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Comparison of flow cytometry and heterotrophic plate count methods for dialysis water microbial monitoring

Rui Lucena[1]✉, Juliane Feurle[1], Célia Gil[2], Aníbal Ferreira[3], Pedro Ponce[4] & Bernard Canaud[5]

Water is an essential component of renal replacement therapy by dialysis. Haemodialysis patients undergoing 4-hour dialysis sessions, thrice weekly, may be exposed to more than 360 L of dialysis fluid per week. Dialysis water and ultrapure dialysis fluids have been established as a prerequisite for online convective therapies (namely haemodiafiltration), to improve biocompatibility of the dialysis system and to reduce inflammation profile of the dialysis patients. The microbial quality of dialysis water should be monitored regularly to demonstrate the effectiveness of the disinfection protocol. Current water and dialysis fluids microbial quality assessment is typically done via heterotrophic plate counts (HPC) and endotoxins analysis. Plate counts methodology provides information on the microbial content of the water only after a considerable incubation time (typically 7 days). Flow cytometry (FCM), as a rapid alternative method, provides the possibility of real-time corrective actions and greater patient safety. In this study, we compared the outcomes of dialysis water microbial quality in an existing dialysis clinic, using two different measurement methods, HPC and FCM, to test the possible benefits of applying FCM as a valid alternative method for dialysis water microbial monitoring. We conclude that FCM offers higher sensitivity than HPC for microbial monitoring of dialysis water, potentially enabling earlier corrective actions. More extensive and larger studies are needed, namely, to evaluate the possible added value of FCM method in dialysis fluids monitoring. If the FCM method is confirmed, it will be necessary to establish maximum allowable levels and typical action levels for dialysis water and dialysis fluids quality when using FCM.

Keywords Haemodialysis, Dialysis water, Microbiological monitoring, Heterotrophic plate count, Flow cytometry, Water microbiology

Typically, a healthy adult consumes 10–12 L of water per week, which is absorbed orally. Water passes through the selective barrier of the gastrointestinal tract, and any remaining toxic contaminants are potentially removed by kidney function. In contrast, patients undergoing conventional 4-hour haemodialysis sessions three times per week are directly exposed to more than 360 L of dialysis fluid per week through an artificial semi-permeable membrane. Dialysis fluids, and consequently any water contaminants present, may pass through the non-selective dialyzer membrane, while limited kidney function cannot effectively excrete potentially harmful substances. Moreover, the today's use of highly permeable membranes, along with the increased prescription of convective-based and online high volume haemodiafiltration therapies, increases the risk of contaminants being infused directly into the bloodstream.

Water used in dialysis must fulfill more stringent quality criteria than drinking water. A review of the literature shows that contamination of water with microorganisms[1–3] and/or chemicals[4–6], is more than just a theoretical risk in haemodialysis, despite considerable efforts made in recent years to improve this situation. Strict standards for dialysis water and ultrapure dialysis fluids were established as a prerequisite for safety of online convective therapies (e.g., haemodiafiltration), improving biocompatibility of the dialysis system, reducing the

[1]Fresenius Medical Care Deutschland GmbH, Bad Homburg, Germany. [2]Unidade Local de Saúde Lisboa Ocidental, Hospital Santa Cruz (Nephrology Department); Medical Director Nephrocare Alverca Dialysis Clinic, Portugal, NephroCare Alverca, Lisbon, Portugal. [3]Nova Medical School Lisboa, Nephrology Department of Hospital Curry Cabral – ULS São José, NephroCare Carregado, Lisbon, Portugal. [4]Country Medical Director NephroCare Portugal, Lisbon, Portugal. [5]Nephrology Department, School of Medicine, and Foundation Ch. Mion, Montpellier University, AIDER-Santé, Montpellier, France. ✉email: rui.lucena@freseniusmedicalcare.com

inflammatory profile of dialysis patients, enhancing responsiveness to erythropoiesis-stimulating agents, and potentially improving survival outcomes[7–12].

The fluid used in dialysis therapy (dialysis fluid) consists of dialysis concentrates and, for the most part, water (common ratios are 1:34 and 1:44). While the concentrate is typically produced commercially with a tightly controlled and consistent composition, the feed water used to produce dialysis water can vary widely in its local composition and quality. The source of dialysis water is drinking water, which undergoes a purification process that includes pressure driven membrane techniques such as reverse osmosis (RO).

Water and dialysis fluid quality assessments have been conducted in several countries for many years. Several multi-center studies developed in the 1990s emphasized the importance of the risk of microbial contamination[13–18].

Possible sources of contamination of dialysis water and dialysis fluids include inadequate disinfection of equipment, machines, and distribution systems[19]. Waterborne Gram-negative bacteria are the main microbial contaminants of dialysis water and dialysis fluid. At first glance, this may seem to have limited clinical relevance, as the likelihood of whole bacteria penetrating a dialysis membrane is rather small, except in cases of membrane leakage. However, these bacteria produce various byproducts that are small enough to enter the bloodstream, even through low-flux membranes. It has been shown that biologically active bacterial fragments can gain access to a patient´s blood during haemodialysis leading to both short-term and long-term medical consequences for the dialysis patient. These bacteria byproducts are also known as pyrogens due to their ability to induce fever in the patient. They trigger an acute phase reaction and potentially fever in the host by activating monocytes and the release of proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor a (TNFa). The most important exogenous pyrogens in haemodialysis are the cell wall component of Gram-negative bacteria, called endotoxins or lipopolysaccharides. Other byproducts of microbial contaminants that have proven biological activity are peptidoglycans and muramyl peptides[20], heat labile polypeptides such as exotoxin A derived from Pseudomonas species[21,22], and haemolysins secreted by *E. coli*[23] or pseudomonads genus[24]. Byproducts of Pseudomonas species are the dominant microbial contaminants in haemodialysis.

Currently, water sampling is primarily based on an offline testing methodology, providing information on the microbial content of the water or dialysis fluid after a specific incubation period. Online water bioburden analyzers are a recent advancement and can be used to monitor and control the microbial content of water and dialysis fluid. In fact, online water bioburden analyzers, which provide real-time bioburden data, may support a dynamic shift to a more effective approach to water quality[25]. However, there is still little regulatory guidance for online testing and open discussions regarding system validation.

