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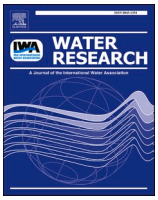

Alternative for HPC22 after repairs in the drinking water distribution system

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ABSTRACT

There is a risk of contamination by (pathogenic) microorganisms from the outside environment into the drinking water during maintenance or pipe breaches in the drinking water distribution system (DWDS) and, consequently, the drinking water distributed to consumers may result in possible detrimental effects on public health. Traditional time-consuming microbiological testing is, therefore, performed to confirm drinking water is not microbially contaminated. This is done by culturing methods of the faecal indicators *Escherichia coli*, intestinal enterococci and the technical parameters coliform bacteria and heterotrophic plate counts at 22 °C (HPC22). In this study, fast methods (adenosine triphosphate (ATP), flow cytometry, enzyme activity and qPCR) were compared as an alternative for HPC22. Using dilution series and field samples, ATP ($ATP_{total\text{-}lab}$ and $ATP_{cell\text{-}mob}$) and enzymatic activity (ALP-2) methods proved to be the more reliable and sensitive than flow cytometry and qPCR methods for detecting microbiological contaminations in drinking water. Significant ($p < 0.05$) and relatively strong correlations ($R^2 = 0.61–0.76$) were obtained between HPC22 and both ATP methods, enzyme activity and qPCR parameters, but relations with flow cytometry were weak ($R^2 = 0.24 – 0.52$). The samples taken after repairs or a calamity from the DWDS showed in general limited variation in the HPC22 count and were in most cases below the guidance level of 1,000 CFU/mL. We recommend that the best performing alternative methods, i.e. $ATP_{total\text{-}lab}$ and $ATP_{cell\text{-}mob}$ and ALP-2, should be included next to HPC22 in additional field studies to further test and compare these methods to be able to decide which fast method can replace HPC22 analysis after maintenance work in the DWDS.

1. Introduction

In many countries heterotrophic plate count (HPC) is included in regulations as a generic microbiological water quality indicator for drinking water and routinely monitored. Next to routine monitoring, HPC can also be included in legislation regarding safeguarding drinking water after repairs in the distribution system (DWDS) as is the case in the Netherlands. During maintenance or pipe breaches in DWDSs there is a risk of contamination by (pathogenic) microorganisms from the outside environment into the drinking water posing a potential public health risk. Therefore, contamination of drinking water with environmental microorganisms, especially those of faecal origin, needs to be avoided. To ensure drinking water safety after maintenance or pipe breaches in the DWDS, legislation in the Netherlands prescribes that water samples have to be taken within 1–24 h after repairs and analysed for four different parameters (Drinkwaterbesluit, 2018; Meerkerk, 2021). Three of them (*Escherichia coli*, intestinal enterococci and coliform bacteria) confirm absence of faecal contamination. The fourth parameter is the HPC determined with a nutrient-rich agar medium incubated at 22 °C for 3 days (HPC22), according to NEN-EN-ISO 6222 as described in the current legislation in the Netherlands, to confirm absence of ingress or contamination with another water type and thus other (non-faecal and non-pathogenic) microorganisms and/or nutrients that enhance microbial growth. In The Netherlands, HPC22 is thus not used as an indicator

Abbreviations: ALP, Alkaline Phosphatase; ATP, Adenosine triphosphate; DWDS, drinking water distribution system; HPC, Heterotrophic Plate Counts; HPC22, Heterotrophic Plate Counts at 22°C; CFU, Colony Forming Units; FCM, Flow cytometry; FCM_{ICC}, Flow cytometry, intact cell count; FCM_{TCC}, Flow cytometry, total cell count; LoA, limit of agreement; TP, (drinking water) treatment plant; WWTP, Wastewater treatment plant.

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of actual public health risk, but as a technical parameter for drinking water quality.

Originally, these microbiological parameters were assessed using time-consuming cultivation-based methods on (selective) agar plates. It would be valuable for water utilities to have methods with a shorter time-to-result, because then they can act faster to undesired water quality changes after repairs, thereby also shortening the inconvenience for the consumers. The Dutch government approved in 2019 the use of RT-qPCR for the detection of *E. coli* within four hours (Heijnen et al., 2024). In addition, the validation of a RT-qPCR for intestinal enterococci is currently at a near-to-implementation stage allowing results within four hours of that parameter as well. However, there is still a need for a fast, sensitive and reliable method that can replace HPC22 as a process parameter after repairs in the DWDS. In recent years, several microbiological methods that determine the general microbiological water quality using adenosine triphosphate (ATP) concentrations, flow cytometry (FCM), 16S rRNA gene copies or enzymatic activity, have become available that provide results faster.

ATP is a measure for active biomass in a sample and can be measured within minutes in the laboratory or in the field and has been used as a parameter to monitor the microbiological drinking water quality (Hammes et al., 2010; Lautenschlager et al., 2013; Liu et al., 2014; Prest et al., 2021; van der Kooij, 2003; van der Wielen and van der Kooij, 2010; van der Wielen et al., 2016). An ATP concentration below 10 ng ATP/L in unchlorinated drinking water in the Netherlands is considered a normal value (van der Kooij, 2003), although each DWDS has its own baseline (van der Wielen and van der Kooij, 2010). In chlorinated drinking water, lower ATP values (approximately 0 – 4 ng/L) are generally observed in the DWDS (Delahaye et al., 2003; Stoddart et al., 2020). Besides ATP, cell counts using FCM have also been used regularly (Gillespie et al., 2014; Prest et al., 2016b; Schönher et al., 2021). When using different dyes or gating, FCM can determine total cell counts (TCC), membrane-intact cell counts (ICC) and high and low nucleic acid cell counts (HNA and LNA). The number of cells in drinking water varies roughly between $1 \times 10^3 - 3 \times 10^5$ cells/mL in the Netherlands (De Roy et al., 2012; Farhat et al., 2020; Hammes et al., 2012). In general, 83 to 97 % of the TTC cells are ICC and 54 to 98 % consisted of LNA cells (van der Wielen et al., 2016). Previous comparisons between HPC22, flow cytometry and microscopic counts have shown that HPC22 only detects between 0.001 and 8.3 % of the bacteria present (Bartram et al., 2003; Burtscher et al., 2009; Hammes et al., 2008). Bacterial numbers in drinking water can also be enumerated by quantifying the 16S rRNA gene copies using qPCR with numbers varying between $1 \times 10^6 - 1 \times 10^7$ gene copies/L in drinking water without a disinfectant residual (Boers et al., 2018). A disadvantage of using 16S rRNA gene copies is that also 16S rRNA genes of dead bacteria can be quantified, thereby overestimating the active bacterial biomass. The enzyme alkaline phosphatase is present in all microbial cells and can also be used as indicator for the total active biomass in drinking water. Studies where alkaline phosphatase have been determined in drinking water are limited, but showed baseline levels of 53.2 ± 1.6 μU/100 mL (Favere et al., 2021).

Previous research in Denmark and Flanders has indicated that some of the above described parameters are not only faster, but might also be more sensitive than HPC22 in detecting contamination with a different water type in drinking water (Favere et al., 2021; Vang et al., 2014). The goal of our study is to extend those laboratory-based studies by investigating more possible contaminant sources (rainwater, groundwater, surface water, sewage water, clay soil and sediment from a DWDS) during maintenance activities in two unchlorinated drinking water types that differ in their biological stability. We compared HPC22 to four alternative, rapid methods that are based on different detection techniques (ATP, alkaline phosphatase enzyme, FCM, qPCR). In addition, our study seems to be the first that subsequently also investigated HPC22 and the alternative methods in samples taken after repairs and calamities in full-scale DWDS. The overall objective of our study is to identify whether alternative parameter(s), determined with a rapid method, can replace HPC22 as a monitoring parameter for the general microbial water quality after maintenance and/or repairs in the DWDS.

