerprinting visualized 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.

1. Introduction

The integrity of drinking water systems is paramount to public health, economic stability, and societal well-being [1]. The microbial safety of drinking water is a priority, as unchecked bacterial proliferation or contamination events can undermine water quality, posing significant health risks and compromising compliance with regulatory standards [2–4]. For operating water treatment works (WTWs), and maintaining drinking water distribution systems (DWDS), ensuring microbial safety entails significant operational and capital costs in order to comply with hygienic and aesthetic standards. Traditional methods like heterotrophic plate count 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 ([5–7]. These constraints indicate necessity for more dynamic, accurate, and real-time methodologies to monitor and ensure water quality. This is especially valid considering significant challenges to water sector which includes; deteriorating infrastructure, climate change, and evolving microbial threats which act to reduce the quality of source waters used to produce treated drinking water [8–10].

Flow cytometry (FCM) has emerged as a robust alternative to culture

based microbial analysis methods, offering rapid and reproducible analysis of microbial populations in water [11–14]. 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. The cell viability metric serves as a valuable ‘direct’ measurement of the efficacy of disinfection [15–17].

Despite its advantages over culture-based methods, FCM's effectiveness is somewhat constrained by challenges associated with data interpretation and standardisation of analysis. Most especially due to the subjectivity in the gating process which is essential for distinguishing cell populations from the background of organic and inorganic particles [18–20]. Previous studies have primarily focused on individual FCM metrics, such as total cell count (TCC) or intact cell count ICC, but have not comprehensively evaluated the full scope of FCM-derived metrics which are inherent within FCM data or how they can be used for microbial water quality assessment. Moreover, there is a lack of consensus on which FCM metrics provide the most reliable indicators of microbial community dynamics, especially across the variable water matrix which

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occurs at different stages of water treatment and distribution. However, one notable advantage of FCM is that it provides a ‘multivariate’ assessment of each single cell, which provides analysts with numerous additional sources of data to detect process changes impacting microbial populations in aquatic systems. These metrics include ICC, TCC, high nucleic acid content (HNA) - or low nucleic acid content (LNA) - bacteria, HNA/LNA as a ratio, Bray–Curtis Dissimilarity Index (BCDI, also known as phenotypic analysis) and the subsequent visualization of the bacterial population using fluorescence fingerprinting analysis [21–24].

Flow cytometry fingerprinting is an approach which transforms two-dimensional histograms into detailed visual representations, allowing for an intuitive and comparative analysis of microbial communities. This technique, coupled with advanced analytical approaches facilitate the detection of microbial community shifts, offering an approach to identify potential water quality issues [22,25,26]. Complementary data on the proportions and ratios of HNA and LNA cells could provide insights into the metabolic status of microbial populations, reflecting to some extent the physiological diversity within water samples. This combination of metrics encompassed within the fluorescent fingerprint and the quantitation afforded by cell counting provides a powerful approach for microbial monitoring [24,27,28]. Applying the BCDI which is a well-established metric in ecological studies—to FCM data, also provides a quantitative means to monitor and understand microbial “taxa” dynamics in terms of their similarity [25,26] without deploying more costly DNA sequencing approaches for taxon identification.

Systematic comparison is needed of multiple FCM-derived metrics to determine their effectiveness, individually and collectively, in assessing microbial water quality across different stages of water treatment and distribution. Addressing this gap could lead to more reliable and comprehensive monitoring strategies, enhancing the ability to detect and respond to microbial water quality issues. In this study, we aim to directly compare three distinct flow cytometric metrics: HNA/LNA ratios, BCDI, and fingerprinting—both individually and collectively, to evaluate which metrics best assess drinking water microbial quality. We hypothesize that the combined application of these analytical metrics can significantly enhance the reliability of microbial quality assessments and useful to inform decision-making processes in drinking water

quality management.

2. Materials and methods

2.1. Study WTW and DWDS

Between August and September 2022, water samples were collected from each stage of the 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 treatment processes are commonly used in the UK for surface waters. These include a Clarifier (CF), Rapid Gravity Filter (RGF), ozonation, Granular Activated Carbon filter (GAC), UV disinfection, and a Chlorine Contact Tank (CCT) (Fig. 1). Each treatment stage within the WTW was monitored weekly for water quality, assessing the microbial state through the FCM metrics: ICC, HNA/LNA, and BCDI—detailed further below and determined using an Accuri C6 flow cytometer (BD, Switzerland). The pre-chlorination, the final water (contact tank outlet), and distributed water at customer taps were all monitored daily for these metrics. Additionally, the pre-contact tank water was analysed every 30 min using an inline BactoSense flow cytometer (bNovate Technologies, Switzerland). The pre-contact tank was selected for enhanced monitoring to understand how microbial populations respond to upstream treatment processes and how well the FCM metrics respond to these perturbations. The post-contact tank was not selected for inline monitoring due to low residual cell count.

The regulatory sampling procedures outlined by Clark [29] were used. Sample taps were flushed for three minutes and flame-sterilized before sample acquisition. Subsequently, taps were flushed for an additional 30 s before the collection of samples into sterile 250 mL sample bottles, containing sodium thiosulphate 0.1 % (w/v), to quench the chlorine. Samples were transported in <8 h maintaining 4–8 °C during transit.

