



Traceability of different brands of bottled mineral water during shelf life, using PCR-DGGE and next generation sequencing techniques



Laura Sala-Comorera^{a,b,*}, Anicet R. Blanch^{a,b}, Arnau Casanovas-Massana^{a,b,1}, Antonio Monleón-Getino^a, Cristina García-Aljaro^{a,b}

^a Department of Genetics, Microbiology and Statistics, University of Barcelona, Avinguda Diagonal, 643, 08028, Barcelona, Spain

^b The Institute of Water Research, C/ Montalegre, 6, 08001, Barcelona, Spain

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ABSTRACT

Natural mineral waters contain indigenous bacteria characteristic of each spring source. Once bottled, these communities change over time until the water is consumed. Bottle material is believed to play a major role in the succession of these populations, but very few studies to date have evaluated the effect of this material on bacterial communities. In this study, we examined the microbial community structure of three natural mineral waters over 3 months after bottling in glass and polyethylene terephthalate (PET) bottles. To this end, we used culture-dependent (heterotrophic plate count) and culture-independent methods (16S rRNA massive gene sequencing, denaturing gradient gel electrophoresis (DGGE) and fluorescent microscopy with vital dyes). Total and viable cell counts increased by around 1–2 log₁₀ units between 1 and 2 weeks after bottling and then remained constant over 3 months for all waters regardless of the bottle material. DGGE fingerprints and 16S rRNA massive sequencing analysis both indicated that different communities were established in the waters two weeks after bottling in the different bottle materials. In conclusion, no differences in total, viable and culturable bacteria counts were observed between mineral waters bottled with PET or glass during shelf life storage. Nevertheless, in spite of changes in the communities, each water brand and material presented a distinct microbial community structure clearly distinguishable from the others, which could be interesting for traceability purposes.

1. Introduction

Natural mineral waters are not sterile environments, but complex ecosystems with high phenotypic and genetic diversity (Casanovas-Massana and Blanch, 2012; Loy et al., 2005; Rosenberg, 2003). In the European Union, Directive 2009/54/EC prohibits the disinfection or bacteriostatic treatment of natural mineral waters, and thus these contain their autochthonous microbiota, which clearly distinguishes them from other mineral waters of different origin (Casanovas-Massana and Blanch, 2012; Hunter, 1993; Loy et al., 2005; Mavridou, 1992; Venieri et al., 2006). The Directive also states that the total aerobic heterotrophic colony count (HPC) in the end product 12 h after bottling cannot exceed 100 CFU ml⁻¹ after incubation for 72 h at 20–22 °C and 20 CFU mL⁻¹ after incubation for 24 h at 37 °C.

The number of heterotrophic bacteria colonies is the most commonly used criterion for microbiological quality assessment. However, there is no evidence in clinical and epidemiological research to link HPCs with an impact on human health (Allen et al., 2004). Many

sources in the literature describe the rapid growth of microorganisms in bottled water samples after the bottling process (Diduch et al., 2016; Falcone-Dias and Farache Filho, 2013; Urmeneta et al., 2000). Some authors have reported an exponential increase in HPCs within several days of bottling, reaching a peak 1–3 weeks after bottling (Bischofberger et al., 1990; Hunter, 1993; Urmeneta et al., 2000). Subsequently, the HPC remains constant or decreases slightly until one year after bottling (Bischofberger et al., 1990). During storage, HPCs fluctuate, and no general trend has been observed that explains the behaviour of HPCs inside the bottle during shelf life (Falcone-Dias et al., 2012).

Although regulations focus solely on the culturable bacteria fraction in mineral water (Anonymous, 2009), mineral water contains an important fraction which is not culturable under the established cultivation parameters in spite of being metabolically active (França et al., 2014; Loy et al., 2005). Thus, routine determination of HPCs underestimates the total number of microorganisms present in mineral water. Consequently, culture-independent methods provide a more accurate

* Corresponding author. Avinguda Diagonal, 643, 08028, Barcelona, Catalonia, Spain.

E-mail address: laura.sala-comorera@ucd.ie (L. Sala-Comorera).