The microbial quality of dialysis water and fluid should be monitored regularly to demonstrate the effectiveness of the water treatment system and disinfection protocol. The frequency of monitoring should be established during the system validation process and is typically performed on a monthly basis. If there is any evidence of microbial contamination in either water or dialysis fluids, the testing frequency must be increased, and sampling should be adjusted according to the situation, along with implementing corrective measures (e.g., disinfection, rinsing).

The microbial requirements for dialysis water and the various dialysis fluids are summarized in Table 1, including assessment via heterotrophic plate counts (HPC), expressed in Colony-Forming Unit (CFU) per mL, and analysis of endotoxins using the Limulus Amoebocyte Lysate (LAL)-test, expressed in Endotoxin Units (EU) per mL. Substitution fluid for convective therapies, such as haemodiafiltration and haemofiltration, or for priming the extracorporeal circuit or bolus administration of fluid during a treatment, shall be sterile and non-pyrogenic and is produced online by a process of ultrafiltration with bacteria and endotoxin-retentive filters. The online process shall be validated by the manufacturer to produce substitution fluid that is sterile and non-pyrogenic.

This table also includes the value of the action level (AL) as a strategy to keep the microbial contamination of a system under control. AL is defined as "…the concentration level of a contaminant, at which action steps should be taken to interrupt the trend towards higher, unacceptable levels"[26]. This approach is particularly important due to the behavior and exponential dynamics of microbial contaminants.

Dialysis units worldwide continue to rely on the century-old heterotrophic plate counts (HPC) method for routine assessment of microbiological quality of dialysis water and dialysis fluids. The main drawbacks of HPC monitoring are mentioned in Table 2.

Contaminant	Maximum allowable level	Typical action level
Dialysis water		
Total viable counts	< 100 CFU/mL	50 CFU/mL
Endotoxin	< 0.25 EU/mL	0.125 EU/mL
Standard dialysis fluid		
Total viable counts	< 100 CFU/mL	50 CFU/mL
Endotoxin	< 0.5 EU/mL	0.25 EU/mL
Ultrapure dialysis fluid		
Total viable counts	< 0.1 CFU/mL	NA
Endotoxin	< 0.03 EU/mL	NA

Table 1. Microbial contamination requirements for dialysis fluids.(Adapted from ISO 23500-1[26]).

Item	HPC (pour plate method / filtration method)	FCM
Sample preparation	Requires a specialized technician and samples must be transported under refrigerated conditions to the laboratory	Sample preparation is usually not needed and no refrigeration step if measurements are done onsite
Incubation time	Typically, 168 h	Typically, 30 min
Accuracy & reproducibility	Not all viable cells will grow on cultivation media (VBNC)	High level of information, accuracy & reproducibility
Normative frame	HPC remains the key variable in assessing microbiological quality of dialysis fluids (e.g., ISO standards)	Still to be considered in some standards (i.e., requires a validation)
Cost perspective	Lower investment, higher running costs	Higher investment, lower running costs

Table 2. Comparison of typical advantages and disadvantages of HPC and FCM methods.

Flow cytometry (FCM) and the ability to measure both total and intact cell populations through DNA staining methodologies are among several alternative methods that challenge this status quo and provide an opportunity for improved water quality monitoring.

FCM is a commonly used technique to detect and measure the physical and chemical characteristics of a population of cells or particles. It can also be used for the determination of bacteria in water or the number of viable bacteria when combined with viability stains[27]. More recently, the technique has been expanded to create flow cytometry fingerprints of bacterial communities, allowing for more detailed characterization of these communities[28,29].

The ability to measure both total and intact cell populations through DNA staining methodologies has rapidly gained attention in the water sector in recent years. A study comparing flow cytometry with culture-based methods for microbial monitoring in drinking water treatment revealed that flow cytometry can provide insights into the bacteriological quality of water that adds significant value over and above that provided by traditional bacterial monitoring[30].

Results from online or offline FCM can be used to rapidly determine biodiversity using the flow cytometric fingerprinting method[31–34]. By applying additional gates on the green fluorescence histogram produced by FCM, bacterial communities with low and high nucleic acid content (LNA, HNA) can be distinguished, and changes in this bacterial fingerprint can be monitored, providing further information on the nature and dynamics of the microbial community studied[35].

Table 2 summarizes the theoretical main differences when comparing HPC and FCM approaches for water microbial monitoring.

Approximately 300 studies conducted between 2000 and 2018 applied FCM to water quality assessment, pointing out that FCM could reasonably and realistically see widespread adoption as a routine method for assessing the quality of drinking water treatment and distribution[36]. Despite the potential for rapid alternative methods to provide real-time corrective actions and greater patient safety, few studies have been conducted to demonstrate their applicability in the field of dialysis[37,38].

To test the real advantages and possible benefits of the FCM method compared to the traditional HPC approach in the context of dialysis water microbial monitoring, we conducted the present study in a real-life setting. Samples were taken from representative sampling points in one fully functional haemodialysis clinic in Portugal, and the outcomes were compared.

Methods

Sampling procedures

We performed a single-center study in an outpatient Portuguese haemodialysis clinic with 35 dialysis stations. One hundred seventy-seven patients were performing dialysis treatments during the study, with a mean age of 70.1 ± 13.7 years (72% having more than 65 years), 67% were male, and 42% were diabetics. The patients were distributed over three shifts daily, with the majority (95%) performing three sessions of 4 h per week and 5% performing four sessions per week. Seventy-five per cent of the patients were submitted to online post-dilution haemodiafiltration with a minimum substitution volume of 21 L, with the remaining performing high-flux haemodialysis.

Samples were taken from representative sampling points of the haemodialysis clinic Water Treatment System (Fig. 1) on a weekly basis from October 2023 to June 2024 (36 weeks).