2.2. Conventional FCM

Samples exceeding total and intact cell concentrations (TCC

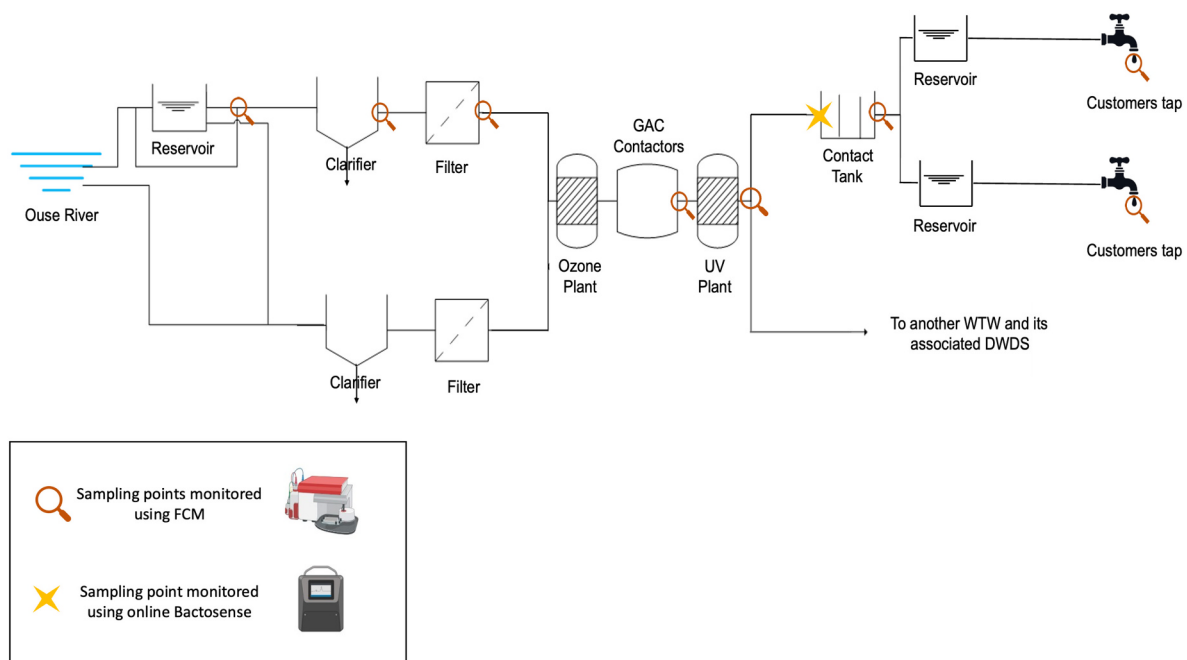


Fig. 1. Flowsheet of the WTW used in the study. River is located in the South of England with a river abstraction from the Sussex Ouse River (UK). Sample points are indicated in orange magnifying glass and yellow star. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

>1,000,000 cells/mL and ICC >800,000 cells/mL) were diluted using sterile Evian™ water filtered through 0.22 µm and 0.1 µm polyethersulfone (PES) filters, and then stained for TCC and ICC. For TCC staining, SYBR Green I (SGI; 10,000× stock; S-7567, Thermo Fisher Scientific, UK) was filtered using 0.22 µm filters (Z290807, Sigma-Aldrich, UK) and diluted to a 100× working concentration in dimethyl sulfoxide (DMSO). For ICC staining, five parts of the 100× SGI were mixed with one part propidium iodide (PI; Thermo Fisher Scientific, UK), resulting in final concentrations of 1× SGI and 3.0 µM PI. Samples were stained in 96-well plates and incubated at 35 °C and 400 rpm for 15 min using a microplate thermoshaker (PHMP; Grant Instruments™, UK). Aliquots of 50 µL were analysed at a flow rate of 66 µL/min using a BD Accuri C6 flow cytometer (Becton Dickinson UK Ltd., UK) equipped with a 488 nm solid-state laser. Green and red fluorescence were measured at 533 nm (FL1) and 670 nm (FL3), respectively. A single primary gate was applied to distinguish bacteria from background signals and intact or total cells, following Prest et al. [30], with the threshold for green fluorescence (FL1-H) set at 480 arbitrary units. Counts within this gate after SGI or SGI/PI staining were used to calculate TCC or ICC per mL, employing a dynamic gating approach to account for variations in microbial populations, particularly after chlorination [18]. High and low nucleic acid content bacteria (HNA and LNA) were identified using a static secondary gate [30,31]. The log reduction values (LRV) were calculated from cell counts.

2.3. Automated and inline FCM

An online flow cytometer (BactoSense; bNovate, Switzerland) equipped with a capillary syringe for particle alignment was used. It features a 488 nm blue 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 (0.1 % in DMSO) and Propidium Iodide (0.01 % in water), while cleaning utilized sterile water containing 0.05 % sodium azide and 0.1 % sodium hypochlorite. Samples (90 µL) were collected every 30 min using the Live/Dead Count (LDC) cartridge to determine ICC. Both offline and inline FCM were used due to their respective strengths. Offline measurements provided lower temporal resolution analysis of cytometric metrics, but are time-consuming and less suitable for continuous monitoring. The inline flow cytometer was positioned at the pre-contact tank to capture high-frequency ICC data, enabling near real-time monitoring of microbial dynamics.

