Journal of Environmental Management 286 (2021) 112151

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: http://www.elsevier.com/locate/jenvman

Research article

Bacterial dynamics in drinking water distribution systems and flow cytometry monitoring scheme optimization

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ARTICLE INFO

Keywords:
Drinking water distribution network
Biological stability
Microbial water quality
Microbial dynamics
Monitoring scheme optimization

ABSTRACT

Water biostability is desired within drinking water distribution systems (DWDSs) to limit microbiologically-related operational, aesthetic and, eventually, health-related issues. However, variations in microbiological quality can take place both spatially along DWDS pipelines and temporally at single locations due to biofilm detachment, water quality seasonality and other processes. In this study, long- and short-term trends of bacterial concentration and community structure were investigated in a secondary branch of an unchlorinated DWDS for several months using high-frequency flow cytometry (FCM) and traditional laboratory monitoring campaigns. Long-term trends of bacterial concentrations and community structures were likely caused by changes in the water physical-chemical quality (i.e. pH and conductivity). Short-term daily pattern, instead, resulted in significant variations between the bacterial concentrations and community structures at different hours, likely due to biofilm detachment and loose deposits resuspension related to changes in the local water flow. These patterns, however, showed broad variations and did not persist during the entire monitoring campaign presumably due to the stochasticity of local instantaneous demand and seasonal changes in water consumption. During periods without sensible long-term trends, the sampling hours explain a comparable or larger fraction of the bacterial community diversity compared to dates. The variations observed with FCM were poorly or not detected by traditional laboratory analyses, as the correlation between the two were rather weak, highlighting the limited information provided by traditional approaches. On the other hand, FCM data correlated with water pH and conductivity, underlining the relation between physical-chemical and microbiological water quality. Such results suggest that the advanced control of the physical-chemical water quality could minimize the microbiological water quality variations. Moreover, monitoring campaign planning should take into account the sampling time to reduce the noise caused by daily fluctuations and/or assess the overall quality variations. Finally, as monitoring costs are one of the barriers which prevent a more widespread use of FCM, a monitoring scheme optimization strategy was developed. Such strategy employs the data from an initial high-frequency sampling period to select the sampling hours which maximize the observed variations of bacterial concentration and community composition.

1. Introduction

The availability of water of high quality at every household is necessary for human wellbeing. With respect to drinking water microbiological quality, such goal is often declined as achieving "biostability", condition in which spatial and temporal microbiological water quality variations are negligible during distribution (Prest et al., 2016b), to control the growth of pathogens, operational and aesthetic issues such as biofouling and discoloration (Li et al., 2015; Vreeburg and Boxall, 2007).

Spatial instability can be caused by several phenomena including the decay of disinfectant residuals, bacterial growth on available substrates and biofilm detachment during distribution, which can result in both an increase in bacterial concentrations (Boe-Hansen et al., 2002; Chan et al., 2019; Gillespie et al., 2014), but also in the variation of microbial populations composition (El-Chakhtoura et al., 2015; Farhat et al., 2020). On the other hand, temporal instability can arise due to seasonality of the source water and within the drinking water distribution system (DWDS), due to changes in the operating conditions, such as water flow, both in drinking water treatment plants (DWTPs) and in

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https://doi.org/10.1016/j.jenvman.2021.112151
Received 24 November 2020; Received in revised form 4 February 2021; Accepted 5 February 2021
Available online 19 February 2021

DWDSs (Bautista-de los Santos et al., 2016; Besmer et al., 2016; Besmer and Hammes, 2016; Pinto et al., 2014). Additionally, the combination of the two processes can produce different effects in different regions of DWDSs (Potgieter et al., 2018).

Full-size monitoring campaigns are needed to verify the water biostability within full-scale DWDSs, as DWDS conditions cannot be reproduced in laboratory (Prest et al., 2016a), and to ensure the protection against possible contamination events due to DWTPs failures or eventual re-contamination events occurring throughout the DWDS. Several methods have been used to monitor the water microbiological quality, either with respect to (i) bacteria concentration as heterotrophic plate counts (HPC), flow cytometry (FCM) and optical counts (Allen et al., 2004; Hammes et al., 2012; Højris et al., 2016), (ii) bacterial community composition based on genetic analyses (Bautista-de los Santos et al., 2016; Burtscher et al., 2009) or (iii) bacterial activity as using adenosine triphosphate (ATP) and available organic carbon (AOC) assays (Van Der Kooij, 2000; Vital et al., 2012).

HPC is used by most water utilities and it is considered the standard by many national and international entities (EC, 1998; Robertson and Brooks, 2003; US EPA, 2009) regardless of its intrinsic drawbacks (Staley and Konopka, 1985; van der Kooij and van der Wielen, 2014). The efficiency of this monitoring method is further reduced by the fact that the sampling frequency adopted by water utilities is often not frequent enough to detect short-term temporal variations (Besmer et al., 2017). Despite the lack of legislative indications outside Switzerland, FCM is recently being adopted by water utilities (Safford and Bischel, 2019) due to its several advantages compared to HPC which include: single-cell information, fast output (<15 min), reproducible results (<10%) (Prest et al., 2013) and the possibility for automation and online use (Besmer et al., 2014). These advantages make the use of FCM feasible for the detection of short-term temporal variations. In addition, FCM can provide information regarding bacterial viability and activity (Safford and Bischel, 2019) and information which correlates with the diversity at species level (Props et al., 2016), useful to highlight bacterial community changes (Farhat et al., 2020; Favere et al., 2020; Props et al., 2018b). Although several studies used FCM to investigate seasonal or other long-term microbiological variations in DWDS (e.g. Nescerecka et al., 2018; Schleich et al., 2019), short-term variations have been studied mostly for short periods (e.g. Farhart et al., 2020; Favere et al., 2020) or with a focus mostly on bacterial concentrations, rather than their community structure (e.g. Prest et al., 2016c). Unfortunately, the further adoption of this technique by water utilities will be likely limited by financial and legislative constraints (Besmer et al., 2017). This is especially relevant in the case of *in situ* FCM: even though real-time data acquisition was tested (Besmer and Hammes, 2016), frequent sampling is likely not feasible for some water utilities, especially considering long-term monitoring. Due to the costs related to long-term monitoring, an optimum between the sampling frequency and usefulness of the information retrieved should be found.

As the bacterial concentrations monitored through HPC and FCM are not necessarily related to hygienic issues (Allen et al., 2004), legislations are progressively moving from defining an upper concentration limit to stating that "no abnormal change" should be detected (Van Nevel et al., 2017b). Unfortunately, the identification of "not abnormal" or acceptable changes in microbiological water quality is not trivial, especially in complex systems as DWDS, due to the fact that several factors can be considered (e.g. relative concentration change, absolute concentration change, different bacterial composition) (Favere et al., 2020; Prest et al., 2016b; Van Nevel et al., 2017b) and the fact that the observed changes depend on the monitoring frequency, but also the locations.