2. Materials and methods

2.1. Contamination experiments

2.1.1. Water sources and contaminants

Drinking water used in the contamination experiments was sampled at two drinking water treatment plants (TP1, TP2). TP1 produces drinking water from aerobic ground water by aeration and limestone filtration for the removal of CO_2 before it is distributed. TP2 uses surface water for drinking water production, which is treated by coagulation and sedimentation with ferric chloride, three-month residence time in a reservoir, rapid sand filtration, ozonation, pellet softening, granular activated carbon filtration and slow sand filtration as final step. At both production locations drinking water is distributed without a disinfectant residual.

Different contamination sources i.e., rainwater, wastewater, groundwater, clay soil, sediment and surface water were used. These contaminants were chosen based on the possibility that they can cause a contamination during repairs and/or calamities in the DWDS. Rainwater was collected in sterile polypropylene bags (LLG Labware, Peterborough, Canada) during three consecutive days in November 2022 and January 2023. The plastic bags were constrained within plastic containers and placed in the open air. After three days of collection the rainwater from the different containers was combined. Sewage water was sampled at the influent of the wastewater treatment plant in the city of Utrecht, the Netherlands. Surface water was sampled at two locations: river water from the Lekkanaal (Nieuwegein, the Netherlands) and ditch water next to the recreational area Gaasperplas near Amsterdam, the Netherlands. The river water was used with drinking water of TP2, whereas ditch water was used with drinking water of TP1. Sediment from the DWDS was obtained by flushing DWDSs where drinking water produced from surface water was distributed. Flushing was performed according to (van Lieverloo et al., 2004). Groundwater and clay soil material were sampled at KWR in Nieuwegein, the Netherlands. At 140 cm depth naturally, emerging groundwater was sampled using a small peristaltic pump (Masterflex L/S, Cole-Parmer, Alessandria, USA). The clay soil was sampled 50 cm below ground surface. All samples were stored and transported at 4 °C and processed within 24 h after sampling.

For the clay soil, a stock mixture was prepared by adding 10- or 100-gram of clay soil to 1-liter drinking water of TP1 or TP2, respectively. To resuspend the clay soil in the water, the mixture was shaken and treated with low energy sonification in a water bath for 5 min followed by overnight incubation at 4 °C while being continuously mixed with a magnetic stirrer. After overnight incubation, the resuspended clay soil mixture was used for the contamination experiments. The sediment from the DWDSs was settled for 30 min after which the water was carefully removed with a pipette and subsequently the remaining sediment volume was determined. Next, a similar volume of drinking water was added to the sediment and continuously mixed overnight at 4 °C with a magnetic stirrer. After overnight incubation, the resuspended sediment mixture was directly used for the contamination experiments.

2.1.2. Dilution series

The different contamination sources were serially diluted in unchlorinated drinking water of TP1 or TP2. Details of these dilution series are shown in Table 1. All dilutions were made on v/v ratio and were mixed for 1 h at room temperature using a magnetic stirrer. The start concentration and dilution steps were tailored to each contaminant source to allow for as many measurable dilutions before the lower detection limit of all tested methods was reached. In some cases the second dilution series of a contaminant source was adapted, depending on the results from the first dilution series to optimize the number of

Table 1
Experimental setup of serial dilution series of six contaminants in unchlorinated drinking water of TP1 and TP2. Given is per contaminant which start concentration (v/v), dilution steps and the range of contaminations that were tested.

	TP1			TP2		
	Start concentration contaminant (v/v)	Dilution steps	Dilution range	Start concentration contaminant (v/v)	Dilution steps	Dilution range
Wastewater	10 %	1:10	0.000001 – 10 %	10 %	1:7	0.000012 – 10 %
Surface water	33 %	1:3	0.015 – 33 %	20 %	1:5	0.00026 – 20 %
Rainwater	100 %	1:2	0.78 – 100 %	100 %	1:2	0.78 – 100 %
Sediment	10 %	1:5	0.00013 – 10 %	10 %	1:4	0.0006 – 10 %
Ground water	50 %	1:2	0.20– 50 %	50 %	1:3	0.0076 – 50 %
Clay soil	0.16 %	1:2 – 1:4	0.00031 – 0.16 %	0.3 %	1:3	0.00014 – 0.3 %

measurable dilutions. The ATP concentration of the clay soil, groundwater and rainwater was used to determine the most optimal dilution series for all methods. For sewage water, surface water, and sediment from the DWDS, it was not possible to measure the ATP concentration on the day of sampling. For these contamination sources, the dilution series was designed based on (historical) knowledge about the ATP concentration in these specific contaminant sources. Each dilution series consisted of eight serial dilutions and the drinking water sample without added contamination source (reference sample). For each dilution, a sample was prepared that was measured in duplicate with each method. The average of these duplicates was used for further data and statistical analysis.

2.1.3. Parameters measured

The following parameters were determined on samples from the dilution series: HPC22, three different ATP parameters (total ATP [$ATP_{total\text{-}lab}$], cellular ATP measured in the lab [$ATP_{cell\text{-}lab}$] and in the field [$ATP_{cell\text{-}mob}$]), four different FCM parameters (total [FCM_{total}], intact [FCM_{intact}], low-nucleic acid [FCM_{LNA}] and high-nucleic acid cell counts [FCM_{HNA}]), two different enzyme activity parameters (BACT-control system [ALP-1] and the ColiMinder system [ALP-2]) and gene copies of the 16S rRNA gene.

HPC22, ATP and ALP-2 were determined for all dilution series. FCM failed for the dilution samples with wastewater in drinking water of TP1 due to technical issues. In addition, FCM_{LNA} and FCM_{HNA} were only determined for the dilution samples of rain and groundwater in drinking water of TP2, clay soil in drinking water of TP1 and TP2 and for sediment in drinking water of TP1. ALP-1 was not determined for dilution samples of wastewater in drinking water of TP1 and surface water in drinking water of TP1 and TP2, as the method was not yet available when these dilution series were measured.

2.2. Field sampling

2.2.1. Locations

Field sampling was done in six different DWDSs in the Netherlands and Belgium after maintenance work was finished (DWDS3 – DWDS8). Details of these six locations are shown in Table 2.

Drinking water sampling from the six DWDSs was done using a standpipe (DWDS3) or sampling cabinet (DWDS4–8). A standpipe is a steel pipe containing a sample valve that was installed on a fire hydrant in the DWDS. A sampling cabinet is a device with a sampling tap that was placed in a secured cabinet and connected directly to the drinking water pipe underground through a ZPE tylene tube.

Before the maintenance activities in the DWDS, a drinking water sample was taken upstream of the maintenance as a reference sample. After the maintenance work was finished, pipes were flushed according to (van der Schans et al., 2016) and samples were taken downstream at different time points after flushing (Table 5). During sampling, drinking water flowed continuously at a low, constant rate of about 0.3 m s^{-1} (Table 2). Drinking water samples of 1 L were taken and stored in gamma sterile high-density polyethylene (HDPE) bottles containing 50 mg/L trisodium-NTA. Samples were transported and stored at 2–5 °C and analysed within 24 h.

2.2.2. Parameters measured

The field samples were analysed with the same parameters as the contamination experiments. ALP-1 results are not available for DWDS3,

Table 2
Information field sampling locations DWDS3 – 8 where repairs and work activities were performed and drinking water samples were taken. The residence time was modelled by the drinking water utility.

