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Culture and molecular methods as complementary tools for water quality management

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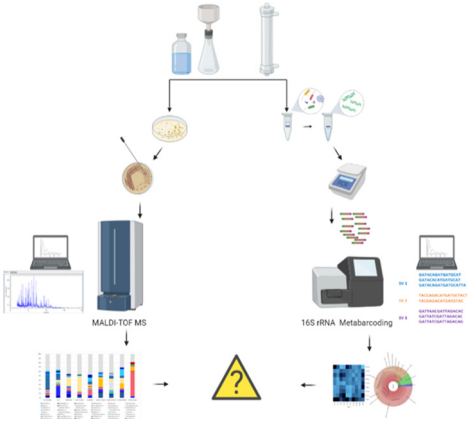
HIGHLIGHTS

- MALDI-TOF MS and metabarcoding analysis yielded different taxonomic results.
- Proteobacteria predominated in the DWTP except in the final drinking water.
- Chlorination strongly reduced bacterial diversity and shaped its taxonomy.
- Temperature affected bacterial diversity in pretreatment and treatment stages.
- Culture and molecular methods are complementary in water quality management.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial communities in a full-scale drinking water treatment plant (DWTP) were characterized using matrix-assisted laser desorption/ionization time of flight mass-spectrometry (MALDI-TOF MS) to identify HPC isolates and the obtained results were compared to 16S rRNA (V4) metabarcoding data acquired in a previous study. Sixty-three samples were collected at nine stages of the potabilization process: river water and groundwater intake, decantation, sand filtration, ozonization, carbon filtration, reverse osmosis, the mixing chamber and post-chlorination drinking water. In total, 1807 bacterial colonies were isolated, 32 % of which were successfully identified to at least the genus level by MALDI-TOF MS using our previously developed Drinking Water Library. Trends in diversity were similar by both approaches, but differences were observed in the detection of taxa, especially at lower hierarchy levels. High bacterial diversity was observed in river and groundwater, where Proteobacteria predominated. The diversity decreased significantly after the chlorination step, where *Bacillus* sp. (Firmicutes) and an unknown genus of Obscuribacteraceae (Cyanobacteria) were the most prevalent genera according to MALDI-TOF MS and metabarcoding, respectively. The two approaches gave similar results for the decantation, sand filtration and mixing chamber steps, where the most abundant taxon was *Flavobacterium*. The combined use of these culture-based and culture-independent methods to characterize microbial populations may help to better understand the role of bacteria in water treatment and quality, which will be of value for DWTP management.

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1. Introduction

Among the Sustainable Development Goals of the United Nations, Goal 6 (Clean Water and Sanitation) calls for universal access to sufficient, safe, and affordable drinking water by 2030 (United Nations, 2020). By reducing levels of organic matter and microorganisms in water, conventional or advanced treatments can greatly improve its quality from source to tap and provide safe drinking water. Although each drinking water treatment plant (DWTP) has its own variations, the most common treatment processes employ a multibarrier approach consisting of coagulation, flocculation, decantation, filtration (e.g., sand or granular/biological activated carbon), with (or without) a final disinfection step (e.g., using UV, chlorine or, chloramine) (Gitis and Hankins, 2018; Betancourt and Rose, 2004).

The recently updated Drinking Water Directive of the European Union (EU 2020/2184) has incorporated the monitoring of viral indicators of fecal pollution (coliphages) (Anonymous, 2020). However, despite such advances, water quality regulation still relies on monitoring a few culturable microorganisms that represent only a minor fraction of the water microbiome.

Heterotrophic bacteria can improve the quality of non-treated water by reducing organic matter and pollutants (Li et al., 2017; Proctor and Hammes, 2015) (Benner et al., 2013; (Skjevrak et al., 2004; (Liu et al., 2016; Wingender and Flemming, 2011)). However, if they proliferate in water systems, they may cause water deterioration or even pose a health risk (Liu et al., 2017, 2016). Moreover, high numbers of heterotrophic bacteria can interfere with coliform detection. Therefore, water quality monitoring has traditionally focused not only on the detection of culturable bacterial indicators of fecal contamination but also on heterotrophic bacteria counts (Bartram et al., 2003). Therefore, obtaining information about overall microbial diversity could shed new light on the functioning of treatment processes.

The wide range of techniques available for the study of microbial communities in DWTPs fall within two broad strategies: culture-dependent (involving the controlled growth of specific microorganisms on selected media) and culture-independent (based on the analysis of nucleic acids previously extracted from samples).

Different culture-based methods for bacterial identification are available. For instance, biochemical phenotyping down to the species level can be performed using API galleries (bioMérieux, France), the Phene Plate System (PhP-Plate Microplate Techniques AB, Sweden) or Biolog (Biolog, US). Widely used, this approach has achieved highly discriminatory bacterial characterization in water samples (Blanch et al., 2007; Hou et al., 2018; Sala-Comorera et al., 2016a, 2016b). However, phenotyping is laborious and time-consuming, especially in assessments of biodiversity, as each identification requires at least 24 h incubation (and up to 120 h) and its application is costly. Recently, the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been proposed as a rapid and robust technology for bacterial identification, involving the analysis of mass spectra of ribosomal proteins extracted from a whole bacterial cell previously isolated and grown. This system has been progressively implemented in routine monitoring, for example, of fermentation processes in the food industry (Angelakis et al., 2011; Kim et al., 2021). It has also been applied to facilitate taxonomic assignment of meiofauna (Rossel et al., 2019) or to identify heterotrophic bacteria present in water during treatment (Sala-Comorera et al., 2017) and in drinking water (Pinar-Méndez et al., 2021), producing results within a few minutes. However, as MALDI-TOF MS was originally designed for clinical diagnostics, mass spectra of target strains may not be available in the database, which limits successful identification.

On the other hand, the use of culture-independent methods, such as 16S rRNA metabarcoding, which allows the study of the total bacteria (culturable and non-culturable), has increased dramatically, thanks to the availability of high-throughput platforms that can simultaneously sequence millions of DNA fragments at a reasonable cost. Although this approach allows an in-depth analysis of the water microbiome, its implementation is still time-consuming. Moreover, the results can be biased by the nucleic extraction protocol or choice of primer, rare groups are not differentiated, and cell viability is not detected (Boers et al., 2019), all of which can complicate the interpretation of data for risk assessment.

Among one of the most challenging issues currently facing drinking water production and water quality management are the uncertainties related to climate change and its impact on freshwater resources. In this context, a consistent monitoring of bacterial communities in DWTPs combined with regulated water quality analysis based on the detection of microbial indicators may help to evaluate the current state of the art in water treatment and distribution systems and their resilience to water stress.

The aim of this work was to combine and compare two different approaches (culture-dependent and -independent) to characterize the bacterial communities in a full-scale DWTP. The culture-based strategy consisted of heterotrophic plate counts (HPC) (European Directive (EU) 2020/2184) and identification by MALDI-TOF MS using a previously developed in-house database (Pinar-Méndez et al., 2021). The culture-independent analysis was based on 16S rRNA metabarcoding (V4 region) data obtained using an Illumina MiSeq platform in a previous study (Pinar-Méndez et al., 2022).

2. Material and methods

2.1. Study site and sampling

Samples were collected from a full-scale DWTP in Sant Joan Despí, 6 km south of Barcelona (Catalonia, North-East of Spain), in the lower basin of the Llobregat River. The anthropogenic impact in the area is high, with widespread industrial and agricultural activities, and the quality of river water is affected by wastewater effluents and industrial discharges. The DWTP has two sources of water: river and groundwater, as described previously (Pinar-Méndez et al., 2022). The water is subjected to sequential treatments as shown in Fig. 1. The water from two treatment lines is mixed in a chamber and treated with chlorine (0.5–1.5 mg/L residual chlorine) in a separate tank before being pumped into the distribution systems.

Seven sampling campaigns were carried out over one year, including the winter of 2018 and summer of 2019, when temperatures ranged from 11.5 °C to 29.5 °C. A total of 63 water samples were collected at nine stages of treatment in the DWTP: river water (RW, $n = 7$) and groundwater (GW, $n = 7$) intake, decantation (DEC, $n = 7$), sand filtration (SF, $n = 7$), ozonization (OZ, $n = 7$), carbon filtration (CF, $n = 7$), reverse osmosis (RO, $n = 7$), mixed chamber (MIX, $n = 7$) and post-chlorination drinking water (DW, $n = 7$).

Sampling sites were classified into two categories according to the microbial load: high or low. For high microbial load samples, corresponding to river water and pretreatment stages (RW, DEC, SF), small volumes of water (2 L) were collected in polyethylene sterile bottles containing sodium thiosulfate (24 mg/L). For samples of the groundwater and conventional/advanced treatments (GW, OZ, CF, RO, MIX, DW), which had a lower microbial load, higher volumes (from 100 to 1100 L/sample) were collected using Rexeed™ 25-A filters (Asahi Kasei Medical Co, Japan) according to the previously described dead-end hollow fiber ultrafiltration (DEUF) method (Gunnarsdottir et al., 2020; Hill et al., 2007; Rhodes et al., 2011). All samples were transported to the laboratory at 4 °C for analysis.

2.2. Sample processing

All samples were processed within 24 h of collection and analyzed for microbial water quality parameters according to the European Directive (EU) 2020/2184. Additionally, all samples were analyzed by MALDI-TOF MS and metabarcoding methods. Sample preparation differed according to the technique and microbial load.