¹ Present address: Department of Epidemiology of Microbial Diseases, School of Public Health, Yale University, New Haven, Connecticut, USA.

description of microbial community dynamics over time inside the bottle (França et al., 2014; Vaz-Moreira et al., 2011).

The reasons for bacterial multiplication and community succession after bottling are still not fully understood. There is some controversy concerning the factors that influence the increase in heterotrophic populations. The concept of “bottle effect”, whereby the ratio of the surface area to volume promotes bacterial growth, was originally proposed by Zobell and Anderson (1936) but subsequently rejected by Hammes (Hammes et al., 2010), contradicting numerous previous reports. It has been suggested that the concentration of available organic matter, large amounts of bicarbonate and total dissolved solids serve as material for the multiplication of microorganisms (Falcone-Dias and Farache Filho, 2013) until the organic material in the water has been depleted (Rosenberg, 2003). In fact, there is a clear correlation between HPC number and the concentration of assimilable organic carbon in bottled mineral water (Diduch et al., 2016). Bacterial regrowth could be explained as the result of reactivation of starving cells initially present (Leclerc and Moreau, 2002). Moreover, a succession of microbial communities has been reported during shelf life, which may grow on the organic matter supplied by dead cells from the former population (Falcone-Dias and Farache Filho, 2013).

The market value of mineral water has increased considerably worldwide in recent decades, prompting the appearance of brand counterfeits. This poses a risk to consumer health and exerts a negative financial and public image impact on genuine brands (personal communication from water companies). Therefore, there is currently a need to develop methods to enable traceability of this product during shelf storage.

Bottle material is believed to play a major role in the succession of bacterial communities, but few studies to date have evaluated the effect of the material on these populations. Some studies have revealed that low molecular substances migrating from PET and PVC plastic promote the growth of bacterial populations (Bischofberger et al., 1990). In contrast, when bottles are reused, residual cleaning agents may interfere with bacterial populations, yielding a bacteriostatic effect (Bischofberger et al., 1990). Moreover, the colour of the bottle material affects microorganism content: lower colony counts have been found in transparent PVC bottles than in dark glass bottles, the colour of which protects bacteria from daylight (Mavridou, 1992). Currently, polyethylene terephthalate (PET) is the most widely used bottle material for mineral waters due to its properties: low weight, colourlessness and transparency, resistance to chemicals, strength, flexibility, impact-resistance and ease of recycling (Spangenberg and Vennemann, 2008; Welle, 2011). PET bottles have replaced glass bottles, which are now mainly used only in the hotel and catering sector (personal communication from water companies).

The aim of this study was to assess the dynamics during shelf storage of microbial communities in mineral waters bottled in different materials, since this information could be used to ensure traceability of mineral waters. To this end, the communities present in three different brands of non-carbonated mineral water bottled in two different materials (glass and PET) were analysed using culture-dependent (heterotrophic plate count) and culture-independent methods (PCR-DGGE, 16S rRNA massive sequencing and fluorescent microscopy with vital dyes) at different time points.

2. Materials and methods

2.1. Sampling and storage of natural mineral waters

Three commercial brands of bottled natural mineral water from three geographically independent Spanish springs (A, B and C) were selected for this study. Each mineral water was bottled on the same day in glass and PET bottles (1.0–1.5 L bottles). For each water and material, 35 bottles were collected at the end of the filling line and transported to the laboratory within 24 h. Bottles were stored under dark

conditions at $20 \pm 2^\circ\text{C}$ and processed on days 1, 7, 15, 21, 30, 45, 60 and 90 after bottling. In addition, one bottle for each water and material from a different bottling batch was purchased randomly from a local retailer and processed similarly to the previous samples.

2.2. Enumeration of total bacteria

Bacterial viability was assessed with LIVE/DEAD® (L/D) BacLight™ (Invitrogen, USA). Briefly, volumes ranging from 10 ml to 50 ml from two different bottles for time analysed were filtered through 0.22 µm pore size black polycarbonate membrane filters (Millipore, German). Filters were covered with 1 ml deionised sterile water containing 3 µl L/D stain mixture and incubated at 37°C in the dark for 15 min. Filters were then dried in the dark at room temperature and observed under an epifluorescence microscope (Leica Microsystem, Germany) equipped with a mercury lamp. A minimum of 20 randomly selected fields were counted at 1008x and green fluorescent cells were considered to be alive (Haugland, 1996).