The water treatment system under study comprises four basic steps: pre-treatment, primary and secondary treatment, and distribution loop. The pre-treatment step prepares the water before it reaches the primary purification device, to prevent fouling or damage to more sensitive down-stream components. The following components are included in the pre-treatment: macroparticle filtration, sedimentation, microparticle filtration, ion exchange and adsorption (via activated carbon). The primary treatment is the main purification process and relies on a reverse osmosis (RO) device, using membrane technology for the removal of dissolved inorganic salts and organic substances as well as microorganisms. The secondary treatment step consists of a second reverse osmosis device installed in series, referred to as double pass device. The final step is the distribution loop, which delivers the purified dialysis water to the dialysis machine and other points of use.

Included sampling points in this study:

- SP1 – at the end of the pre-treatment step, i.e., after activated carbon filters.
- SP2 – primary treatment, i.e., after RO first stage permeate.
- SP3 – secondary treatment step, i.e., after RO second stage permeate (dialysis water supply).

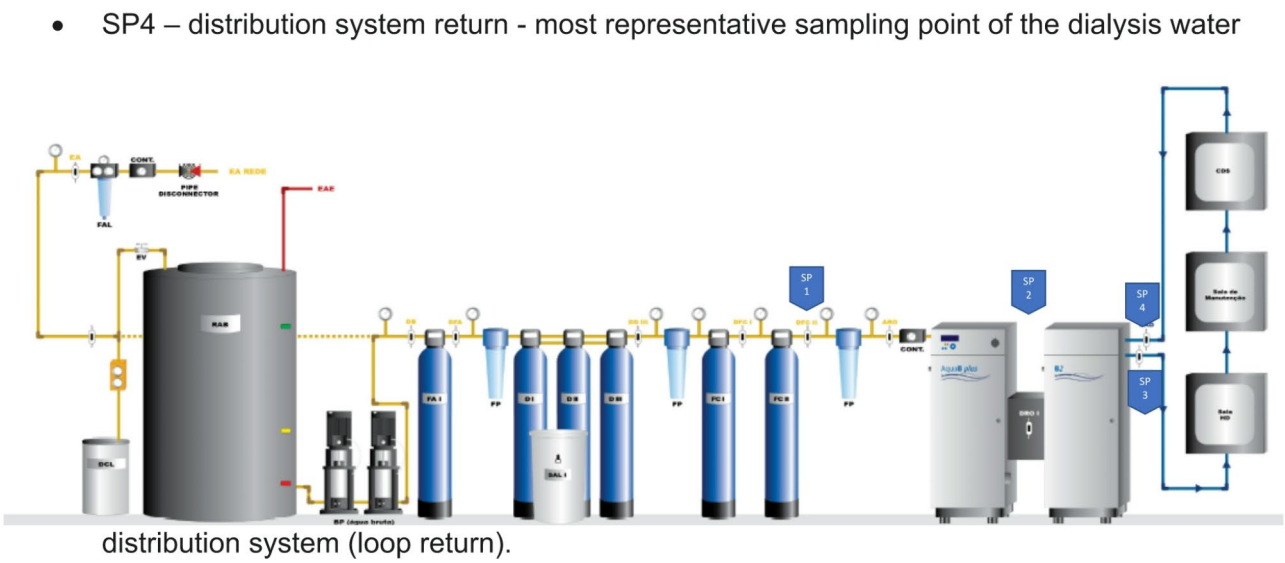

Fig. 1. Dialysis water treatment system and reference sampling points (drawing generated in Adobe Illustrator 2025, version 29.0.1; https://www.adobe.com/uk/products/illustrator.html).

Prior to taking samples for HPC and FCM assessment, free and total chlorine measurements were carried out using the N, N–diethyl-p-phenyldiamine (DPD) colorimetric method and a portable photometer (Macherey-Nagel GmbH & Co. KG, Germany). This was done to confirm that no chlorine residuals were present that would falsify the HPC results, so that sodium thiosulphate dose for quenching chlorine was not needed.

Samples referred in Fig. 1 were taken at the appropriate sampling valve at the end of the working week. The exterior of the sampling valves was disinfected with a sterile gauze wetted with 70% isopropyl alcohol. The sampling valves were opened, and fluid was allowed to run to waste for 60 s, prior to the aseptic collection of the samples.

Samples for HPC assessment were aseptically collected (sterile gloves and face masks were used), using 100 mL sterile bottles, refrigerated (at < 10 °C without freezing) and transported to the laboratory for analysis within 4 h of sampling.

Samples for FCM measurements were aseptically collected (sterile gloves and face masks were used), using 5 mL sterile vials and processed immediately (i.e., onsite).

A pre-test was performed using the following reference microorganisms: two Gram-positive microorganisms (*Bacillus subtilis* and *Enterococcus faecalis*) and three representative microorganisms of Enterobacteriaceae family: *Klebsiella pneumoniae*, *Salmonella enterica* and *Salmonella typhimurium*.

Endotoxins assessment was performed for all sampling moments of the study.

HPC assessment

Heterotrophic plate counts (HPC) were determined in accordance with ISO 23500-3[39], namely via pour-plate technique procedure, using a low nutrient Agar (Reasoner's Agar No. 2 - R2A). Samples were incubated at 17 °C to 23 °C for a period of 168 h (i.e., 7 days). Analyses were performed in duplicate and expressed as CFU/mL.

FCM assessment

FCM was used to determine the total bacterial cell concentration. FCM analysis was conducted using the AQU@ Sense MB cytometer (BWT Aqua AG, Switzerland) in offline mode (i.e., manual mode) with grab samples. The system's microfluidic sample preparation enabled full automation, including precise sampling and DNA-staining (using PI and SYBR Green I) in a continuous batch process. Analysis was performed immediately after sampling, and the AQU@Sense MB reported intact/living cell counts as ICC/mL (Intact Cell Count per Milliliter).

Cells falling within the ICC gate were classified as intact, while those outside the gate, including dead cells, biological debris, and background, were excluded from the quantification. Additionally, SYBR Green I fluorescence intensity allowed further interpretation, distinguishing between High Nucleic Acid (HNA) cells, which are more active, and Low Nucleic Acid (LNA) cells, which indicate a stressed state.

For quantification, 5 mL of the cell suspension was collected in a screw-cap tube (AXYGEN®, SCT-5ML-S) and attached to the automated flow cytometer with special in-built option.