2.3. Heterotrophic bacteria plate count

HPC was performed by mass inoculation according to the ISO Standard 6222:1999. Water samples concentrated by the DEUF method (Rexeed)

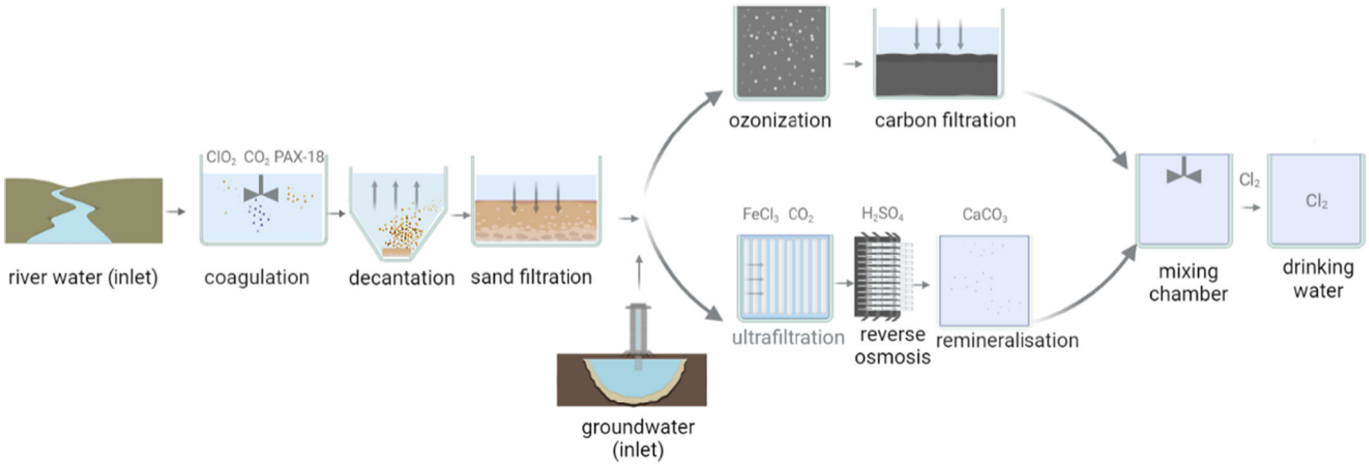


Fig. 1. Schematic diagram of the stages in a drinking water treatment plant in Sant Joan Despí (Barcelona, Spain).

were eluted as described by Hill et al. (2007) and Gunnarsdottir et al. (2020). The final eluate was used for mass inoculation. For water samples with a higher microbial load that did not require DEUF concentration, direct mass inoculation was performed. Different sample volumes (0.001–1 mL) were inoculated in duplicate in ISO Water Plate Count Agar (Oxoid, UK) and incubated at 22 ± 2 °C for 72 h ± 3 h.

2.4. Analysis of bacterial communities by MALDI-TOF MS

The bacterial composition of water samples was analyzed using the Microflex LT MALDI-TOF MS device (Bruker Daltonics, Germany). In HPC dilution plates containing well-isolated colonies (from 10 to 130), a total of 30 colonies per sample (where possible) were randomly selected for identification purposes and diversity studies (Bianchi and Bianchi, 1982). To achieve fresh pure cultures, bacterial isolates were subcultured in Water Plate Count Agar and incubated at 22 ± 2 °C for 72 h ± 3 h or until growth (maximum 7 days). Experiments were carried out in a biosafety level 2 cabinet (BSL-2). Samples for MALDI-TOF MS analysis were prepared using the formic acid extended direct transfer method recommended by Bruker Daltonics under a fume extraction cabinet, as previously described (Pinar-Méndez et al., 2021).

The resulting spectrum patterns were classified according to log score values and consistency category and identified by matches with the reference mass spectra database. This study was conducted using two databases simultaneously: the Bruker Daltonics (BDAL) library (8468 reference spectra) and the Drinking Water Library (DWL) (319 reference spectra), the latter being previously developed in our laboratory specifically for the identification of bacteria associated with water for human consumption (Pinar-Méndez et al., 2021). Results were interpreted according to the Bruker score values: highly probable species identification (green; ≥2.300–3.000), secure genus and probable species identification (also green; 2.000–2.299), probable genus identification (yellow; 1.700–1.999) and unreliable identification (red; ≤1.699). Another approach to taxonomic assignment (Bruker Daltonics) classifies the top ten best matches according to categories of consistency: A (species consistency: top 10 matches >2.000 are of the same species, or >1.700 are of the same genus); B (genus consistency: top 10 matches >1.700 are of the same genus but not of the same species); C (no consistency: top 10 matches <1.700, or >1.700 are not of the same genus).

2.4.1. Unidentified isolates

A fraction of colonies that could not be identified were subcultured, incubated as described above and submitted to further analysis. If the second attempt at identification failed, isolates were reprocessed but using a different sample preparation method for an improved protein extraction. Thus, the acid/acetonitrile extraction method was performed following Bruker's instructions, and 1uL of protein suspension was spotted in triplicate on a MALDI 96 target plate, which was air-dried, and samples were covered with 1uL of matrix solution for the analysis. If the colonies remained unidentified, they were treated separately for clustering purposes (**Fig. S1**). First, for the non-identified isolates, a mini Main Spectrum Profile (mMSP) ad hoc library was created using MALDI Biotyper 3.1 software offline (Bruker Daltonics, Germany). Thus, 9 mass spectra per isolate were checked for the following criteria: 3000 to 10,000 Da and maximum error tolerance of 500 ppm. The mMSP library was used as a database for offline analysis of the unidentified isolates one-by-one and score values ranging from 3.000 to 1.700 were grouped into a cluster, assuming a maximum resolution at genus level.

BioNumerics 7.6 software (Apply Maths, Belgium) was used to help with the visualization of the large MALDI-TOF spectra datasets by creating a dendrogram. For clustering purposes, similarities between all spectra were analyzed using the peak-based Pearson correlation coefficient and clustered by the UPGMA clustering algorithm to generate the dendrogram. The maximum distance level cut-off for clustering spectra was set at 50.

2.5. Comparison of culturable bacterial communities with 16S rRNA metabarcoding data for the total bacteria

The results obtained by MALDI-TOF MS were compared with previously obtained 16S rRNA (V4) metabarcoding data derived from the same samples (Pinar-Méndez et al., 2022) (**Fig. S2**).

2.6. Statistical analyses

Data were analyzed using R version 4.0.5 and RStudio version 1.2.1335. All samples were $\log_{10}$ transformed for statistical purposes. The Shapiro-Wilk Normality test was run to check if data fitted a normal distribution. The Kruskal–Wallis test was used to look for differences in HPC between all the DWTP samples, and then the Mann-Whitney *U* test was run to find out which samples were different. The alpha diversity metrics of Shannon (diversity index) and Observed (richness) were calculated at the genus level to assess bacterial diversity, including all isolates (identified and non-identified). To determine seasonal differences in HPC and diversity, the samples were grouped according to the ambient temperature during the sampling campaign (low ≤18 °C and high ≥22 °C) and the Mann-Whitney test was used to determine possible significant differences between groups.

3. Results

3.1. Heterotrophic bacteria

The studied drinking water fully met all the quality standards of the EU Directive (data not shown). All samples gave positive HPC results except for two: one after the DEC stage and the other after OZ. The total number of culturable heterotrophic bacteria differed significantly between all the treatment stages (Kruskal-Wallis test, $p < 0.01$). Average values ranged from 0.027 $\log_{10}$ CFU/mL to 4.62 $\log_{10}$ CFU/mL (Fig. 2A). When comparing the two water sources, HPC values were 4 $\log_{10}$ units higher in RW compared to GW (4.47 $\log_{10}$ CFU/mL and 0.37 $\log_{10}$ CFU/ml, respectively). After DEC, the HPC remained high (4.62 $\log_{10}$ CFU/mL). A decrease of about 1-log was observed after the SF stage (3.63 $\log_{10}$ CFU/mL), and a further decrease of 2 $\log_{10}$ units after the subsequent processes of OZ (2.18 $\log_{10}$ CFU/mL) and CF (2.16 $\log_{10}$ CFU/mL). The HPC after the advanced treatment (1.48 $\log_{10}$ CFU/mL) was 3 $\log_{10}$ units lower compared to RW. MIX samples (2.23 $\log_{10}$ CFU/mL) gave similar HPC values to those of CF, followed by a strong reduction of >2 $\log_{10}$ units after the final chlorination (0.027 $\log_{10}$ CFU/mL).

Seasonal differences in HPC were found for some stages in the DWTP (Fig. 2**B**). Thus, RW, OZ and DW samples presented significantly higher HPC values at high temperature (HT) than low temperature (LT) (Mann-Whitney *U* test, $p < 0.05$). Conversely, in GW, DEC, SF, CF and MIX samples, counts were slightly higher at LT, although the differences were not statistically significant. Interestingly, bacterial regrowth was observed after CF at LT, which accounted for a 2-log increase with regard to the previous stage (OZ).

3.2. Analysis of bacterial communities by MALDI-TOF MS analysis

3.2.1. Identification of bacterial isolates using BDAL and DWL databases

A total of 1807 colonies were isolated from the DWTP stages and further analyzed by MALDI-TOF MS: GW (211), RW (210), DEC (182), SF (203), OZ (180), CF (207), RO (211), MIX (211) and DW (192). The DWL database (Pinar-Méndez et al., 2021) was used to improve identification, as with only the BDAL database, more than half of the isolates (1010 out of 1807) remained unidentified (56 %) (Fig. 3). The simultaneous use of both databases gave better results, reducing unreliable identification to 38 % (686 out of 1807), and classification at the genus level increasing to 32 % and species level to 30 %.