This study aims at investigating the short- and long-term variations of microbiological water quality within DWDS with respect to both bacterial populations concentration and composition to deepen the understanding about DWDS microbial water quality dynamics. For this reason, high-frequency FCM and traditional laboratory monitoring campaigns were carried out for 5 months at a DWDS secondary branch. Furthermore, a monitoring scheme optimization strategy for the detection of microbiological water variations is proposed.

2. Materials and methods

2.1. Study site and sampling location

This study was conducted on a DWDS of an Italian city with around 27,000 inhabitants supplying on average about $2{\cdot}10^6$ m^3/y of water. The network, made predominantly of steel pipes, extends for 36.5 km and it comprises of a main conduit and a grid of secondary pipes, which deliver the water to the households. No reservoirs are present throughout the DWDS as DWTPs pumps are activated automatically based on water consumption. No other pumps are present throughout the DWDS. Furthermore, no pipes flushing was conducted on the network during the monitoring campaign. The drinking water is supplied by two DWTPs: while DWTP1 employs only a granular activated carbon (GAC) filter, DWTP2 treats groundwater through a static sand trap and a GAC filter and supplies on average the 67% of water to the city.

DWDS sampling was carried out thanks to a continuous bleed applied directly to a secondary branch of the network placed in a mostly residential area. Based on the analysis of previous samples (data not shown), chemical data suggest that the water at the sampling location originates almost completely from DWTP2.

2.2. Monitoring campaign

Microbiological water quality at the DWDS sampling location was monitored by both online flow cytometry and laboratory analyses between May and October 2019.

2.2.1. Online flow cytometry

Samples (volume: 260 μL of which 90 μL for analysis) were taken automatically from the bleed every 2 h, for a total of 1423 samples, and analyzed with a BactoSense® (Sigrist-Photometer AG, Switzerland) flow-cytometer equipped with a 488-nm solid-state laser, a side-scatter detector (SSC: 488/10) and two fluorescence detectors (FL1: 525/45, FL2: 715 LP). Before analysis, samples were mixed with SYBR Green I and incubated for 10 min at 37 °C.

2.2.2. Laboratory microbiological and chemical analyses

Microbiological quality was monitored through traditional laboratory analyses. Manual sampling was performed about 3 times per day, 3 days per week at the same time as flow cytometric analyses, for a total of 98 samples. Samples were collected in sterile plastic bottles, kept in the dark, refrigerated at 4 °C and plated within maximum 6 h from collection. All samples were analyzed for the detection of *Pseudomonas aeruginosa* and psychrophilic and mesophilic heterotrophic plate counts (HPC), while 28 samples were also analyzed for *Escherichia coli,* total coliforms and intestinal enterococci according to the Italian standard procedures (6020 B IRSA-CNR; 7050 IRSA-CNR, APAT-IRSA/CNR, 2003), similar to those reported in Standards Methods (APHA/AWA/-WEF, 2012). For the detection of *P. aeruginosa*, 250 mL of water were filtered on a 0.45 μm sterile filter (Sartorius AG, Germany) and incubated for 48 h at 37 °C in Pseudomonas Agar Base (VWR International, Belgium). HPC were, instead, analyzed with 2 replicates incubated in Plate Count Agar (VWR International, Belgium). Other than the standard incubating conditions equal respectively to 48 h at 37 °C and 72 h at 22 °C for mesophilic and psychrophilic HPC, the incubation was prolonged to 7 days for both types of HPCs. *E. coli,* total coliforms and intestinal enterococci were analyzed by filtering 100 mL of water on a 0.45 μm sterile filter and incubated respectively in Tryptone Bile X-Glucuronide Agar (VWR International, Belgium), m-LES Endo Agar (Biolife Italiana, Italy) and Slanetz Bartley Agar Base (VWR International, Belgium) for 48 h or, in the case of *E. coli,* 24 h. Results were reported as CFU (Colony Forming Units) per unit of filtered volume.

Additionally, 33 samples were analyzed also for pH (pH 538, WTW, Germany) and conductivity (Cond 3210, WTW, Germany) in accordance to the Italian standard procedures (2060 IRSA-CNR; 2030 IRSA-CNR, APAT-IRSA/CNR, 2003).

2.3. Data analysis

Data was processed and analyzed in R (v3.6.3) (R Core Team, 2020). Flow Cytometric Standard files were loaded using the *flowcore* package (v1.50.0) (Ellis et al., 2019) and cleaned from anomalous values regarding the dynamic range, flow rate stability and signal acquisition thanks to the *flowAI* package (v1.12.7) (Monaco et al., 2016). Successively, background noise due to debris was excluded from the analyses by drawing a polygonal gate on the FL1 and FL2 graph, after hyperbolic arcsine transformation, in order to recover the cells signal and calculate total cell concentration (TCC) [cells/µL]. Such gate was drawn based on 10 random samples uniformly spread throughout the entire monitoring period. The same samples were used to select the threshold on the FL1 signal to estimate the low nucleic acid (LNA) and high nucleic acid (HNA) cells concentrations [cells/µL] and calculate the percentage of HNA cells (HNA%) as the ratio of between HNA cells and TCC (Amalfitano et al., 2018; Prest et al., 2013). Advanced fingerprinting was carried out thanks to the *flowFDA* package (v0.99) (Clement and Thas, 2014) discretizing FL1, FL2 and SSC in 128 evenly distributed bins after the data was rescaled to a [0,1] range (Props et al, 2016, 2018b).

Following this step, the *Phenoflow* package (v1.1.1) (Props et al., 2018a) was used for additional analyses. The phenotypic diversity index (D2) [arbitrary units, a.u.] was calculated for each sample thanks to the function *diversity_rf* using the standard settings (Props et al., 2016). Furthermore, "phenotypic community types" were calculated as described by Props et al. (2018b). In short, samples were first reduced dimensionally through principal component analysis to retain 90% of their variability and then clustered, maximizing the silhouette index, through k-medoid clustering. To account for sample size differences, this workflow was repeated for 100 bootstrap samples, after which the most frequent result for each sample was selected. In addition, the advanced fingerprint was used for the analysis of the beta diversity, estimated through the Bray-Curtis dissimilarity using the *vegan* package (v2.5-5) (Oksanen et al., 2019). Prior to the estimation of beta diversity, resampling to the lowest cell count (n = 1609) was carried out to exclude possible interferences due to the different cell count in different samples (Favere et al., 2020).

The entire monitoring campaign was divided into 6 periods based on a visual assessment of the TCC trend and sampling interruptions (due to malfunctioning or external reasons), labeled from A to F (Figure S1; Table S1). Furthermore, sampling hours were grouped considering a typical water demand pattern of a comparable municipality (Candelieri, 2017) (Figure S2).