2.4. Data analysis

2.4.1. Bray Curtis Dissimilarity Index (BCDI)

We selected the BCDI as a key fingerprint-derived metric due to its significant ecological relevance for monitoring population dynamics. BCDI is a unitless measure ranging from 0 (identical fingerprints) to 1 (completely dissimilar fingerprints with no shared patterns), providing an intuitive framework for microbial community comparison [32]. In this study, cytometric fingerprints obtained from FCM data capturing fluorescence and scatter properties of individual microbial cells stained with specific dyes—served as the units of analysis for BCDI. Species numbers and identities were inferred from patterns in these fingerprints rather than direct species identification, leveraging high-resolution data to distinguish microbial populations based on phenotypic characteristics. We collected a substantial set of samples: weekly from interstage water, daily from final and tap water, and hourly from pre-contact tank water ($n = 1128$ in August 2022; $n = 897$ in September 2022). This frequent sampling enabled detailed temporal analysis of microbial community dynamics over the water treatment and distribution system.

2.4.2. FCM fingerprinting

Cell concentration data for ICC, TCC, HNA, and LNA were extracted from .FCS files using FlowJo software (v10.7.2), facilitating FL1 vs. FL3

dot plot extraction. Initial FCM scatter plots were generated with BD Accuri C6 software, capturing cellular characteristics and background information. These .FCS files were then transformed into grayscale pixel images via FlowJo, focusing on the ICC fingerprint assessment of cell viability WTW due to use of oxidative treatments like ozone and chlorine. Image comparison was conducted using ImageJ (v1.54d) with the Cytometric Histogram Image Comparison (CHIC) method. Fingerprinting followed guidelines adapted from Koch et al. [26], utilizing the BCDI index to compare images based on pixel (cell) density and distribution. Fingerprint analysis preparation was described previously [18]. Scatter plots were standardized into 300 × 300-pixel images with a 64-channel grayscale resolution to ensure uniformity and precision in image comparison. Results visualization and significance assessment were performed using R (version 2022-02-3), employing non-metric multidimensional scaling (NMDS) and vector analysis. NMDS is an ordination technique that reduces complex, high-dimensional data into a lower-dimensional space (typically two dimensions) based on the ranks of dissimilarities between samples rather than their absolute differences. Predictor variables were correlated with CHIC fingerprints using Pearson's rank correlation. Two-dimensional stress values were calculated to assess how well the NMDS configuration reproduced observed dissimilarities among data points, with stress values interpreted as follows: <0.05 indicating excellent fit; 0.05–0.1, good fit; 0.1–0.2, acceptable with caution; 0.2–0.3, poor fit. Cluster validation involved the Silhouette Value and Within-Cluster Sum of Squares (WSS). A Silhouette Value approaching +1 indicated strong cluster coherence and differentiation, assessing clustering quality and cluster count suitability. WSS, calculated as the sum of squared distances from each object to its cluster centroid, measured cluster compactness; lower WSS values indicated tighter, more cohesive clusters. The optimal number of clusters was identified using the elbow method, determining where additional clusters did not significantly decrease WSS.

2.4.3. Statistical tests

The Dunn's test was applied for pairwise comparisons among inter-stage 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 metrics, 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).

3. Results and discussion

3.1. Comparison of FCM analysis metrics

3.1.1. Cell count

Fig. 2-A displays temporal trends in the measurements, while Fig. 2-B compares measurements across each treatment and distribution stages. Cell counts 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), and pre-CCT (10 to 2.3×10^6 cells/mL). Final water exhibited counts 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. The average LRVs were 0.5 between raw water and post-RGF, 0.6 between post-RGF and post-GAC, ~0 between post-GAC and post-UV (as expected), and 1 between pre-CCT and final water. An increase of 0.4 log cells/mL was observed from final water to tap water (Table 1; Dunn's test, $p < 0.001$). This demonstrates a progressive decrease in median ICC, illustrating the cumulative effect of each treatment process in reducing cell abundance. Physical treatments (RGF and GAC) and chlorine dosing (pre-CCT) played significant roles in this reduction followed by a slight increase in cells within the distribution system [33,34].