Sample name	Source water	Chlorine residual	Sampling date	Sampling reason	Sampling method	Residence time (hours)	Pipe material	Cultivation	Flushing velocity /time	Disinfection
DWDS3	Ground water	Yes	17 April 2023	Leaking fire hydrant	Standpipe	ND	FGG*	Residential area	0.57 m s^{-1} (30 min), followed by 0.25 m s^{-1}	Sodium hypochlorite
DWDS4	Surface water	No	01 May 2023	Replace part PVC-O	Sampling cabinet	ND	PVC-O	Residential area	ND	Chlorine dioxide**
DWDS5	Surface water	No	22 May 2023	Calamity/ leakage	Sampling cabinet	ND	HPE	Residential area	ND	Chlorine dioxide**
DWDS6	Ground water	No	12 June 2023	Replace part PVC-O	Sampling cabinet	24	PVC-O	Residential area	0.3 m s^{-1}	–
DWDS7	Ground water	No	20 June 2023	Replace part PVC-O	Sampling cabinet	24	PVC-O	Residential area	0.3 m s^{-1}	–
DWDS8	Surface water	No	08 May 2023	Building new residential area	Sampling cabinet	***	PVC	Residential area under construction	0.25 m s^{-1}	–

- No disinfection.
ND Not determined.
* FGG is made out of cast iron.
** The final step of the treatment plant consisted of addition of a low concentration of chlorine dioxide before the drinking water entered the clear water reservoir. The chlorine dioxide was below the detection limit the moment the drinking water left the reservoir and was distributed to the consumers.
*** Waterpipe not in use. Water demand of 3 L/hour via sampling box.

as the system was not yet available, and yielded negative results for DWDS6 and DWDS7. The negative results were omitted from further data and statistical analysis.

2.3. Analyses

HPC22 was determined according to NEN-EN-ISO 6222 using the pour plate method where 1 mL of the water and/or diluted sample was incubated on Plate Count Agar (3,564,474, Bio-Rad Laboratories, Veenendaal, the Netherlands). Agar plates were incubated for 68 ± 4 h at 22 °C after which colonies were counted.

ATP was determined using the luciferin-luciferase reaction. $ATP_{total\text{-}lab}$ and $ATP_{cell\text{-}lab}$ were determined with the laboratory method (CEN-EN, 2014; van der Wielen and van der Kooij, 2010) and with a mobile ATP measurement kit (LuminUltra, New Brunswick, Canada), respectively. For the laboratory method the sample was first measured with addition of luciferase to measure the amount of free ATP. Next a lysis buffer (Celsis International B.V., the Netherlands) was added to measure the amount of total ATP. The ATP concentrations were calculated from the produced light, measured with a luminometer (Celsis Advance II, Celsis International B.V., the Netherlands), using a calibration curve. $ATP_{cell\text{-}lab}$ was calculated by subtracting the free ATP from the $ATP_{total\text{-}lab}$. $ATP_{cell\text{-}mob}$ was measured according to the manufacturer's instructions. The water sample was filtered over a quenchgone filter after which a lysis buffer (UltraLyse) was passed through the filter. The eluate was twice diluted with Luminase before it was measured with the luminometer provided by the manufacturer. The generated light was converted to ATP concentrations using an internal standard.

FCM_{total}, FCM_{intact}, FCM_{LNA} and FCM_{HNA} were determined using a flow cytometer (FACSCalibur, BD, Franklin Lakes, USA) as described previously (van der Wielen and van der Kooij, 2013). The water sample was incubated with SYBR green and propidium iodide to distinguish between total and membrane-intact cells. In addition, for each sample the number of HNA cells and LNA cells was determined by defining a window in the FCM software based on SYBR green fluorescence (Prest et al., 2013).

The enzyme alkaline phosphatase activity (ALP) was measured using a prototype of the BACTcontrol system (microLAN, the Netherlands) (ALP-1) and with the ColiMinder system (VWMs GmbH, Austria) (ALP-2). In both systems, the microbial ALP in the sample converts 4-methylumbelliferyl phosphate to 4-methylumbelliferone, upon which fluorescent light is emitted that is detected by the sensor in the system. Samples were measured from lowest to highest expected biomass concentration, and each measurement was automatically followed by a cleaning procedure.

For ALP-1, the system and reagents were handled according to the manufacturer's instructions. Enzymatic activity was expressed as pmol/min. The day before a dilution series was measured, a measurement was performed using cleaning solution, to control that contaminations were removed from the system and sampling tubes. For ALP-2, a total activity reagent kit was used (RK-TA-500, VWMs GmbH, Austria) according to the manufacturer's instructions. The total microbial activity is expressed as μU/100 mL. The day before a dilution series was measured, the device was cleaned and a blank of ultrapure water was measured as a negative control. After the dilution series was finished for both ALP-1 and ALP-2, several cleaning procedures were performed followed by a measurement of ultrapure water until a blank measurement met the requirements.

To determine the number of 16S rRNA gene copies, DNA was isolated from the water samples conform NEN 6254 (14.1) using the PowerBiofilm™ DNAeasy isolation kit (Qiagen, Venlo, the Netherlands). In short, 300 μl of each sample was directly added to a bead tube of the PowerBiofilm™ kit, containing 350 μl of buffer MBL, after which the manufacturer's instructions were followed. To each water sample an internal control was added to determine the recovery of the DNA isolation and possible PCR inhibition. Results were corrected with the yield of this internal control. DNA was eluted in elution buffer from the PowerBiofilm™ kit and stored at −20 °C for further analyses. For the qPCR analysis, the following was mixed: 25 μl iQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 0.4 mg/mL Bovine Serum Albumin) and 0.2 μM of forward primer (331F: 5′- TCC TAC GGG AGG CAG CAG T −3′) and reverse primer (518R: 5′- ATT ACC GCG GCT GCT GG −3′) in 50 μl. The PCR-protocol consisted of 5 min at 95 °C; 44 cycli of 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C; followed by 10 min at 72 °C, 10 s at 95 °C and 1 min at 65 °C.

2.4. Data analysis and statistical analysis

The last sample in the dilution series that still showed a measurable decrease in the HPC22 or alternative parameter was determined by comparing whether the measured value from each dilution of all individual dilution series had a higher or equal concentration (including standard deviation) than the next measured samples. One exception to this was made for the 16S rRNA gene copies in groundwater in the TP1 series, due to an outlier of one of the dilution samples.

A statistical analysis was done on the $\log_{10}$ transformed data with RStudio (version 2022.07.2 + 576) and packages ggplot2, reshape2, scales, ggpattern, plyr, dplyr, ggpubr, gridExtra and patchwork. Correlations were determined using the Pearson and Spearman correlation coefficients. Relative trueness, which is the degree of correspondence between the results obtained by HPC22 and the results obtained with the alternative methods on similar samples, was determined according to NEN-EN-ISO 16,140–2. The results were subsequently visualized in Bland-Altman plots, made according to the same NEN-standard, and that can be used to evaluate the agreement between two methods. The mean difference in the estimated bias and the limit of agreement (LoA) is derived from the variation in the difference between HPC22 and the alternative method. A higher variation in the difference between the two methods results in a larger LoA. For these analyses, the data was $\log_{10}$-transformed after which the data was normalized to compare parameters with different units. The data was normalized according to min-max normalization (Equation 2.1).

$$X_{normalized} = \frac{x - \text{Lowest measurement of method}}{\text{Highest measurement of method} - \text{Lowest measurement of method}} \quad (2.1)$$

In which x denotes the measurement result and $X_{normalized}$ the normalized measurement result.

