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

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ABSTRACT

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

1. Introduction

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

The majority of bacteria, typically < 1%, are not culturable under

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Table 1
Overview of source water types and disinfection practice for the 213 WTW included in this study.

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

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

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

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

Table 2
Drinking water treatment trains of the three selected WTW.

	Treatment train sampling points					
Conventional	Surface water (loch)	Coagulation	Dissolved air flotation (DAF) clarifier	Rapid gravity filtration (RGF)	Chlorination (Ct^a = 8 mg·min/L)	Clear water tank (CWT) storage
Direct depth filtration	Surface water (loch)	Coagulant flash mix	–	RGF	Chlorination (Ct^a = 14 mg·min/L)	CWT storage
Membrane filtration	Surface water (river)	Coagulation	–	Submerged hollow fibre (HF) ultrafiltration (UF) membrane	Chlorination (Ct^a = 12 mg·min/L)	Chloramination

[a] Ct calculated as: average free chlorine residual at disinfectant stage outlet (mg/L) × residence time at typical flow (as determined by tracer test, AWWA t_{10} methodology) (mins) = Ct (mg·min/L).

Table 3
Microbial water quality data for all samples between January 2014 and December 2016 (n = 39,340).

Parameter	Units	Min	Lower quartile	Median	Upper quartile	Max
Intact cells	Cells/mL	< 100	100	240	720	73,322,000
Total cells	Cells/mL	< 100	700	6000	1,054,598	89,912,000
% Intact cells	%	0	1	4	20	100
HPC 22	CFU/mL	0	0	0	0	300
HPC 37	CFU/mL	0	0	0	0	300
Coliform[a]	CFU/100 mL	1	1	1	2	8400
E. coli[a]	CFU/100 mL	0	0	0	0	10
Enterococci	CFU/100 mL	0	0	0	0	4
Free chlorine[b]	mg/l	0.03	0.40	0.60	0.74	2.20
Total chlorine[b]	mg/l	0.03	0.62	0.77	0.97	2.20
pH		5.8	7.7	7.9	8.2	10.8
Post flush temp	°C	0.0	6.7	9.6	13.0	29.0
Total organic carbon (TOC)[c]	mgC/l	0.20	0.30	0.70	1.30	31.0
Turbidity[c]	NTU	0.20	0.20	0.20	0.20	6.10

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

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

There have been a number of comprehensive reviews outlining the key advantages and suitability of FCM for various areas of application (Van Nevel et al., 2017; Safford and Bischel, 2019; Wang et al., 2010). Van Nevel et al. (2017) demonstrated that there was no conclusive link between HPC and FCM using a large dataset from a variety of drinking water sources (chlorinated and unchlorinated) (n > 1800). However, key questions remain concerning where FCM be used to provide genuine benefit? Accordingly, the aim of this paper was to investigate the potential of flow cytometry in two core areas: (1) the relationship between FCM and regulated indicator organisms and (2) the suitability of FCM as a tool for monitoring bacterial removal and inactivation throughout multiple barriers of treatment. To achieve this water samples from 213 active drinking water sites were sampled over 3 years for indicator organisms, FCM and HPC (n = 39,340). To the authors' knowledge, this is the largest FCM dataset of its kind in the world.

2. Materials and methods

2.1. Sampling procedures

Water quality data from final water samples at 213 WTW across Scotland were analysed over a period spanning January 2014 until December 2016. For raw water, microbial water quality data was available between October 2015 and December 2016. These WTW represented a wide range of different source waters, treatment processes and disinfection practices (Table 1). In order to understand the variation in process performance, three WTW were selected for further investigation involving inter-stage sampling. These WTW represent the three most common treatment configurations used in Scottish Water, namely conventional coagulation-clarification-filtration (Conventional), direct depth filtration (Direct Filtration) and membrane filtration (Membrane) (Table 2). Each sample was collected at least in triplicate and performed according to regulatory sampling procedures. Prior to sampling, sample taps were flushed for 3 min followed by flame sterilisation. A subsequent 30 s flushing step was employed before the sample was collected into a sterile 500 mL sample bottle (Aurora Scientific, Bristol, UK.) containing a pre-aliquoted sodium thiosulphate dose for quenching of chlorine. Samples were refrigerated and transported to the laboratory for analysis within 24 h of sampling.

2.2. Standard chemical analysis

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

2.3. Standard microbiological analysis

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

2.4. Flow cytometry fluorescence staining and analysis

Flow cytometry analysis was carried out using a BD Accuri C6 flow cytometer with a 488 nm solid-state laser equipped with an auto-sampler (Becton Dickinson UK Ltd., Oxford, UK). Sample volumes of 25 µL were analysed at a fast flow rate of 66 µL min^{-1} in accordance with the rapid method described previously (Nevel et al., 2013). In cases where the event rate was > 1500 s^{-1}, the samples were diluted with 0.1 µm filter sterilized mineral water (Evian, Évian-les-Bains, France). To prevent large particulates entering the FCM system raw water samples were briefly shaken before filtration through 11 µm pore size filters (Grade 1 Whatman filters, GE Healthcare) to remove large particles which may clog the flow cytometer. The green fluorescence was collected in the FL1 channel at 533 nm and red florescence in the FL3 channel at 670 nm with the trigger set on FL-1. A signal threshold on the green fluorescence at FL1 = 600 was applied. No compensation was used. All staining parameters were as described elsewhere (Gillespie