2.3. Enumeration of culturable heterotrophic bacteria

For heterotrophic enumeration, samples ranging from 10 µl to 500 ml from two different bottles for time analysed were filtered through 0.22 µm pore size polycarbonate membrane filters (Millipore, Germany), in triplicate. Filters were incubated on R2A plates (Pronadisa, Spain) for 7 days at $20 \pm 2^\circ\text{C}$ and colonies were enumerated.

2.4. DNA extraction

Three litres from each brand and timepoint was filtered through 0.22 µm pore size polycarbonate membrane filters (Millipore, German). DNA was extracted from the filters applying mechanical disruption through bead beating and phenol/chloroform purification using a modification of a previous protocol (Griffiths et al., 2000) (See Supplementary Material). A negative control using a polycarbonate membrane filter was performed for each DNA extraction batch.

2.5. DGGE and sequence analysis

The V3 hypervariable region of the 16S rRNA gene was amplified by nested PCR (see Supplementary Material for primers and PCR conditions). Amplimers were analysed by DGGE using a DCode system (Bio-Rad, USA) as previously described (Ballesté and Blanch, 2011) (see Supplementary Material for details).

The positions and relative signal intensities of detected bands in each gel track were determined with FPQuest Software v.5.10 (Bio-rad, USA). Cluster analysis of DGGE patterns was performed using FPQuest Software v.5.10. Normalisation was achieved by applying an internal lane standard as reference to every 1st, 6th and 11th lane on the DGGE gels. For cluster analysis, unweighted-pair group method analysis (UPGMA) and Dice distance were used with 1% band position tolerance and 1% optimisation. Following DGGE analysis, a jackknife analysis was conducted to determine how accurately DGGE fingerprints of bottled water communities could be assigned to each water brand. To perform jackknife analysis, bottled water community fingerprints were manually assigned to their respective water and material group. The software then removed each fingerprint from the data set individually and queried the data set to determine the water and material group to which the fingerprint was most similar. User-set parameters included maximum-similarity coefficients and ties spread equally among groups. The internal accuracy of classification was calculated as the percentage of community fingerprints assigned to the group to which the sample was known to belong. The Shannon-Weaver index (H') was used as an estimate of microbial diversity (Tiodjio et al., 2014).

2.6. Illumina MiSeq sequencing and analysis

The V3-V4 hypervariable region of the bacterial 16S rRNA gene (460-bp size on average) was amplified using primers described elsewhere (Klindworth et al., 2013). The Nextera XT index kit was used for library construction. A total of 24 libraries were pooled at equimolar concentrations and sequenced in a single run at the Centre for Genomic Regulation (PRBB, Barcelona) using the Illumina MiSeq sequencing platform (2 × 300 bp paired end, 600 cycles), employing V3 chemistry, following the manufacturer's instructions (Illumina MiSeq, USA).

Raw FASTQ files were assessed for quality and trimmed and only those reads with a quality higher than Q20 were processed further using the Mothur pipeline V.1.36.1 (Schloss et al., 2009). A 97% similarity cut-off was used to cluster the sequences into operational taxonomic units (OTUs). Chimeras were removed using UCHIME, and OTUs observed less than 10 times in at least one sample group were also removed from the analysis. Taxonomic classifications were assigned using the Silva SSU Ref database v128 from the Ribosomal Database Project, employing Mothur software. A phylogenetic tree was generated using the Neighbour Joining method and the same software. The OTU classification tables and tree were imported into the R environment (R Development Core Team, 2016) for analysis. Shannon diversity indices were calculated after normalising the reads, using Mothur and the R Vegan package (Oksanen et al., 2017). For normalisation, the relative abundance of OTU sequences in a sample was calculated and then sequences were normalized to the minimum number of sequences in a sample (45,000). UniFrac distance metrics were calculated using the Phyloseq R package (McMurdie and Holmes, 2013).