For the FCM data two outliers were identified in the undiluted rainwater series and removed from the dataset. The concentration of these two outliers was below the background concentration of 0.4 - 1.8 $\times 10^5$ total cells in the measured drinking water.

3. Results

3.1. Artificial contaminations

The results showed that of all parameters tested the HPC22 detected the lowest percent contaminant concentration (vol/vol or weight/vol) in

Table 3
Lowest contaminant concentration (in percentages) that could be detected with HPC22 and the alternative methods. The actual values corresponding to each percentage are given in the supplementary data (Table S2).

Water source	HPC22	$ATP_{total-lab}$	$ATP_{cell-mob}$	FCM_{intact}	FCM_{total}	FCM_{LNA}	FCM_{HNA}	ALP-1	ALP-2	16S rRNA
Wastewater										
TP1	0.00001 %	0.001 %	0.001 %	–	–	–	–	–	0.001 %	–
TP2	0.00009 %	0.0042 %	0.0042 %	0.029 %	0.029 %	0.2 %	0.0042 %	0.0042 %	0.00009 %	0.0006 %
Surface water										
TP1	1.2 %	3.7 %	3.7 %	3.7 %	3.7 %	3.7 %	1.2 %	–	3.7 %	–
TP2	0.032 %	0.032 %	0.032 %	0.16 %	0.16 %	0.16 %	0.0064 %	–	0.8 %	–
Rainwater										
TP1	0.78 %	1.56 %	1.56 %	ND	ND	ND	ND	ND	ND	ND
TP2	0.78 %	3.1 %	6.3 %	ND	ND	ND	ND	ND	ND	ND
Groundwater										
TP1	≤0.39 %	0.39 %	0.39 %	≤0.39 %	≤0.39 %	≤0.39 %	≤0.39 %	0.78 %	≤0.39 %	0.39 %
TP2	≤0.023 %	0.069 %	0.21 %	0.21 %	0.21 %	0.62 %	0.069 %	0.62 %	0.069 %	0.21 %
Clay soil										
TP1	0.0012 %	0.0049 %	0.0012 %	ND	ND	ND	ND	ND	0.0003 %	0.039 %
TP2	0.011 %	0.0037 %	0.011 %	ND	ND	ND	ND	0.0012 %	≤0.00005 %	0.0037 %
Sediment										
TP1	0.0001 %	0.0032 %	0.0006 %	0.016 %	0.016 %	0.016 %	0.0006 %	0.016 %	0.0006 %	0.016 %
TP2	0.0006 %	0.0006 %	0.0006 %	0.039 %	0.039 %	0.039 %	0.0098 %	0.0024 %	≤0.0002 %	0.0024 %

ND: No decrease detected.
-: No measurement.

eight out of twelve artificial contamination dilution series (Table 3, Table S2). The lowest percent contamination concentration measured with HPC22 depended on the type of contamination and ranged from 0.00001 % (wastewater) to 1.2 % (surface water). The ALP-2 parameter was the most sensitive of the alternative parameters tested in eight of the twelve dilution series (Table 3, Table S2). Another alternative parameter (once FCM_{intact}, FCM_{total} and FCM_{LNA}; twice $ATP_{total-lab}$ and $ATP_{cell-mob}$; and three times FCM_{HNA}) was as sensitive as ALP-2 in four of these dilution series. The lowest percent contamination concentration detected with ALP-2 ranged from 0.00009 % (wastewater) to 3.7 % (surface water). Moreover, ALP-2 was unable to detect any rainwater contamination. Two ATP parameters detected the lowest contaminant concentration in four ($ATP_{total-lab}$) or three ($ATP_{cell-mob}$) of the twelve dilution series of the alternative parameters tested. In two out of these three or four dilution series another alternative parameter (three times ALP-2, and twice FCM_{HNA}) was as sensitive as ATP. In contrast to ALP-2, the two ATP parameters were able to detect all contaminants in the drinking water tested. The lowest percent contamination concentration measured by ATP ranged from 0.0006 % (sediment) to 6.3 % (rain). One or more of the FCM parameters detected the lowest percent concentration in five of the twelve dilution series, but in three of these five dilution series another alternative parameter was as sensitive as the FCM parameter(s). In four of these five dilution series FCM_{HNA} measured the lowest contaminant concentration of the FCM parameters analysed and in the fifth dilution series FCM_{HNA} measured an equal contaminant concentration. The lowest measured percent contaminant concentration with the FCM parameters ranged from 0.0006 % (sediment) to 3.7 % (surface water). Moreover, FCM parameters were unable to detect rain and soil as contaminations in the drinking water tested. The percentage contaminant concentration with 16S rRNA gene copies ranged from 0.0006 % (wastewater) to 3.7 % (surface water). 16S rRNA gene copies could not be detected with a rain contamination. The lowest percent concentrations measured with the ALP-1 method ranged from 0.0012 % (clay soil) to 0.78 % (groundwater). The ALP-1 method could also not detect rain as contamination, nor one of the two dilution series with soil.

Table 4
Correlations calculated for HPC22 and the alternative methods for the contamination experiments ($p < 0.001$).

Method	All data		
	Pearson R^2	Spearman R^2	Data points (N)
$ATP_{total-lab}$	0.73	0.72	214
$ATP_{cell-lab}$	0.68	0.61	214
$ATP_{cell-mob}$	0.76	0.73	214
FCM_{intact}	0.35	0.24	194
FCM_{total}	0.44	0.27	194
FCM_{LNA}	0.28	0.24	188
FCM_{HNA}	0.52	0.48	188
ALP-1	0.38	0.36	112
ALP-2	0.71	0.65	199
16S rRNA	0.68	0.61	157

All parameters determined with an alternative rapid method showed a significant ($p < 0.05$) correlation with HPC22 (Table 4 and Fig. 1). The results from the ATP methods, the ALP-2 method and 16S rRNA gene copy numbers showed the strongest correlation with the HPC22 results ($R^2 = 0.61$ - 0.76). In contrast, the results from ALP-1 and the various FCM parameters showed weaker correlations with HPC22 results ($R^2 = 0.24 – 0.44$).

The calculated mean difference and the LoA between the alternative parameters and HPC22, using Bland-Altman analysis, are shown in Fig. 2 and Table S1. The calculated mean difference was quite close to zero indicating that on average the values of the alternative parameters corresponded well with those from HPC22. There is, however, some difference in the LoA between the various comparisons. The $ATP_{total-lab}$, $ATP_{cell-mob}$, and ALP-2 data had the smallest LoA with HPC22 compared to the other methods (Table S1). The largest LoA was found with the ALP-1 and 16S-rRNA gene copy data. At low mean HPC22 values, the difference in values between FCM_{HNA} and ALP-2 and HPC22 is positive (i.e. FCM_{HNA} and ALP-2 had higher normalized values than HPC22 when measuring samples with a low contaminant concentration). In contrast, at higher mean HPC22 values the difference in values between the two alternative parameters and HPC22 is negative (i.e. FCM_{HNA} and ALP-2 had lower normalized values than HPC22 when measuring samples with a high contaminant concentration). This effect is smaller for ALP-1 and both ATP parameters.