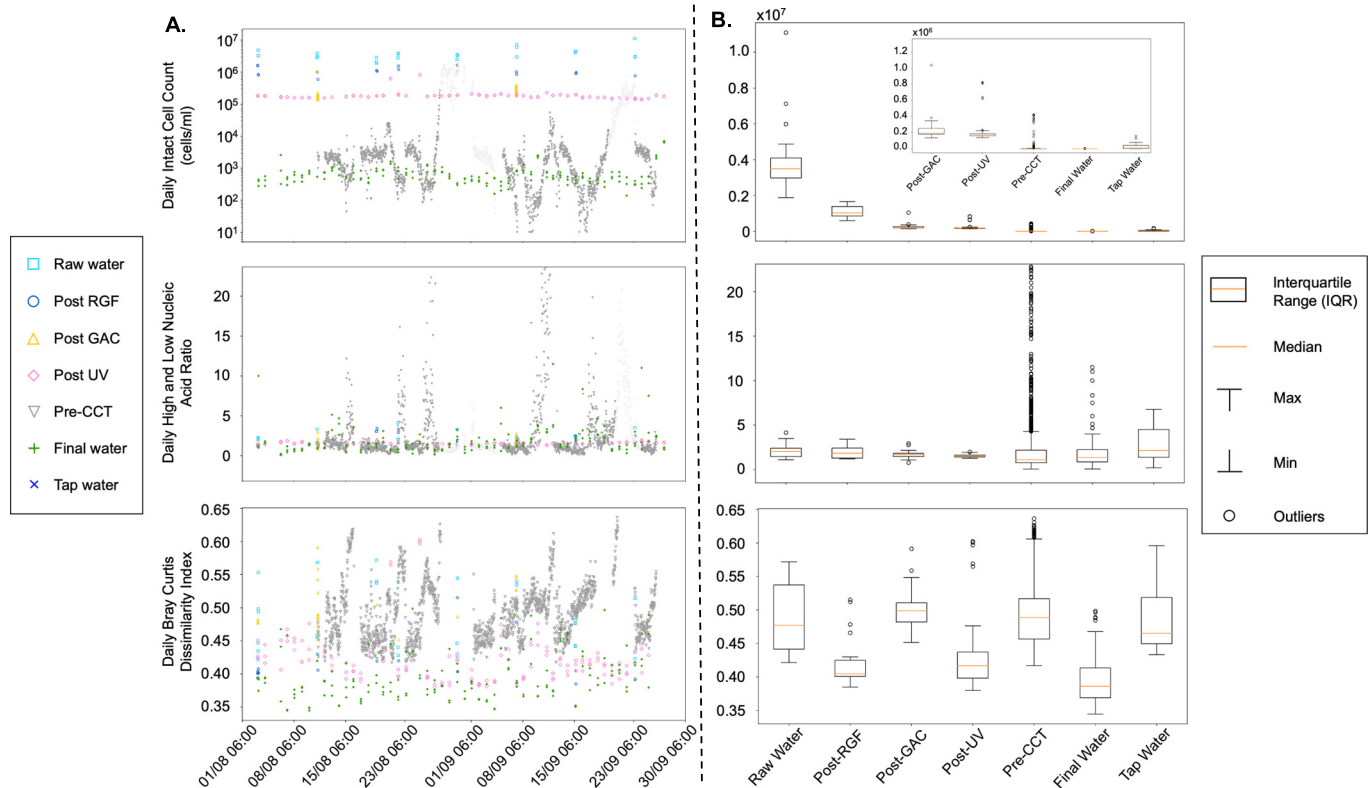


Fig. 2. A. Daily results of the measurement of the water cell count, HNA/LNA ratio and BCDI at each stage of a WTW. B. Distribution of the measurements at each stage of the WTW. The middle orange bar represents the median of the measurements. The outliers represent the data falling outside the main distribution 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 stages.

3.1.2. HNA/LNA

The daily HNA/LNA ratio showed minimal variation across treatment stages (Fig. 2-A). Mean HNA/LNA ratios were 2.0 ± 0.8 for raw water, 2.0 ± 0.7 post-RGF, 1.7 ± 0.4 post-GAC, 1.5 ± 0.2 post-UV, 1.9 ± 1.8 for final water, and 2.8 ± 2.0 for tap water. A notable deviation occurred at the pre-CCT stage, where hourly measurements using the inline system showed higher variability in HNA/LNA (4.0 ± 0.1), suggesting that offline measurements may not provide resolution to fully reflect the dynamic nature of processes affecting microbial populations in raw water or WTW samples which were typically in order of minutes in terms of events and hours in terms of trends. The median HNA/LNA ratio remained relatively consistent across treatment stages, ranging from 1.5 to 2.8 (Fig. 2-B), which was not statistically significant. Pairwise comparisons using Dunn's test ($p > 0.05$, Table S-1) showed no significant differences in the HNA/LNA ratio, except between the pre-CCT and final water stages, where a significant decline was observed ($p < 0.01$) due to disinfection stage. Despite overall similarity in median ratios, the interquartile range (IQR) was broader for pre-CCT (0.1–584), final water (0.1–11.5), and tap water (0.2–6.8), compared to narrower ranges for interstage waters: raw water (1.1–4.2), post-RGF (1.2–3.4), post-GAC (0.8–2.9), and post-UV (1.3–2.0). This is suggestive of a more varied response in the HNA/LNA ratio due to chlorine disinfection. Given that each water type has distinct HNA/LNA signature, we recommend its application for quantifying process changes within each WTW / DWDS but caution in comparing between different systems – due to high likelihood of site specific effects. The HNA/LNA ratio was effective in detecting changes resulting from oxidative treatment, showing more pronounced variability in waters subject to chlorine disinfection or chlorine residual.