Prior to each round of measurements, a sterility blank was performed using a sterile saline solution. All blanks showed no growth (i.e., no positive results). After each measurement, a rinse of the cytometer was done using a cleaning solution (BWT Aqua AG, Switzerland) to avoid cross-contamination of the following samples.

Figure 2 represents a single measurement results overview from the display of the FCM device.

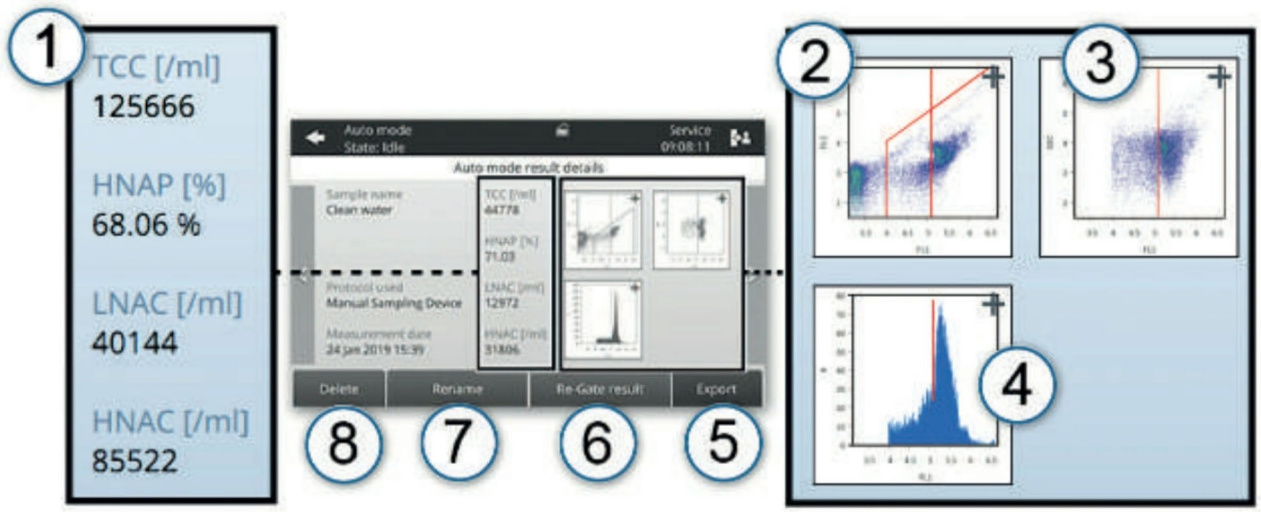

Fig. 2. Display of a single measurement results with the FCM device used in this study (courtesy of BWT Aqua AG, Switzerland). 1. Measured parameters are displayed. 2. The FL2 vs. FL1 dot plot shows all detected events according to the amplitude of their fluorescence signals FL1 (535 nm, X-axis) and FL2 (715 nm, Y-axis). The red polygon defines the gates. 3. The SSC vs. FL1 dot plot shows only cells inside the gates, according to their fluorescence signal FL1 (535 nm) and scattered light signal SSC (488 nm). 4. The FL1 histogram shows all cells inside the gates, binned according to their fluorescence in FL1. 5. Export saves this result to a USB stick. 6. Re-Gate result allows to move the gates and recalculate cell counts. 7. Rename: ability to rename the result. 8. Delete: the result is deleted permanently (requires confirmation). FL1 - Fluorescence signal 1 = Green fluorescence emission of the SYBR Green I (525 nm). FL2 - Fluorescence signal 2 = Red fluorescence emission of Propidium Iodide (715 nm). SSC - Side scatter signal = Scattered light, increases with the size of the cell.

Endotoxins assessment

The kinetic chromogenic LAL test measures endotoxin levels by detecting the color change that occurs when endotoxins trigger a reaction with a chromogenic substrate in the presence of Limulus Amebocyte Lysate (LAL). The rate of color formation, monitored over time, is directly proportional to the concentration of endotoxins in the sample.

Samples were aseptically collected in a 15 mL non-pyrogenic tube (Greiner, DE) and transported to the laboratory where they were processed within 48 h of collection.

Endotoxins were measured using the Kinetic-QCL™ Kinetic Chromogenic LAL (Lonza) according to the manufactures protocol. Results were obtained using the Genesis software package.

Data analysis

Descriptive analyses were conducted to examine the FCM vs. HPC values in the 4 different sampling points. Means, medians, interquartile ranges and standard deviations were calculated.

Differences between the FCM and HPC were assessed for statistical significance using the Mann-Whitney test.

Scatter plots were performed with the mean value and the corresponding confidence interval for 4 sampling points individually and for sampling points 2, 3 and 4 combined.

The generalized linear estimation (GLM) model was used to analyze the cell counts (i.e., ICC/CFU) from FCM and HPC approaches at the three different dialysis water sampling points (SP2, 3 and 4). The two GLM models were built using robust estimation and the identity link function with two dependent variables: cell counts to study the difference in means and the logarithm of cell counts to study the ratio of geometric means. The 0 values have been replaced by 0.5.

The level of significance was considered less than 0.05.

All reported p values are two-tailed and $p < 0.05$ was considered statistically significant.

All analyses were performed using IBM SPSS Statistics v.26.

All FCM data analysis were carried out using the BWT AQU@Sense MB software.

Results

We conducted a pre-test phase to identify possible problems with the sampling process, transport of samples for HPC assessment to the laboratory and onsite FCM measurements. Reference liquid solutions with selected microorganisms were used for this purpose. For the selected microorganisms, we chose two Gram-positive representative microorganisms (*Bacillus subtilis* and *Enterococcus faecalis*) and three representative microorganisms of Enterobacteriaceae family (a large family of Gram-negative bacteria): *Klebsiella pneumoniae*, *Salmonella enterica* and *Salmonella typhimurium*. Samples and assays were performed on October 25th, 2023, and the outcomes are presented in Table 3.

Microorganism	FCM (ICC/mL)	HPC (CFU/mL)
Bacillus subtilis	310	76
Enterococcus faecalis	280	27
Klebsiella pneumoniae	440	21
Salmonella enterica	290	2
Salmonella typhimurium	320	1

Table 3. FCM and HPC outcomes during pre-test phase.