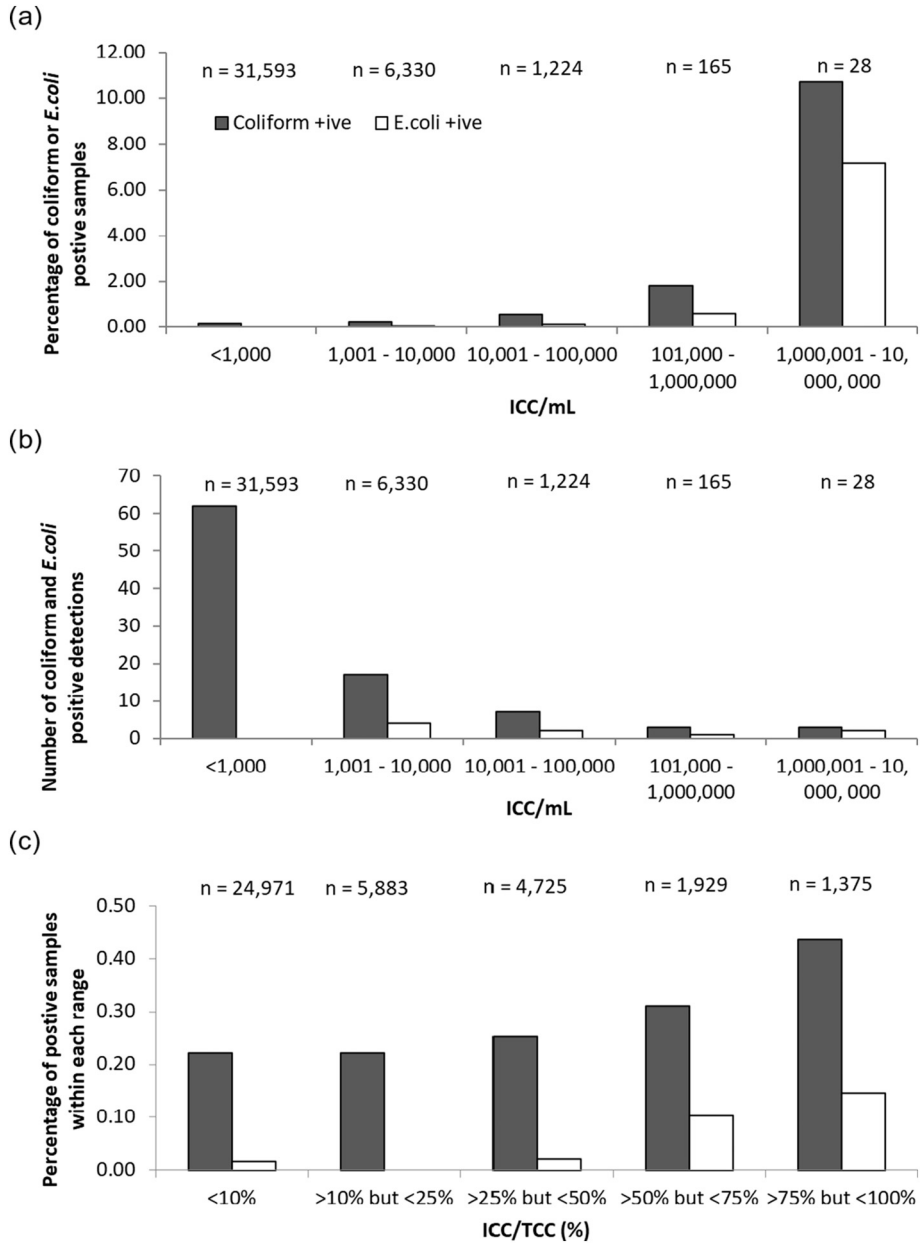

Fig. 1. (a) Intact cell counts grouped on a logarithmic scale with the percentage of coliform or E. coli detections within each group and (b) the number of positives within each range; (c) The ratio of intact to total cells (ICC/TCC%) with respect to the percentage of coliform and E. coli detections. n is the number of samples within each group.

et al., 2014). For the analysis of TCC and ICC the fixed gate described previously (Gatza et al., 2013) was used as a template.

2.5. Data analysis

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

3. Results and discussion

3.1. Water quality

Over the sampling period only 92 samples were positive for coliforms resulting in a compliance of 99.76%. Of these samples, 9 were positive for *E. coli* (99.97% compliance) (Table 3). The coliform detections occurred at 58 individual WTWs from a variety of treatment configurations: Conventional = 27; Direct filtration = 30; Membrane = 23; Other (including borehole abstraction) = 12. In total, 93% of the coliform detections were associated with surface water (SW) sources.

Large variations in TCC and ICC in final water were observed from the FCM data, reflected by a broad interquartile range (IQR) of 640 cell/mL for ICC (267% of median) and 1,053,898 cells/mL for TCC (176% of median). This difference was largely a reflection of the impact chlorine-based disinfection has on cell integrity, resulting in much lower ICC levels. The HPC data was skewed as a significant proportion of the samples did not result in any colony growth, a feature common with culture-based techniques and a limitation in their use for analysing removal processes (Kong et al., 2015). Post-flush water temperatures and total organic carbon (TOC) were representative of typical seasonal