3.1.3. BCDI

The daily BCDI values across treatment stages were broadly similar

(Fig. 2-A), ranging from 0.35 to 0.65, indicating relatively small changes in microbial composition. Mean BCDI values were 0.49 ± 0.05 for raw water, 0.42 ± 0.04 post-RGF, 0.50 ± 0.03 post-GAC, 0.42 ± 0.04 post-UV, 0.49 ± 0.04 pre-CCT, 0.40 ± 0.04 for final water, and 0.49 ± 0.05 for tap water. Median BCDI values showed a similar pattern (Fig. 2-B). However, the IQRs differed significantly (Dunn's test, $p < 0.05$): raw water (0.095), post-RGF (0.024), post-GAC (0.029), post-UV (0.039), pre-CCT (0.060), final water (0.045), and tap water (0.069). Variability in IQR highlighted the phenotypic plasticity of drinking water microbial populations temporally in response to vagaries of treatment and raw water quality.

Previous studies have demonstrated the individual roles these metrics show in microbial assessment. Cell count serves as a metric for detecting system anomalies such as distribution system ingress or leakage [35–37] and evaluating treatment efficacy ([6,14]. The HNA/LNA ratio provides insights into microbial populations' reactivity to treatments or regrowth [38] based on nucleic acid content [39]. The BCDI, a precursor for microbial fluorescence fingerprinting, helped track changes in microbial community composition and diversity over time without needing species identification, though it is probably is a simplistic representation of the actual community structure [25,40], due to spectral overlap between different taxonomic groups. High-frequency data used here and elsewhere reveal important sub-daily variations and inter-parameter links, which could be important for promptly detecting subtle water quality changes. Most studies however do not provide effective proactive monitoring due to lack of real time or rapid data analysis. Given the complementary nature of these FCM metrics, we hypothesized that a lead-lag analysis would uncover any temporal relationships between these metrics. This could offer new insights into how changes in one parameter might influence changes in others or respond to process or source water quality changes - through better use of existing multivariate data within fluorescence FCM fingerprints.

Table 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 <0.01, and 3 stars *** indicate a p-value inferior to 0.001.

	Raw water	Post-RGF	Post-GAC	Post-UV	Pre-CCT	Final water	Tap water
ICC							
Raw water							
Post-RGF	***						
Post-GAC	***	***					
Post-UV	***	***	***				
Pre-CCT	***	***	***	***			
Final water	***	***	***	***	***		
Tap water	***	***	***	***	***	***	
HNA/LNA							
Raw water							
Post-RGF	0.744						
Post-GAC	0.214	0.337					
Post-UV	***	0.191	***				
Pre-CCT	***	***	***	***			
Final water	***	*	0.059	*	0.1		
Tap water	0.4	0.251	0.061	***	***	*	
BCDI							
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	***	

3.2. Cross-correlation (lead-lag test)

To understand the temporal relationships among cell count, HNA/LNA ratio, and BCDI, we performed a lead-lag analysis using cross-correlation. Lead-lag analysis is a statistical method that examines whether changes in one time series (e.g., cell count) precede or follow changes in another time series (e.g., HNA/LNA ratio), and quantifies the time delay between these changes. This approach helps identify potential causal relationships and predict future trends in microbial populations, which is important for timely interventions in water treatment and supply processes. We conducted the cross-correlation analysis at the post-GAC/UV, pre-CCT, and final water stages (Figs. 3 and 4), calculating Spearman's rank correlation coefficients under varying time lags. The time lags ranged from -10 to 10 days for post-GAC/UV and final water, and -48 to 48 h for pre-CCT. These stages were selected due to their substantial dataset sizes ($N = 153$ for post-UV, 1990 for pre-CCT,

and 163 for final water) and their significant influence on final microbial water quality.

At the post-UV stage, the analysis revealed a negative correlation between ICC and HNA/LNA ratio with a lag of 5 days ($\rho = -0.6$). This indicates that an increase in ICC precedes a decrease in the HNA/LNA ratio after five days. This could suggest that an initial rise in ICC leads to resource depletion or environmental changes that favour the persistence of LNA cells over HNA cells. Alternatively, the reduction in the HNA/LNA ratio might be due to the aging of HNA cells or their transition to a less active state (Fig. 4). This finding contrasts with previous studies where bacterial growth was associated with an increase in HNA cells [27,28,41,42]. The discrepancy may stem from site specific differences in water source, treatment processes, or specific microbial communities present in the water at this site.

Additionally, a positive correlation between HNA/LNA ratio and BCDI was observed ($\rho = 0.4$, $p < 0.001$) with no significant time lag, suggesting that changes in microbial community structure are immediately reflected in the nucleic acid content ratio. This is logical since both BCDI and HNA/LNA ratio are influenced by cell fluorescence characteristics; thus, alterations in fluorescence due to shifts in community composition impact both metrics simultaneously. At the pre-CCT stage, analysis revealed that peaks in cell count ($>2 \times 10^4$ cells/mL), marked in Fig. 3, corresponded with elevated HNA/LNA ratios (>10) and higher BCDI values (>0.55). The lead-lag analysis showed a positive correlation between cell count and HNA/LNA ratio ($\rho = 0.5$, $p < 0.001$), with cell count leading by 48 h. This implies that increases in cell abundance precede changes in the nucleic acid content of the microbial population, possibly indicating that rapid microbial growth initially increases cell numbers before affecting the metabolic status or activity levels within the community. The HNA/LNA ratio responding to changes in BCDI within a day ($\rho = 0.4$, $p < 0.001$) suggests that as the microbial community deviates from its baseline state (higher BCDI), there is an associated increase in HNA cells, reflecting a shift towards more active or stressed microbial populations.

