Heterotrophic monitoring at a drinking water treatment plant by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry after different drinking water treatments

Laura Sala-Comorera, Anicet R. Blanch, Carles Vilaró, Belén Galofré and Cristina García-Aljaro

ABSTRACT

The aim of this work was to assess the suitability of matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) for routine heterotrophic monitoring in a drinking water treatment plant. Water samples were collected from raw surface water and after different treatments during two campaigns over a 1-year period. Heterotrophic bacteria were studied and isolates were identified by MALDI-TOF MS. Moreover, the diversity index and the coefficient of population similarity were also calculated using biochemical fingerprinting of the populations studied. MALDI-TOF MS enabled us to characterize and detect changes in the bacterial community composition throughout the water treatment plant. Raw water showed a large and diverse population which was slightly modified after initial treatment steps (sand filtration and ultrafiltration). Reverse osmosis had a significant impact on the microbial diversity, while the final chlorination step produced a shift in the composition of the bacterial community. Although MALDI-TOF MS could not identify all the isolates since the available MALDI-TOF MS database does not cover all the bacterial diversity in water, this technique could be used to monitor bacterial changes in drinking water treatment plants by creating a specific protein profile database for tracking purposes.

Key words | drinking water, heterotrophic bacteria, identification, MALDI-TOF MS, water treatment

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INTRODUCTION

In efforts to protect human health, drinking water is one of the most extensively monitored natural resources (Eichler *et al.* 2006; Poitelon *et al.* 2009). In drinking water treatment plants, water is subjected to different treatments so as to ensure adequate chemical and microbiological quality (European Commission 1998). The different treatments exert a selective pressure which some groups of microorganisms can overcome and under certain conditions some can even grow. Fecal indicators and heterotrophic bacteria, among other microorganisms, should be monitored in the treated water supplied by drinking water treatment plants.

According to different drinking water regulations worldwide, fecal indicator bacteria (mostly *Escherichia coli*) should be absent in 100 mL water to certify there is no fecal pollution. Furthermore, according to the Spanish regulations concerning drinking water (140/2003), the total aerobic heterotrophic colony count (HPC) in treated water must not exceed 100 CFU/mL after incubation at 22 °C for 3 days. Additionally, the HPC should be routinely monitored at 22 °C so as to ensure that no abnormal changes in it occur during distribution. The HPC does not indicate the sanitary conditions of water, but it reflects the overall bacteriological

doi: 10.2166/wh.2017.090

quality of water resources and the drinking water treatment process. For example, when continuously monitored, changes in HPC may reflect failures in the water treatment or regrowth of microorganisms in water distribution systems (Bartram *et al.* 2003; Allen *et al.* 2004; Diduch *et al.* 2016).

Although not included in the regulations, some water treatment drinking companies are increasingly interested in gaining knowledge of the heterotrophic communities routinely present at each treatment step (personal communication). Knowledge of the heterotrophic microbial populations and their dynamics through the different treatments and under different temperature conditions could be used to rapidly detect failures and thereby further improve water management in drinking water treatment plants. Furthermore, such information could reflect on the activities of waterworks, because the populations could be associated with biofilm formation or bacterial regrowth (Carter *et al.* 2000). Although new genomic techniques allow us to determine the diversity of the dominant bacterial populations in water samples (Hong *et al.* 2010; Pinto *et al.* 2012), they do not provide the necessary information on the culturability of bacteria nor, consequently, on the bacteria which are routinely monitored by the HPC according to the current regulations.

The characterization of the HPC by traditional methods, such as biochemical test galleries, is extremely time-consuming and therefore a novel methodology for routine water monitoring is needed. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) has emerged in recent years as a rapid proteomic method for the identification of bacteria from clinical settings (Welker 2011). The cost of the equipment is high, although there is minimal consumable cost after the initial investment (Bizzini & Greub 2010). MALDI-TOF MS is a library-dependent method based on spectral analysis of bacterial proteins, mainly ribosomal, using intact cells with no previous treatment (Welker 2011). It has been reported that the current commercial MALDI-TOF database could identify some bacterial strains from water samples (Sala-Comorera *et al.* 2016a, 2016b). The aim of this work was to assess the suitability of MALDI-TOF MS for routine heterotrophic bacteria monitoring of the different treatments performed in a drinking water treatment plant. The study was performed considering two different seasons, and it was complemented with the assessment of the diversity and population similarities using a biochemical phenotypic method (PhenePlate™ system).

METHODS

Sampling

Samples were collected from a drinking water treatment plant in Spain (Sant Joan Despí, Barcelona). In the plant (Figure 1), surface water from the River Llobregat is pre-oxidized using chlorine dioxide and suspended solids are removed by settling, followed by sand filtration. Then, groundwater is incorporated into the treatment process. From here, the water may be treated using two processes: one includes ozonization and granular activated carbon filtration to oxidize and remove organic constituents and residual disinfectants; the other includes the use of ultrafiltration and reverse osmosis. The two lines converge in a