While using the BDAL database alone, unreliable identification at the different DWTP stages varied from 32 to 71 %, compared to 30 to 46 % when using the extended database (BDAL + DWL) (**Fig. S3**), the rate being even lower in DW (19 %). Accordingly, combining both databases led to an improvement in results in all DWTP samples and the classification rate at genus or species level ranged from 54 up to 81 %.

3.2.2. Characterization of bacterial isolates

The bacterial community composition in the DWTP was highly diverse and differed at each stage, not only at the genus level but also at the phylum level. Four phyla were detected (Fig. 4A). Overall, Proteobacteria predominated, except in DEC, SF and CF samples, in which the proportion of Bacteroidota was higher, and in DW, where Firmicutes was clearly dominant. Bacteroidota was well represented throughout the DWTP and was the second most dominant phylum. In contrast with its abundance in chlorinated DW, Firmicutes was scarcely found during the previous treatments, with the exception of OZ samples, in which 14.4 % of the isolates were affiliated to this phylum. Actinobacteriota was also detected in the treatment samples, but to a low extent (4.7 % of the isolates).

At the genus level, a total of 57 genera were successfully identified by MALDI-TOF MS (Fig. 4B). Analysis of GW and RW revealed each contained 24 different genera, *Aeromonas* (22 %) and *Pseudomonas* (32 %) predominating, respectively. The first change in bacterial composition was observed in the pretreatment stages, as *Flavobacterium*, poorly represented in RW (5 %), was the most abundant genus in DEC (19 %) and SF (34 %). Further shifts were observed during the conventional treatments, with a reduction of *Flavobacterium* and an increase of *Bacillus* (13 %) and *Rheinheimera* (9 %) after OZ, and a higher relative abundance of *Chryseobacterium* (20 %) *and Acidovorax* (12 %) after CF. Regarding the advanced treatment, the predominant genus in RO samples was *Comamonas* (17 %), not detected upstream except after SF (1 %). The genus profile in MIX samples was similar to that of CF (*Rheinheimera* 15 %, *Acidovorax* 13 %, *Flavobacterium* 11 %, *Chryseobacterium* 11 %), with little influence from the RO stage. Overall, the most notably shift in composition was observed in DW, in which *Bacillus* was clearly dominant (58 %), and *Chryseobacterium* (9 %) and *Paenibacillus* (6 %) were also detected.

3.2.3. Unidentified isolates

The unidentified isolates (686 colonies) were grouped into a total of 91 clusters (C:565 colonies). 121 colonies had a single protein profile (S:80) or

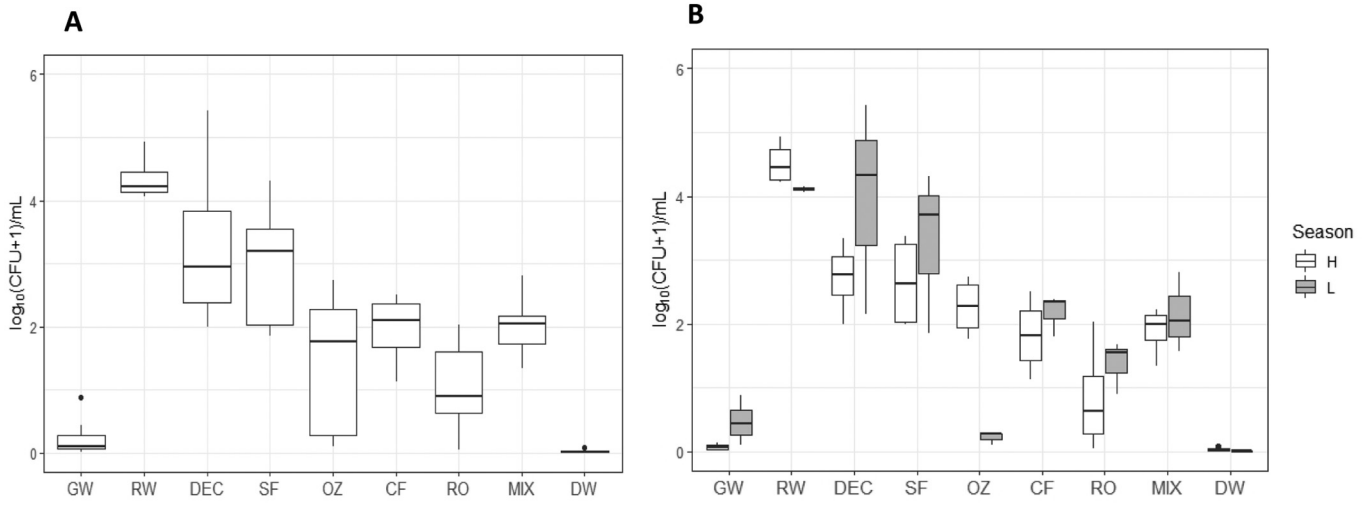
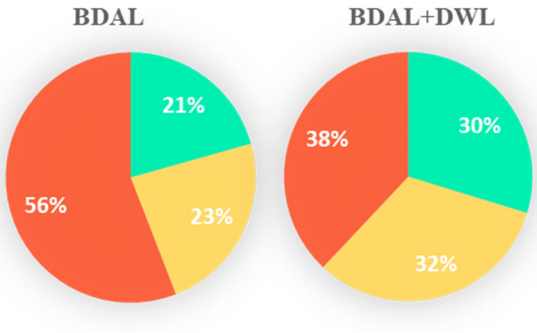


Fig. 2. Boxplot charts displaying the distribution of heterotrophic bacteria plate counts ($\log_{10}$ CFU/mL) in water samples from different stages in the DWTP: groundwater (GW), river water (RW), decantation (DEC), sand filtration (SF), ozonization (OZ), carbon filtration (CF), reverse osmosis (RO), mixing chamber (MIX) and post-chlorination drinking water (DW). Boxplots in (A) show total counts and in (B) the results are grouped by the ambient temperature when sampling was carried out: high (H) or low (L). Summary of data includes minimum score (lower whisker), first, median and third quartiles (box) and maximum scores (upper whisker). Statistically significant outliers are represented by dots.



	species	genus	not reliable
BDAL	373	424	1010
BDAL+DWL	537	584	686

Fig. 3. Identification of the 1807 water bacterial isolates when using the Bruker Daltonics (BDAL) database alone or in conjunction with the Drinking Water Library (DWL). Isolates were classified according to scores based on MALDI-TOF MS analysis: highly probable species (≥2.300–3.000; green), secure genus and probable species (2.000–2.29; yellow), probable genus (1.700–1.999; yellow) or unreliable identification (≤1.699; red).

exhibited altered or stopped growth, probably due to stress (S:41) (Fig. 5). One of the most abundant clusters, C-79 (green), was only detected at HT in the DEC (10 %), SF (10 %), CF (2 %) and RO (3 %) stages, and was not found in source water or the final DW. C-47 (light brown) was only detected at LT in OZ (5 %) and DW (13 %) samples. Other clusters had less seasonal association, such as C-31 (dark brown), which in CF samples was more abundant at LT (26 %) than HT (7 %), but was also detected in low relative abundance (2–7 %) in DEC, SF, OZ, and MIX stages in both seasons.

3.2.4. Alpha diversity analysis

Alpha diversity metrics were calculated at the genus level to assess bacterial diversity. For this analysis, the clusters described in the previous section were included, as they represent possible genera (**Fig. S4**). Changes in diversity were observed throughout the treatment, a decrease occurring from source waters to the final DW. Statistically significant differences in bacterial communities were found between some treatment stages (Kruskal Wallis, $p < 0.01$). RW presented the highest diversity indices, followed by GW, while the lowest values corresponded to DW, where after the chlorination procedure diversity and richness was significantly lower compared to source water (Tukey HSD test, $p = 0.002$). Small differences in diversity were observed between treatment stages, but they were not statistically significant. Nevertheless, all treatment stages clearly differed from DW.

3.2.5. Seasonal variation

In order to detect possible seasonal variations in bacterial diversity in the DWTP stages, samples were grouped into two categories according to ambient temperature, high (HT; ≥22 °C) and low (LT; ≤18 °C), but only small differences were observed in some samples, without statistical significance (Fig. 6). Species richness (number of observed species) and the Shannon index followed a similar trend. The Shannon index revealed a small increase in diversity at HT in the DEC, SF, OZ, CF and MIX stages, and at LT in RW, RO and DW, whereas GW samples remained quite stable between seasons. Richness increased slightly at HT in DEC, SF, CF, and MIX samples and at LT in RW and RO samples, but no differences were observed for GW, OZ and DW.