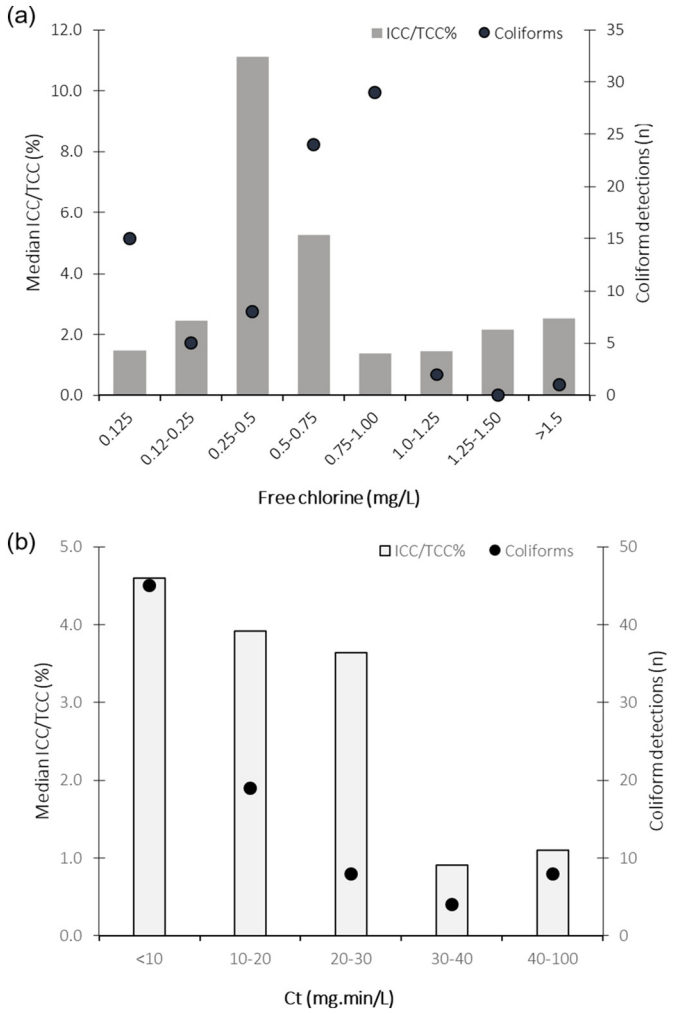

Fig. 2. (a) The median percentage of intact cells (ICC/TCC%) and positive coliform samples against the residual free chlorine (n = 33,854 - only sites with Ct data); (b) The median ICC/TCC% against the tracer derived Ct at the disinfection stage of each WTW and related coliform detections.

variations in climate and water quality across Scotland and was similar to that described elsewhere (Golea et al., 2017).

3.2. Relating FCM data to coliform detections

To elucidate potential links between FCM and regulatory monitoring, only samples containing a positive coliform result were analysed (n = 92) to prevent skewing of the data by the high proportion of blank results observed. Importantly, coliforms were detected in samples that appeared in the lowest cell-count band of the FCM (< 1000 ICC/ml), such that it is currently not possible to set an FCM threshold or prescribed value that could be used to replace regulatory sampling. To demonstrate, while the percentage of samples where no coliforms were detected was high at 99.76% for samples where the FCM cell counts were < 1000 ICC/mL (Fig. 1a), there were still > 60 samples where coliforms were observed (Fig. 1b). Five times fewer positive coliform samples were observed for cell counts between 1000 and 10,000 ICC/mL, but coliform compliance remained at 99.7% overall due to the much lower number of samples in this category. As the ICC concentration increased above 10,000 cells/mL, the frequency of positive coliform samples began to increase relative to the number of samples, where a maximum value of 10.71% of FCM samples resulted in positive coliform detections for the highest recorded intact cell counts (1000,000-10,000,000 ICC/mL). Similar observations were seen when the coliform data was considered relative to the proportion of intact cells (ICC/TCC percentage) (Fig. 1c), although the relationships were less pronounced. Above an ICC/TCC of 50%, the coliform detection rate increased by almost 1.5-fold to 0.31% and for those samples where the intact cell ratio was > 75% the relative coliform detections almost doubled to 0.44% compared to samples where the percentage ICC was < 50% (Fig. 1c). *E. coli* detections were consistent with the coliform data, but the trend was less pronounced due to the relatively small number of positive samples (n = 9). These results show that although the probability of coliform detections increased with increasing cell counts, the highest frequency of coliforms occurred at < 1000 ICC/mL and show that an FCM count threshold is not suitable for regulatory purposes from a classical hygienic perspective.

However, FCM provides positive bacterial counts for all samples, avoiding the large non-detected sample sets with zero culturable counts associated with indicator organism measurements. Relationships between each of the FCM parameters of TCC, ICC, and ICC/TCC% with coliform counts were poorly correlated, with a maximum Pearson correlation coefficient observed of $R = 0.13$ for the ICC ratio (ICC/TCC%) against coliforms (See Table S1, Supporting information). Spearman correlations were somewhat higher for the ICC/TCC against coliforms, showing there was a weak correlation ($\rho = 0.35$), while the ICC and TCC had lower ρ values of 0.32 and 0.23 respectively. The results were comparable to Pearson coefficients obtained by Ellis et al. (2013) for culture-based bacteriological failures against surrogates such as average temperature ($R = 0.23$) and rainfall ($R = 0.17$), supporting the view that most parameters cannot be accurately linked to bacterial detections from culture based methods. Given that most regulatory regimes worldwide are heavily reliant on culture methods for bacterial detection this further confirms the view that FCM is not an appropriate replacement, but should rather be considered as an additional diagnostic tool.

3.3. Chlorine residual and contact time

Assessment of the efficacy of disinfection was undertaken by analysing samples that were taken following storage and before the distribution network. These samples therefore represent the water quality leaving the WTWs. Analysis of the relationship between the chlorine residual and positive coliforms showed that free chlorine below 1.0 mg/L was coincidental with nearly all of the positive coliform samples obtained from plate counting (Fig. 2a). Only three instances were observed where coliform failures occurred above 1.0 mg/L free chlorine (Fig. 2a), aligning to previously reported instances of coliform growth in elevated chlorine concentrations (Chevallier et al., 1996). From the FCM, a maximum ICC/TCC of 11.1% was observed at a free chlorine concentration of 0.25–0.50 mg/L with a minimum ratio of 1.4% at elevated concentrations of 0.75–1.0 mg/L. This was consistent with previous trials on three WTW with final waters that had chlorine values in the range of 0.5–3.0 mg/L (residual concentration range 0.42–0.51 mg/L) where the ICC/TCC varied between 2.6 and 6.0% (Nescerecka et al., 2014). When the distribution network is considered, Gillespie et al. (2014) showed that the free chlorine residual had a more obvious influence on ICC/TCC% and a threshold of 0.50 mg/L limited the increase in cell viability. The results also align with the increasing detection of indicator organisms in water distribution systems as the chlorine concentration in the network decreases (Ellis et al., 2013). In these cases, detections at the network extremities were linked to microbial regrowth due to loss of chlorine residual in the network, rather than the direct impact of the disinfection process at the WTWs.