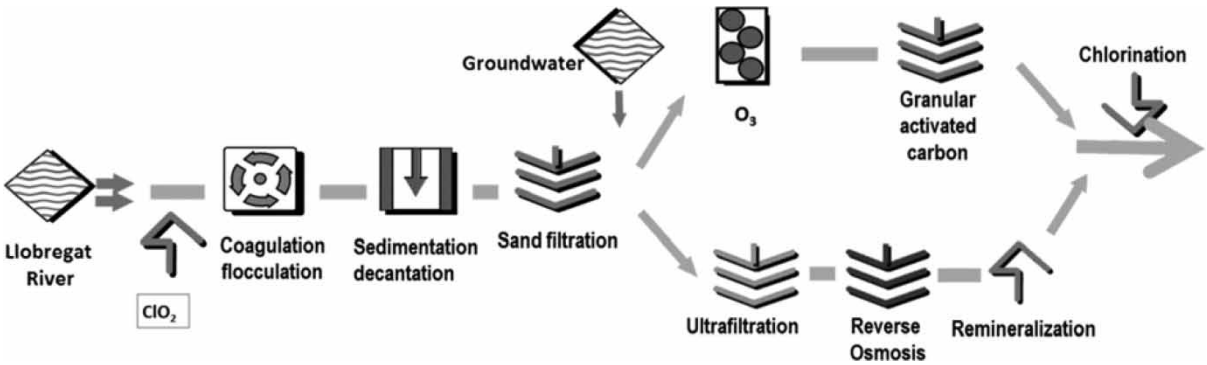


Figure 1 | Scheme of the water drinking treatment plant.

mixing chamber. Eventually, the water is chlorinated before leaving the drinking water treatment plant. The resultant disinfected water enters a distribution system which supplies 21 municipal pipeline distribution networks. The treatment plant serves a population of 1,200,000 from the Barcelona metropolitan region.

Samples were collected from five different stages of the water treatment process: raw surface water (R), after sand filtration (S), after membrane ultrafiltration (U), after reverse osmosis (O), and from the drinking water reservoir after the chlorination (T).

Volumes of water between 0.001–0.1 mL of raw surface and sand filtration water, 100–350 mL from ultrafiltration and reverse osmosis water, and 1 L of treated water were filtered so as to obtain isolates. Immediately after sampling, sterile sodium thiosulfate was added so as to neutralize chlorine from treated water. Water samples were kept cold before filtration and they were analyzed within 3 h of collection. Membrane filtration was performed using 0.45 μm pore size membranes (Millipore, Germany), following standard techniques. The membranes with the filtrated samples were incubated on Water Plate Count Agar (ISO) plates (Oxoid, UK) at 22 °C ± 2 °C for 3 days. The medium was appropriate for the cultivation of microorganisms of water, according to ISO 6222:1999 – Water quality: Enumeration of culturable micro-organisms, colony count by inoculation in a nutrient agar culture medium. The isolates were sub-cultured on the same medium. Two sampling campaigns were performed. The first sampling was performed in winter, when the temperature of the water was at its lowest (around 13 °C); while the second was carried out in summer, when the temperature of the water was at its highest (around 22 °C). The HPC counts from the different steps were determined weekly during both sampling campaigns. In summary, a total of 25 HPC counts for each season were performed. For diversity studies, 30 strains from each step were isolated from the plates following the procedures previously reported (Bianchi & Bianchi 1982) to assess the heterotrophic community diversity at two different sampling occasions in the two different seasons.

Mass spectrometry analysis

All the isolated strains were analyzed by MALDI-TOF MS using the procedure detailed below. A small quantity of the colony growth on the Water Plate Count Agar (ISO) was transferred to a metallic MALDI-TOF MS plate and 1 μL of matrix (saturated alpha-cian-4-hydroxycinamic acid-50% acetonitrile–2.5% trifluoroacetic acid) was added to cover the specimen preparation. The plate was then left to dry for 5 min at room temperature. Measurements were performed using a Mircroflex II mass spectrometer (Bruker Daltonick GmbH, Germany) equipped with a 60 Hz laser. The proteic spectra obtained with the spectrometer were processed using the Bruker MALDI Biotyper v2.0 software (Bruker Daltonik GmbH) and compared with the spectrum database. The Biotyper software compares each sample mass spectrum to the reference mass spectra in the database, and calculates an arbitrary logarithmic unit score value between 0 and 3 reflecting the similarity between the sample and the reference spectrum. The identification score criteria used were those recommended by the manufacturer: <1.700 was interpreted as not a reliable identification; 1.700 to 1.999 indicated identification at the genus level; while a score ≥2.000 identified the species level (Schulthess *et al.* 2013).

Indices of population diversity and similarity

Simpson's diversity index (Di) was used to calculate the diversity of the bacterial populations at the different sampling points and for each season (Hunter & Gaston 1988). Di is a relative measure of the distribution of isolates into different phenotypes; while the similarity between populations was calculated using the coefficient of population similarity (Sp) (Kühn *et al.* 1991). The populations are considered similar when the Sp value is greater than 0.2 (Kühn *et al.* 1991). The diversity indexes were calculated using the biochemical profiles obtained with the miniaturized biochemical phenotyping method, the PhenePlate™ system, PhP-48 plates (PhPlate Microplate Techniques AB, Sweden) as previously described (Casanovas-Massana & Blanch 2012). Briefly, the PhenePlate system is a miniaturized biochemical phenotyping method based on evaluation of the kinetics of several biochemical reactions performed in microtiter plates. The comparison of the bacterial populations from the various samples was analyzed using the unweighted-pair group method analysis (UPGMA) with average linkage. The index calculations were performed

using the software PhPWin® (PhPlate Microplate Techniques AB, Sweden).

Statistical analyses

Statistical analyses for the differences in HPC counts in the two different campaigns was performed using the Mann–Whitney–Wilcoxon test with median concentrations using Statgraphic software (Statgraphics.net, Spain).