3.2. Field sampling before and after maintenance work in the DWDS

Different trends for HPC22 and the alternative parameters were observed for the different DWDSs (Figure S1). The HPC22 values of the reference sample in DWDS3 were higher compared to the samples taken after maintenance. DWDS3 is located in Belgium, and is the only one fed with chlorinated drinking water. The HPC22 in DWDS3 decreased from 24 ± 1.4 to 9 ± 1.4 CFU/mL three hours after flushing and fluctuated between 9.5 ± 0.7 and 62 ± 0 CFU/ml thereafter. The alternative parameters decreased up to two hours after flushing and subsequently

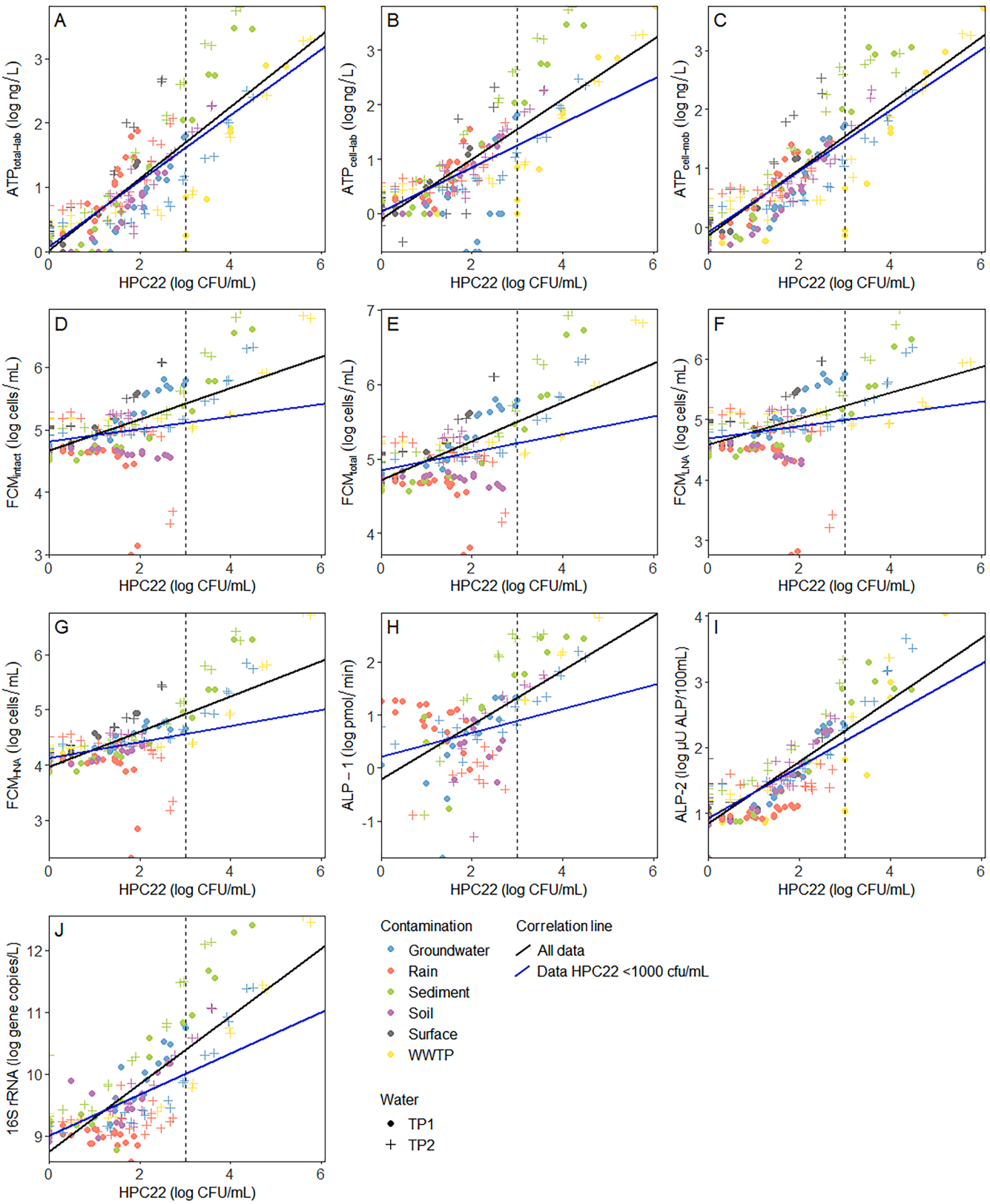

Fig. 1. Comparison of HPC22 versus the alternative methods for the contamination experiments. The black dotted line at 3 log CFU/mL for HPC22 indicates the Dutch guideline value of 1000 CFU/mL. The black line indicates the correlation line using all samples and the blue line indicates the correlation line using only the samples with a HPC22-value of <1000 CFU/mL.

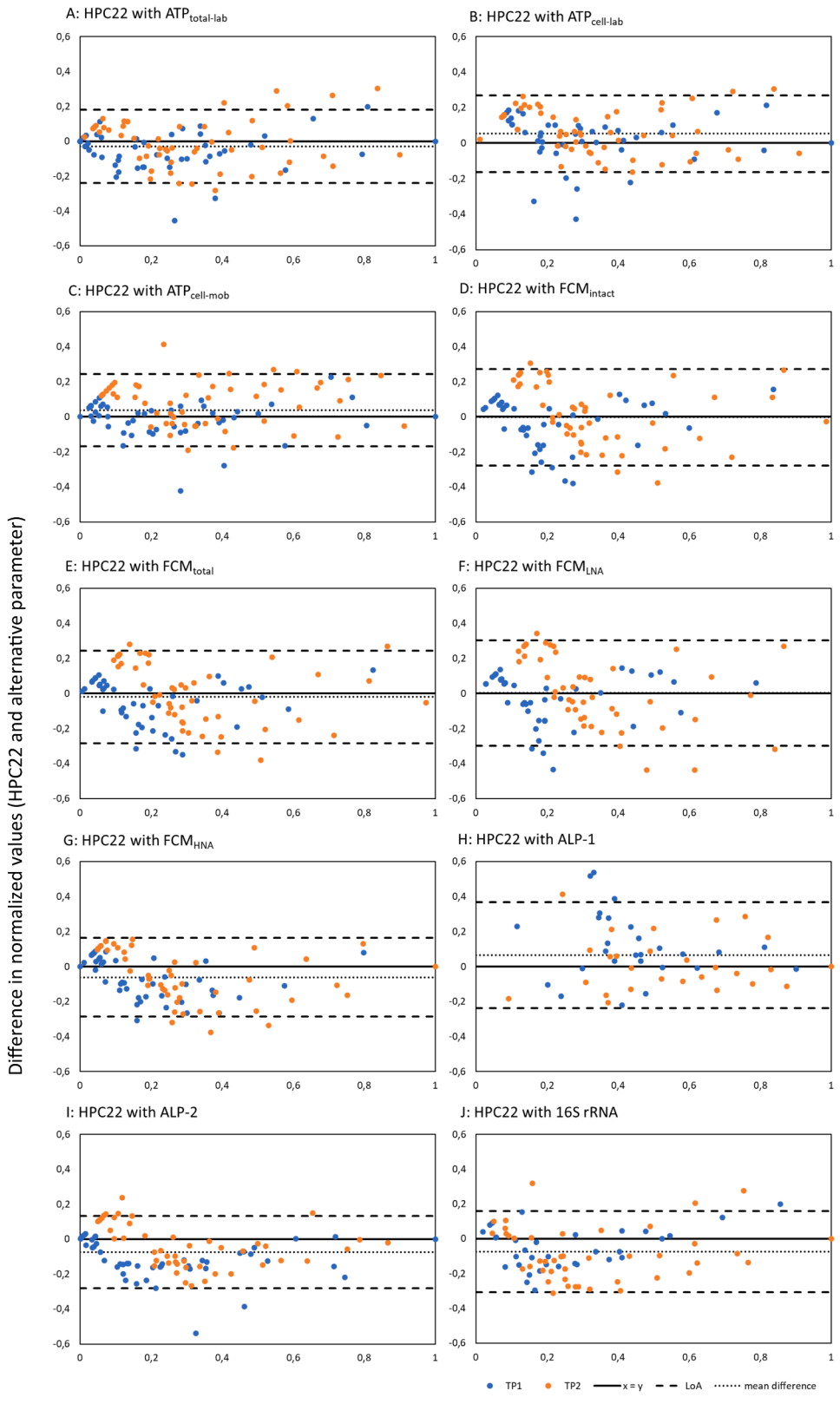

Fig. 2. Relative trueness, the degree of correspondence between the results obtained by the reference method (HPC22) and with the alternative methods, visualized with Bland-Altman plots. Dotted line: mean difference between the two parameters. Intermittent lines: lower and upper limit of agreement (LoA). A higher variation in the difference between two parameters results in a larger LoA. The calculated mean difference was quite close to zero indicating that on average the values of the alternative parameters corresponded well with those from HPC22.

fluctuated during the rest of the monitoring period (Figure S1).