There were stronger relationships between microbes and the product of the chlorine concentration (C) and the disinfection contact time (t), the Ct value. In the present study, bacterial detections were more likely when Ct values were lower. Similarly cell viability (ICC/TCC) increased as the Ct decreased, although the important threshold was different between the FCM and culture data (Fig. 2b). For coliforms,

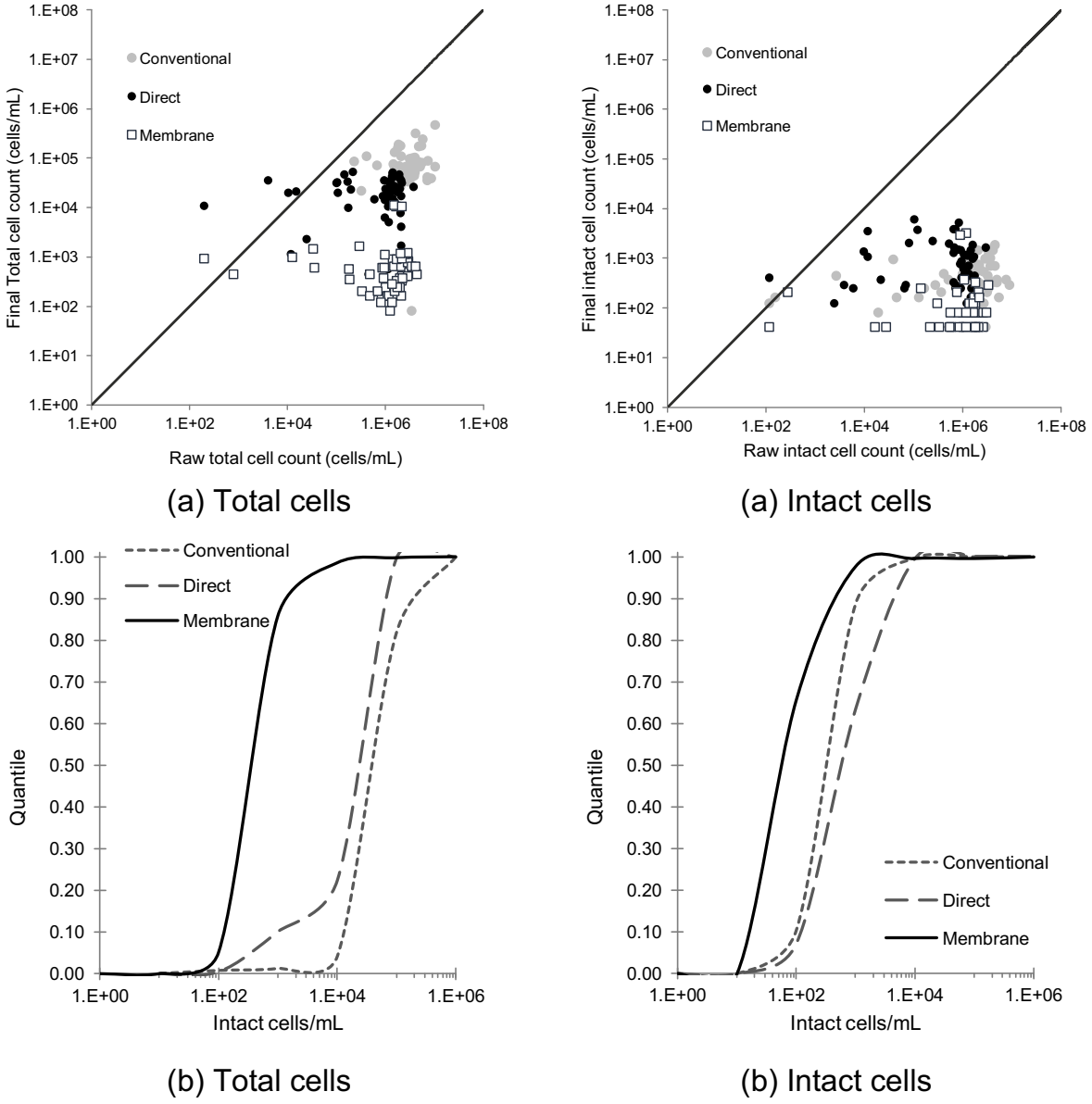

Fig. 3. (a) Raw and final intact and total cell counts for each of the WTW, solid line represents the y = x; (b) Cumulative frequency distributions of intact cell count at each of the three WTW.

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

3.4. Detailed assessment of treatment performance

For each of the WTW types investigated in more depth, the final cell counts were relatively stable irrespective of the raw water inlet counts, showing there was no relationship between the TCC or ICC microbial loading into the WTW and the bacterial levels seen in the final treated water (Fig. 3a). However, the conventional WTWs received water containing more TCC than for the other two sites, with 84% of samples containing > 1×10^6 cells, while this was 62 and 67% for the Direct Filtration and Membrane site, respectively. As a result of the higher loading, final TCCs at this WTWs were higher than for the other two WTWs. This was not the case for the ICCs, where again the loading ono the WTWs was highest for the Conventional site, with more samples containing > 1×10^6 cells. However, ICC in final water were of the same order of magnitude as for the Direct Filtration WTWs, while the