Small differences in taxonomy at the genus level were also associated with seasons (Fig. 7). At LT, DEC and SF samples were mostly dominated by *Flavobacterium* (33 % and 60 %, respectively), whereas at HT, when genus diversity was higher, the relative abundance of *Flavobacterium* was lower (5 % and 12 %, respectively). In conventional treatment stages, the temperature also affected the taxa. Thus, the dominant genus in OZ samples was *Bacillus* (22 %) at HT and *Rheinheimera* (18 %) at LT. In CF samples, the high relative abundance of *Chryseobacterium* at HT (32 %) was reduced at LT (3 %). In the advanced treatment, RO samples showed different profiles according to temperature: at LT, *Comamonas* (19 %) and *Flavobacterium* (16 %) were predominant, whereas the latter was not detected at HT. Moreover, two genera were only found at HT in RO samples: *Sphingopyxis* (10 %) and *Arthrobacter* (9 %), both of which were detected in a few stages upstream only in very low abundance (<1 %). In MIX samples, *Acidovorax* (21 %) and *Flavobacterium* (20 %) were well represented at LT, whereas at HT the predominant genera were *Chryseobacterium* (20 %) and *Rheinheimera* (20 %). Finally, DW did not show large differences in genus composition, being dominated by *Bacillus* in both seasons (HT: 70 %, LT: 57 %), although *Aeromonas*, *Chryseobacterium* and *Ewingella* were only detected at HT, and *Blastomonas*, *Brevibacillus*, *Flavobacterium* and *Methylobacterium* only at LT.

3.3. Comparison of culturable bacterial communities with the total bacteria detected by 16S rRNA metabarcoding

The study of culturable bacterial communities provides very useful information on metabolically active cells. However, culturable bacteria represent only a minor fraction of the entire water microbiome and other approaches are needed to provide complementary and more in-depth information. Thus, the identified heterotrophic bacterial populations were compared with the data obtained in a previous 16S rRNA metabarcoding study (Pinar-Méndez et al., 2022).

3.3.1. Detection of MALDI-TOF MS-identified genera in metabarcoding reads

The 57 genera identified by MALDI-TOF MS in samples from the different water treatments were compared with metabarcoding data (presence/

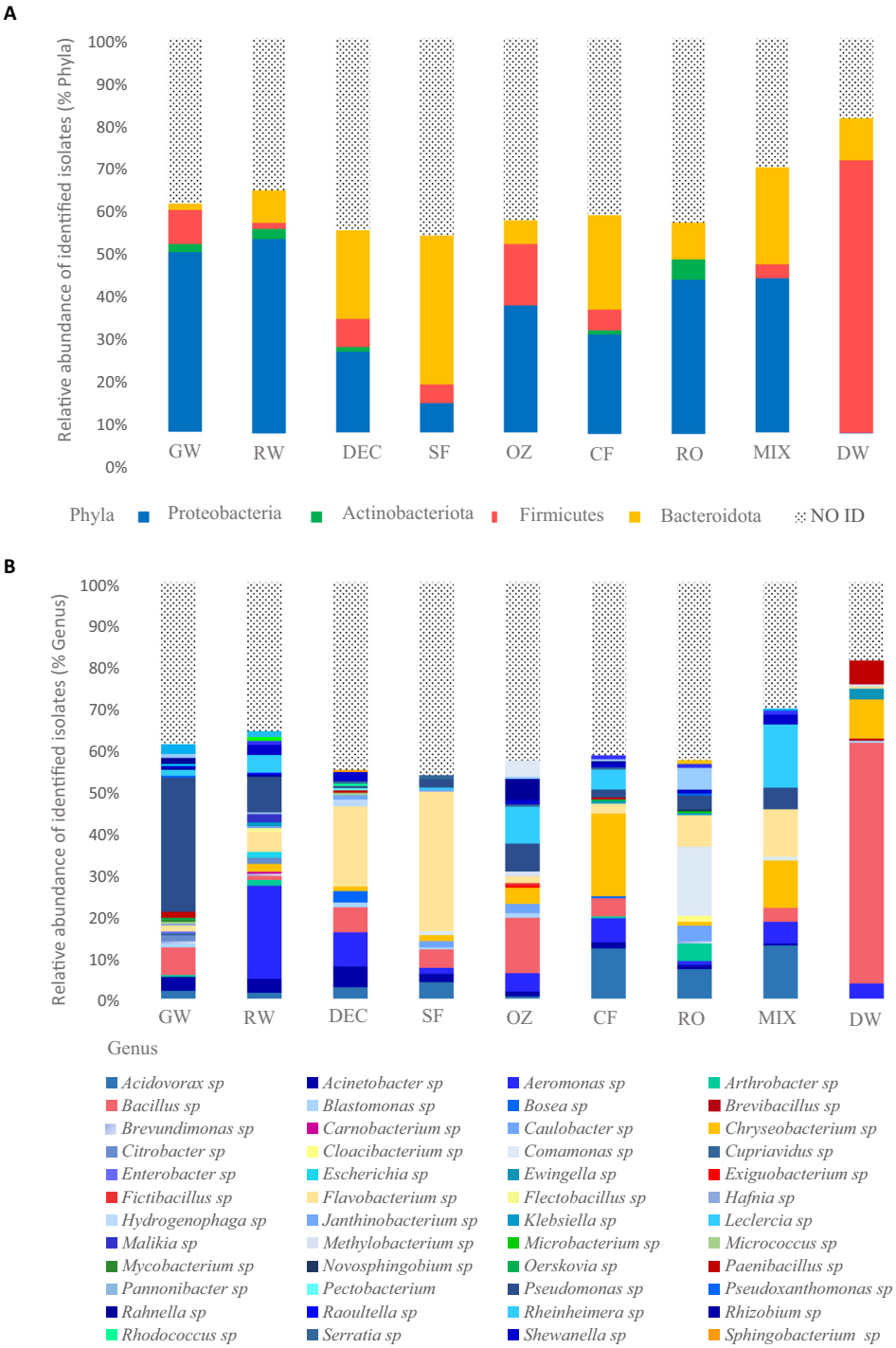


Fig. 4. Patterns of relative abundance of isolates identified at the A) phylum level and B) genus level, and their distribution along the treatment stages: groundwater (GW), river water (RW), decantation (DEC), sand filtration (SF), ozonization (OZ), carbon filtration (CF), reverse osmosis (RO), mixing chamber (MIX) and post-chlorination drinking water (DW). Dots represent unidentified fractions (NO ID).

absence and relative abundance reads) (Table 1). On 41 occasions (27 different genera), MALDI-TOF MS allowed the detection of a genus that was missed by metabarcoding (boxes edged in black). Conversely, on 152 occasions, 33 genera were only detected by metabarcoding (boxes edged in white).

It is also noteworthy that the most abundant genera in each matrix according to MALDI-TOF MS analysis were all successfully detected by metabarcoding, although with different relative abundance. Thus, in GW, *Pseudomonas* abundance was higher by MALDI-TOF MS (32 %) than by metabarcoding (1 %); a similar difference was observed in *Aeromonas* in RW (22 % versus 0.4 %, respectively). In DEC samples, relative abundance of *Flavobacterium* was similar by MALDI-TOF MS (19 %) and metabarcoding (14 %), and in SF samples, *Flavobacterium* was the prevailing genus by both techniques (33.5 % and 15 %, respectively). In conventional treatments, at the OZ stage *Bacillus* was predominant according to MALDI-TOF MS analysis (13 %), whereas metabarcoding indicated a very low abundance (0.005 %). Similarly, 19.8 % of the CF isolates corresponded to *Chryseobacterium*, whose incidence was much lower according to metabarcoding results (0.08 %). In the advanced treatments, *Comamonas* was identified in RO samples by both MALDI-TOF MS (16.6 %) and metabarcoding (2 %). In MIX samples, *Rheinheimera*