For the relationship between BCDI and cell count at the pre-CCT stage, the lead-lag analysis presented two scenarios: BCDI preceding cell count by approximately 53 h ($p < 0.001$) or responding concurrently ($p < 0.001$). When BCDI leads, it suggests that changes in community composition occur before increases in cell numbers. Conversely, when changes are concurrent, it implies that alterations in cell count and community structure happen simultaneously, possibly in response to immediate environmental changes or treatment processes such as fluctuations in nutrient availability or disinfection efficacy. The occurrence of both scenarios indicates the complexity of microbial dynamics and suggests that multiple factors may influence the timing and sequence of these changes – requiring further work to disentangle.

At the final water stage, determining lead and lag calculations were challenging due to the lower frequency of sampling and the complexity introduced by chlorination, which can rapidly alter microbial communities. Chlorination may cause immediate and drastic changes in microbial viability and community composition, making it difficult to capture lead-lag relationships without high-frequency data. This limitation suggests that high-frequency sampling is important for effective lead-lag analysis in chlorinated waters to accurately detect and interpret rapid microbial shifts. Our findings suggest that ICC often serves as a leading indicator for changes in HNA/LNA ratio and BCDI, highlighting its potential as an early warning signal for shifts in microbial populations. The sensitivity of ICC to changes in microbial load allows for the prediction of subsequent alterations in community structure and metabolic activity. This insight is valuable for water treatment operators, as it emphasizes the importance of monitoring ICC to anticipate and respond to microbial fluctuations proactively.

While our analysis provides valuable insights, it is important to acknowledge the lead-lag relationships observed may be influenced by the specific conditions of the sampled WTW and DWDS, and may not be universally applicable. Additionally, the temporal resolution of

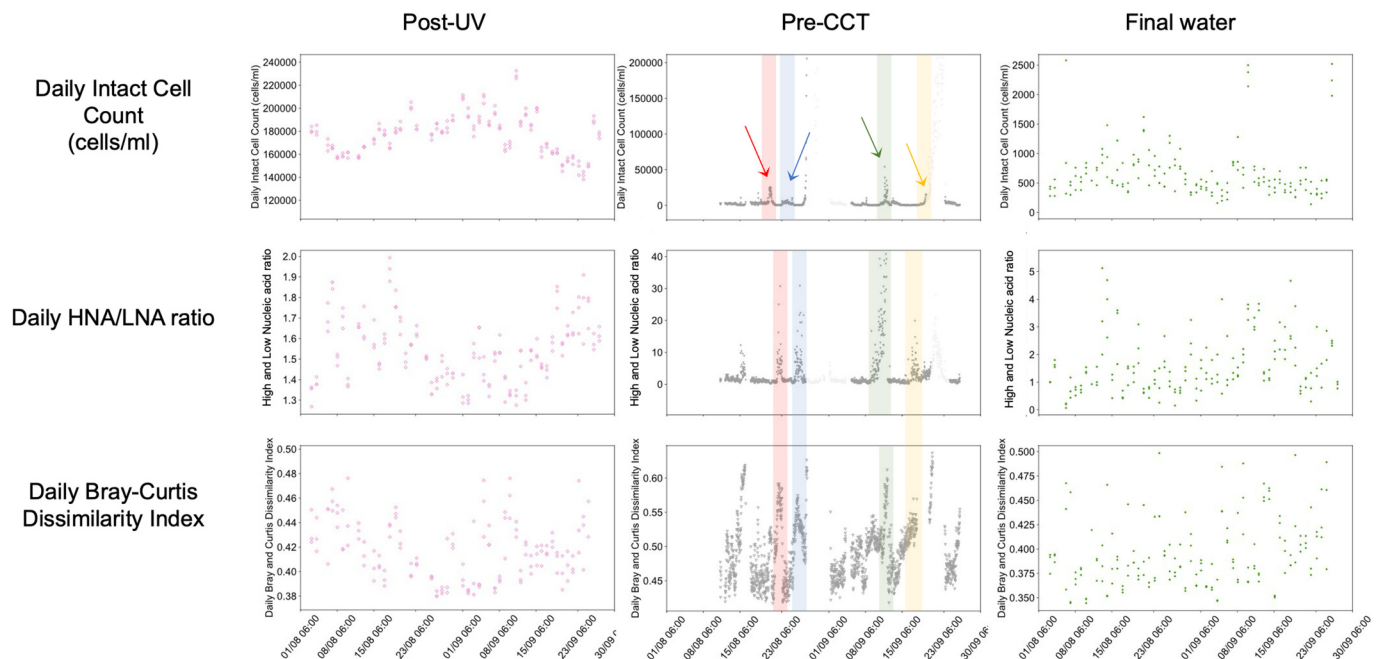


Fig. 3. Daily results of the water cell count, HNA/LNA ratio, and BCDI at the Post-UV, Pre-CCT, and Final water stage of the WTW. Coloured arrows and background on the Pre-CCT graphs represent the periods for which each metrics showed a peak almost simultaneously.