Sampling point	Mann–Whitney test	FCM (ICC/mL) ($n=36$)	HPC (CFU/mL) ($n=36$)	p-value
SP1	Mean (SD)	46,080.28 (15,146.18)	250.53 (94.81)	< 0.001
	Median (Min.–Max.)	42,265.00 (27,120–95,010)	300.00 (31–300)	
SP2	Mean (SD)	10.00 (11.95)	1.97 (2.80)	0.012
	Median (Min.–Max.)	10.00 (0–50)	0.50 (0–10)	
SP3	Mean (SD)	8.61 (10.99)	0,28 (0.70)	< 0.001
	Median (Min.–Max.)	10.00 (0–40)	0.00 (0–3)	
SP4	Mean (SD)	43.33 (21.65)	11.81 (12.05)	< 0.001
	Median (Min.–Max.)	40.00 (10–100)	7.50 (0–50)	

Table 4. FCM vs. HPC for the different sampling points – SP1 to SP4 (Mann–Whitney test).

As previously mentioned, for this study samples were taken from four sampling points of the Dialysis Water Treatment System (Fig. 1) on a weekly basis from October 13th, 2023, up to June 12th, 2024 (36 weeks).

FCM and HPC outcomes for the different sampling points are shown in Table 4.

There is a significant difference between FCM and HPC microbial outcomes for all sampling points (SP1, SP2, SP3 and SP4), meaning that there is a higher sensitivity (i.e., recovery of microbial load/bioburden) when using FCM.

Scatter plots with the mean value and the corresponding confidence interval individually for the 4 sampling points (SP1, SP2, SP3 and SP4) and for the combined SP2, 3 and 4 (dialysis water sampling points) are presented in Figs. 3, and 4, respectively.

Median, mean, and geometric mean of number of cell counts in the dialysis water sampling points (i.e., SP2, 3 and 4) are presented in Table 5.

As it is shown in Table 6, there is a mean increase for dialysis water samples (i.e., SP2, 3 and 4) of 15.96 cells per mL when using the FCM compared to HPC.

The detection of cells in dialysis water samples is 4.36 times higher in FCM compared to HPC, as reflected in Table 7.

Endotoxins assessment was performed for all sampling moments of the study. The results are presented in Table 8.

As already stated, FCM was used to determine the total bacterial cell concentration. SYBR Green I fluorescence intensity allowed further interpretation, distinguishing between High Nucleic Acid (HNA) cells and Low Nucleic Acid (LNA) cells. LNA cells have been reported to be smaller and adapted to oligotrophic (low nutrient) environments. Percentage of samples with HNA are presented in Table 9.

Discussion

In this exploratory study, we investigate the potential of the flow cytometric method (FCM) compared to conventional heterotrophic plate count (HPC) method for monitoring microbial contamination and bioburden in dialysis water, with the aim of providing a faster approach to facilitate action based on the results.

During the pre-test phase, we observed differences in the results obtained for the various reference microorganisms when using HPC and FCM to assess microbial load (see Table 3).

The microbial outcomes for dialysis water showed significant differences when comparing FCM and HPC methods at all sampling points (Table 4), revealing a higher sensitivity of FCM over HPC for microbial monitoring of dialysis water (p-values for SP1 < 0.001, SP2 0.012, SP3 < 0.001 and SP4 < 0.001). In our study we calculated the magnitude of this difference in dialysis water sampling points. The detection of cells in SP2, 3 and 4 was 4.36 times higher in FCM compared to HPC. This info could be of relevance for future guidance regarding the establishment of maximum allowable levels and action levels when using FCM.

If the typical action level for dialysis water quality established for HPC (50 CFU/mL) had been adopted for the FCM method (i.e., 50 ICC/mL), we could have triggered timely corrective actions at sampling point SP2 – primary treatment (i.e., after RO first stage). At this sampling point, we identified a maximum value of 50 ICC/mL during the study period, while the same sample moment yielded only 10 CFU/mL using the HPC method. Regarding sampling point SP4, the distribution system return (the most representative sampling point of the dialysis water distribution system), the scenario is even more critical. If the maximum allowable level for dialysis water quality (< 100 CFU/mL, established for HPC) is adopted using the FCM method, we identified a