RESULTS

The water treatment plant showed a reduction of around 4 $\log_{10}$ units in the HPC (Table 1). Sand filtration reduced the HPC in 1–2 $\log_{10}$ ($p = 7.3 \times 10^{-6}$ in winter and $p = 2.7 \times 10^{-8}$ in summer, Mann–Whitney–Wilcoxon test), whereas no significant changes were observed after ultrafiltration treatment ($p = 0.84$ in winter and $p = 0.68$ in summer). After reverse osmosis, the counts dropped sharply ($p = 2.9 \times 10^{-9}$ in winter and $p = 7.2 \times 10^{-8}$ in summer) and, although reverse osmosis filtration would be expected to eliminate all bacterial cells, heterotrophic bacteria were still detected. It is not possible to establish the origin of the isolates after RO, since RO membranes retain bacteria very effectively and, in general, do not allow bacteria or viruses to pass through the membrane. However, the detection of microorganisms after RO was not unexpected, since the pipes that connected the RO system to the remineralization tank are covered but are not a sterile system, and microorganisms could eventually recolonize the system (Sala-Comorera *et al.* 2016a, 2016b). Moreover, the bacterial community in the permeate water must be adapted to selective pressures exerted by that water, with low nutrient levels, and chemical parameters of the permeate water (conductivity 75 µS/cm and TOC <1 mgC/L). As previously, after the chlorination step, the HPC decreased significantly in winter ($p = 1.0 \times 10^{-8}$) and summer ($p = 8.0 \times 10^{-10}$). Raw and treated water showed similar values in both seasons, whereas the concentration after sand filtration, ultrafiltration, and reverse osmosis was between 0.5 and 1 $\log_{10}$ units higher in summer than in winter. A total of 277 colonies were isolated from the water treatment process for further characterization: 133 isolates corresponding to winter and 144 to summer.

Identification of the isolates by MALDI-TOF MS

All the isolates representing the microbial cultivable communities suspended in the water column after the different treatments applied in the water treatment plant were characterized by MALDI-TOF MS. This methodology allowed the identification of the isolates into different levels of classification from phylum to species. The dominant phyla (Figure 2) for all the sampling sites combined were, in descending order: *Proteobacteria* (51%), *Firmicutes* (35%), *Bacteroidetes* (9%), and *Actinobacteria* (5%). Despite the dominance of *Proteobacteria* in the first stages, when moving along the water treatment process, the relative abundance of *Proteobacteria* decreased. In contrast, the relative abundance of *Firmicutes* increased in both sampling occasions, becoming the dominant population after chlorination.

In winter sampling (Figure 2), at least 85% of the isolates retrieved from the raw water and the first steps (sand

Table 1 | Seasonal HPC at the different treatment steps and the distribution of the isolates used in the study

	Winter		Summer	
Step	**$\log_{10}$ (CFU mL^{-1})**	**No. of isolates**	**$\log_{10}$ (CFU mL^{-1})**	**No. of isolates**
Raw water (R)	4.66 (±0.55)	26	4.50 (±0.51)	30
After sand filtration (S)	2.27 (±0.23)	24	3.05 (±0.67)	25
After ultrafiltration (U)	2.34 (±0.34)	25	2.95 (±0.41)	29
After reverse osmosis (O)	0.97 (±0.32)	29	1.32 (±0.25)	30
After chlorination treatment (T)	<0.03	29	<0.13	30

The HPC counts were determined weekly during 1 year ($N = 25$ for each group). Values of standard deviation for colony counts are indicated in brackets.