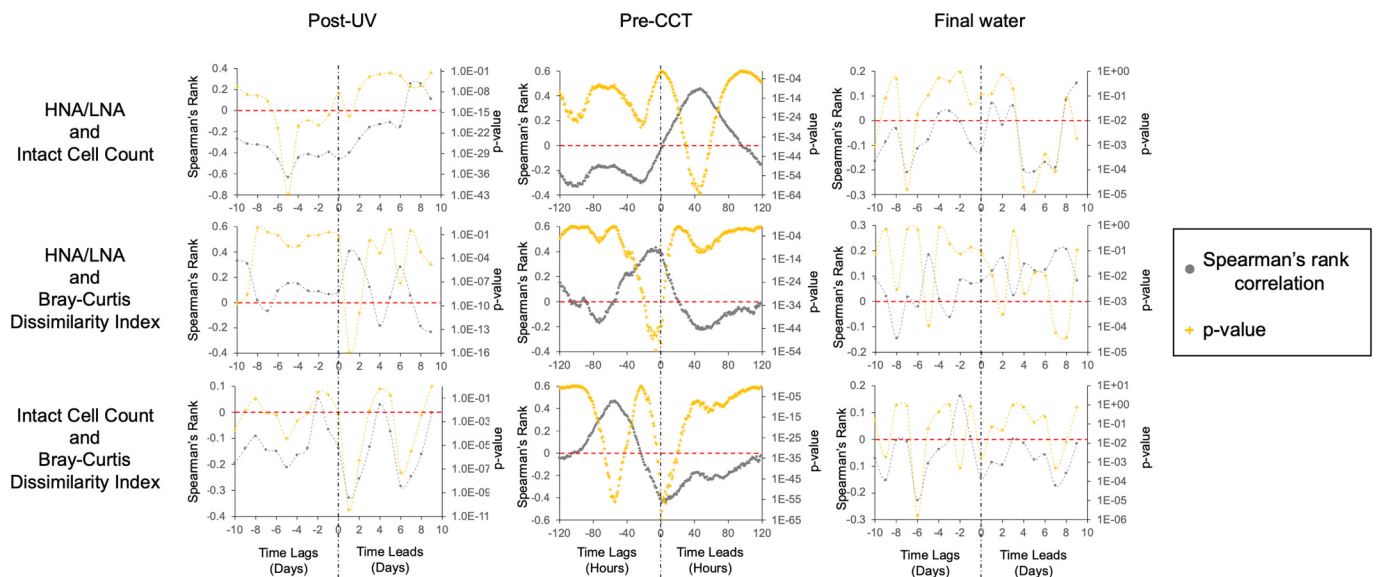


Fig. 4. Pair wise cross-correlation between the different metrics in a at the Post-UV, Pre-CCT, and Final water stage of the WTW. The grey dotted line represents the Spearman's rank correlation at each time lag. The yellow dotted line represents the p -value associated with each Spearman's rank correlation. Note that below 0 the time is seen as a time lag and above zero the time is seen as a lead time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sampling, especially at the final water stage, may have been insufficient to capture rapid microbial dynamics caused by chlorination. Variability in microbial communities and environmental conditions across different facilities could affect the generalizability of our findings. Further studies with increased sampling frequency and across multiple treatment facilities are necessary to validate these findings and enhance their applicability to broader contexts.

3.3. Multivariate analysis

We applied multivariate statistical methods to assess how cell count,

HNA/LNA ratio, and BCDI influence microbial water quality dynamics during treatment and distribution. BCDI values ranging from 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 (Fig. 5). The NMDS analysis, utilizing BCDI values, segregated data into distinct clusters with strong intra-cluster homogeneity. Cluster 1 included post-UV and select post-GAC samples, showing uniformity. Cluster 2 comprised raw and post-RGF water along with some post-UV and post-GAC samples, indicating similar features. Cluster 3 grouped all pre-CCT samples, while