No decrease in HPC22, cell counts, ATP, 16S rRNA gene copies, or ALP activity was measured after maintenance in DWDS4, DWDS5 and DWDS8 (Figure S1).

HPC22 decreased from 180 ± 28.3 to 9.5 ± 6.4 CFU/mL during the first two hours after flushing in DWDS6 and subsequently remained relatively stable up to 23 h after flushing (Figure S1). Thereafter, HPC22 started to increase to $2.3 \pm 1.2 \times 10^4$CFU/mL after 49 h. The alternative parameters showed the same trend as HPC22 in DWDS6 during the first 27 h after flushing, except for ALP-1 which showed values under the detection limit. A strong increase after 27 h after flushing was observed for the ATP concentration and 16S rRNA gene copies, which was similar to the increase in HPC22 after 23 h. $FCM_{intact,}$ $FCM_{total,}$ $FCM_{LNA,}$ FCM_{HNA} and ALP-2 only slightly increased after 27 h.

A decrease in HPC22 was detected in DWDS7 up to 2 h after flushing to 92 ± 4.2 CFU/mL (Figure S1). Thereafter, HPC22 increased to 1700 ± 0 CFU/mL at 48 h after flushing. Some of the alternative parameters ($ATP_{total\text{-}lab}$, FCM_{intact} and 16S rRNA gene copies) also showed a decrease during the first two hours after flushing, but these values remained relatively stable thereafter, which is in contrast with the HPC22 data. DWDS7 was the only DWDS where the values for HPC22, FCM_{total}, $ATP_{total\text{-}lab}$ and ALP-2 showed much higher values than in the samples taken after flushing.

The correlation data between HPC22 and the alternative parameters are shown in Table 5 and Fig. 3. HPC22 did not significantly ($p > 0.05$) correlate with FCM_{total}, except for DWDS6 and DWDS7. In addition, HPC22 only correlated significantly ($p < 0.05$) with ALP-1 for all locations combined, but not for the individual locations. Moreover, the ATP and 16S rRNA gene parameters showed the strongest correlation with HPC22 using all datapoints, although the correlation strength was relatively low ($R^{2:}$ 0.31 – 0.47). For the location specific correlation coefficients, ATP parameters, 16S rRNA gene copies and ALP-2 showed the strongest correlation with HPC22 (R^2: 0.23 – 0.91). The different FCM parameters and ALP-1 showed very weak correlations with HPC22 when all data points (R^2: 0.05 – 0.17) or location specific data points (R^2: 0.25 – 0.37) were used.

4. Discussion

4.1. Evaluation of the different microbiological methods using artificial contaminations

The overall objective of our study was to identify alternative parameter(s) that can be determined relatively fast and that can replace HPC22 as a monitoring parameter for the general microbial water quality after maintenance and/or repairs in the DWDS. In the first step of our study, we determined HPC22 and alternative parameters on artificial contaminations made in the laboratory. The suitability of the alternative parameters were scored based on three criteria: (i) sensitivity compared with HPC22, (ii) correlation with HPC22, (iii) ability to detect different contamination sources. For all contamination sources HPC22 was the most sensitive parameter and able to detect the lowest contaminant concentration, followed by ATP and ALP-2. In addition, ATP and ALP-2 showed the strongest correlation with HPC22. $ATP_{total\text{-}lab}$, $ATP_{cell\text{-}mob}$, and ALP-2 also had the smallest LoA in the Bland-Altman plots, indicating less variation in the difference between the alternative method and HPC22 results. Whereas ATP was able to detect a contamination with all different contamination sources, ALP-2 did not detect a contamination with rainwater. The FCM-parameters, ALP-2 and 16S rRNA gene copies were less sensitive than ATP and ALP-2 and the correlation with HPC22 was lower than with ATP or ALP-2. The lower sensitivity of FCM and 16S rRNA gene copies is probably caused by the fact that the background level of cells in drinking water is too high to detect bacteria from other water types that might ingress in the distribution system during maintenance. Furthermore, the FCM parameters could not detect clay soil and rainwater contamination, ALP-1 did not

Table 5
Correlation coefficients for the different locations of field samples (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, – = $p > 0.05$) with the number of data points (N).

DWDS		Time after flushing (hours)	Pearson R^2										Spearman R^2									
			$ATP_{total\text{-}lab}$	$ATP_{cell\text{-}lab}$	$ATP_{cell\text{-}mob}$	FCM_{intact}	FCM_{total}	FCM_{LNA}	FCM_{HNA}	ALP-1	ALP-2	16S rRNA	$ATP_{total\text{-}lab}$	$ATP_{cell\text{-}lab}$	$ATP_{cell\text{-}mob}$	FCM_{intact}	FCM_{total}	FCM_{LNA}	FCM_{HNA}	ALP-1	ALP-2	16S rRNA
3–8	R^2		0.39***	0.46***	0.43***	0.08**	–	0.1**	0.05*	–	0.04*	0.47***	0.34***	0.4***	0.31***	0.07**	–	0.09**	0.06*	0.17**	0.04*	0.44***
	N		100	100	102	102	102	102	102	48	102	84	100	100	102	102	102	102	102	48	102	84
3	R^2	0, 1, 2, 3, 22, 26, 29, 49	0.4*	0.4*	0.28*	–	–	–	–	–	–	0.38*	–	–	–	–	–	–	–	–	–	-
	N		14	14	16	16	16	16	16	0	16	16	14	14	16	16	16	16	16	0	16	16
4	R^2	0, 9, 11, 13, 16, 34, 38	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	-
	N		16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
5	R^2	0, 1, 2, 3, 19, 23, 27, 49	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	-
	N		14	14	14	14	14	14	14	13	14	14	14	14	14	14	14	14	14	13	14	14
6	R^2	0, 1, 2, 3, 5, 19, 23, 27, 49	0.91***	0.79***	0.9***	0.28*	0.37**	–	0.31*	–	0.36**	0.78***	0.76***	0.25*	0.4**	–	–	–	–	–	–	-
	N		18	18	18	18	18	18	18	0	18	18	18	18	18	18	18	18	18	0	18	18
7	R^2	0, 1, 2, 3, 5, 19, 23, 27, 48	0.57***	0.53***	0.55***	0.3*	0.31*	–	0.3*	–	0.7***	–	0.44**	0.4**	0.23*	0.24*	–	–	–	–	0.49**	-
	N		18	18	18	18	18	18	18	0	18	0	18	18	18	18	18	18	18	0	18	0
8	R^2	0, 1, 2, 3, 5, 22, 26, 30, 48	0.28*	0.69***	–	–	–	0.25*	–	–	0.54***	–	0.38**	0.82***	–	–	–	–	–	–	0.47***	-
	N		14	14	14	14	14	14	14	13	14	14	14	14	14	14	14	14	14	13	14	14