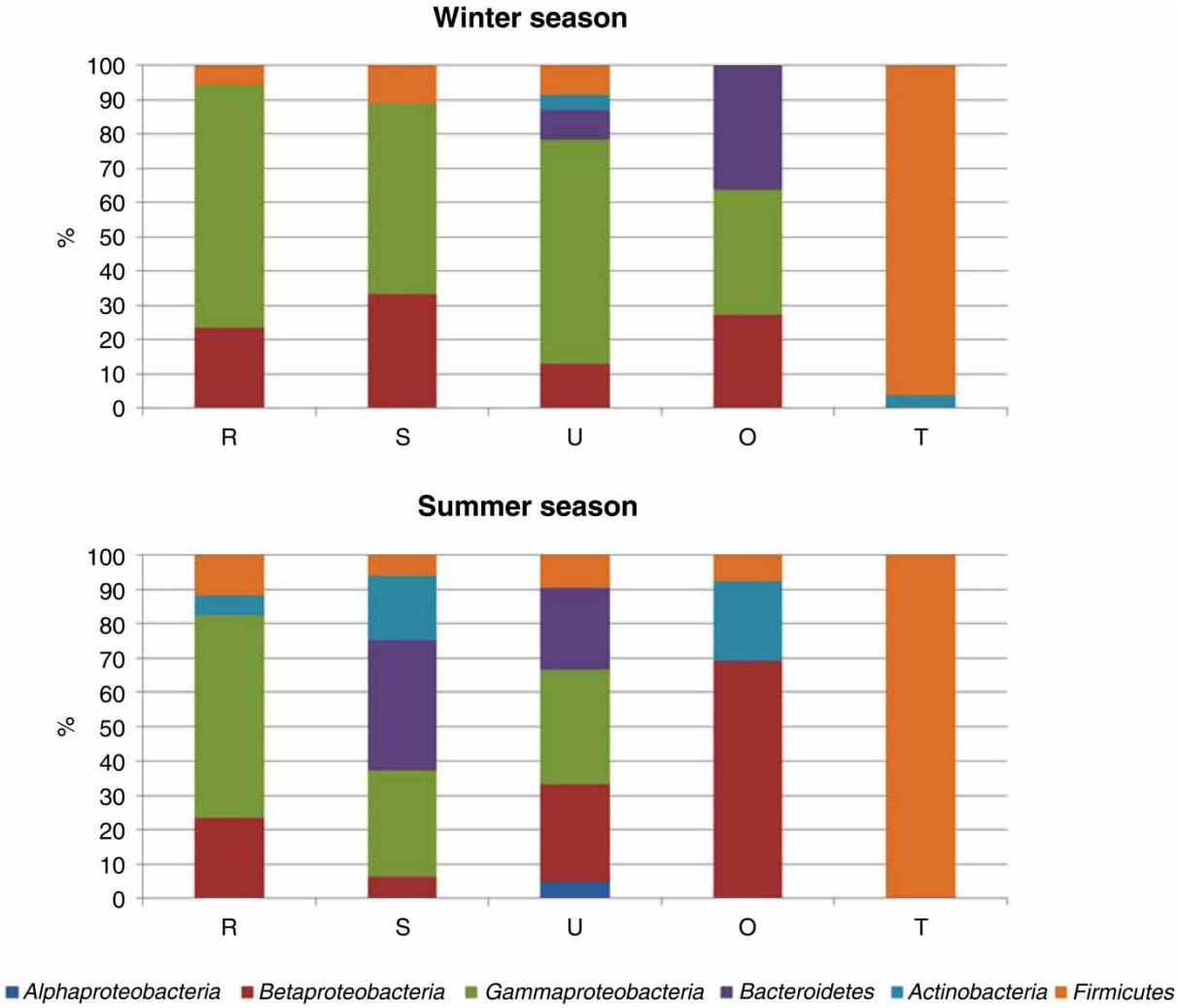


Figure 2 | Relative abundance of bacterial phyla at the five sampling locations in each season. The dominant phylum, *Proteobacteria*, is divided into classes: *Alphaproteobacteria, Betaproteobacteria*, and *Gammaproteobacteria*. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water (mixture of the treatment lines ozone/granular activated carbon and ultrafiltration/reverse osmosis, followed by chlorination).

filtration and ultrafiltration) were assigned to *Betaproteobacteria* (20%) and *Gammaproteobacteria* (65%). The number of isolates belonging to the *Proteobacteria* phylum decreased after the ultrafiltration treatment. Among the strains isolated after the ultrafiltration, an increased abundance of isolates affiliated to the phylum *Bacteroidetes* was observed, yet no member of this phylum was observed in the raw water or after sand filtration. Members of the phylum *Actinobacteria* were only isolated in the ultrafiltration step. *Firmicutes*, which represented less than 10% of the community in the early treatments, represented 96% of the community after the chlorination step.

In summer sampling (Figure 2), the isolates affiliated to the *Proteobacteria* phylum represented a smaller part of the overall community (63%) than in winter. *Betaproteobacteria* represented 20% of the isolates, *Gammaproteobacteria* 41%, and *Alphaproteobacteria* 2%, this class was not identified in winter sampling. *Betaproteobacteria* greatly increased in the reverse osmosis, and constituted 69% of the isolates after this step. During sand filtration and ultrafiltration, the content of *Bacteroidetes* was relatively stable; this phylum was not detected in the other stages of the treatment. *Actinobacteria* abundance increased in the successive treatments from raw water to the reverse osmosis, accounting for nearly 20%

of the reverse osmosis effluent community; however, it was not present after the ultrafiltration step. The isolates affiliated to the *Firmicutes* phylum dominated the community of the treated water, as in winter sampling.

MALDI-TOF MS analysis allowed us to identify at genus or species level 65% of all the strains isolated from the water samples according to the database available. The percentage of the genera identified at each treatment and the percentage of not identified are shown in Table 2. The percentages of the identifications differed in the treatments analyzed in this study. After some treatments, sand filtration and reverse osmosis, more than 50% of the isolates could not be identified with any taxon, according to the commercially available database, whereas the isolates from ultrafiltration and treated water in both sampling occasions showed the highest identification score and isolates could be classified with high confidence. Regarding the identification level (species or genus), a larger number of strains from raw water, sand filtration, and ultrafiltration were classified at species level in both sampling occasions; whereas the majority of isolates from reverse osmosis and treated water could only be classified at genus level.