2.4. Monitoring scheme evaluation

Hypothetical alternative monitoring schemes were evaluated through the index $R_{beta \times \Delta TCC}$ which was calculated as the product between R_{beta} and $R_{\Delta TCC}$. With reference to the maximum amplitude of the daily variations which can be observed in each specific monitoring scheme, R_{beta} and $R_{\Delta TCC}$ represent the amplitude observed in the hypothetical alternative monitoring schemes compared to the ones witnessed in the full monitoring campaign for fingerprints and TCC respectively. R_{beta} was, then, calculated as the ratio between the observed maximum and the true maximum daily beta diversity, while $R_{\Delta TCC}$ as the ratio between the observed maximum and the true maximum TCC daily variation.

2.5. Statistical analyses

Statistical differences between groups were tested through the use of non-parametric Conover and Dunn tests with Bonferroni adjustment, implemented respectively in the packages *conover.test* (v1.1.5) (Dinno, 2017a) and *dunn.test* (v1.3.5) (Dinno, 2017b), depending on the homoscedasticity of the data, assessed thanks to Levene tests, as implemented in the *car* package (v3.0-3) (Fox and Weisberg, 2019).

Monotonic and linear correlations between variables were calculated respectively using the Spearman and Pearson correlation coefficients. The differences between the HPCs at different incubation times were carried out using a paired Wilcoxon test. To test the difference between the beta diversity among groups, the multivariate homogeneity of groups dispersion (PERMDISP) was assessed and tested thanks to the function *betadisper*, while the permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were conducted using the functions *adonis* and *anosim* considering 999 permutations. Furthermore, the monotonic correlation between the diversity data and other variables was tested through Mantel tests considering ranked data and euclidean dissimilarity of the tested variable. All functions used to analyze multivariate and diversity data can be found in the package *vegan* (Oksanen et al., 2019).

3. Results and discussion

3.1. Bacterial concentration dynamics

Five months of FCM sampling at 2-h intervals revealed the presence of both short- and long-term variations in the bacterial concentration of the water at the sampling point (Fig. 1). Such trends allowed for the distinction of three groups: (i) periods A and E, (ii) periods B, D and F and (iii) period C (Tables S2, S3). Such concentrations lie in the same range as other studies involving treated or untreated groundwater (Besmer et al., 2016; Nescerecka et al., 2018; Van Nevel et al., 2016). While periods A and E can be considered as the TCC "baseline" as TCC does not show any long-term trends, period B was affected by an increase in TCC which culminated during period C and carried over to period D. Period F, instead, even though presenting an increase of TCC with respect to period E and levels not statistically different from periods B and D, consisted of a 5-day event where the bacterial population gradually increased and then decreased (Figure S1).

Long-term seasonal variations in the microbial abundance, likely caused by changes in the chemical water quality and temperature, have been reported by others (Nescerecka et al., 2018; Prest et al., 2016c; Schleich et al., 2019). The long-term TCC variation reflected also on the median SSC (Figure S3) indicating an increase in cell biovolume, complexity and granularity (Hammes et al., 2010; Safford and Bischel, 2019). The increase in SSC could be caused by the presence of more

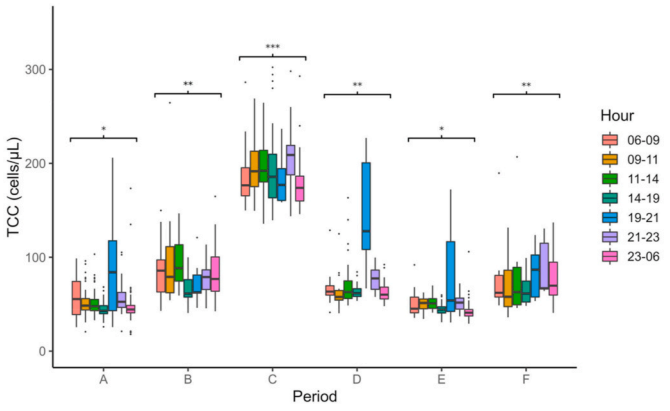

Fig. 1. TCC for each group of hours interval during the different periods. A different number of asterisks indicates groups with significantly different concentrations assessed through a Conover test with Bonferroni adjustment (Table S3).

nutrients, condition known to result in increased cell sizes (Chien et al., 2012). Noteworthy, the SSC associated to LNA cells remained always below the HNA one in the same sample, which, as observed by Bouvier et al. (2007), is not strictly necessary. Furthermore, during periods B and C, the SSC associated to LNA cells reached values higher than the one of HNA cells during baseline conditions indicating their high biovolume, complexity and granularity.

Focusing on the TCC differences across different groups of hours, it is clear the presence of important short-term variations in the water delivered by the DWDS. Differently from Besmer et al. (2014), but in accordance with Nescerecka et al. (2014) and Farhat et al. (2020), TCC did not show a gradual overnight increase, supporting the grouping of these hours. Such difference is likely due to the fact that Besmer and coworkers sampled from a building tap, not directly from the DWDS, which possibly affected the bacterial concentration observed. Periods A, D and E show a clear peak in TCC between the hours 19–21. Such group of hours was, also, characterized by significantly higher variability than the other times of the day (p-value < 0.001). This variability could not be attributed to different days of the week or the occurrence of weekends, but it is likely due to the diversity of flow conditions which affected biofilm detachment and loose deposits resuspension (Chan et al., 2019; Lehtola et al., 2006; Liu et al., 2014; Nescerecka et al., 2014). The same behavior was not observed during periods B, C and F where, instead, the group of hours did not show significant differences in their variability (p-values, respectively, equal to 0.17, 0.97 and 0.91) and in their TCC (p-values > 0.08, with the exception of 0.02 for the groups of hours 11–14 and 14–19 in period B). The different behavior could be caused by an alteration of the water consumption due to the occurrence of the holiday season coincident with periods B and C. Noteworthy, period F also coincides with the city patron holiday.

Noticeably, neither the TCC daily trend observed during this monitoring campaign or either the ones carried out by Nescerecka et al. (2014) and Farhat et al. (2020) follow the typical water demand pattern (e.g. Candelieri, 2017). While some events might have occurred within samples, it is unlikely that a consumption peak was missed due to the high sampling frequency. A more likely explanation is that TCC variations are caused by local variations of the water flow which are affected by the instantaneous demand of small groups of consumers leading to higher unpredictability in such patterns (Trifunović, 2006).