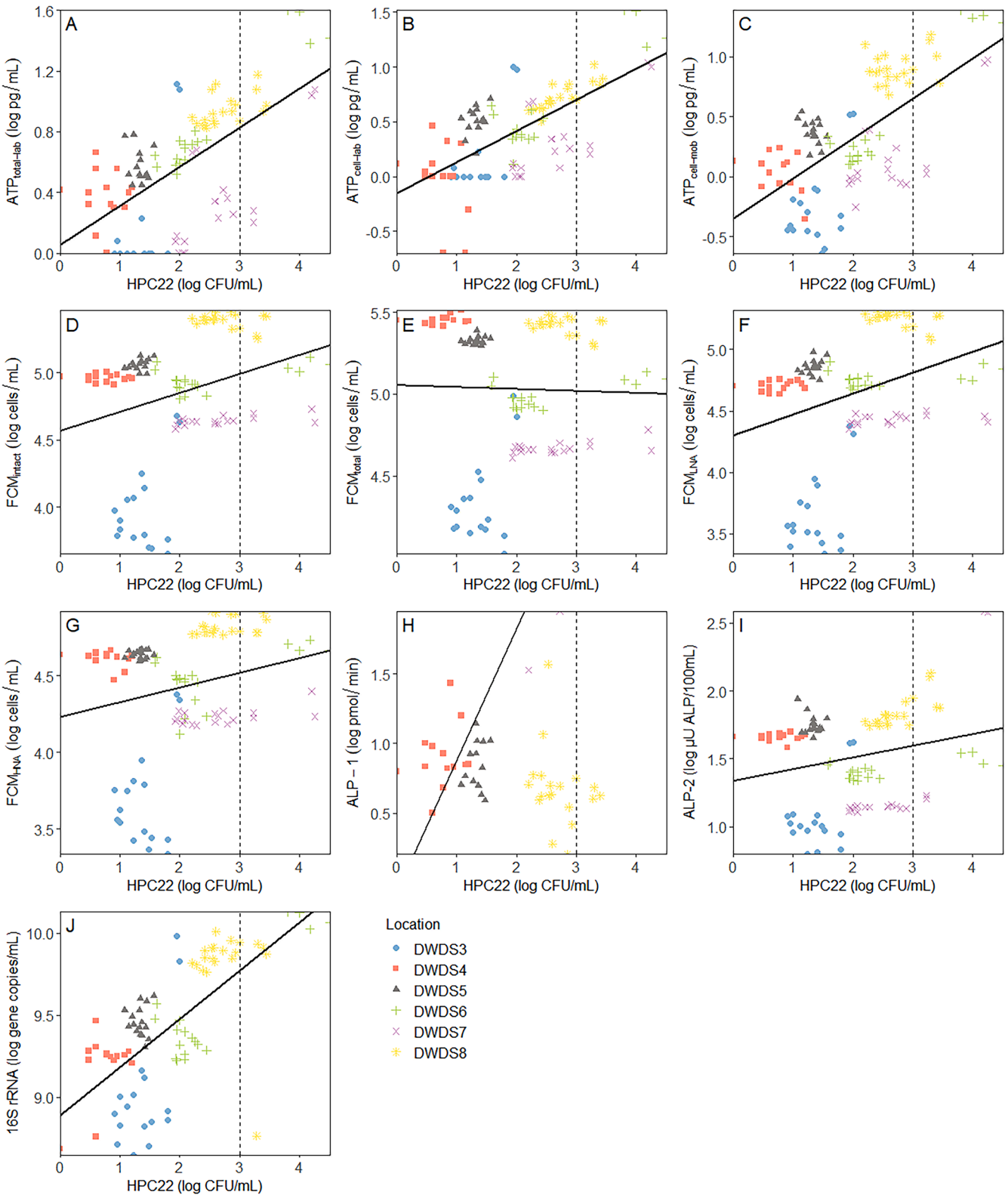

Fig. 3. Comparison of HPC22 versus the alternative methods for the field samples. The dotted vertical line at 3 log CFU/mL for HPC22 indicates the Dutch guideline value of 1000 CFU/mL. The black line indicates the correlation line.

detect rainwater and clay soil contamination and 16S rRNA gene copies could not detect rainwater contamination. Based on the sensitivity, correlation with HPC22 and the ability to detect all contaminant sources criteria, we conclude that the ATP-method, especially ATP$_{total-lab}$ and ATP$_{cell-mob}$, is the best performing alternative method to HPC22 for detecting contamination after repairs in the DWDS.

Vang et al. (2014) also investigated ATP as possible alternative for HPC22 to detect drinking water contamination and showed that HPC22 was 100 to 1000 times more sensitive than ATP in detecting a contamination with wastewater or surface water in drinking water. We

observed comparable results, as the HPC22 method detected a 100 times higher wastewater dilution than the ATP assay. Moreover, we investigated additional contamination sources (rainwater, groundwater, clay soil and sediment) than those studied by Vang et al. (2014) and showed that for these contamination sources HPC22 was the most sensitive method as well. However, the sensitivity between HPC22 and ATP differed between contamination sources and varied between a 30 to 100 times higher dilution detected with HPC22 than ATP. The lower sensitivity of ATP compared to HPC22 is probably caused by higher background levels of ATP in drinking water of the Netherlands, due to which a bacterial increase is more difficult to detect. It is, therefore, important to include multiple possible contamination sources when deciding on a possible alternative method for HPC22 to determine the microbial water quality after repairs in the DWDS. A more recent study also tested different microbiological parameters to determine contamination of drinking water with groundwater, rainwater or wastewater effluent (Favere et al., 2021). The alternative microbiological parameters determined in that study were, however, only compared to plate counts of faecal indicator bacteria and not to the HPC22 parameter. Thus, a direct comparison between their and our study is not possible. Still, it was shown in the study of Favere et al. (2021) that ATP and ALP-2 were the most sensitive methods in detecting a contamination with rainwater or groundwater, followed by FCM and microscopy, which is in line with the results obtained from our study.

4.2. Evaluation of the different microbiological methods using field samples

In the second part of our study, HPC22 and alternative parameters were determined in samples from different full-scale DWDS after maintenance work. We observed that HPC22 exceeded the Dutch guidance value of 1000 CFU/mL in 1.4 % of these full-scale samples after work activities in the DWDS. Surprisingly, however, a deterioration of the water quality was not detected in these DWDSs with the alternative parameters, except for DWDS6. HPC22 seems a more sensitive parameter to detect aberrated drinking water quality in full-scale DWDS after repair or maintenance than the alternative parameters determined in our study. This agrees with the results of the artificial contaminations, which demonstrated that HPC22 was a more sensitive parameter than the alternative methods. However, the low percentage of exceedances in the field samples asks for further field studies, before a definite conclusion can be drawn whether HPC22 is also a more sensitive parameter than the alternative methods in field cases.

In general, the average ATP concentrations and total cell counts observed in our study were in the range of previously reported values for these two parameters in unchlorinated and chlorinated drinking water sampled from full-scale DWDS without repairs (Delahaye et al., 2003; Højris et al., 2016; Liu et al., 2013a; Prest et al., 2016a; van der Wielen and van der Kooij, 2010; Vang et al., 2014). Depending on the study, the correlation between ATP and HPC varied from strong (Deininger and Lee, 2001) to moderate (van der Kooij, 1992) to weak (Berney et al., 2008). The number of 16S rRNA gene copies obtained in our study ($1.2 \times 10^9 - 4.1 \times 10^9$ gene copies/L) was higher than previously reported values ($1 \times 10^6 - 1 \times 10^7$ gene copies/L) (Boers et al., 2018). The high HPC22 values observed during the whole sampling period at DWDS8 is more often seen at DWDS locations that are under construction and not in use (personal communication Marco Dignum, Waternet). In the Netherlands, there is a preference to use PVC-U pipes in the DWDS due to its lower biomass production potential compared to other plastic materials like PE (Hambsch et al., 2014; Learbuch et al., 2019, 2021). These new PVC-U pipes often contain a coating layer or other substances that may lead to enhanced regrowth. After a few weeks these substances have, however, been biodegraded and from that moment the PVC-U has a much lower regrowth potential (van der Kooij and Veenendaal, 1993).