A total of 25 genera were identified from all the sampling locations and, in general, a decrease in genus diversity was observed with water treatment procedures (Table 3). However, after ultrafiltration, an increase in genus diversity was found in both samplings in comparison with the number of genera identified in the previous step. It should be noted that the percentage of not identified isolates after sand filtration was high. For this reason, the number of genera isolated did not represent the real diversity at this point in the particular period analyzed. Raw water showed the highest population diversity (12 genera). Most of the genera were detected in both seasons, such as *Acidovorax* sp., *Acinetobacter* sp., *Aeromonas* sp., *Bacillus* sp., and *Ralstonia* sp. Genera such as *Janthinobacterium* sp., *Pantoea* sp., and *Shewanella* sp. were isolated in winter; whereas *Dickeya* sp., *Lysinibacillus* sp., *Massilia* sp., and *Micrococcus* sp. were identified in summer. Some of the isolates identified after sand filtration were also present in the raw water, but two different new genera were detected in sand filtrated water in winter: *Pseudomonas* sp. and *Staphylococcus* sp. Meanwhile, in the corresponding summer isolates, three new genera were present: *Chryseobacterium* sp., *Elizabethkingia* sp., and *Staphylococcus* sp. Following water treatment, the majority of the genera identified in the ultrafiltration effluent were also observed in the sand filtration effluent and the raw water. Members of *Pseudomonas* sp. were numerically the most frequently encountered in the winter community, whereas the community in the summer was composed of members of different genera, primarily *Acidovorax* sp., *Aeromonas* sp., *Elizabethkingia* sp., and *Pseudomonas* sp. The number of genera identified in the reverse osmosis effluent was greatly reduced compared to the samples from the early treatment stages. Reverse osmosis reduced the microbial diversity to four genera in each season; the community was dominated by *Flavobacterium* sp. and *Pseudomonas* sp. in the winter and *Acidovorax* sp. in the summer. Chlorination resulted in a selective pressure that permitted only a few genera of bacteria to overcome the treatment, two per season. *Bacillus* sp. was the largest group found in the treated water in winter, together with *Kocuria* sp., whereas *Bacillus* sp. and *Lysinibacillus* sp. were the most common in summer.

The MALDI-TOF MS analysis also allowed us to identify some isolates at the species level. A total of 40 species belonging to 25 genera were identified, most of them

Table 2 | Percentages of isolates identified at taxonomic levels by MALDI-TOF MS at each treatment step

	Winter					Summer				
	R	S	U	O	T	R	S	U	O	T
Species	58	21	72	17	34	37	44	52	3	50
Genus	8	17	20	21	59	20	20	21	40	37
No reliable identification	34	62	8	62	7	43	36	27	57	13

R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water (mixture of the treatment lines ozone/granular activated carbon and ultrafiltration/reverse osmosis followed by chlorination).

Table 3 | Seasonal distribution of the heterotrophic community after the different treatment steps based on MALDI-TOF MS spectra at the genus level

	% Community in:									
	Winter					Summer				
	R	S	U	O	T	R	S	U	O	T
Acidovorax sp.	4			7		8	4	14	30	
Acinetobacter sp.	15		4			13	4	3		
Aeromonas sp.	15					18	16	11		
Arthrobacter sp.									3	
Bacillus sp.	4		8		90	3		8	3	70
Chryseobacterium sp.							16	3		
Comamonas sp.								3		
Cupriavidus sp.								3		
Dickeya sp.						3				
Elizabethkingia sp.							8	11		
Flavobacterium sp.			8	14						
Janthinobacterium sp.	4	13	12	3						
Kocuria sp.					3					
Lysinibacillus sp.						3				17
Massilia sp.						3				
Microbacterium sp.									7	
Micrococcus sp.			4			3	12			
Pantoea sp.	4									
Pseudomonas sp.		13	52	14				11		
Ralstonia sp.	8					3				
Serratia sp.			4							
Shewanella sp.	12	8								
Sphingopyxis sp.								3		
Staphylococcus sp.		4					4			
Wautersiella sp.								3		
No reliable identification	34	62	8	62	7	43	36	27	57	13

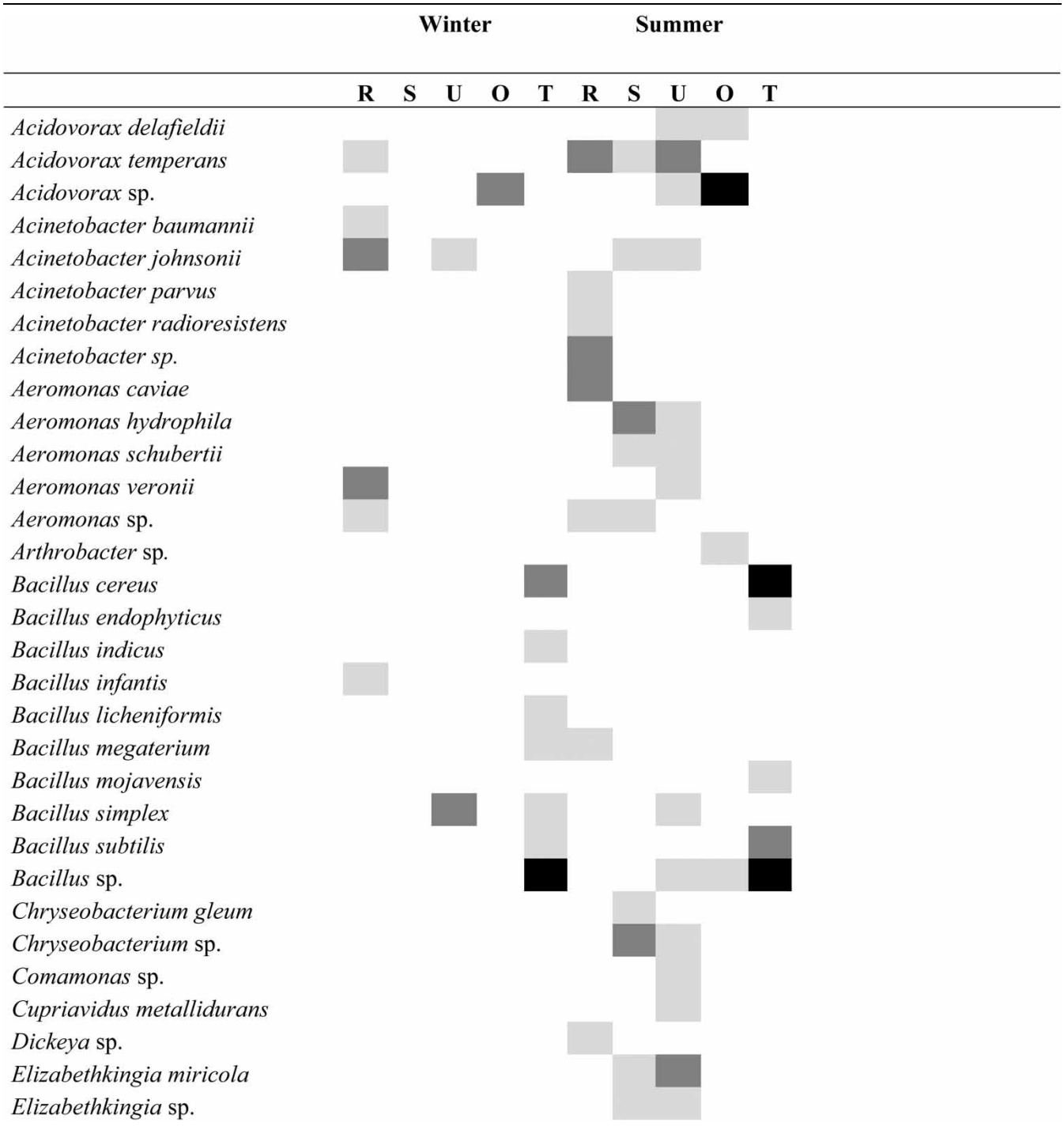
R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water (mixture of the treatment lines ozone/granular activated carbon and ultrafiltration/reverse osmosis followed by chlorination).