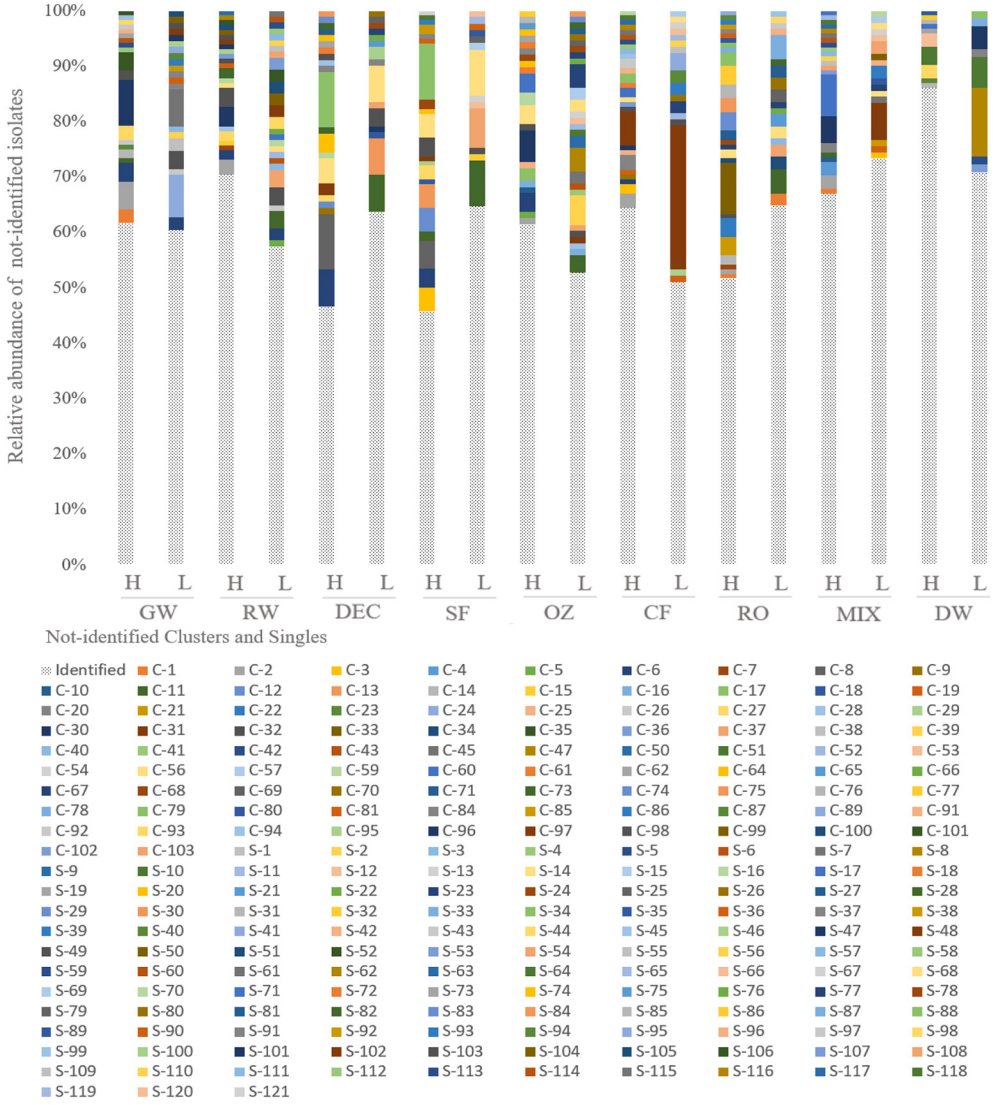


Fig. 5. Distribution of unidentified isolates in the DWTP: groundwater (GW), river water (RW), decantation (DEC), sand filtration (SF), ozonization (OZ), carbon filtration (CF), reverse osmosis (RO), mixing chamber (MIX) and post-chlorination drinking water (DW). Samples are grouped according to the ambient temperature during the sampling campaign: high (H) or low (L). The colors show the relative abundance of all isolates, which were either single (S) or grouped in clusters (C). Dots represent the identified fraction previously shown in Fig. 4.

represented 15 % of the culturable fraction and was also detected by metabarcoding (3 %). Finally, in DW samples, culturable *Bacillus* predominated (57.8 %) according to MALDI-TOF MS analysis and was also identified by metabarcoding but with much lower abundance (0.05 %).

Regarding the total genera in the final DW, some genera were only detected by MALDI-TOF MS but not by metabarcoding, in abundances ranging from 0.5 to 3.9 %: *Arthrobacter*, *Brevibacillus*, *Carnobacterium*, *Enterobacter*, *Leclercia*, *Micrococcus*, *Oerskovia*, *Rahnella*, *Rhodococcus*, *Sphingobacterium*, *Streptomyces* and *Wautersiella*. On the other hand, metabarcoding revealed a greater diversity of bacteria in DW not detected by MALDI-TOF MS, the relative abundance reads ranging from 0.003 to 2 %: *Acidovorax*, *Bosea*, *Brevundimonas*, *Cloacibacterium*, *Comamonas*, *Hafnia*, *Hidrogenophaga*, *Janthinobacterium*, *Klebsiella*, *Mycobacterium*, *Novosphingobium*, *Pseudomonas*, *Pseudoxanthomonas*, *Raoultella*, *Rheinheimera*, *Rhizobium*, *Serratia*, *Shewanella*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis*, *Stenotrophomonas*, *Variovorax* and *Yersinia*.

3.3.2. Fluctuation of the six most abundant genera along DWTP stages

According to MALDI-TOF MS analysis, the most abundant genera recovered from all DWTP samples were *Bacillus* (206 out of 1807 isolates), *Flavobacterium* (166), *Aeromonas* (124), *Pseudomonas* (123), *Chryseobacterium* (106) and *Acidovorax* (87).

With the aim of tracking their fluctuation along the different treatment stages, and to evaluate the capacity of MALDI-TOF and metabarcoding techniques to detect their presence, these genera and their abundance were compared in a schematic diagram of the DWTP (Fig. 8).

Flavobacterium was the only genus identified by both approaches at all treatment stages, from source to tap. The genera *Acidovorax* and *Pseudomonas* were detected by both methods at all DWTP stages but in DW metabarcoding revealed a low abundance (0.04 % and 1 %, respectively). *Aeromonas* was identified by both techniques at all the stages except GW, where it was detected only by metabarcoding. *Bacillus* was tracked from inlet water to outlet DW, with a massive increase after chlorination, although its detection and abundance differed according to the technique. Analysis by MALDI-TOF MS provided positive results for *Bacillus* in GW, RW, SF, CF, MIX and DW, but by metabarcoding only in DEC, OZ and DW samples. Finally, *Chryseobacterium* was detected in RW (but not in GW), SF, OZ, CF, RO, MIX and DW by both approaches, and in DEC only by MALDI-TOF MS.

Likewise, to gain insight into the DWTP locations of the most abundant taxa, the six most abundant genera (>5 %) according to MALDI-TOF MS

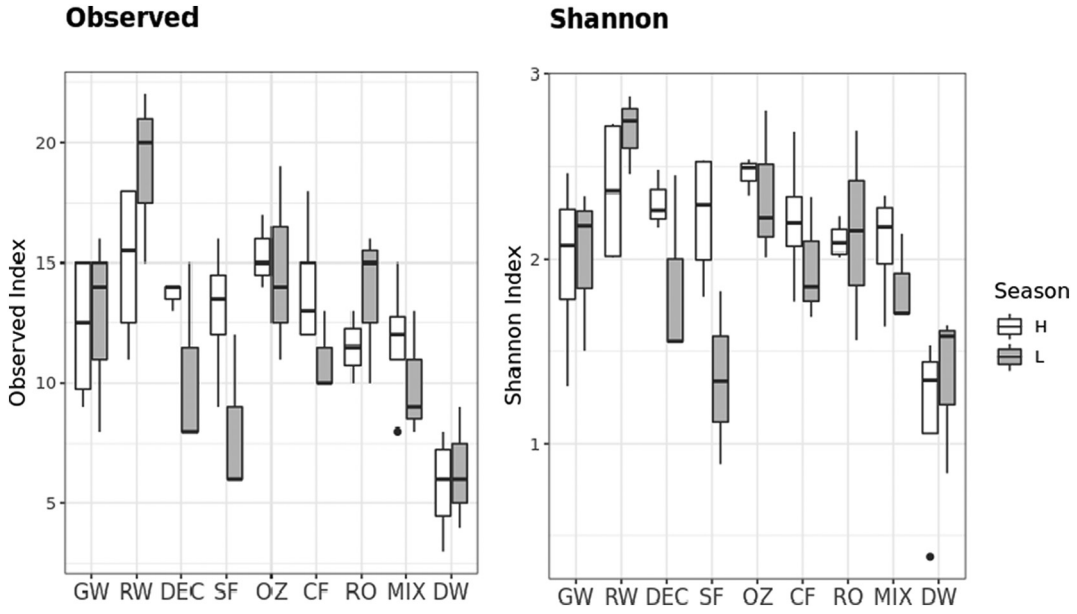


Fig. 6. Boxplots representing alpha diversity indices of genus richness (Observed) and diversity (Shannon) for all DWTP samples: groundwater (GW), river water (RW), decantation (DEC), sand filtration (SF), ozonization (OZ), carbon filtration (CF), reverse osmosis (RO), mixing chamber (MIX) and post-chlorination drinking water (DW). Samples are grouped according to the ambient temperature during the sampling campaign: high (H, white) or low (L, grey).

(same genera as in Fig. 8) and metabarcoding (Pinar-Méndez et al., 2022) were compared. Their hotspots are depicted in **Fig. S5**.

4. Discussion

The bacterial communities in a full-scale DWTP in Barcelona (Catalonia, North-East Spain) were compared via two identification methods, MALDI-TOF MS (culture-dependent) and high-throughput amplicon sequencing of the 16S rRNA gene (culture-independent), using metabarcoding data obtained in a previous study (Pinar-Méndez et al., 2022).

The studied water sources (river and groundwater) and the final drinking water fully met the quality standard requirements of the European Drinking Water Directive. HPC values decreased progressively along the successive DWTP treatments from source to tap (from 4.62 $\log_{10}$ to 0.027 $\log_{10}$ CFU/mL), with a significant reduction after chlorination. During the warmer seasons, the abundance of heterotrophic bacteria in RW, OZ and DW samples increased significantly. However, at the OZ stage, it should be noted that besides temperature, the level of residual ozone may be another influential seasonal factor, as the DWTP operational procedure stipulates a lower ozone dosage in summer (mean: 0.05 ppm) than in winter (mean: 0.22 ppm). This is to avoid the formation of bromate, an ozonation by-product and potential human carcinogen produced at higher temperatures (Von Gunten, 2003). Therefore, lower residual disinfectant may contribute to bacterial regrowth in the warmer seasons (Li et al., 2018). Additionally, although CF samples did not show seasonal variation, at low temperatures they had higher HPC values than samples from the preceding OZ stage. This may indicate the establishment of a microbial biofilm community in the carbon filters, as the HPC values of the CF effluent were 2 log higher than in the CF influent. Although drinking water regulations do not stipulate upper limits for the HPC, they require that there should be no abnormal changes (Anonymous, 2020). In this context, it is of interest that the two identification methods gave different results in terms of bacterial abundance and diversity, even in water samples with a normal HPC, as observed by other researchers using multiparametric approaches (Lautenschlager et al., 2013; Prest et al., 2014).