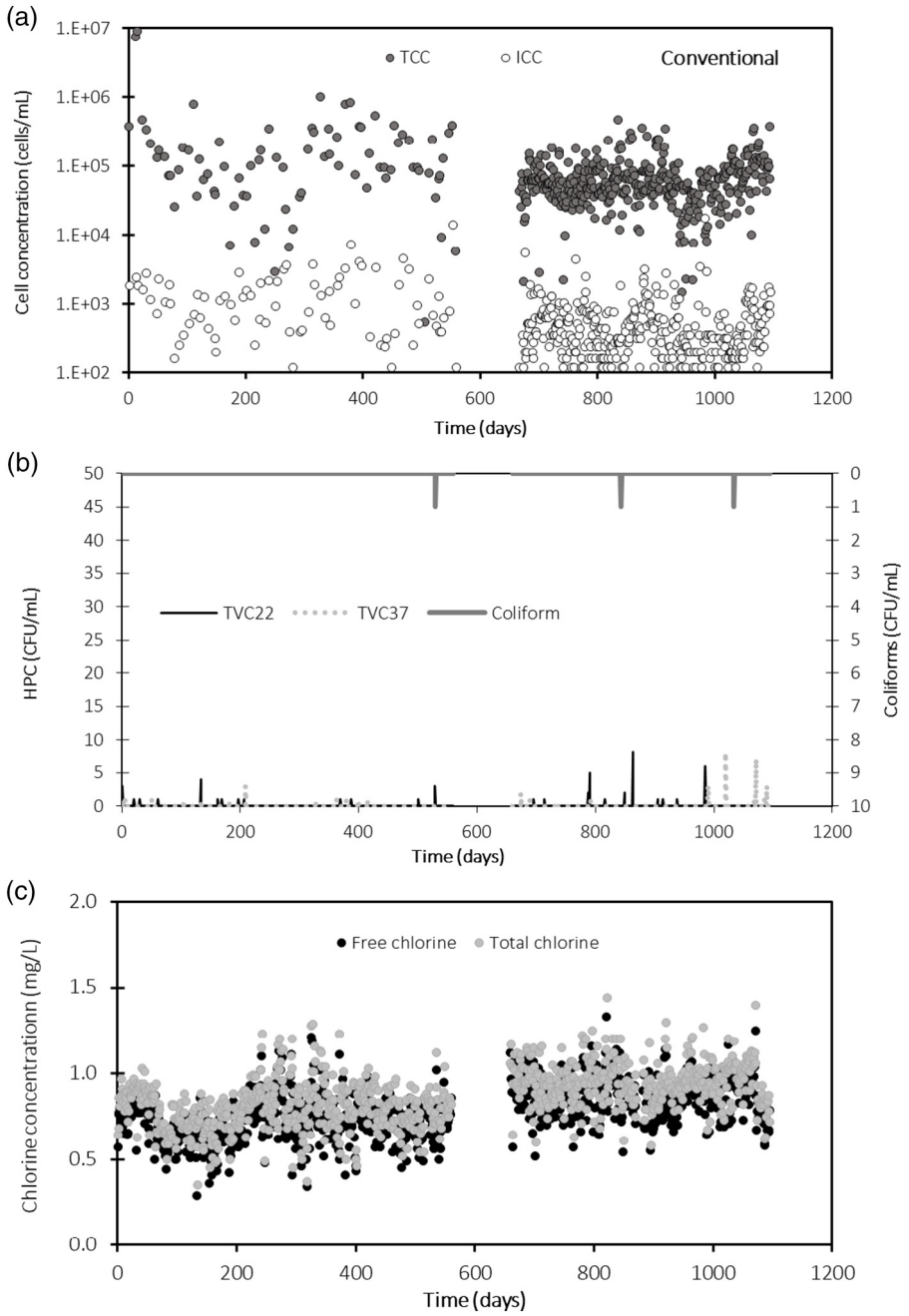

Fig. 4. Final water quality trends for the Conventional WTW, frequency of Flow Cytometry analysis was increased from every 3 days to daily for the second half of the study period (n = 1103) for: (a) Flow cytometric cell concentrations (total and intact); (b) culture derived data, and; (c) final water chlorine concentrations.

Membrane WTWs contained the lowest range of ICCs showing that the treatment processes themselves were controlling bacterial cell. This observation was consistent with that seen by Besmer and Hammes (2016) who have shown that even significant bacterial increases (up to five-fold), measured at higher resolution (20 min online sampling) in the source water, were not seen in the final produced water from the WTW.

The increased granularity of data obtained from the FCM enabled effective assessment of the robustness of performance across the three WTWs (Fig. 3b). For example, final treated water samples containing < 1000 ICC cells/mL were observed in 98.5%, 88.9% and 64.6% of cases for the Membrane, Conventional and Direct Filtration WTW respectively, showing a clear distinction in removal of viable bacteria between the different treatment types. The profile for the TCC in final water was similar as for the ICC, with the Membrane WTWs resulting in lower cells counts than for the other two WTWs. In this case the 90th percentile was 1000 cells/mL for the membrane site, 15,000 cells/mL for the direct filtration site and 150,000 cells/mL for Conventional WTWs. These results were in-line with the higher loading of TCC onto the Conventional WTWs compared to the Direct Filtration site.

There were a total of nine positive coliform detections seen in treated water over the sampling period across the three WTW: three at the Conventional site; two at the Direct Filtration site; and four at the Membrane site (Figs. 4–6). At the Conventional WTW, no specific changes in cell concentration were observed for TCC or ICC when the three coliform detections occurred. For the Direct Filtration WTW, there was an increase in the final water ICC after 850 days, increasing from 5×10^2 to 1×10^4 cells/mL (Fig. 5). It was during this period that two failures at this site occurred. At the Membrane WTW, the cell counts in