Compared to TCC, HPC allowed to detect <1% of the bacteria present in the water (Table S2), as reported by previous studies (van der Kooij, 2003; Van Nevel et al., 2017b). Moreover, HPC showed higher variability within each periods (coefficients of variation: HPC 0.4–2.53, TCC 0.24–0.43) which allowed to discriminate only period C from periods A and E (Table S3) indicating the lower sensibility of HPC with respect to FCM, similarly to what found by Prest et al. (2016c) and Jie et al. (2017). Furthermore, the concordance ratio of the sign of the variations of HPC and TCC in consecutive samples was in the range 0.46–0.6, suggesting that even a resource-intensive sampling campaign based on HPC would not be able to properly characterize microbial water quality dynamics, in accordance to what reported by Besmer et al. (2017). Furthermore, congruently to the low concordance ratios found and similarly to other studies (Nescerecka et al., 2014; Siebel et al., 2008), low correlations between TCC and mesophilic and psychrophilic HPC with standard incubation were found (Pearson's r equal to 0.25 in both cases). However, in case of extended incubation such coefficients increased respectively to 0.48 and 0.47 due to the fact that a larger number of colonies had time to develop (p-value < 0.001). Separating the data for each period made possible to observe how such relationship varied in different periods (Figure S4). Such behavior is likely due to the difference of the bacterial communities present in each period. For this reason, even though an extended incubation was shown to generically increase the values of correlations between TCC and HPCs, the correlations obtainable are not only site-specific, but also, time-specific and, as shown by Van Nevel et al. (2017), extremely weak correlations are found in case data from multiple sources is used. In any case, the time required to obtain a result with traditional plating methods makes this technique infeasible for an efficient management of the DWDS.

3.2. Bacterial community dynamics

The bacterial communities were studied with respect to samples HNA% and, both, alpha and beta diversity, which represent respectively the intra- and inter-sample variability. These properties are useful to discriminate samples not only based on their concentration, but also on the characteristics of the bacteria present in the water allowing the identification of bacterial population changes (Favere et al., 2020; Prest et al., 2014; Props et al., 2018b).

In coincidence with the increase of TCC during periods B and F, an increase in HNA% was also observed (Figure S5). However, differently from TCC, this increase was not found in period D, which, conversely, had significantly lower HNA% with respect to the baseline periods. As some LNA bacteria are likely to be obligate oligotrophs unable to grow in rich media (Wang et al., 2009), the increase of HNA, again, suggests a higher availability of nutrients which favored the growth of HNA bacteria and/or the passage from LNA to HNA (Bouvier et al., 2007; Proctor et al., 2018; Rubbens et al., 2019). Furthermore, by observing HNA% at hours group level a general daily trend can be identified: HNA% generically increased between 06 and 19, dropped between 19 and 21 and re-increased during the night. The daily trend, as explained in Section 3.1 regarding TCC, is likely due to the consumers' water demand which alters the water microbiological quality due to biofilm detachment and loose deposits resuspension.

Before assessing the samples alpha and beta diversity, all samples were analyzed to identify phenotypic community types present (Props et al., 2018b). Such analysis identified 2 different phenotypic types, as shown in Figure S6, with "type 1" predominantly present in periods A, with the exception of an event close to its beginning, D and E, and "type 2" in periods B, C and F. Except for the onset of the event in period A, a persistent change between the two types occurred after a period with frequent switches between community types (e.g. the onset of period B), indicating that the transition occurred in a period longer than the interval between two measurements.

Alpha diversity was assessed through the phenotypic diversity index D2 which accounts for both the effective number of phenotypical states (richness) and their relative abundance (evenness) within each sample (Props et al., 2016). The index D2 showed an average value of 1946 $\pm$ 247 a.u., slightly lower but in the same range of the tap water results obtained with comparable settings in Props et al. (2018b), indicating a similar richness and evenness of the two microbial communities. During the monitoring period the diversity index was not stable, but, as presented in Fig. 2a, its values showed negligible uncertainty and a negative correlation with TCC in each period (p-value < 0.001), regardless of the phenotypic community type. As taxonomic and phenotypic indices are strongly related (Props et al., 2016), such result indicates that the increase of TCC is due to a limited number of taxa which affect the community richness and evenness. It can also be noted how the relationship between the index D2 and TCC varies in the different periods, such as periods C or F compared to B, D and E (Spearman's *rho*, respectively, -0.27, -0.87, -0.55, -0.58, -0.62), or also within a single period (period A). However, as shown in Fig. 2b, when phenotypic community "type 1" was present, the increase in TCC resulted in lowered HNA%; while, in the presence of phenotypic community "type 2", a different behavior can be observed. These differences are likely due to the diversity of the phenotypic characteristics of the additional cells with respect to the ones present at lower TCC. Declines in bacterial community evenness are generically not wanted in the distributed water, especially in correspondence to increases in the cell concentrations, as such behavior has been linked with lower resistance against invasive species (De Roy et al., 2013; Van Nevel et al., 2013).

Beta diversity was, instead, evaluated thanks to the estimation of Bray-Curtis dissimilarity and analyzed using principal coordinates