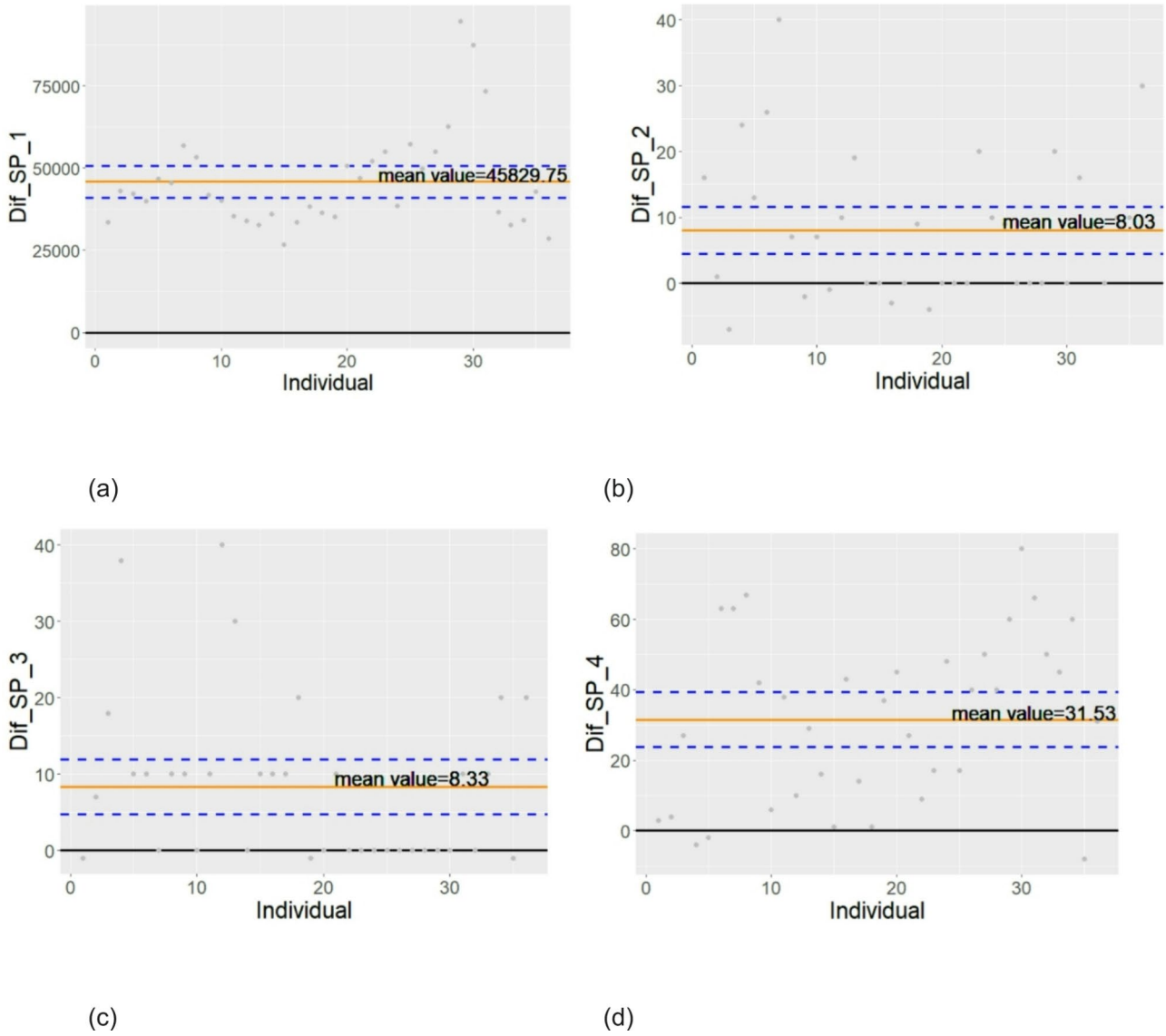

Fig. 3. Scatter plot with the mean value and the corresponding confidence interval for sampling points SP1 (**a**), SP2 (**b**), SP3 (**c**), and SP4 (**d**).

maximum value of 100 ICC/mL (FCM method) at this sampling point during the study period, while the sample taken in parallel yielded only 50 CFU/mL using the HPC method. The water used in this center complies with local and international standards (e.g., ISO 23500) for dialysis water. In cases where the produced water has higher microbial purity, the advantage of FCM over HPC may not be as pronounced.

During the 36 weeks of monitoring, we were able to identify in dialysis water sampling points (i.e., SP2, 3 and 4) values equal to or greater than 50 ICC/mL using the FCM method 15 times, whereas the HPC method identified a value equal to 50 CFU/mL only once during the same period. In water distribution systems, due to the surface to volume ratio, more than 95% of the entire biomass is located on the walls, with less than 5% in the water phase[40]. This aspect might explain the variation obtained in the outcomes during the 36 weeks regardless of the method used. A possible explanation for the differences obtained in the results of FCM over HPC is the viable but not culturable phenomenon (VNBC). When applying the HPC method, counts vary depending on the incubation duration and culture conditions. However, not all viable cells will grow on cultivation media. One of the biggest obstacles in monitoring the microbiological quality of dialysis water and dialysis fluids is the extended incubation time required for the HPC method (i.e., 168 h). FCM, as a rapid alternative method (with approximately 30 min per sample), allows timely corrective actions and enhances patient safety. No strong correlation can be established between the cell concentration in the water and that in the biofilm, although most of the cells found in the water phase originate from biofilms[19]. This fact also makes it impossible to establish strong correlations between cell numbers and endotoxins concentrations. The average endotoxins concentrations at the dialysis water sampling points SP2, SP3 and SP4 (Table 8) were below the typical action level established by international standards (i.e., <0.125 EU/mL).