isolated once or twice at the different sampling sites (Table 4). The heterotrophic community of the treatment plant was composed of a large number of species, each of which represented a small fraction of the total community. The heterotrophic community underwent changes not only at the phylum level, but also with respect to the predominant genera and species. It should be noted that some species were found after at least three different consecutive treatments, but these species were different in each season: we identified *Janthinobacterium lividum* among the winter isolates, but *Acidovorax temperans* in summer.

Indices of population diversity and similarity

The isolates were further typed biochemically for comparison using the PhenePlate system, so as to calculate the Simpson diversity index (Di) of the samples. However, only 174 out of 277 isolates could be subjected to clustering

Table 4 | Number of isolates identified by MALDI-TOF MS at each point, at the species (cut-off score ≥2.000) and genus (cut-off score ≤2.000) levels



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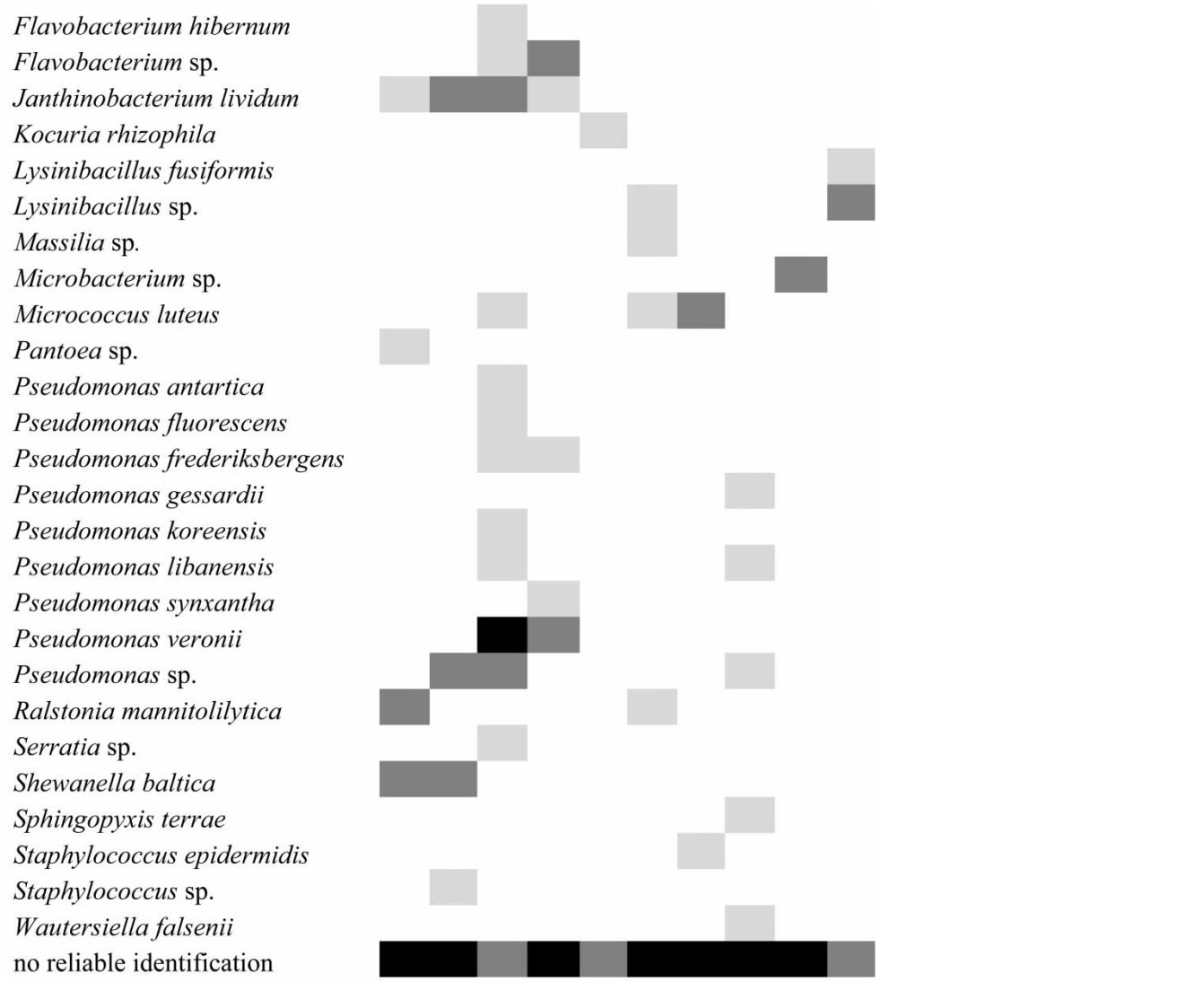