2.7. Statistical analyses

Principal component analysis (PCA) of the DGGE fingerprints was performed to analyse temporal variations in bacterial community structure based on the relative band intensity and positions using GelCompar II Software (Applied Maths, Belgium). Differences in cell median concentrations between PET and glass bottles were evaluated using the Mann-Whitney-Wilcoxon test with Statgraphics software (Statgraphics.net, Spain). For 16S rRNA massive sequencing analysis, the Benjamini-Hochberg correction was used to adjust the p-value ("fdr" method) for multiple hypothesis testing. The Kruskal-Wallis test was used to assess differences between OTUs and taxonomic classifications of the different mineral water groups, because the data did not present a normal distribution. Discriminant analysis was performed using linear discriminant analysis in order to discriminate between mineral water groups using the R functions `lda()` and `svm()` and artificial neural networks. Finally, an analysis of differential proportions was performed to detect the most and least differentially expressed OTUs using a binomial test of proportions in R, adjusting the p-value ("fdr" method) for multiple hypothesis testing. Differences in the group's communities retrieved from Illumina experiment were assessed by anova using weighted UniFrac distance (vegan package, R), and amova using Bray Curtis distance (Mothur).

3. Results

3.1. Total, viable and culturable heterotrophic bacteria counts

Changes in the number of total, viable and culturable cells in the 3 mineral water brands examined are shown in Fig. 1. Total, viable and culturable cell counts were similar one day after bottling regardless of the water brand and material, ranging from 10^3 to 10^4 , 10^2 to 10^3 and < 0.02 to 0.5×10^0 , respectively. From this point onwards, all counts increased, showing a maximum increase between one and two weeks after bottling in both materials. Subsequently, total and viable cell counts remained stable in all waters and materials except in B PET-bottled water, where a slight increase was observed from day 21

onwards. In the case of culturable counts, no general trend was observed. A and B PET-bottled water presented a decrease in culturable counts as the water bottles aged, whereas in their glass-bottled counterparts, the number of culturable cells remained constant. It should be noted that culturable bacteria represented less than 1% of the population 1 day after bottling and reached the highest counts at 15 or 21 days. From day 21 onwards, after the initial increase in all counts analysed, no significant differences were observed between materials in viable and culturable counts in A and C bottled water ($p > 0.05$, Mann-Whitney-Wilcoxon test). In B bottled water, no differences were detected in viable counts ($p = 0.67$, Mann-Whitney-Wilcoxon test), whereas culturable counts in B glass-bottled water were higher ($p = 0.012$, Mann-Whitney-Wilcoxon test). Regarding total counts, there were no statistically significant differences in B and C water, but total counts after 21 days were different between materials in A water ($p = 0.010$, Mann-Whitney-Wilcoxon test).

3.2. DGGE analysis

3.2.1. Analysis of DGGE fingerprints

An analysis of DGGE fingerprints showed that each brand of water had a distinct fingerprint that varied after bottling (Fig. 2).

A dendrogram analysis of A water showed that the water samples grouped into two main clusters according to bottle material (Fig. S1). One day after bottling, differences were detected in communities from different bottle materials, with a Dice similarity value of 54% (6 bands out of 18 bands in common). All the DGGE fingerprints from day 7 onwards were very similar (76–96%) in A PET-bottled water (Fig. 2a). All the A PET-bottled water samples shared 5 bands (219, 292, 480, 241 and 202). The identity of these bands is shown in Table 1. Band 202 could not be extracted due to the low concentration. Interestingly, band 219 affiliated to *Hyphomicrobium* sp was consistently the most prominent band in all PET-bottled waters (Table 1). Conversely, the fingerprints of A glass-bottled water changed throughout the experiment (Fig. 2b). The Dice coefficient ranged from 65 to 87%, but again certain bands were detected in all experiments (271, 281, 292) (see Table 1 for taxonomic affiliation of bands). Band 292 affiliated to *Rhodococcus* sp. was detected exclusively in A water bottled in both materials.