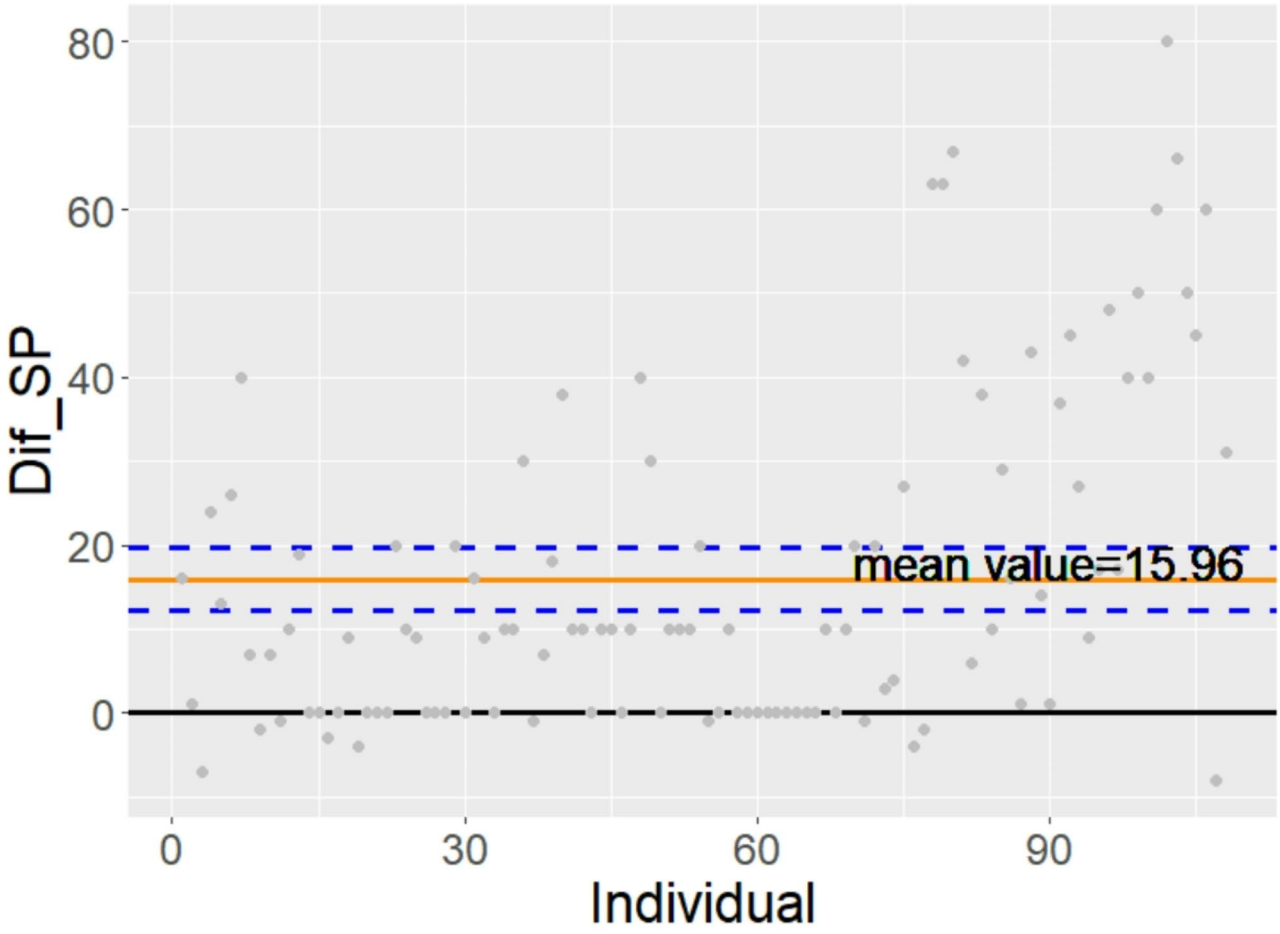

Fig. 4. Scatter plot with the mean value and the corresponding confidence interval for the combined SP2, 3 and 4 (dialysis water sampling points).

Median		Mean		Geometric mean	
FCM (ICC/mL)	HPC (CFU/mL)	FCM (ICC/mL)	HPC (CFU/mL)	FCM (ICC/mL)	HPC (CFU/mL)
10.00	1.00	20.65	4.69	7.23	1.66

Table 5. Median, mean, and geometric mean of cell counts in SP2, 3 and 4.

	Adjusted B	CI for adjusted B	*p*-value
Method	15.96	11.46–20.47	< 0.001

Table 6. FCM vs. HPC (GLM model for cell counts in SP2, 3 and 4).

	Exp(B)	CI for Exp(B)	*p*-value
Method	4.36	2.80–6.77	< 0.001

Table 7. FCM vs. HPC (GLM model for Ln of cell counts in SP2, 3 and 4).

Comparison of LNA/HNA ratios (Table 9) demonstrated the occurrence of both types of bacteria in all sampling points, as has been reported also for various other aquatic samples[36]. We observed a rather high variability without any distinct pattern. The slightly higher abundance of HNA-type cells at SP4 compared to the other more upstream sampling points, may be an indication of higher nutrient content in that environment of

Sampling point	Endotoxins assessment (EU/mL)					
	Min	Max	av.	σ	*n*	AL (EU/mL)
SP1	2.794	25.000	17.138	7.013	36	n.a.
SP2	0.005	0.121	0.010	0.020	33	0.125
SP3	0.005	0.094	0.008	0.016	33	0.125
SP4	0.005	0.125	0.023	0.034	32	0.125

Table 8. Endotoxins assessment for the different sampling points. AL: typical action level as established by international norms[26].

Sampling point	HNA (%)				
	Min	Max	av.	σ	*n*
SP1	39.0%	83.1%	60.5%	10.3%	36
SP2	0.0%	100.0%	45.3%	46.5%	36
SP3	0.0%	100.0%	33.8%	45.0%	36
SP4	50.0%	100.0%	92.4%	15.6%	36

Table 9. FCM assessment (percentage of samples with HNA in the different sampling points).

an 18-year-old ring installation including an established biofilm, which would favor a less oligotrophic bacterial population[41].

Certain FCM devices have an online mode, providing a real-time bioburden data and supporting a dynamic shift toward a more effective water quality approach. However, we did not use the FCM device in online mode, but rather in offline mode, due to the need to cover multiple sampling points on the different days we collected samples.

Further studies are needed to demonstrate FCM applicability and its superior value in the field of dialysis, including its use in monitoring the quality of dialysis fluids, which was beyond the scope of this study (we only addressed FCM on dialysis water, not dialysis fluids). However, we do not expect major challenges to apply FCM on dialysis fluids monitoring (i.e., preliminary dialysis fluids FCM analysis done by the authors did not show any procedural interferences). A similar study as the current one could easily be set up to perform FCM assessment and comparison with HPC. Such a study comparing HPC and FCM for microbial assessment of dialysis fluids, particularly ultrapure dialysis fluid, should consider using the membrane filtration technique for HPC rather than the pour-plate technique. In treatment scenarios where the microbial profile of dialysis water exceeds the maximum allowable levels, it might be needed to test ultrapure dialysis fluids to ensure the integrity of bacteria and endotoxin-retentive filter (BERF) in a timely and accurate manner. This highlights the potential added value of using FCM instead of HPC as an intervention tool in contamination events in the dialysis machine fluid pathway.

If the FCM method is considered for dialysis water quality monitoring, it will be necessary to establish maximum allowable levels and typical action levels for dialysis water quality when using FCM. Moreover, running FCM in online mode would be extremely valuable, as it would provide a more comprehensive and continuous view of microbial dynamics within the water treatment system. This could allow for a detailed characterization of microbial loads during different phases of the RO or distribution loop system such as supply mode, rinse mode, disinfection mode and idle times (where little or no water is consumed due to absence of dialysis treatments).