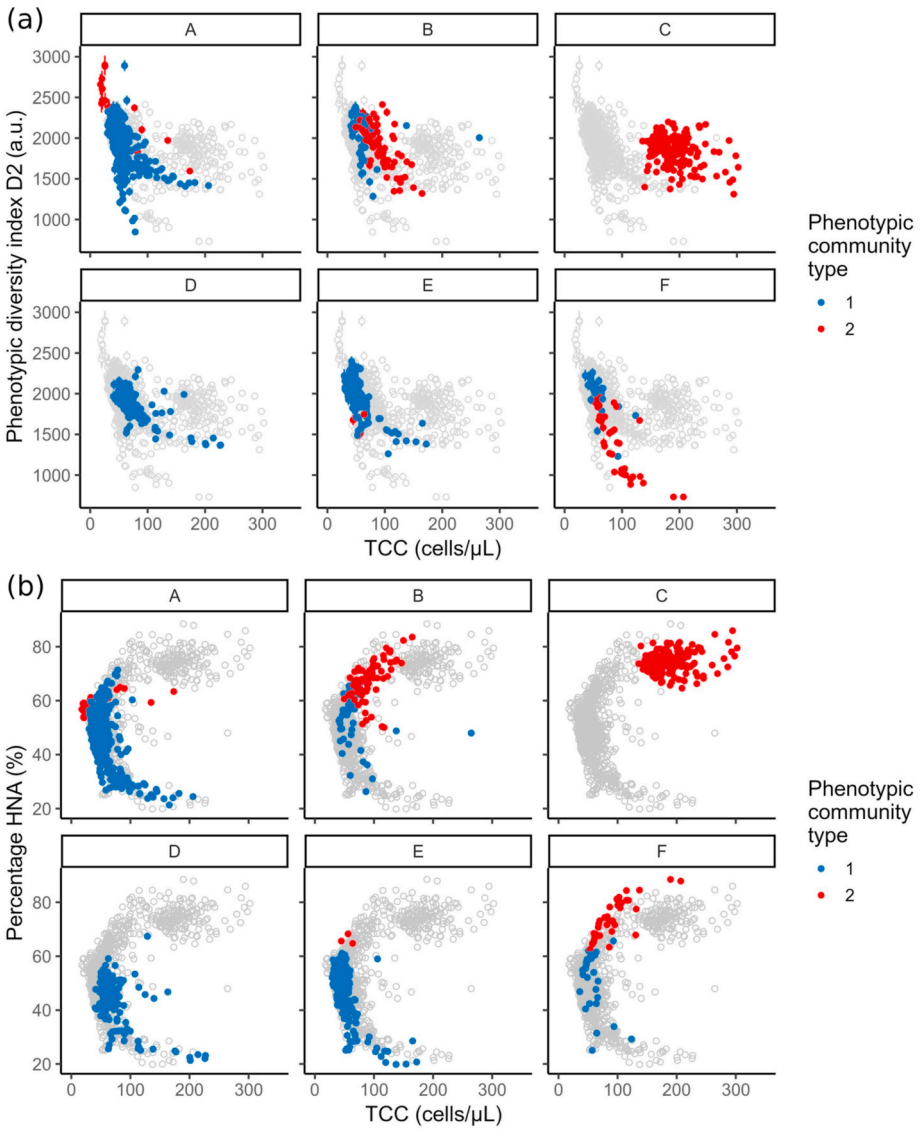

Fig. 2. Phenotypic diversity index D2 (a) and HNA% (b) as a function of TCC. The labels on the top of each panel indicate to which periods the filled markers belong to, while grey empty markers represent the samples not belonging to the specific period. Vertical bars present in (a) indicate the 95% confidence interval estimated through bootstrapping.

analysis (PCoA) plots, shown in Figs. 3 and 4, and several multivariate statistics summed up in Table 1. Bray-Curtis dissimilarity provides an easily interpretable and bounded measure of the difference between two fingerprints and is widely used in ecology (Favere et al., 2020; Legendre and Legendre, 2012). Period A was tested both including and excluding the event characterized by the presence of phenotypic community "type 2", as such event could be considered an outlier influencing period A results. Figs. 3 and 4 show how phenotypic clustering leads to comparable results to the analysis of Bray-Curtis dissimilarity, as the clusters belonging to the different phenotypic community types show a clear separation in the PCoA plot indicating the diversity of the bacterial communities. However, the discrete nature of phenotypic community types makes its result less detailed, especially during periods in which a modification of the bacterial community is present (periods B and F), in accordance with what reported by Favere et al. (2020). In addition, the beta diversity analysis confirmed the peculiarity of the event in period A, highlighted by phenotypic clustering: such samples are shown in the same region as the ones belonging to period C indicating high similarity between these samples. The reason for such event was investigated with the aid of the water utility managing the DWDS, but no evident reason was found as no interventions or costumer complaints were reported and no conventional physical-chemical analyses were carried out during those days.

The Mantel tests in Table 1 demonstrate that only a limited correlation is present between samples beta diversity and time differences in periods A, D and E, suggesting the stability of the bacterial community during these periods. Differently, periods B and C show higher Mantel coefficients due to the presence of monotonic correlations between the beta and temporal diversity. The presence of a stronger correlation can also be seen in Fig. 3: periods B and C show a clear trend between the start and the end of the period, while periods A, D and E do not. The Mantel test does not detect properly the non-monotonic trend present in period F, which can be seen in Fig. 3, leading to a coefficient similar to period A. As the beta diversity was calculated after resampling to the minimum sample size, such diversity analysis is not influenced by the different TCC in each sample. Nonetheless, it can be observed how the increase of TCC in periods B, C and F coincides with alterations in the bacterial community. As no changes in water treatment were done during this monitoring period and, thus, it can be excluded as the cause for this behavior, a possible cause could be due to the onset of physical-

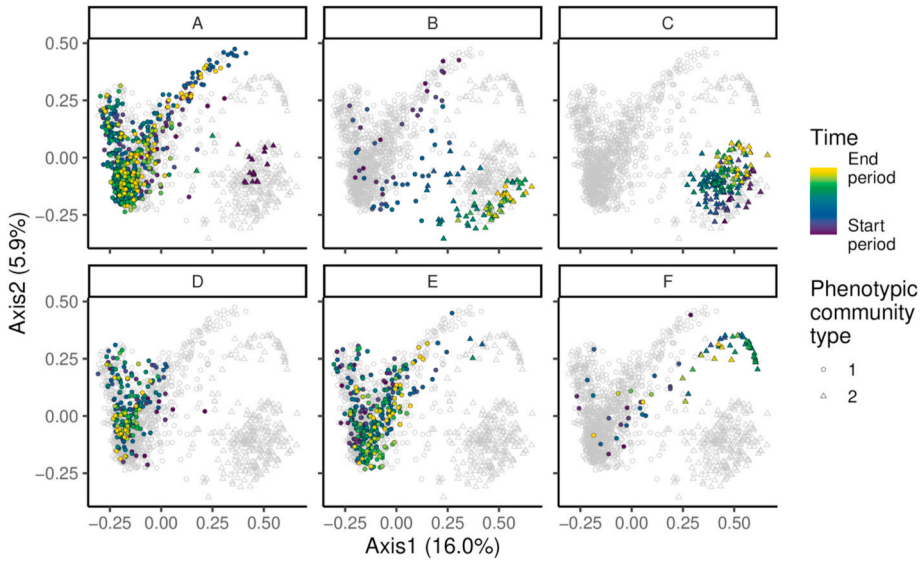

Fig. 3. Principal coordinates analysis (PCoA) of beta diversity time trends estimated through Bray-Curtis dissimilarity. The labels on the top of each panel indicate to which periods the filled markers belong to, while grey empty markers represent the samples not belonging to the specific period.

chemical conditions within the DWDS which increased bacterial growth leading to higher TCC and to the changes in the fingerprints, similarly to what observed by Buysschaert et al. (2019).

Beta diversity was also analyzed with respect to sampling dates and hours groups. Before the analysis, the groups of hours used beforehand were modified, as shown in Fig. 4, to obtain a more balanced design and obtain valid results in case of groups dispersion heterogeneity (Anderson and Walsh, 2013). Such operations are also supported by the similarity of these groups position in the PCoA plot (Fig. 4). The different groups of hours show a significant distinction (p-value < 0.001) in case the samples belonged to phenotypic community "type 1", while a much weaker distinction was found for "type 2" (p-value $= 0.06$). The PERMDISP analysis shows, as reported in Table 1, that during periods A, D and E, the dispersion of the samples of the various groups of hours is significantly different, while this is not verified in case of periods B, C and F. During periods A, D and E, the groups of hours which include mealtimes (06–11, 11–14 and 19–23) showed an average higher dispersion than the others. On the contrary, sampling dates show no significant difference in their dispersion with the exception of periods C and, to a lesser extent, E. The results of PERMANOVA analysis showed in Table 1 indicate that, during periods with a limited correlation between beta diversity and time (periods A, D and E), the hour groups explain a comparable or larger share of the beta diversity, compared to the sampling date. On the contrary, the sampling dates explain most of the variability in periods B, C and F, in accordance with the monotonic trends detected by the Mantel test. The high percentage of variation due to the sampling dates in period F supports the conclusion of the presence of a trend, even though non monotonic, during this period, as deduced from Fig. 3.