MALDI-TOF MS is described as a rapid identification tool and a reliable alternative to amplicon sequencing (Kraková et al., 2017), although its resolution capacity depends directly on the available reference spectra. In the present work, to analyze the diversity of culturable bacteria, 1807 isolates from HPC plates were analyzed by MALDI-TOF MS using the Bruker BDAL database extended by our previously developed DWL (Pinar-Méndez et al., 2021). The rate of identification at the different treatment stages varied from 54 % to 81 %. Such differences can occur when predominant taxa are not represented in the libraries, which increases the percentage of misidentification. Thus, to improve identification power, it is advisable to customize databases with taxa of interest, as previously reported (De Carolis et al., 2014; Kim et al., 2016; Seuylemezian et al., 2018). Accordingly, DW samples contributed the lowest percentage of unidentified isolates (19 %), probably because in the DWL, 92 out of 319 main spectrum profiles (MSPs) corresponded to DW samples, whereas process water was represented by only 28 MSPs. Notably, in SF samples the rate of identification increased by 24 % when both databases were used, although they still accounted for 46 % of unidentified isolates. According to the alpha diversity indices, SF samples had a low diversity and therefore the unidentified fraction may correspond to numerous isolates of only a few taxa not represented in the database.

The bacterial diversity was influenced by the treatment applied, especially chlorination, which caused a strong reduction in the final drinking water. Nevertheless, the results need careful interpretation, as the identified and unidentified isolates (clusters or singles) were evaluated using alpha diversity indices with a cut-off at the genus level.

Trends in diversity differed according to the identification method used. Metabarcoding indicated that diversity was highest in GW followed by RW, with a gradual reduction during the successive treatments until CF; after a temporary increase, it was drastically reduced in the DW. According to MALDI-TOF MS results, diversity was highest in RW followed by GW, and after a similar decrease in subsequent stages, underwent an increase in OZ samples; only small differences in diversity were observed between the other stages, except the post-chlorination reduction.

Regarding taxonomic assignments, MALDI-TOF MS was able to identify 30 % of isolates at the species level, while in some cases metabarcoding could not provide classification beyond the genus level due to the small size of the sequenced DNA fragment. A total of 36 % of reads were unsuccessfully assigned to genera, and 6 % of reads corresponded to Candidatus (sequence-based potential new taxa as-yet uncultured).

At the phylum level, MALDI-TOF MS identified Proteobacteria as predominant in source water (51 % on average), its relative abundance decreasing during the pretreatment stages, when Bacteroidota increased,

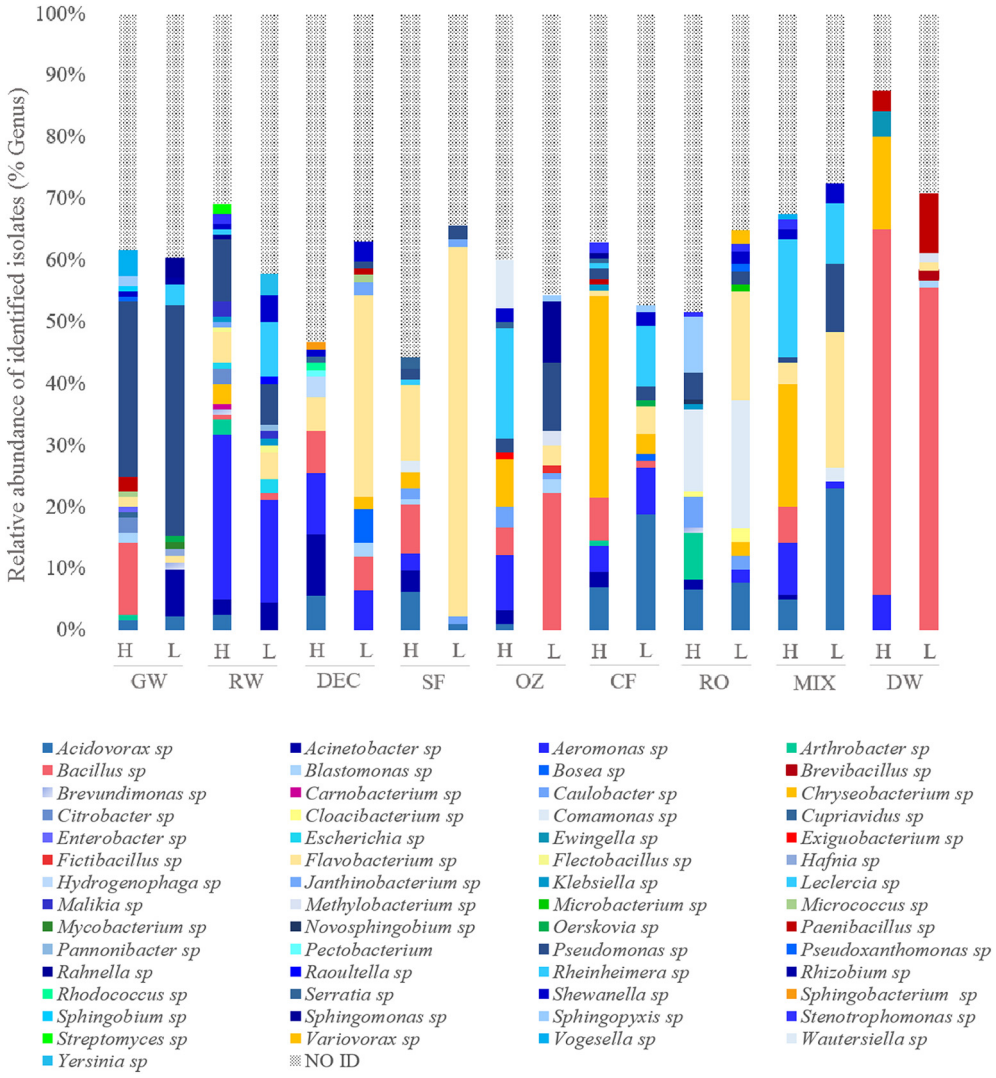


Fig. 7. Seasonal variation in genus diversity in the 63 DWTP samples: groundwater (GW), river water (RW), decantation (DEC), sand filtration (SF), ozonization (OZ), carbon filtration (CF), reverse osmosis (RO), mixing chamber (MIX) and post-chlorination drinking water (DW). Samples are grouped according to the ambient temperature during the sampling campaign: high (H) or low (L). Dots represent the unidentified fraction (NO ID).

and prevailing again in conventional and advanced treatments. Both phyla predominated throughout the treatment until chlorination, when Firmicutes clearly increased its relative abundance (64.1 %), becoming the dominant phylum in the final DW. The proportion of Firmicutes was <8 % in source water and treatment samples, except at the OZ stage, when it reached 14 %. Similar trends have been described by other researchers (Atnafu et al., 2021; Li et al., 2017; Sala-Comorera et al., 2017). The domination of Firmicutes in drinking water may be attributed to the greater resistance of Gram-positive bacteria to chlorine disinfection in water systems compared to Gram-negative bacteria, which are more susceptible to disinfectants (Mir et al., 1997).

Succession was also observed at the genus level: *Aeromonas* and *Pseudomonas* predominated in source water, *Flavobacterium* in pretreatment stages, *Bacillus* in OZ, *Chryseobacterium* in CF, *Comamonas* in RO, *Rheinheimera* in MIX and *Bacillus* in DW. All these genera have been previously reported in drinking water environments (Atnafu et al., 2021; Fish and Boxall, 2018; Sala-Comorera et al., 2017, 2020).

In the studied system, spore-forming bacteria belonging to the *Bacillus* genus resisted all treatments and became dominant in the final drinking water, increasing after the disinfection procedures of ozonization and chlorination. Different species of *Bacillus* were identified in the DW: *B. cereus*, *B. cibi*, *B. horneckiae*, *B. indicus*, *B. idriensis*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. mycoides*, *B. simplex*, *B. pumilus*, *B. sonoriensis*, *B. thuringiensis* and *B. weihenstemphanensis*. However, reflecting the difficulties in differentiating between *Bacillus* species, and perhaps influenced by the degree of sporulation, the MALDI-TOF identification scores ranged widely from 1.707 to 2.459 (Shu and Yang, 2017). Moreover, as some species within this genus are closely related, their identification with the 16S rRNA sequencing reference method was also challenging, and most isolates were not classified beyond the genus level. *B. cereus* is included in the Risk Group Database (ABSA, 2020) as a human and animal pathogen, but in this study it was not possible to safely distinguish it from other similar species such as *B. thuringiensis*, *B. mycoides and B. weihenstemphanensis*, none of which are human pathogens. On the other hand, some surfactin-producing *Bacillus* species have been reported as control agents against *Legionella pneumophila* due to the antagonistic activity of the biosurfactant (Loiseau et al., 2015) and some species are used in bioremediation of aquaculture water (Hlordzi et al., 2020).

MALDI-TOF MS analysis was able to detect taxonomic changes related to seasonality in some of the treatment stages. Temperature has been described as a modulator of microbial dynamics, which may disrupt the community composition in water (Degerman et al., 2013). For instance, both DEC and SF samples presented a clear predominance of *Flavobacterium* at low temperatures, whereas at higher temperatures, there was a greater diversity of genera. Conversely, *Chryseobacterium* was more prevalent in CF at high than at low temperatures.

Table 1
Heatmap showing the relative abundance of the 57 bacterial genera identified by MALDI-TOF MS and their presence/absence and abundance according to metabarcoding analysis in all the DWTP stages. Data are illustrated by a color scale spanning from green (not detected) to red (high relative abundance). Boxes edged in black indicate identification only by MALDI-TOF MS, while boxes edged in white correspond to exclusive identification by metabarcoding. Stars indicates differences in taxon classification by metabarcoding: **Escherichia-Shigella*, ***Hafnia-Obesumbacterium*, ****Methylobacterium-Methylorubrum*, ****Microbacteriaceae, ******Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*.