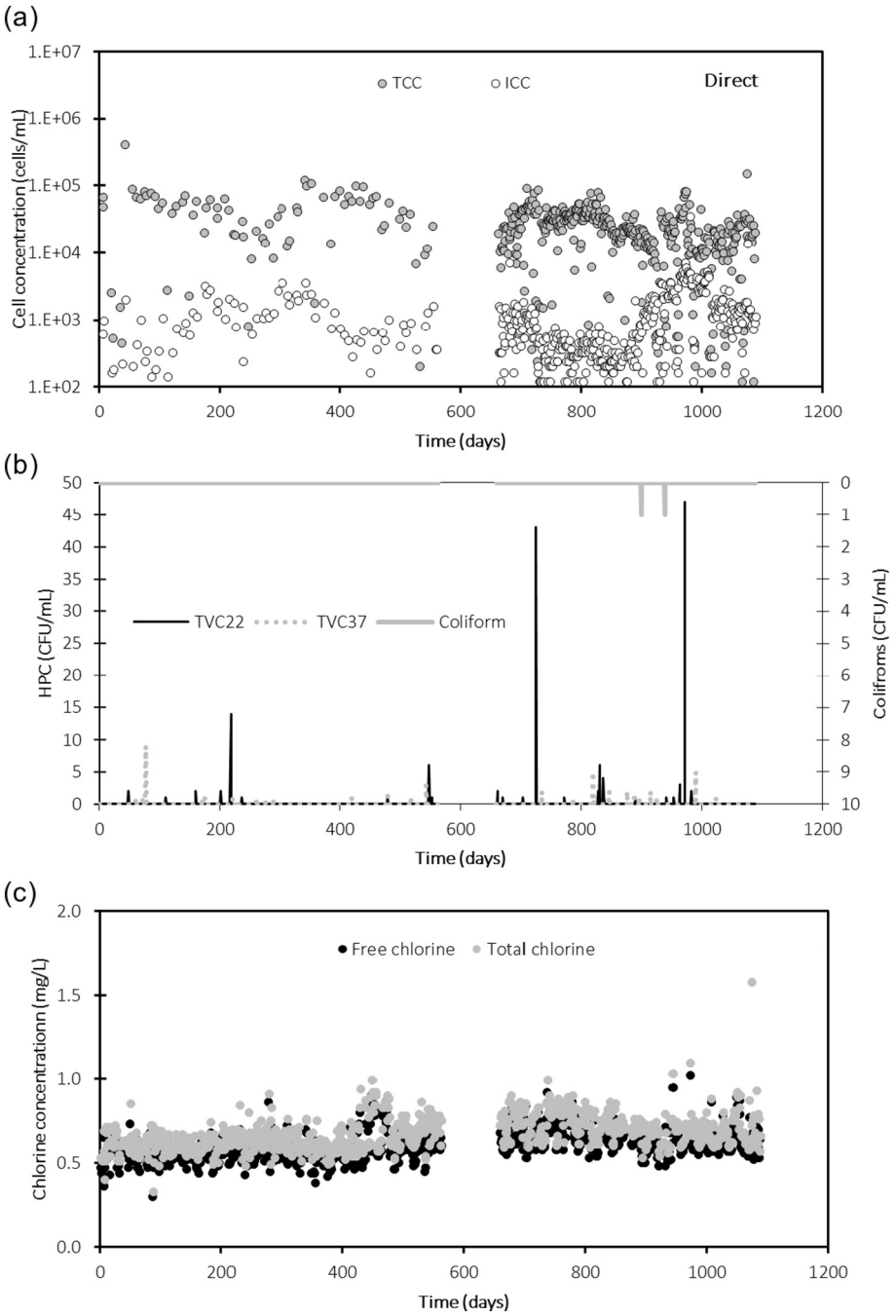

Fig. 5. Final water quality trends for the Direct filtration WTW, frequency of sampling was increased from every 3 days to daily for the second half of the study period (n = 1095) for: (a) Flow cytometric cell concentrations (total and intact); (b) culture derived data, and; (c) final water chlorine concentrations.

the final treated water were the lowest of the three WTW studied, having median cell counts of < 100 cells/mL compared to 360 and 320 cells/mL at the conventional and direct sites respectively (Figs. 4–6). There was a large spike in the intact cells on day 854 (1.66×10^5 cells/mL) in the final water. No coliforms were concurrently detected but 11 days later a positive sample was observed. There were no obvious explanations for the other three detections. The results show that while there were some co-incidental occurrences of higher FCM cell counts with positive culture based samples, most of these detections could not be explained by a higher frequency of cells as measured by the FCM. In fact, seven of the nine coliform events occurred when the ICC concentration was < 1000 cell/mL, a result consistent with the observations seen from the overall data (Fig. 1).

With respect to the HPCs, the data was again sporadically spread, with many non-detected samples. However, the frequency of positive samples was greater than for the coliform detections. The highest frequency of HPC positive detections were at the Membrane WTW = 116 (57 at 22 °C, 59 at 37 °C); Direct filtration WTW = 59 (31 at 22 °C, 28 at 37 °C); and Conventional WTW = 48 (25 at 22 °C, 23 at 37 °C). This was the opposite order to the cell concentrations observed using FCM and again demonstrates the changing picture that can be developed with the richer data sets generated when using a non-culture-based technique such as FCM. This would, for example, enable investigation into other important features of a WTW system, such as process age, operational practice and process transients. The HPC were also poorly associated with the FCM cell count data, with no trends apparent between the two parameters. Likewise, the coliform and HPC counts were not coincidental, with only one of the coliform detections having a positive