1 isolate, 2–4 isolates, ≥5 isolates

The genus of the isolates that could not be identified at the species level is indicated with sp.

R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water (mixture of the treatment lines ozone/granular activated carbon and ultrafiltration/reverse osmosis, followed by chlorination).

and population analysis, because a total of 103 isolates were regarded as non-typeable, due to their weak or very weak reaction with the PhenePlate system plates. All the isolates belonged to the *Bacillus* genus, which were isolated from the treated water and were non-typeable by the PhenePlate system.

A high value of Di (maximum 1) indicates an even distribution of the isolates into many different biochemical phenotypes, whereas a low value (minimum 0) indicates there are few biochemical phenotypes that dominate the population. High values of Di (>0.82 in all cases) were found for the sampling sites in both sampling occasions (Table 5). Consequently, there was no dominant phenotype among the populations. The diversity of the bacterial community in the raw water was almost identical in both sampling occasions and it was particularly rich. Di

Table 5 | Simpson diversity of the heterotrophic populations analyzed for both sampling occasions

Population	Winter diversity (Di)	Summer diversity (Di)
R	0.98	0.97
S	0.97	0.87
U	0.94	0.88
O	0.82	0.88
T	ND	ND

ND: not determined, R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis, T: treated water (mixture of the treatment lines ozone/granular activated carbon and ultrafiltration/reverse osmosis, followed by chlorination).

decreased slightly over the successive treatments the water was subjected to during winter, whereas in the summer, the diversity decreased after sand filtration and then it remained stable over the following treatments.

The heterotrophic community associated with the winter sampling was significantly different at the stages analyzed, according to the Sp values, which were lower than 0.2 (Figure 3). The difference in clustering could be ascribed to the variation in relative abundance of the genera among treatments. For the summer samples, the highest similarity indexes were recorded after sand filtration, ultrafiltration, and reverse osmosis, with Sp values above 0.2 (Figure 3). Although variations in the percentages of genera were observed in the summer, there were no significant changes

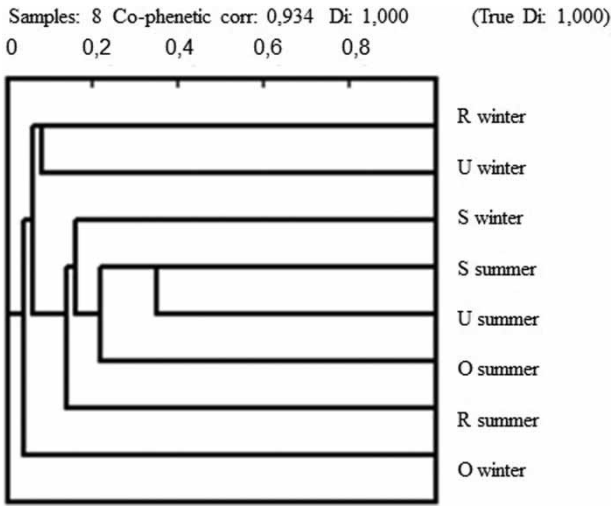

Figure 3 | Dendrogram of the clustering analysis of the similarity of populations (Sp) for each treatment step. R: raw water, S: after sand filtration, U: after ultrafiltration, O: after reverse osmosis.

in either the composition or structure of these populations as shown by the clustering analysis and similarity population indexes. Low similarity was observed in the raw samples between sampling occasions (Sp = 0.08). Consequently, the heterotrophic community of the water that was treated in the plant was not constant during the year.

DISCUSSION

The present study explores the utilization of a novel technology, MALDI-TOF MS, so as to characterize the heterotrophic bacteria isolated in routine analyses after five treatment steps in a drinking water treatment plant at two moments of the year (when the temperature of the water was at its lowest and highest). The characterization showed a potential shift from predominantly Gram-negative bacteria in the raw water and the drinking water treatment steps to mostly Gram-positive bacteria in the chlorinated water in both sampling occasions performed in this study. The obtained results showed that the phylum *Proteobacteria* dominated within the different communities which is in agreement with previous studies that have demonstrated the preponderance of members of the *Proteobacteria* phylum in drinking water systems (Poitelon *et al.* 2009, 2010; Pinto *et al.* 2012). Therefore, members of the *Betaproteobacteria* class are particularly sensitive to chlorination (Poitelon *et al.* 2010), which could explain the decrease in *Betaproteobacteria* with the progress of the water treatment.