Genera	GW		RW		DEC		SF		OZ		CF		RO		MIX		DW	
	MALDI	16S	MALDI	16S	MALDI	16S	MALDI	16S	MALDI	16S	MALDI	16S	MALDI	16S	MALDI	16S	MALDI	16S
Acidovorax	1.9	0.007	1.4	0.8	2.7	0.5	3.9	0.6	0.6	1	12.1	0.3	7.1	0.8	12.8	0.2	0	0.04
Acinetobacter	3.3	0.2	3.3	2.0	4.9	0.8	2.0	0.7	1.1	0.2	1.4	0.02	0.9	2.0	0.5	0.004	0	0
Aeromonas	0	0.02	22.4	0.4	8.2	0.2	1.5	0.3	4.4	2	5.8	3	0.9	0.8	5.2	4	3.6	2
Arthrobacter	**0.5**	0	**1.4**	0	0	0	0	0	0	0	**0.5**	0	**4.3**	0	0	0	0	0
Bacillus	**6.6**	0	**1.0**	0	6.0	0.010	**4.4**	0	13.3	0.005	**4.3**	0	0	0	**3.3**	0	57.8	0.05
Blastomonas	**0.9**	0	0	0	1.1	0.3	0.5	0.3	1.1	0.5	0.0	0.1	0	0.6	0	0.1	**0.5**	0
Bosea	0	0.006	0	0.03	2.7	0.2	0	0.1	0	0.2	0.5	0.06	0	0.06	0	0.08	0	0.03
Brevibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.5**	0
Brevundimonas	0.5	0.008	0.5	0.2	0	0.03	0	0.2	0	0.2	0	0.2	0.5	4.0	0	0.3	0	0.009
Carnobacterium	0	0	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Caulobacter	0	0.07	0.0	0.08	0	0.1	1.5	0.2	2.2	0.06	0	0.0	3.8	7.0	0	0.2	0	0
Chryseobacterium	0	0	1.9	0.01	**1.1**	0	1.5	0.01	3.9	0.4	19.8	0.08	0.9	0.06	11.4	0.1	9.4	0.9
Citrobacter	1.4	0.008	**1.4**	0	0	0	0	0.02	0	0	0	0.01	0	0.02	0	0.01	0	0
Cloacibacterium	0	0.008	0	0.2	0	0.08	0	0.2	0	0.06	0	0.02	1.4	0.1	0	0.01	0	0.003
Comamonas	0	0.4	0	0	0	0	1.0	0.02	0	0.2	0	0	16.6	2.0	0.9	0.1	0	0.8
Cupriavidus	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0
Enterobacter	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*Escherichia **	0	0	1.4	0.003	0	0.007	0	0.009	0	0	0	0	0	0	0	0	0	0
Ewingella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**2.6**	0
Exiguobacterium	0	0	0	0	0	0	0	0	0.6	0.001	0	0	0	0	0	0	0	0
Fictibacillus	0	0	0	0	0	0	0	0	**0.6**	0.0	0	0	0	0	0	0	0	0
Flavobacterium	1.4	0.05	4.8	11.0	19.2	14.0	33.5	15.0	1.7	9.0	2.4	7.0	7.6	8.0	11.4	7.0	0.5	2.0
Flectobacillus	0	0.002	1	0.008	0	0.02	0	0.008	0	0	0	0.002	0	0.03	0	0.003	0	0
*Hafnia***	**0.5**	0	0	0	0	0	0	0	0	0	0	0.001	0	0	0	0	0	0.1
Hydrogenophaga	0	0.8	0	2.0	1.6	5.0	0	2.0	0	1.0	0	3.0	0	0.6	0	2.0	0	0.2
Janthinobacterium	0	0	0.5	0.009	1.1	0.02	0.5	0.1	0	0.05	0	0.06	0	0.8	0	0.07	0	0.01
Klebsiella	0	0.003	1.0	0.02	0	0.01	0	0.02	0	0.8	0.5	0.004	0.5	0.002	0	0	0	0.2
Leclercia	0	0	0	0	0	0	**0.5**	0	0	0	0	0	0	0	0	0	0	0
Malikia	0	0	1.9	0.9	0	1	0	1	0	0	0	0.2	0	0.3	0	0.2	0	0
*Methylobacterium****	0	0	0	0	0	0	0	0	1.1	0.03	0	0	0	0	0	0	**0.5**	0
*Microbacterium*****	0	0	0	0	0	0	0	0	0	0	0	0	0.5	0.09	0	0	0	0
Micrococcus	**0.5**	0	0	0	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0
Mycobacterium	0.5	0.002	0	0	0	0.02	0	0.02	0	0.3	0	0.02	0	0.009	0	0.003	0	0.2
Novosphingobium	0	0.03	0	0.5	0	1.0	0	0.3	0	0.2	0	0.2	0.5	0.3	0	0.1	0	0.8
Oerskovia	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paenibacillus	**1.4**	0	0	0	**0.5**	0	0	0	0	0	**0.5**	0	0	0	0	0	5.7	0.004
Pannonibacter	0	0	0.5	0.003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pectobacterium	0	0	0	0.002	**0.5**	0	0	0.002	0	0	0	0	0	0	0	0	0	0
Pseudomonas	32.2	1.0	8.6	3.0	0.5	2.0	2.0	3.0	6.7	8.0	1.9	2.0	3.3	5.0	5.2	3.0	0	1.0
Pseudoxanthomonas	**0.5**	0	0	0.1	0	0.007	0	0	0	0.003	0	0	0.5	0.02	0	0	0	0.004
Rahnella	0	0	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Raoultella	0	0	**0.5**	0	0	0	0	0.005	0	0	0	0	0	0.008	0	0.02	0	0.01
Rheinheimera	1.4	0.3	4.3	0.6	0	0.8	0	1	8.9	6.0	4.8	4.0	0	0.2	15.2	3.0	0	0.6
*Rhizobium******	**0.5**	0	0	0.06	0	0.1	0	0.08	0	0.05	0	0.1	0	0.2	0	0.2	0	0.005
Rhodococcus	0	0	0	0	**0.5**	0	0	0	0	0	0	0	0	0	0	0	0	0
Serratia	0	0	0	0	**0.5**	0	**1**	0	**0.6**	0	**0.5**	0	0	0	0	0	0	0.07
Shewanella	0.5	0.01	2.4	0.06	2.2	0.02	0	0.03	1.1	0.9	1.0	0.3	0.9	1.0	2.4	0.2	0	0.003
Sphingobacterium	0	0	0	0	**0.5**	0	0	0	0	0.001	0	0	0	0	0	0	0	0
Sphingobium	0.5	0.006	0	0.002	0	0	0	0.003	0	0.2	0	0	0	0.05	0	0	0	0.06
Sphingomonas	1.4	0.05	0	0.09	0	0.09	0	0.4	5.0	0.5	0.5	3.0	0	0	0	1.0	0	0.3
Sphingopyxis	0.9	0.02	0	0.01	0	0.6	0	0.006	0.6	0.2	0.5	1.0	5.2	1.0	0	0.5	0	0.04
Stenotrophomonas	0	0	1.0	0.02	0	0.002	0	0.001	0	0.01	1.0	0.01	0.9	0.005	0.9	0.006	0	0.006
Streptomyces	0	0	**1.0**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Variovorax	0	0	0	0.006	0	0.07	0	0.03	0	0	0	0.6	0.9	0.4	0	0.3	0	0.02
Vogesella	2.4	0.04	0	0.02	0	0.003	0	0.004	0	0.06	0	0.05	0	0.5	0.5	0.09	0	0
Wautersiella	0	0	0	0	0	0	0	0	**3.9**	0	0	0	0	0	0	0	0	0
Yersinia	0	0	1.4	0.007	0	0.005	0	0.02	0	0.02	0	0.02	0	0.006	0	0.01	0	0.2

At the phylum level, the two techniques provided similar results, showing DWTP domination by *Proteobacteria* except in the final water, in which chlorination exerted a strong selective pressure, resulting in lower diversity and a taxonomic shift to Cyanobacteria (metabarcoding) or Firmicutes (MALDI-TOF MS). At lower taxonomic levels, the results differed to a greater extent, although the most abundant genera identified by MALDI-TOF MS were all detected by metabarcoding. In DEC, SF, RO and MIX samples, the most abundant genera were the same according to both methods: *Flavobacterium* in the pretreatment stages and MIX, and *Comamonas* (or uncultured Comamonadaceae by metabarcoding) in RO. The taxa identified in the other treatments varied (Pinar-Méndez et al., 2022). For instance, in GW the dominant genus was *Candidatus Omnitrophus* (25 %) according to metabarcoding and *Aeromonas* (22 %) by MALDI-TOF MS, while the final DW was dominated by uncultured members of Obscuribacteraceae (31 %) or by *Bacillus* (58 %), respectively.