B water presented a more diverse community than A and C waters, based on the number of observed bands one day after bottling in both materials (21 bands) (Fig. 2). The fingerprints of these two samples (B PET 1 and B glass 1) were quite similar (Dice coefficient = 73%) and formed one separate cluster with sample B PET 7 (Fig. S1b). B PET-bottled water displayed greater temporal variations during the study from 15 days after bottling, with Dice similarity coefficient values ranging from 50 to 92%. Five bands (155, 241, 253, 254 and 255) were detected in 6 out of 8 samples (see Table 1 for band affiliation). In B glass-bottled water, the fingerprints were more stable than those from PET-bottled water over the three months analysed from 7 days after bottling onwards (Fig. 2d), and formed a well-defined cluster for the entire set of samples analysed (Fig. S1b). Bands 476 and 255 were detected in all the 8 samples analysed, and were affiliated to *Acidovorax radicus* and to an uncultured bacterium associated with the microbiota of the water in a drinking water treatment plant, respectively (Table 1). Two bands were detected in common in both materials (255 and 243). Band 255 was visualised in 88% of the bottles analysed; however, this band was very faint in glass-bottled water samples. Finally, band 243 was detected in 69% of the samples, and was affiliated to *Schlegelella*.

C PET- and glass-bottled water also showed similar fingerprints one day after bottling (Fig. 2e and f) (61% Dice similarity coefficient). DGGE fingerprints remained constant in glass-bottled water for 15 days, whereas in PET-bottled water samples, the DGGE fingerprint changed within a week after bottling although it subsequently remained stable. The number of bands was fairly constant for C PET-bottled samples, and between 7 and 8 bands were visualised throughout the study period. Bands 480, 476 and 473 were present in all the C PET samples