The characteristics of treated water depend on the water source, the treatment processes, storage and distribution devices (Eichler *et al.* 2006; Vaz-Moreira *et al.* 2013). No water processing step affected each group studied identically. The obtained results by MALDI-TOF MS complemented the performed biochemical fingerprinting support changes on the heterotrophic composition and structure of heterotrophic communities throughout the water treatment. This supports that this methodology is valid to detect changes among the heterotrophic community in the different treatment steps. Previous studies have shown that after flocculation and sand filtration no major changes in the structures occurred (Eichler *et al.* 2006; Tian *et al.* 2014). However, other studies have stated that established microbial communities on the surfaces of biofiltration materials, such as sand or granular activated carbon,

affect the microbial community in the effluent water (Lautenschlager *et al.* 2014), which is in agreement with the obtained results showing that communities after sand filtration differed from those in the raw water. A chemical disinfection step is useful in water treatment plants so as to avoid the regrowth of microorganisms, especially pathogens. In this study, chlorination greatly changed the structure of the bacterial community and plays a substantial role in determining its final composition. Even though disinfection is effective against the vast majority of the indigenous bacteria, the *Firmicutes* phylum remains functional. It is difficult to prove that the appearance of *Firmicutes* was caused by chlorination, because the treated water was a mixture of two treatment lines, including a granular activated carbon treatment step. However, this is in agreement with previous studies in which *Firmicutes* also dominated after disinfection treatment (Norton & LeChevallier 2000; Poitelon *et al.* 2010).

Concerning the identification of the isolates, some of them belonged to genera that had already been reported in drinking water treatment plants or drinking water distribution systems. Although previous studies used other culture or culture-independent methods so as to isolate or characterize the community, genera identified by MALDI-TOF MS in the present study are in agreement with these studies. To the best of our knowledge, this is the first study in which the heterotrophic community is reported by MALDI-TOF MS. Accordingly, previous studies have reported the isolation of members of the genera *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Comamonas*, *Chryseobacterium*, *Flavobacterium*, *Janthinobacterium*, *Micrococcus*, *Pseudomonas*, and *Ralstonia* from river or fresh water from the Netherlands or USA (Norton & LeChevallier 2000; Bereschenko *et al.* 2008; Vaz-Moreira *et al.* 2011). Meanwhile, genera such as *Acidovorax*, *Camamonas*, *Flavobacterium*, and *Pseudomonas* have previously been identified in granular activated carbon effluent (Norton & LeChevallier 2000; Poitelon *et al.* 2010) and *Acidovorax*, *Flavobacterium*, *Janthinobacterium*, *Pseudomonas* after ultrafiltration (Bereschenko *et al.* 2008). Members affiliated to *Acidovorax*, *Janthinobacterium*, and *Pseudomonas* have also been identified as part of the biofilm attached to membranes of reverse osmosis (Bereschenko *et al.* 2008).

Biochemically clustering by the PhenePlate system gave additional information so as to understand the similarities between microbial communities in both sampling occasions. High levels of diversity were found in all the samples, which were in agreement with MALDI-TOF MS results, confirming the complexity of the microbial community. Some bacterial species were removed during the treatment process, so bacterial species and the diversity decreased as expected throughout the treatments, whereas, some bacterial species stated above were found consecutively in different treatments. In contrast to the rest of the samples, the high Sp value obtained for populations at three sampling points in the summer revealed that a similar community was established after the sand filtration, ultrafiltration, and reverse osmosis treatments in the sampling occasions studied. This could be explained by biofilms forming in the network that distributes the water to the different treatments, which could influence the microbial populations in the water column.

The applied MALDI-TOF MS allowed a rapid characterization of the heterotrophic communities after each treatment step, and it would therefore be an appropriate method for the rapid monitoring of the cultivable community in a drinking water treatment plant in future. However, in this characterization of the heterotrophic community, an important percentage of the isolates could not be identified, so the creation of a database for each microorganism would be necessary because the current protein profile database does not cover the bacterial diversity found in water samples. For example, a high number of isolates could not be identified, especially in some treatments, such as sand filtration and reverse osmosis. MALDI-TOF MS system has a further advantage that the mass spectra from unidentified isolates could be added to the database. This methodology could also be useful to identify only recurrent or abnormal strains isolated routinely in minutes, with the regular resources of a laboratory of any waterworks company. Better knowledge of the microbial community will provide the opportunity for improved management of drinking water plants, since all the decisions at the different stages could determine the final bacterial composition.

CONCLUSIONS

MALDI-TOF MS is a useful technique to rapidly characterize the heterotrophic microorganisms which are detected by

routine culture techniques in a drinking water treatment plant as stated in the regulations. Monitoring and characterizing these communities with MALDI-TOF MS allowed mapping of their own community. Accumulating data on heterotrophic communities is important to further understand the dynamics and ecology of the populations in the different processes in a drinking water treatment plant. Furthermore, identification of their own heterotrophic communities may help detection of failures in the treatment systems.

ACKNOWLEDGEMENTS

Laura Sala-Comorera is the recipient of a FPU grant from the Spanish Ministry of Education: FPU12/00614. This study was supported by the *Generalitat de Catalunya* regional authorities via the award SGR-2014-007 and by the Spanish Ministry of Science and Innovation via CGL2011-25401. We thank Jordi Vila from the Department of Clinical Microbiology, Hospital Clínic, CRESIB/IDIBAPS, School of Medicine, University of Barcelona, for help with MALDI-TOF MS. No conflict of interest is declared.

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First received 7 April 2016; accepted in revised form 7 September 2017. Available online 10 October 2017