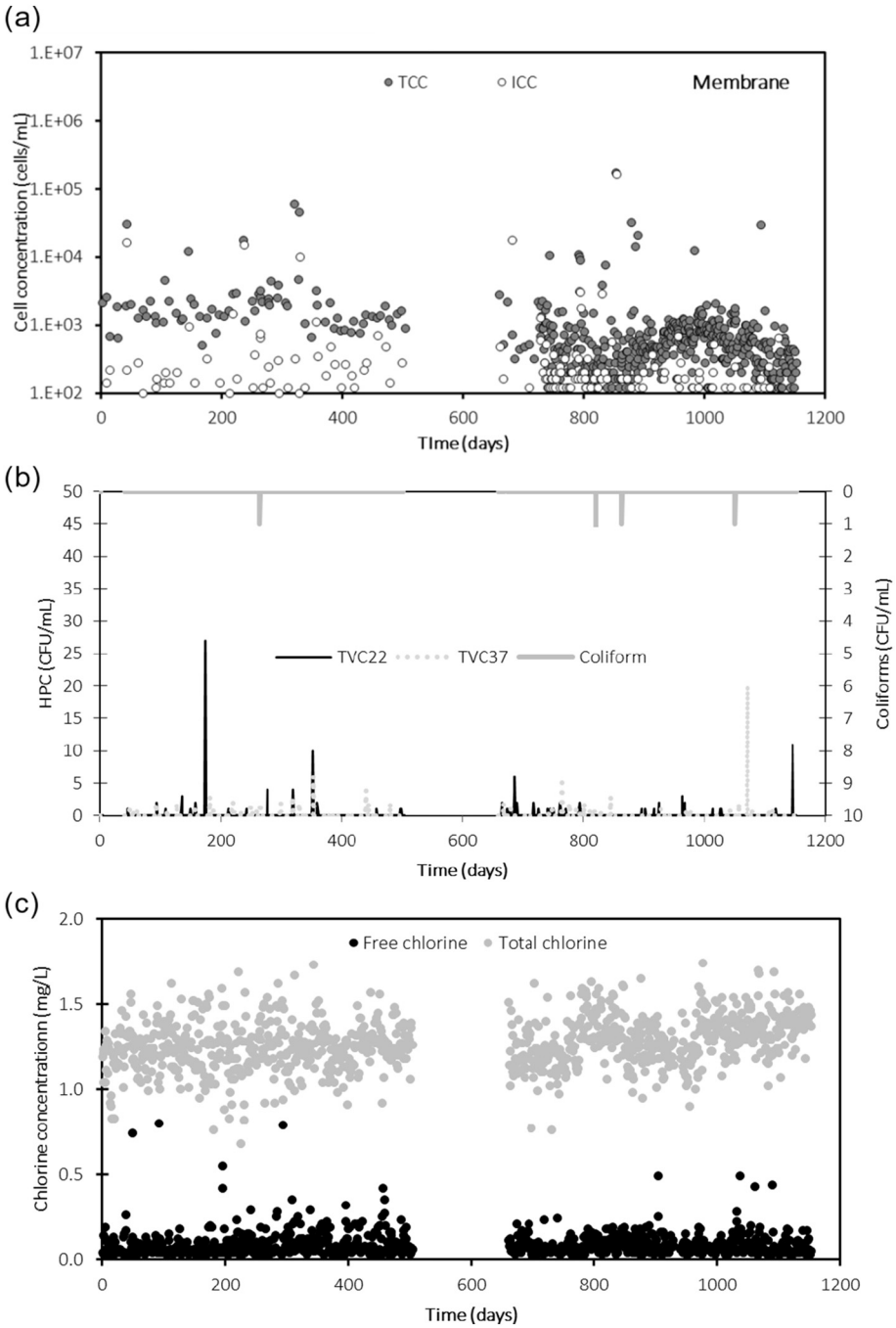

Fig. 6. Final water quality trends for the Membrane WTW, frequency of sampling was increased from every 3 days to daily for the second half of the study period (n = 1203) for: (a) Flow cytometric cell concentrations (total and intact); (b) culture derived data, and; (c) final water chlorine concentrations: note that this site practiced chloramination, hence the difference between the free and total chlorine. TVC22 and TVC37 are total viable counts at 22 and 37 °C, respectively.

corresponding HPC count (this was the first detection at the Conventional WTW on day 530 (Fig. 4b)).

3.5. WTW log removals as an indicator of bacteriological performance

As noted, one of the opportunities provided by FCM is the ability to provide appropriate removal data for water safety plan risk assessments. Bacterial removal rates across whole WTWs and between treatment stages were therefore calculated for the three selected sites. The median TCC log removal (LogR) across the Conventional and Direct Filtration WTW was similar at 1.73 and 1.76 LogR, respectively (Fig. 7). The ICC removal was much higher at both of these sites with logR of 3.77 and 3.22 for the Conventional and Direct Filtration sites respectively. These data show that at clarification sites, the processes do not provide a complete barrier for removal of cells and rely on the chlorination stage to significantly reduce the viable cell concentration. The presence of the DAF process at the Conventional WTWs facilitated enhanced removal of intact cells, although this was not the case for total cells. While not established directly in this work, this can be attributed to improved capture of viable cells during the extended coagulation/flocculation stage (Ho et al., 2012). These results contrast with those seen for the membrane WTWs that provides an absolute barrier to particulates. In this case, both the TCC and ICC LogR was much higher at 3.55 and 4.17, respectively. This data agrees with the rankings

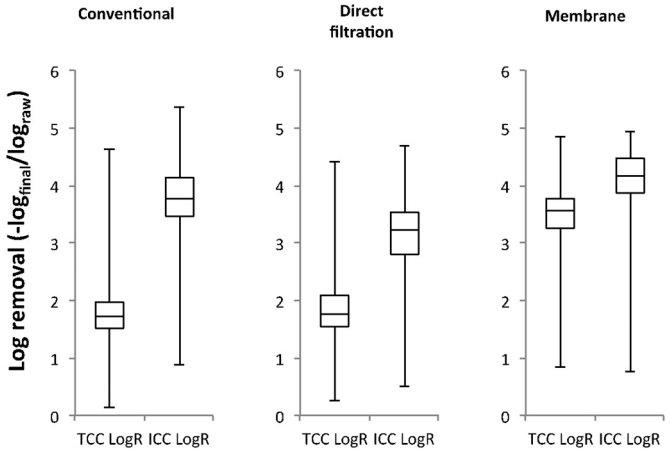

Fig. 7. Log removal of total and intact cells across three WTW between November 2015 and December 2016.

established previously when only final water cell counts were considered (Fig. 3b).

To investigate the microbial removal at these sites in more detail and understand where the microbe removal occurred, inter-stage sampling was carried out (Fig. 8). Overall, ICC LogR for the Conventional, Direct Filtration and Membrane WTWs were 3.03, 2.29 and 4.47 respectively. Equivalent figures for the TCC logR were 1.6, 1.7 and 3.5. These results were within the ranges shown from the long-term evaluation (Fig. 7). The removal across the rapid gravity filters at the two depth filtration sites were 0.26 and 0.50 TCC LogR and 0.27 and 0.52 for ICC logR for the Conventional and Direct Filtration WTWs, respectively. However, when the combined removal across the DAF and RGF was considered, overall TCC and ICC LogR were 0.85 and 0.88, a removal greater than that seen for the Direct Filtration site and that there was no apparent difference in the removal of intact and total cells. This shows the beneficial role that effective coagulation and clarification processes can have in reducing cell and particle loads onto filters, as well as producing particles that are effectively removed by depth filter capture mechanisms (Ho et al., 2012). Previous assessment of RGF has shown a higher average LogR of 1.07 ± 0.82 from operational sites (Helmi et al., 2014; Hijnen, 2008). The present results also sit at the lower end of the LogR range suggested by WHO for high rate filtration of between 0.2 and 4.4 (WHO, 2011). Variation in particle and bacteria removal can be expected across processes due to the impacts of contact time, media choice, media arrangement, temperature (changes of which can affect bed expansion), time in run and backwash rates (Hijnen, 2008). However, the current data indicates that previously utilised removal credits may be over estimates and that more work is required especially around the different aspects of the batch operating cycle of filters, a task well suited to FCM.