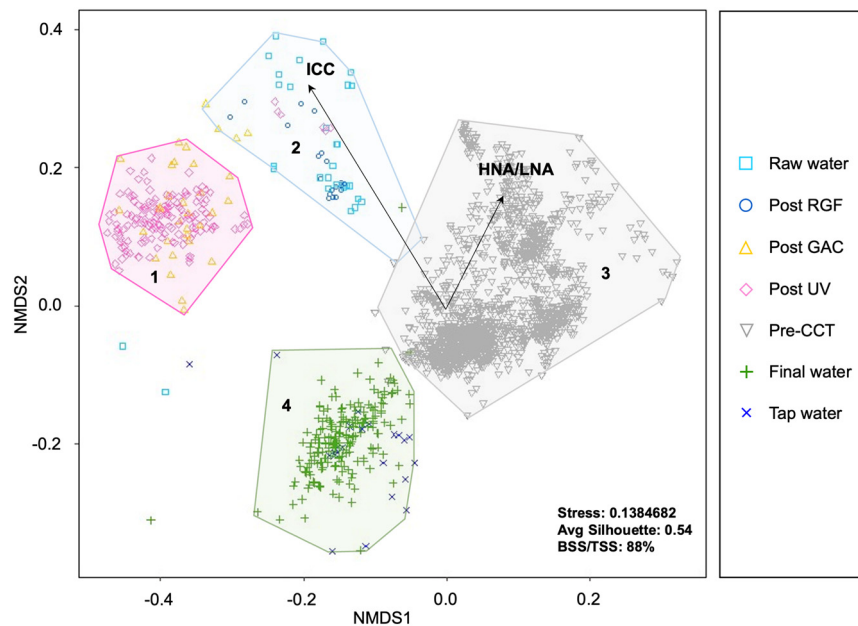


Fig. 5. Non-Metric Dimensional Scaling (NMDS) plot of daily measurements from different stages of a WTW, including raw water, Post-RGF, Post-GAC, PostUV, Pre-CCT, Final water, tap water). The Bray Curtis Dissimilarity Index (BCDI), as applied in the CHIC analysis in Koch's study, was used to position the various sampling points on the graph. The BCDI, a measure of distance, facilitates the evaluation of the dissimilarities and similarities among and within the samples. The vectors of ICC and HNA/LNA ratio were included to determine their influence on the sample dissimilarities. Hierarchical clustering was applied to categorize similar sampling points. Metrics such as 2D stress, the average silhouette, and the BSS/TSS ratio were provided to gauge the plot's capacity to accurately depict the data and the clustering's effectiveness.

Cluster 4 consisted of final and tap water, each displaying consistency. This clustering statistically validates BCDI's utility in discerning microbial water quality across various treatment stages.

Correlation vector analysis revealed that ICC significantly distinguished Clusters 3 and 4 (chlorinated, low-cell-count waters) from Clusters 1 and 2 (unchlorinated, high-cell-count waters), with a clear orientation towards the latter. This differentiation shows the sensitivity of microbial populations to chlorine-based disinfection and demonstrates how its impact can be detected automatically in an FCM user- and gating-independent manner [18]. The vector for the HNA/LNA ratio differentiates chlorinated water clusters, highlighting a higher prevalence of HNA-content cells 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 characteristics of low-cell-count waters. Our findings align with those of Chan et al. [21,25] and Favere et al. [22], who used BCDI associated with NMDS to detect changes in microbial population structure in water in response to process or environmental changes. Integrating cell count, HNA/LNA ratio, and BCDI into an analytical framework affirms the distinct yet complementary roles each parameter for evaluating the impact of water treatment on microbial populations. NMDS plots and hierarchical clustering refine microbial water quality analysis, enabling treatment adjustments to promote water safety through monitoring. These tools aid in addressing microbial variability due to source changes or inefficiencies. Notably, cell count acts as a key daily predictor of shifts in water quality.

3.4. Implications for microbial water quality assessment

This study shows that combining ICC, HNA/LNA ratio, and BCDI enhances differentiation of microbial populations in chlorinated and unchlorinated water, improving water quality assessments across treatment stages. Specifically, ICC effectively identifies microbial loads during early treatment, serving as a sensitive indicator of changes in microbial abundance. The HNA/LNA ratio provides valuable insights into microbial community structure post-chlorination, even when

residual ICC levels are low. BCDI offers comprehensive characterization across all treatment stages, detecting subtle changes in community composition.

These findings have practical applications for water treatment professionals. By using these metrics together, operators can more accurately monitor microbial dynamics, enabling precise adjustments to treatment processes. This integrated approach supports proactive monitoring and rapid responses to microbial fluctuations. However, the study has limitations including the work being undertaken at a single WTW over two months, its scope may affect generalizability to other facilities or conditions. Future research should validate these findings across multiple WTWs with varied treatment processes and source waters. Long-term monitoring would assess the consistency of observed patterns over different seasons and conditions. Integrating metagenomic sequencing or other molecular methods could provide deeper insights into microbial communities, complementing FCM data. Combining FCM metrics with other microbial assessment techniques could further enhance predictive capabilities, advancing water treatment efficiency and early warning systems.

4. Conclusion

In conclusion, combining multiple FCM-based metrics: ICC, HNA/LNA ratio, and BCDI—enhances microbial water quality assessment across water treatment and supply. ICC serves as a direct indicator of changes in microbial load, the HNA/LNA ratio provides insights into microbial community structure post-chlorination, and BCDI offers a versatile tool for characterizing microbial communities throughout the treatment process. Fluorescence fingerprinting and cluster analysis provides opportunity for gate free, user independent approaches to 'phenotypic' characterization based on FCM data. Applying these methods in a real-world context offers a practical framework for water treatment professionals to select appropriate microbial monitoring techniques based on specific treatment stages and anticipated microbial loads. This integrated approach enables more subtle management of microbial populations, supports proactive decision-making, and has the

potential to improve water treatment outcomes and public health protection. As the water industry continues to face challenges from evolving microbial threats and environmental changes, adopting advanced monitoring strategies like those presented in this study could help maintain drinking water quality.

CRedit authorship contribution statement

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

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.

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Data availability

Data for this manuscript is available on figshare at: <https://doi.org/10.6084/m9.figshare.26395141>

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