An additional challenge for FCM as method for dialysis water monitoring, apart from system validation, limits definition and implementation in regulatory standards, certainly lies within the cost perspective: initial investment and costs for the flow cytometer cartridge refill have to be balanced against the current cost for standard HPC assays, which are often outsourced to external labs, where they are performed on routine basis with relatively high sample numbers. Our initial calculation model shows that for a single dialysis center a switch from HPC to FCM, using offline measurements, for regular dialysis water and fluids monitoring is not financially attractive. Assuming four dialysis water sampling points (RO inlet, primary treatment, secondary treatment, and loop return) and a monthly sampling rhythm, this would sum up to only 48 samples taken per year, or 96, when running duplicates. While pure reagent costs per sample, calculated by taking the cost of one refill of the measurement cartridge divided by number of samples that can be run with one cartridge is below 3 EUR, the allocation of initial invest (around 81,000 EUR for the device incl. two measuring cartridges plus initial installation and qualification) renders this method not attractive for a single clinic. On the other hand, combining clinics into regional clusters, sharing the flow cytometry device and its associated qualification and servicing costs, might lead to a much more attractive picture. Additionally, one could envisage not only dialysis water monitoring but also utilizing FCM for regular dialysis fluid monitoring, to double annual sample numbers per clinic and increase device utilization, thereby reducing per-sample costs. As shown in Fig. 5, per-sample costs might in those cases be in the range or even below traditional HPC assay costs, with the non-quantifiable advantages such as faster turnaround (especially relevant during validation and troubleshooting) and the more complete picture of the microbial state not even considered in the calculation.

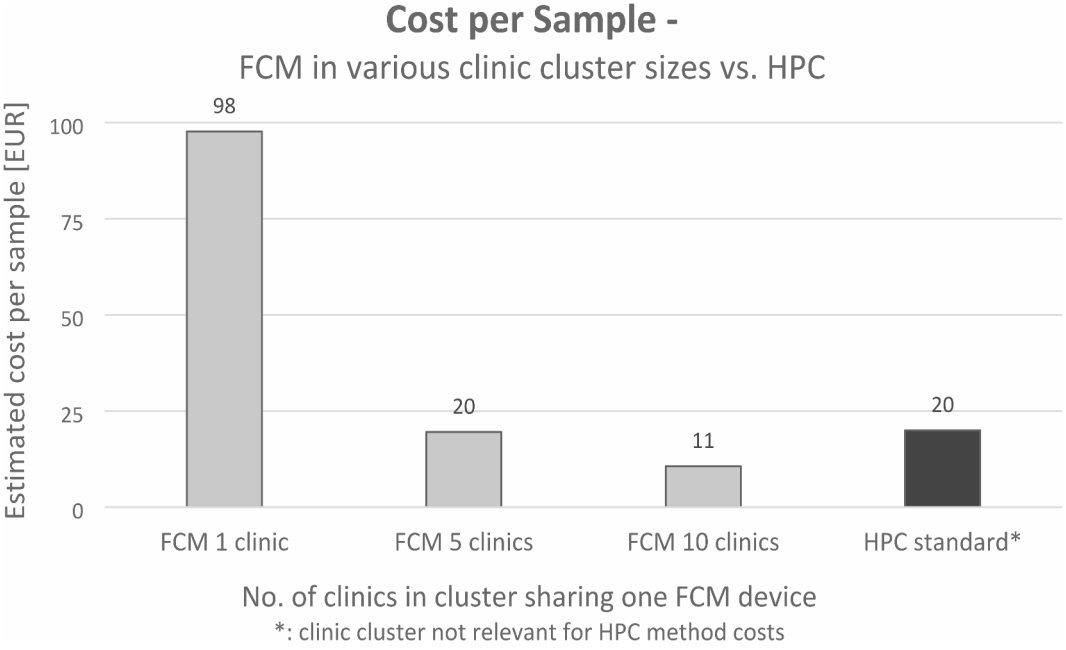

Fig. 5. Cost estimate per sample (FCM vs. HPC). Assumptions: 4 sampling points per clinic for dialysis water monitoring plus 4 additional dialysis fluids samples monitoring, each measured in duplicates, monthly sampling; initial invest for FCM device incl. spare cartridge, installation and qualification of 81,000 EUR, plus full supplier validation package (18,000 EUR); 10-year device lifetime period with respective "per-sample allocation"; annual maintenance and service cost (5,000 EUR); 1,000 samples per cartridge refill, cartridge lifetime 9 months (if less than 1,000 samples are analyzed within 9 months, the rest is "wasted"); cost of cartridge refill of 2,900 EUR, as per manufacturer information; the authors assumed an average cost for external HPC assay of 20 EUR per sample. Sample transport and logistics costs were not considered for either method.

As technology advances and adoption increases, costs may decrease, making FCM a more accessible and practical tool for routine dialysis water and fluid monitoring.

Conclusions

Currently, the use of FCM may be considered as an add-on rather than a replacement for traditional HPC methods, as it has not yet been established as reference method by pharmaceutical or industry standards.

According to our evaluation, FCM is a much faster and higher sensitivity method for microbial monitoring of dialysis water compared to the classical HPC. Clearly the risk of contamination is reduced, and the possible operator influence on the results is minimized. More relevant is the "real-time" evaluation that we can obtain with FCM allowing immediate corrective actions if needed. This is particularly relevant when performing troubleshooting activities or microbial assessment after an important technical intervention in the water treatment system hydraulic circuit (e.g., intervention that obliges the RO permeate outlet or distribution system to be opened). The current approach implies waiting more than 7 days to receive the HPC results from the lab (possibly releasing a risk assessment). Up to the time that HPC outcomes are available several treatments have already been performed.

Microbial assessment using FCM allows for a different approach regarding validation and disinfection strategies given that the results are available approximately 30 min after sampling. This will have a significant impact regarding the time required for validation procedures of the water treatment system. The same rationale applies when facing exogenous contaminations (i.e., sampling contamination). FCM allows false positives to be identified in real time, whereas HPC takes several days to detect this phenomenon.

The main challenge towards establishing FCM as a true HPC alternative, lies in reducing the initial investment costs, e.g., by increasing device utilization through regional device sharing (clinics clusters), and to setting maximum allowable levels, as well as redefining typical action levels when using FCM. However, due to its faster results, high sensitivity and reliability, further studies are warranted to validate its use in real world practice. Since there is still discrepancy between HPC and FCM, it is recommended that a working group be formed within standardization bodies to address this issue.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

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Author contributions
R.L.: study conceptualization and design, sampling, measurements and data acquisition; R.L., J.F.: data analysis and writing original draft; R.L., J.F., C.G.: study protocol; J.F.: cost analysis; A.F., P.P. and B.C.: methodology validation, supervision and critical review.R.L. serves as corresponding author. All authors contributed equally to manuscript editing and writing. All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to R.L.

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