Cell removal across the membrane at the Membrane WTW was 3.01 and 3.38 for TCC and ICC LogR with cell counts that were below 1000 cells/mL for both parameters, levels that were not seen for the conventional and direct filtration WTWs. These results were comparable to the 3.11 LogR observed by Hammes et al. (2008) following UF membrane treatment of GAC permeate. The performance of the membrane was consistent with them acting as an absolute barrier for particles above the pore size of the ultrafiltration membrane, around 0.01 µm. Bacterial cells can be in the size range of 0.5 to 100.0 µm, although typical cells are no larger than 2 µm (Madigan et al., 1997). Accordingly, positive cell counts post membrane were attributed to the non-sterile environment downstream of membrane systems (Hammes et al., 2008) including biofilm growth in the downstream tanks (Zanetti et al., 2010). The other cause of detection would be a loss of membrane filtration integrity (Krahnstöver et al., 2018). The regular monitoring of the permeate by FCM offers great potential as an easy approach for membrane filtration integrity monitoring through the identification of a sudden increase in cells.

The chlorination stage was the most significant contributor to intact cell log reduction at 0.63 and 1.14 ICC LogR for the conventional and

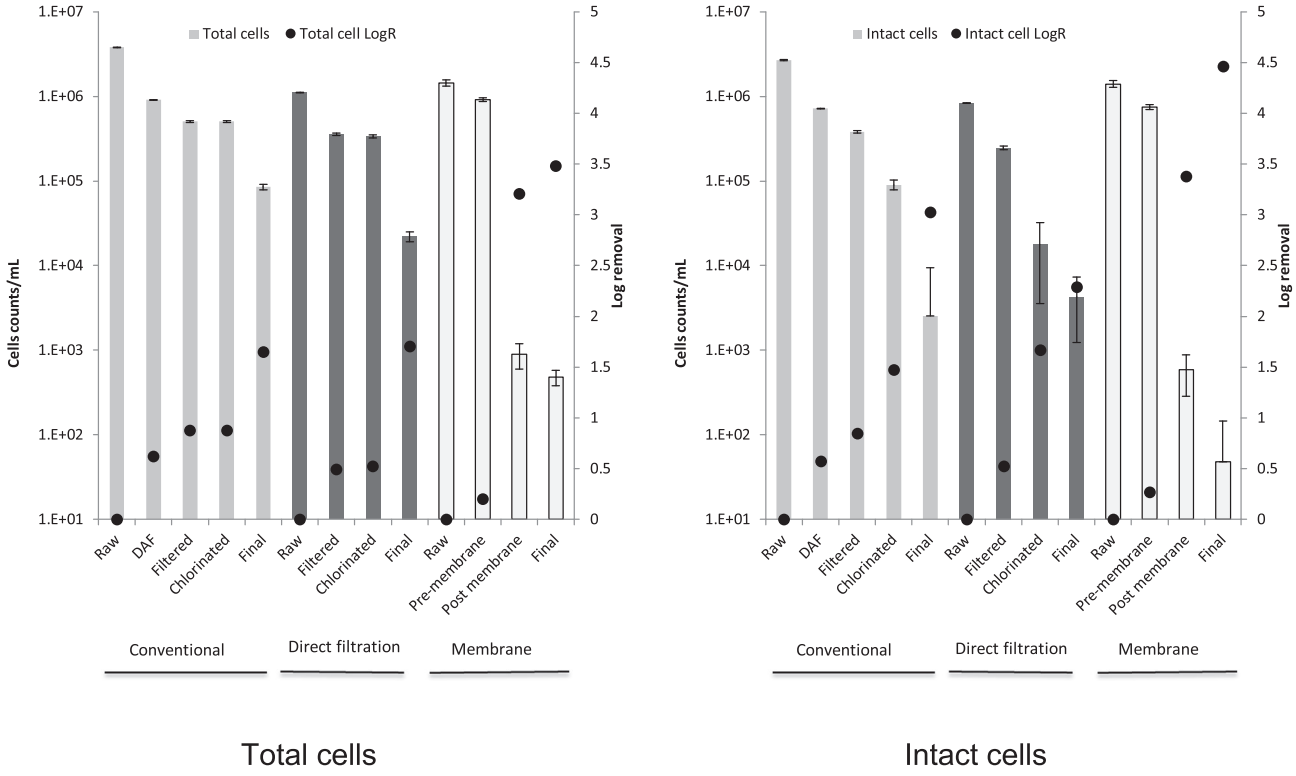

Fig. 8. TCC and ICC with corresponding cumulative log removals (circles) for the three study sites: conventional, direct filtration and membrane WTWs.

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

4. Conclusions

It has been demonstrated in this study that FCM will best benefit the water industry by its use in tandem with current regulatory methods. While there was an increasing probability of coliform detection with increasing cell counts, most positive coliform samples were coincidental with low FCM counts of < 1000 ICC/mL. Furthermore, the FCM results did not distinguish between different types of microorganism present in the sample. As a result FCM cannot be used to provide hygienic information about water quality and so is not a suitable replacement for indicator organism monitoring. However, the sporadic detection of bacteria using culture based methods makes FCM the most suitable tool for process monitoring. The 'Ct value' was identified as the key factor for suppression of intact cells and reducing the number of coliform detections in final drinking water, with Ct > 30 mg·min/L being effective at reducing both variables to a minima. FCM was able to provide accurate bacterial cell reduction across treatment processes, providing data that can be used for risk assessments in water safety plans Furthermore, FCM offers opportunities for rapidly diagnosing where critical bacteriological barriers are not performing effectively. It was shown that raw water cell counts were not related to final treated water intact cell numbers. Instead, the final water cell counts were mostly driven by individual treatment process with membrane treatment having the highest removal efficiency of ICC and TCC. Chlorine disinfection was the main way by which ICC was reduced for the Conventional and Direct Filtration WTWs. This reinforces the importance of maintaining each treatment barrier without reliance on physical or chemical processes alone to deal with microbiological challenges. In the future, it would be beneficial to develop FCM (or align it with other techniques) to be able to quickly and accurately distinguish between different types of bacteria based on their pathogenicity, such that the hygienic risk of the water can be more rapidly assessed.

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

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