By observing the PCoA plot in Fig. 4, it is also possible to notice an intra-day pattern in the samples belonging to phenotypic community "type 1". This is also supported by the pairwise ANOSIM analysis reported in Table 2: even though all differences can be considered as significant (p-value < 0.001), the R statistics for the couples "23–03" and "03–06", "19–23" and "06–11", and "14–19" and "11–14" is one order of magnitude lower than the other combinations and below 0.1, indicating the high similarity of their center and dispersion (Buttigieg and Ramette, 2014). To the best of our knowledge, the detection of cyclical intra-day patterns in the DWDS bacterial community composition has never been highlighted before through FCM, confirming what observed with molecular and genetic techniques which, however, do not allow for online use (Bautista-de los Santos et al., 2016; Sekar et al., 2012).

Such results suggest that a certain value of daily variability of the bacterial community within DWDS is to be expected. Furthermore, even though the daily degree of variability might be constant across days, this might not be true at different hours. The cause of such diversity could be due to variations at the DWTP and/or to phenomena occurring within the DWDS, including stagnation in pipes, biofilm resuspension and loose deposit resuspension, likely affected by the different local water consumption and by its variability (Bautista-de los Santos et al., 2016; Farhat et al., 2020; Prest et al., 2014). For example, the water uses connected with mealtimes can lead to greater fluctuations of the local water flow compared to the other groups of hours, causing an increased sample diversity. For these reasons, sampling times should then be taken into account in the monitoring planning in order to observe the full diversity (e.g. sampling at different hours) or to avoid spurious variation in the data (e.g. sampling at a specified time).

Beta diversity variations were tested against changes of the plate counts values. No significant differences were found between samples characterized by the detection of *P. aeruginosa* and the ones, taken in the same period and groups of hours, with no detection, likely due to the low counts (2–4 CFU/250 mL). Furthermore, no significant correlation was found between the samples beta diversity and the ratio between mesophilic and psychrophilic HPC, used as an indicator of the cultured community composition. The absence of a correlation between beta diversity and the diversity of the ratio between HPCs is likely due to the fact that HPC are composed of copiotrophic bacteria, whose change does not reflect the one of the entire bacterial community (Burtscher et al., 2009; Hoefel et al., 2005). The lack of the detection of fecal contamination indicators demonstrates that the observed variations are not due to a fecal contamination, but are, instead, to be attributed to the DWTP and/or the DWDS operation. This comparison further underlines the limited sensibility of standard plating methods to detect changes in the total bacterial community composition.

3.3. Correlations with physical-chemical water quality

Microbiological parameters showed significant correlations with pH and conductivity as summarized in Table 3. Both water characteristics were found to be significantly correlated with changes in TCC and, furthermore, the composition of the bacterial populations. Except for the correlation between alpha and beta diversities and pH, which show a weakly-significant monotonic correlation and a non-significant linear

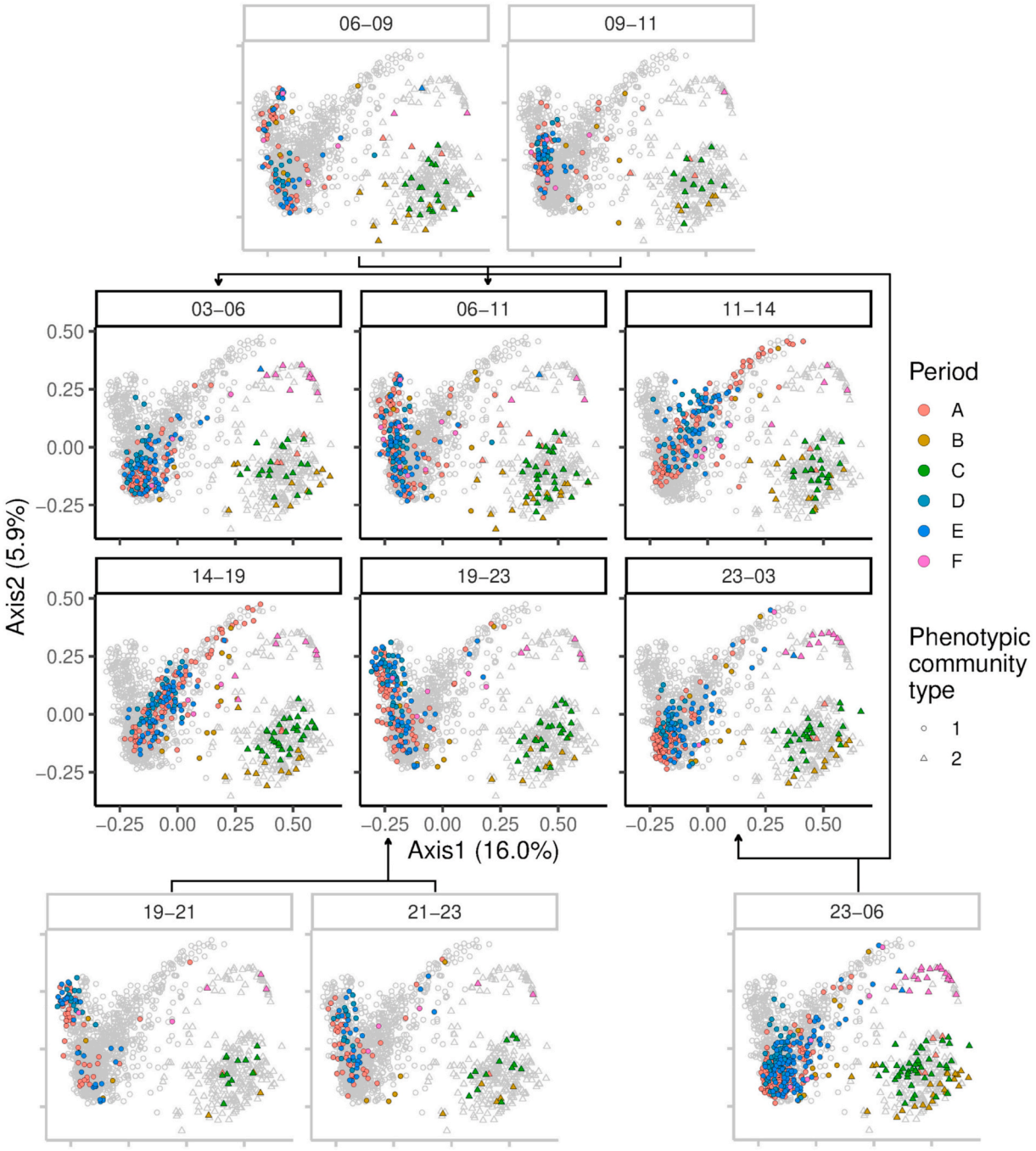

Fig. 4. Principal coordinates analysis (PCoA) of beta diversity estimated through Bray-Curtis dissimilarity. Plots with grey axes and the connected arrows illustrate the merging and splitting operation described in the text. The labels on the top of each panel indicate to which periods the filled markers belong to, while grey empty markers represent the samples not belonging to the specific period.