A previous study on drinking water from DWDSs in the Netherlands demonstrated that HPC22 increased in the drinking water due to regrowth in the distribution system environment, but that ATP and FCM methods were not able to detect this regrowth in the drinking water sampled from the DWDS, because the ATP concentration and cell counts in drinking water were not enhanced compared to the baseline values (van der Wielen et al., 2016). This observation is similar to our observation that HPC22 were enhanced but that the alternative parameters remained stable. Others showed a weak or no correlation between FCM and HPC22 (Bartram et al., 2003; Burtscher et al., 2009; Hammes et al., 2008; Hoefel et al., 2003). In addition, (Hoefel et al., 2003) determined that HPC results were 2 – 4 log orders of magnitude below that reported by the FCM assays for raw waters. They stated that the absence of a linear correlation between HPC and cell counts may be due to different waters differ in the presence of bacteria capable to be cultured with the HPC method. Based on these observations that HPC22 is capable to regrow in drinking water systems, we hypothesize that the higher HPC22 values 23 to 49 h after repair or maintenance in the DWDS is not caused by contamination of drinking water with another source, but by regrowth of HPC22 bacteria in the DWDS. This hypothesis is supported by the observation that HPC22 values remain high during 23 to 49 h after the repair or maintenance, because a contamination would likely lead to high HPC22 directly after flushing, but declining HPC22 values over time as the contamination is diluted with drinking water that has a lower HPC22 (van der Wielen et al. 2016).

Another unexpected observation was that at DWDS6, an increase in microbial abundance after 27 h was observed with HPC22, FCM_{total}, 16S rRNA gene copies, ATP and ALP-2. We hypothesize that the increase in these parameters after 27 h was caused by a low flow and refreshment of the DWDS as these samples were taken at a dead end in the DWDS. This stagnant water can facilitate growth, detachment of microbial cells from the biofilm on the pipe wall or resuspension of loose deposits (including the microbial cells attached to these loose deposits) due to a sudden increase in flow caused by flushing (Besmer and Hammes, 2016; Liu et al., 2013b; Nescerecka et al., 2018).

4.3. Application value for drinking water utilities

Next to sensitivity, correlation with HPC22 and the ability to detect different contamination sources, other criteria are important for utilities as well before deciding to switch to an alternative parameters for HPC22 after repairs in the DWDS. A summary of the performance of HPC22 and the alternative methods based on additional criteria is given in Table 6. For the ATP, FCM and enzymatic activity parameters, the short time-to-result is an important beneficial value compared to HPC22 as drinking water utilities can act quicker in response to potential water quality issues after repair or maintenance in the DWDS. Besides the fact that the methods to determine these parameters are rapid, they are relatively easy in use and, therefore, have potential to be used as a routine monitoring tool for drinking water quality. The apparent discrepancy we observed between the laboratory-based and field studies stresses the need that, in order to identify a reliable alternative to detect possible contamination of drinking water with other sources, drinking water utilities and researchers should not solely rely on laboratory-based studies to draw conclusions on possible alternative parameters to measure the microbial drinking water quality. It remains important to investigate these parameters in both dilution series and field samples before reliable conclusions can be drawn.

Based on our results, we advise drinking water utilities to incorporate ATP or ALP-2 measurements for a specific period, next to HPC22, to determine possible aberration of the drinking water quality after maintenance or repair in the DWDS. Subsequent analysis of the ATP and ALP-2 data and comparison with the HPC22 data can be performed to determine whether HPC22 can be reliably replaced by ATP or ALP-2 in the future and to set signal values that indicate aberrated water quality. Finally, it remains important to stress that next to HPC22, ATP or ALP-2, methods to detect possible faecal contamination using *E. coli* and intestinal enterococci are still required to ensure that the drinking water is

Table 6
Alternative methods that were tested and their characteristics, based on experience obtained during this study (sample preparation time, analysis time, maintenance, data analysis) and on the manufacturers information (technical lower detection limit).

Method	Type	Technical lower detection limit	Sample preparation time (min)	Analysis time (min)	Maintenance	Data analysis
HPC22	Culture	1 CFU/mL	30	5760 (3 days)	Basic	Basic
$ATP_{total\text{-}lab}$	ATP	1 ng/L	2	2	Basic	Basic
$ATP_{cell\text{-}lab}$	ATP	1 ng/L	2	2	Basic	Basic
$ATP_{cell\text{-}mob}$	ATP	1 ng/L	<10	2	Basic	Basic
FCM	Flow cytometry	1000 cells/mL	15	2	Medium	Medium
ALP-1	Enzymatic	0.36 μU/100 ml	5	25	Medium	Medium
ALP-2	Enzymatic	0.1–20 pmol/min	5	30	Medium	Medium
16S rRNA	Molecular	100 LOD	60	96 (4 h)	Medium	Medium

safe and fulfills the legal requirements after these repairs in the DWDS.

5. Conclusions

This study demonstrated that $ATP_{total\text{-}lab}$ and $ATP_{cell\text{-}mob}$, followed by the ALP-2 method (which did not detect a rainwater contamination) are promising candidates to potentially replace HPC22, as they provide fast and reliable results for detecting microbiological contaminations in drinking water. Although these methods are not as sensitive as HPC22, they show potential to be sensitive enough to detect contaminations that can be expected after maintenance work in the DWDS. More specifically, the conclusions of this study were:

- HPC22 was the most sensitive method and was able to detect the lowest contaminant concentration.
- Of the alternative methods, $ATP_{total\text{-}lab}$, $ATP_{cell\text{-}mob}$ and ALP-2 detected the lowest contaminant concentration, followed by the FCM parameters and finally 16S rRNA gene copies and ALP-1.
- HPC22, $ATP_{total\text{-}lab}$ and $ATP_{cell\text{-}mob}$ detected all contaminant sources in drinking water, whereas the other alternative methods could not detect rainwater and/or soil as contamination source.
- The strongest significant correlations were obtained between HPC22 and both ATP methods and ALP-2.
- Samples taken after repairs or a calamity in the DWDS showed in general limited variation in the HPC22 count in time, indicating that contamination with a source that has higher HPC22 values did not occur. The lack of variation in HPC22 also made it difficult to make an extensive comparison between HPC22 and the alternative methods.
- $ATP_{total\text{-}lab}$ and $ATP_{cell\text{-}mob}$ performed best compared to HPC22 and should, together with next best-performing method ALP-2, be included in additional field studies (next to HPC22) to further test and compare these methods. The results from such field studies can then be used to decide on which method can reliably be implemented as an alternative for HPC22 after maintenance work in the DWDS.

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CRediT authorship contribution statement

Marcelle J. van der Waals: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Nikki van Bel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization. **Frits van Charante:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Jeroen van Rijn:** Methodology, Investigation. **Anita van der Veen:** Methodology, Investigation. **Paul W.J.J. van der Wielen:** Writing – original draft, Writing – review & editing, Supervision, Validation, Conceptualization, Methodology.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122264.

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