Additionally, some genera were identified only by one of the two techniques. For instance, metabarcoding detected a greater variety of Proteobacteria at the genus level in DW. Despite the use of disinfectant residuals, certain chlorine-resistant taxa remained in the final treated water. According to other studies, the class Gammaproteobacteria is more resistant to free chlorine than Alphaproteobacteria (Mathieu et al., 2009). Moreover, some of the genera reported in the present study are associated with biofilm formation, such as *Bosea*, *Comamonas*, *Methylobacterium*, *Pseudomonas*, *Sphingobium* and *Sphingomonas* (Fish and Boxall, 2018). It is

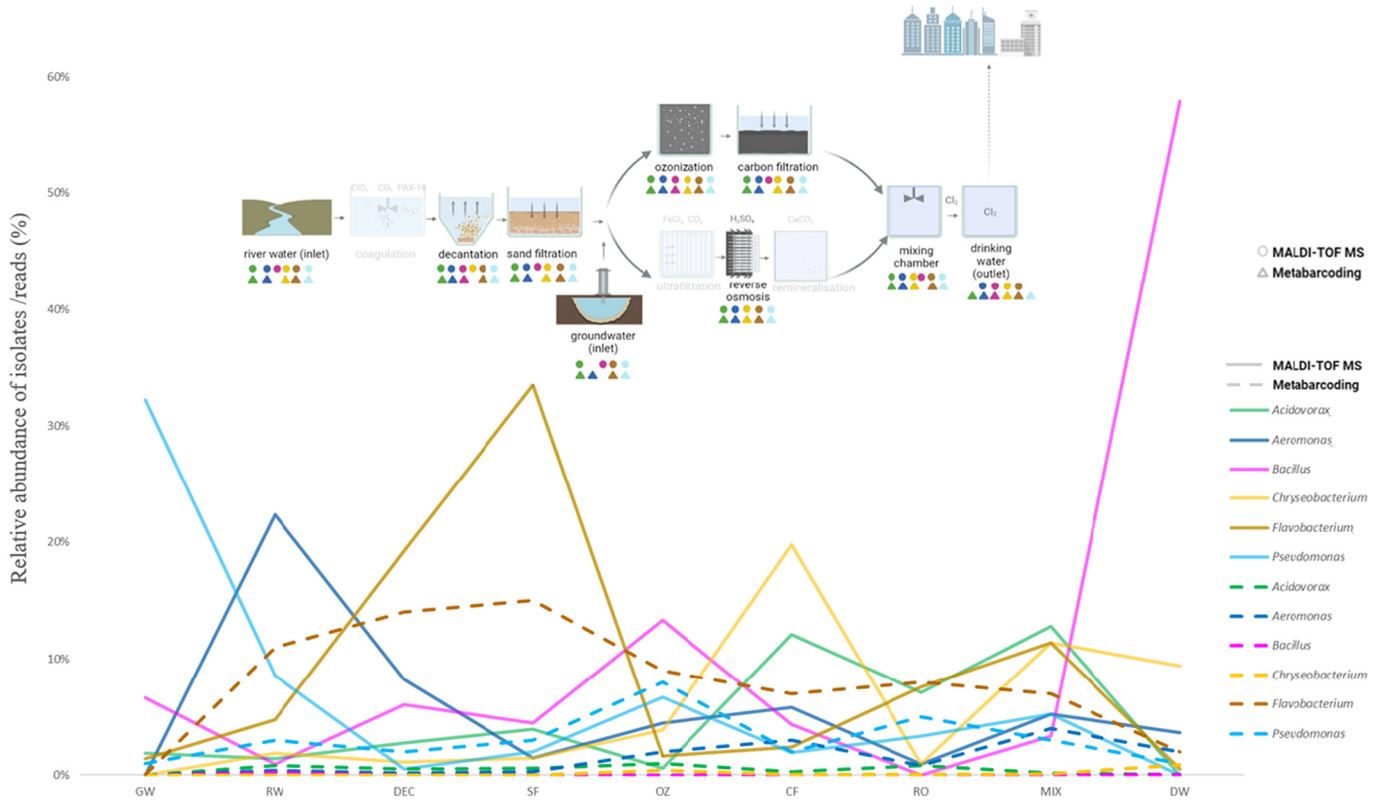


Fig. 8. Fluctuation graph of the relative abundance of the six most abundant genera in the DWTP stages according to MALDI-TOF MS results (solid line) and relative abundance reads by metabarcoding (dotted line). In the schematic diagram of the DWTP the genera are indicated by a circle for MALDI-TOF results or a triangle for metabarcoding. Samples corresponded to groundwater (GW), river water (RW), decantation (DEC), sand filtration (SF), ozonization (OZ), carbon filtration (CF), reverse osmosis (RO), mixing chamber (MIX) and post-chlorination drinking water (DW).

important to detect which taxa are seeding from biofilms into the distribution network to prevent possible regrowth on water pipes, which is also favored by high chlorine concentrations or rechlorination (Fish and Boxall, 2018; Mathieu et al., 2009). Water quality can be subsequently affected by bacterial mobilization into the water column. However, only a few isolates of these taxa were detected by MALDI, perhaps hindered by the dominance of *Bacillus* at this stage.

The most abundant genera detected in DW, *Bacillus* (Firmicutes) according to MALDI-TOF and unknown Obscuribacteraceae (Cyanobacteria) according to metabarcoding, have high resistance to disinfection procedures. However, the Cyanobacteria group includes a large proportion of uncultured strains. In the NCBI database, about 49 % of cyanobacterial genomes correspond to uncultured metagenome-assembled strains (Dextro et al., 2021) and may therefore be undetectable by culture-based techniques such as MALDI-TOF MS. Conversely, *Bacillus* was identified by metabarcoding, although in very low relative abundance.

Both technologies have benefits and shortcomings. Metabarcoding analysis provided a more detailed view of the bacterial communities in the DWTP water samples than MALDI-TOF MS, revealing a higher diversity and additional taxa. However, amplicon sequencing is currently unable to differentiate between live or dead cells, so the resulting data cannot provide a reliable assessment of hazards associated with specific taxa. Moreover, the results may be strongly influenced by nucleic acid extraction procedures, be biased against rare taxa, and samples low in DNA, such as chlorinated water, may be contaminated by the DNA reagent (Salter et al., 2014). To overcome the analytical problem of low microbial biomass in the DWTP, ultrafiltration by Rexeed was used, which concentrates large volumes of water and thus allows a more representative characterization of bacterial communities. This method allowed sufficient microbial biomass to be recovered for both MALDI-TOF and metabarcoding analyses.

As a cultured-based method, MALDI-TOF MS can focus on the viable microorganisms of a microbial community, although the characterization results can be skewed by other factors. Thus, sample processing (direct plating, filtration/concentration procedures), media nutrient composition, growth conditions (aerobic or anaerobic, incubation time and temperature), the protein extraction method as well as the representativeness of the mass spectra database are all factors that can impede identification. However, with a suitable database, proteomics studies by MALDI-TOF MS represent a rapid and reliable microbial identification strategy that provides data directly from isolated bacteria in a few minutes. Easy to use and low cost, it can be applied in routine bacterial monitoring in DWTPs and distribution systems, and the data interpretation does not require advanced bioinformatics skills. Nevertheless, culturable bacteria represent a minor fraction of the overall microbiome, so the evaluation of bacteria only by culture-based methods may underestimate the microbial diversity in a water system.

Both MALDI-TOF MS and metabarcoding detected shifts in the bacterial community composition in the DWTP that could be explained by bacterial attachment to or detachment from biofilms, the displacement or removal of certain taxa by the treatments, or selective microbial growth. Although these changes may influence water quality, they were not reflected in the quantitative data of HPC analysis required by the water safety regulations. Therefore, the information generated by the combined application of these proteomic and metagenomic tools in addition to the monitoring of regulated microbial indicators should increase our understanding of how DWTPs function and improve water quality management. However, a more complete understanding of the complexity of microbial dynamics in a DWTP would require knowledge about the metabolic activity and functions of the resident microbiota (Douterelo et al., 2014). Furthermore, the characterization of the bacterial populations in specific events, such

as contamination episodes, in which the water quality required by the regulations is not met, may help to identify specific bacterial indicators of the impaired treatments to be used as an early alert for water managers of the alteration of the established microbiota in the DWTP. For this, either a qPCR approach could be developed based on the 16S rRNA sequences obtained from the amplicon sequencing, or the detection of these specific genera by MALDI-TOF MS (if culturable), could be used as routine tests.

5. Conclusions

The HPC in analyzed samples decreased along the successive water treatments, increasing with ambient temperature in some processes. Both MALDI-TOF MS and 16S rRNA metabarcoding revealed a high bacterial diversity in the DWTP, with the highest values obtained in source water and the lowest in the final drinking water. Bacterial monitoring by the two techniques provided insights into the taxa present in the different DWTP stages, with low-abundance species being detected and some genera identified by only one approach. Spore-forming bacteria of the *Bacillus* genus (Firmicutes) resisted all treatments and was the dominant genus in drinking water according to MALDI-TOF MS, whereas metabarcoding indicated that members of the unculturable Obscuribacteraceae (Cyanobacteria) were more abundant. Monitoring microbial fluctuations between treatment stages and seasons is of interest for drinking water management and can help assess the impact of specific events on treatment effectivity and water quality. Overall, the combined use of multiple approaches constitutes a powerful monitoring tool of bacterial community dynamics in DWTPs, although they are not meant to be used on a routinely base due to the current cost and turnaround time. The obtained results from both technologies can be used to have a deeper knowledge of water microbiome inside a DWTP and to identify potential indicator species of the regular operation of the DWTP. Further research is needed in order to identify them.

CRediT authorship contribution statement

A.P.-M.: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Funding acquisition. **B.G.**: Conceptualization, Resources, Project administration, Supervision, Writing – review & editing. **A.R.B.**: Conceptualization, Methodology, Resources, Project administration, Supervision, Writing – review & editing. **C.G.-A.**: Conceptualization, Methodology, Resources, Formal analysis, Supervision, Project administration, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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