Table 1
Summary of beta diversity analysis results for each period: p-values are enclosed in brackets, while the other entries represent the Mantel correlation coefficient and the percentage of explained variance [R^2] in the PERMANOVA analysis.

		Periods						
		A	A (no event)	B	C	D	E	F
Mantel test		0.22 (0.001)	0.20 (0.001)	0.76 (0.001)	0.49 (0.001)	0.06 (0.005)	0.11 (0.001)	0.22 (0.001)
PERMDISP	Hours groups	(0.05)	(<0.001)	(0.21)	(0.54)	(<0.001)	(<0.001)	(0.53)
	Dates	(<0.001)	(0.08)	(0.68)	(0.014)	(0.59)	(0.04)	(0.49)
PERMANOVA	Hours groups	0.11 (0.001)	0.18 (0.001)	0.08 (0.03)	0.06 (0.001)	0.29 (0.001)	0.20 (0.001)	0.17 (0.01)
	Dates	0.38 (0.001)	0.18 (0.001)	0.68 (0.001)	0.49 (0.001)	0.10 (0.06)	0.16 (0.001)	0.45 (0.001)

Table 2
Pairwise ANOSIM R statistics between the samples characterized by phenotypic community "type 1" in the different groups of hours.

		Sampling hours group					
		03–06	06–11	11–14	14–19	19–23	23–03
Sampling hours group	03–06						
	06–11	0.21					
	11–14	0.16	0.26				
	14–19	0.17	0.36	0.04			
	19–23	0.26	0.07	0.30	0.38		
	23–03	0.02	0.17	0.13	0.15	0.21	

Table 3
Correlations between physical-chemical and microbiological water quality indices. p-values are reported within brackets.

	pH		Conductivity	
	Spearman	Pearson	Spearman	Pearson
TCC	0.45 (0.02)	0.52 (0.004)	−0.68 (<0.001)	−0.76 (<0.001)
HNA%	0.44 (0.02)	0.45 (0.02)	−0.71 (<0.001)	−0.74 (<0.001)
Alpha diversity (phenotypic diversity index D2)	−0.37 (0.05)	−0.29 (0.14)	0.43 (0.027)	0.39 (0.05)
Beta diversity (Bray-Curtis dissimilarity)	0.15 (0.047)	0.10 (0.13)	0.44 (0.001)	0.42 (0.003)

one, the other correlations showed similar values, and indicating that a monotonic linear relationship between the variables can be assumed. Even though both HNA% and Bray-Curtis dissimilarity reflect the bacterial community composition, the different results obtained are due to the fact that Bray-Curtis dissimilarity has been estimated using data regarding FL1, but also FL2 and SSC, and it is thus more sensitive to changes in the community structure with respect to the overall variation of FL1 assessed through HNA% (Prest et al., 2013). Correlations bearing the same sign, as the ones in Table 3, were found between pH and conductivity, and cells concentrations and HNA% in other studies (Jie et al., 2017; Prest et al., 2016c). Unfortunately, as only pH and conductivity were measured, we cannot exclude the variation of other physical-chemical characteristics which could have affected bacterial cells characteristics, concentrations and communities, as speculated in Sections 3.1 and 3.2. These results highlight the link between the physical-chemical and the microbiological water quality within DWDSs, suggesting the possibility to act on the former to manage the latter in order to minimize operational issues (e.g. biocorrosion), aesthetic issues and ensure safer water for consumers (Hull et al., 2019; Prest et al., 2016b; Waak et al., 2019).

3.4. Implications for DWDS management and monitoring

Due to the consumers demand, the operating conditions in DWDSs pipes vary widely within each day. The influence of local changes of such conditions, due to local instantaneous demands, on microbiological water quality leads to sensible variations in both bacterial concentrations and community structure during the day. Such changes, even though not being harmful to consumers per se (Allen et al., 2004), can highlight the presence of uncontrolled processes (e.g. bacterial growth, biofilm detachment and loose deposit resuspension, trigger of biocorrosion phenomena, aesthetic deterioration) which are highly undesirable in a DWDS.

Even though DWDS management can be optimized to take into account water quality and pumps scheduling (Mala-Jetmarova et al., 2017), the minimization of microbiological water quality fluctuations is likely challenging due to its susceptibility to local operating conditions. Nonetheless, the entity of the microbiological water quality variations can provide an indirect estimate of the DWDS infrastructure conditions and be used to assess the effect of maintenance operations used to control the presence of bacteria and biofilms, similarly to what done by Van Nevel et al. (2017a) and Del Olmo et al. (2021).

The correlation between microbiological and physical-chemical water quality (Table 3; Jie et al., 2017; Prest et al., 2016c) suggests that a possible approach to control the microbiological variations of the delivered water is the reduction of the changes of physical-chemical water quality through improved water treatment. Nonetheless, this approach would not address the water quality variations which could occur during distribution due to the variation of operating conditions.

FCM enables to observe variations of the bacterial concentration and community composition which are not necessarily detected by traditional laboratory analysis based on HPC monitoring. In any case, this technique is unfortunately of little help in the assessment of the respect of HPC-based legal standards as EC (1998) and US EPA (2009), due to the limited correlation and concordance. Furthermore, due to the non-specificity of FCM, laboratory methods are still required to assess the presence of pathogen indicators such as *E. coli* and coliforms bacteria (Cheswick et al., 2019). Despite this, FCM automated solutions allow utilities to monitor the water microbiological water quality without the need for human intervention, thus extending monitoring outside the working hours of water system operators. In addition, FCM potentially enables a smart management of DWTPs and DWDSs if the detected variations are linked to specific actions.

The findings of this study highlight how substantial variations of microbiological water quality can have a limited duration and can occur without any explicit cause (e.g. DWTP and DWDS failures, maintenance work, etc.). Standard microbiological and physical-chemical monitoring is not helpful to detect their occurrence as the temporal (and spatial) resolution is too coarse, not allowing to detect short-lived events.

Regardless of the sampling frequency, when planning a monitoring campaign, it is necessary to take into account that bacteria concentration and community structure can vary significantly throughout the day and decide the most suitable sampling strategy depending on the goal of the monitoring campaign. For example, in case the goal is to verify the presence of a long-term trend, sampling could be performed over a long-time span (e.g. weeks) at a fixed time of the day to reduce the effect caused by the daily variability. On the other hand, sampling at different hours in a short timeframe would, instead, ensure to assess the overall daily microbiological water quality fluctuations and the need to implement corrective actions. In this sense, FCM can support an effective planning of the monitoring campaigns, performed by both FCM itself and standard methods, according to the final purpose, i.e. DWDS (and DWTP) management and verification of regulation compliance.

3.5. Monitoring scheme optimization

Due to the costs connected with long-term monitoring at high sampling frequency, a trade-off between the monitoring cost and the retrieved information is required. In case of FCM, an optimum should be found with respect to both cell concentrations and bacterial communities diversity, as both are relevant to ensure the delivery of high quality water (Favere et al., 2020). Furthermore, as high FCM cell concentrations do not necessarily imply health risks and no universal absolute upper limit exist (Prest et al., 2016b; Van Nevel et al., 2017), an optimal monitoring scheme should focus more on the detection of changes of the microbiological water quality, rather than just the maximum cell concentrations.

Hypothetical alternative monitoring schemes were evaluated by comparing the full monitoring campaign, described before, with hypothetical scenarios in which a lower number of samples per day was analyzed (number of samples = 2–6). Fig. 5 illustrates the ratio $R_{beta \times \Delta TCC}$ obtained comparing the amplitude of daily beta diversity and TCC variations of the full monitoring campaign with the ones which would have been observed in hypothetical scenarios with different

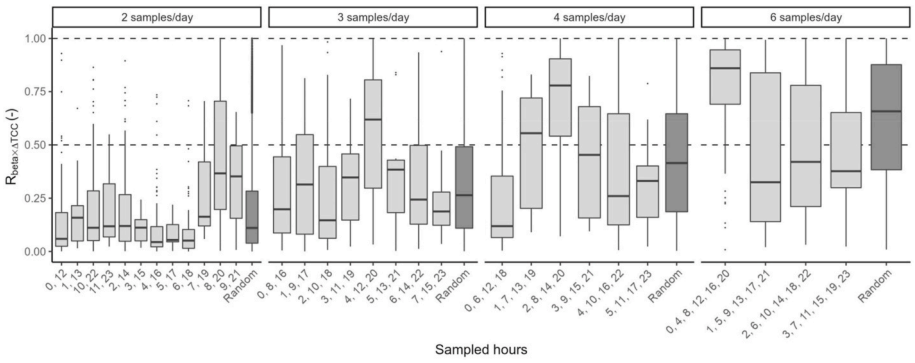

Fig. 5. Daily $R_{beta\times\Delta TCC}$ values based on the number of samples per day and the sampling hours.

sampling schemes. This metric expresses the ability of the specific monitoring scheme to capture the variability of beta diversity and TCC. As expected, $R_{beta\times\Delta TCC}$ increases with a higher number of samples per day but, regardless on the number of samples per day selected, the choice of sampling hours leads to significant differences (p-value $<$ 0.001) in the value of $R_{beta\times\Delta TCC}$. A strategy with random hours sampling, on one side, prevents from selecting a combination of hours resulting in the lowest $R_{beta\times\Delta TCC}$ values, but, on the other side, does not maximize the observed microbiological water quality variations, resulting thus in average and suboptimal performances.

Even though the values shown in Fig. 5 are specific to the case study investigated, microbiological water quality has been often linked with cyclic patterns both at buildings taps (Besmer et al., 2014), in DWDS (Favere et al., 2020) and at DWTPs outlet (Besmer and Hammes, 2016). The presence of such patterns could be exploited by an optimized monitoring scheme to maximize the information obtained and minimizing costs. On the basis of these considerations, a monitoring scheme optimization strategy based on the following steps is proposed:

1. Initial high-frequency sampling: an initial high-frequency sampling should be carried out to properly characterize the microbiological quality variations. Such initial period should last until the variation of the means of TCC in each hour and the beta diversity among all the hours drops below a defined threshold (e.g. 5% which was achieved after approximately one month in this case study).
2. $R_{beta\times\Delta TCC}$ estimation and sampling scheme selection: the $R_{beta\times\Delta TCC}$ ratio between the high-frequency campaign and hypothetical scenarios with lower sampling frequency should be calculated and used to select the optimal monitoring scheme by taking into account both monitoring costs and the $R_{beta\times\Delta TCC}$ values.
3. Monitoring and detection of changes: microbiological monitoring should be carried out with the selected combination of number of samples per day and sampling hours.

Due to the possible variability of these patterns, their eventual change should be investigated. Useful tools for this task could be the change of the detection methods, as, for example, the ones described in Aminikhanghahi and Cook (2017). If a change is detected, the whole strategy should be iterated from step 1 to redefine the new optimal sampling scheme. The complete procedure might not be necessary in all cases, as seasonal trend might be present within the DWDS and previous daily patterns might reoccur, as exemplified by the behavior of periods A, D and E in Figs. 1 and 3. In this case, an equivalence tests (Wellek, 2010) could be used to verify the novelty of the pattern, eventually leading to the reuse of an old pattern, further limiting the costs associated with the initial high-frequency sampling. In case the collection and analysis of 2 samples per day over long periods would still not be feasible due to the costs or operational constraints, the collection and analysis of the samples could be carried out only for a few selected and equally distant days. In this case, it could, also, be possible to take into account (if present), systematic differences between weekdays to maximize the odds of observing the maximum variations possible.

4. Conclusions

Long term FCM and plate count high-frequency monitoring allowed to investigate the water quality in an unchlorinated DWDS secondary branch with respect to both microbial abundance and bacterial community composition, highlighting both short- and long-term variations. In the periods when long-term microbiological quality variations were absent, FCM revealed consistent cyclic daily patterns for both cell concentrations and, for the first time, bacterial community composition, resulting in higher hourly than daily differences. Such dynamics could not be properly observed using plate counts monitoring, underlining its low sensitivity. Instead, FCM data showed significant correlation with the water pH and conductivity suggesting a link between microbiological and physical-chemical water quality which could eventually be exploited for DWDS management. These findings stress the importance of a carefully planned monitoring campaign both with respect to the number of samples per day and the time of sampling to properly characterize the microbiological water quality dynamics. For these reasons, we propose a monitoring scheme optimization strategy to ensure the detection of most the microbiological water dynamics, limiting however the number of analyzed samples. Such optimization scheme will be particularly relevant for future studies and water utilities applying high-frequency long-term microbiological water monitoring.

Author contribution

Marco Gabrielli: Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing; Andrea Turolla: Methodology, Writing – review & editing; Manuela Antonelli: Conceptualization, Writing – review & editing, Supervision, Project administration

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.

Acknowledgments

This research has been funded by CAP Holding S.p.A (CAP), which manages the integrated water service for the metropolitan area in the outskirt of Milan city. The authors would like to thank the CAP laboratory staff for their helpful cooperation. The authors would also like to thank Cristina Baronchelli for her precious contribution in the intensive sampling campaign.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.112151.

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