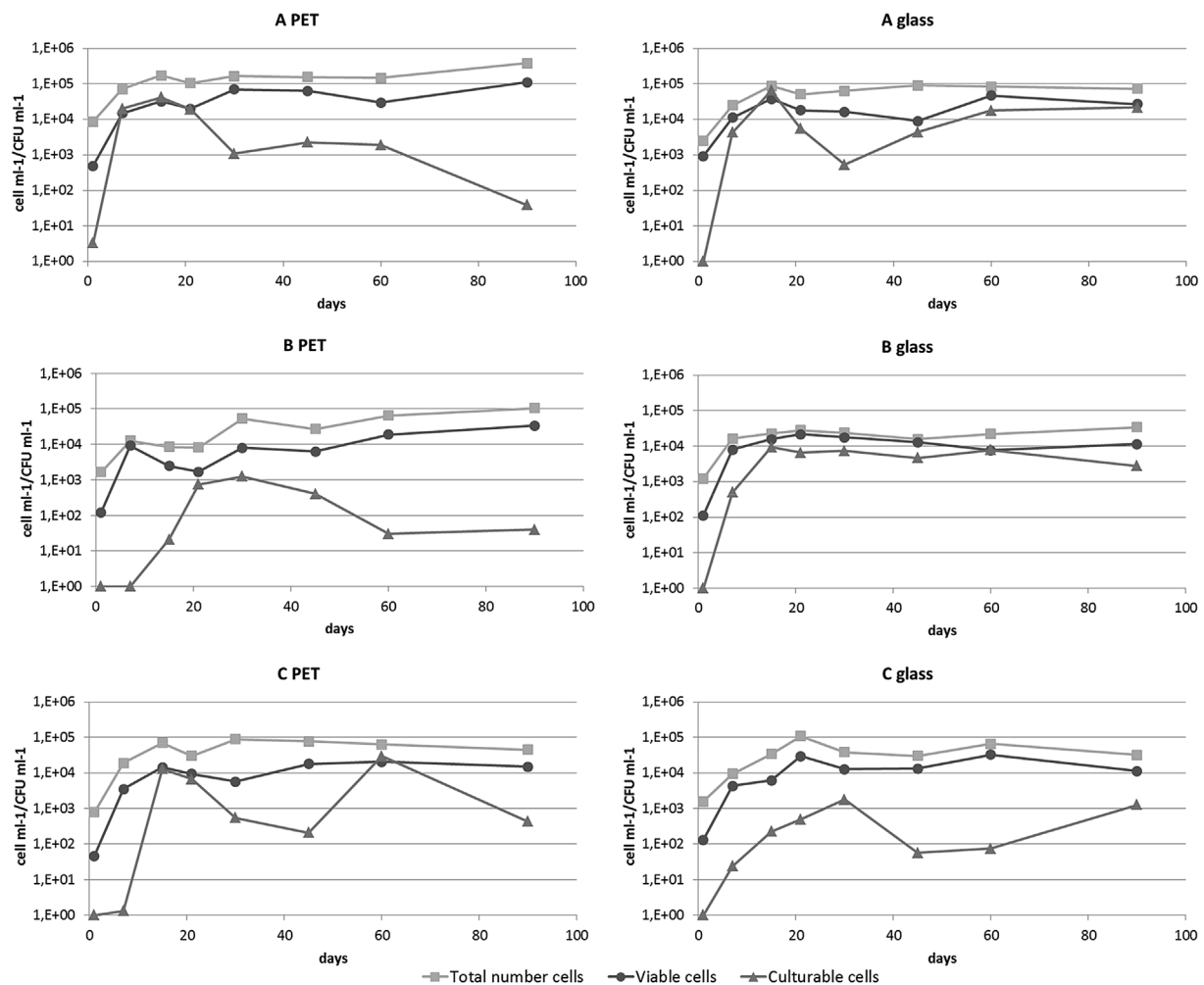


Fig. 1. Changes in the number of total and viable cells and culturable counts in PET- and glass-bottled mineral water (A, B and C). Bacterial viability and total number cells were determined with LIVE/DEAD® (L/D) BacLight™ and culturable heterotrophic bacteria were enumerated using R2A plates.

(Table 1). Band 281 acquired importance in the samples after long-term storage. The fingerprints of C glass-bottled water samples were less stable over time and displayed a larger number of bands (14 bands on average) than their PET-bottled counterparts (Fig. 2). Band 476 was common to all glass-bottled samples. Band 436 appeared 7 days after bottling, whereas band 181 was detected 15 days after bottling and both bands were subsequently detected on all days (Table 1). A total of 4 bands (281, 431, 476 and 480) were observed in common in C PET- and C glass-bottled samples. Band 476 was detected in all the C water samples, whereas bands 281, 431 and 480 were present in 13 out of 16 samples.

A cluster analysis of DGGE fingerprints for all samples (different water brands and materials) revealed a tendency towards clustering by bottle material and origin, supporting the information obtained from a cluster analysis of each water brand individually (Fig. 3). Interestingly, one day after bottling, samples clustered more closely together. Thereafter, each water and material changed differently; communities from different brands and materials did not tend to converge after long-term storage (90 days), but instead preserved their own community according to the cluster analysis, except C glass-bottled waters, which grouped into three different clusters depending on the number of days after bottling. Moreover, 60 days after bottling, B PET-bottled water clustered separately from the other B PET-bottled water samples.

A jackknife analysis indicated that not all the mineral waters presented the same internal accuracy for a fingerprint-based classification (Table 2). Higher rates of internal accuracy were observed for A water

than for B and C waters. Fingerprints for A PET-bottled water were always correctly classified (100%), whereas A glass-bottled water fingerprints were often misclassified as A PET-bottled water. In total, 88% percent of the samples were correctly classified as A glass-, B glass- and C PET-bottled water fingerprints. Fifty percent of B PET-bottled water DGGE fingerprints were identified as A glass-bottled water. C glass-bottled water fingerprints were most often misclassified as A PET-, B glass- and C PET-bottled water (12%, 12% and 13%, respectively).

A multivariate analysis of DGGE fingerprints revealed differences between mineral water brands (Fig. 4): axes x, y and z explained 31.6%, 15.9% and 11.7% of bacterial community variance, respectively. This analysis demonstrated the existence of distinct bacterial communities depending on the water brand and bottle material. A water (PET-bottled and glass-bottled) and C water (PET-bottled) were clearly distinct from the other brands, whereas B PET- and glass-bottled water and C glass-bottled water were not.

3.2.2. DGGE diversity analysis

Shannon diversity patterns over time were very similar for all the mineral water brands tested (Fig. S2). Diversity was higher after bottling and decreased after day 15 in B and C waters. From this point, the diversity indices remained constant. As shown by the number of DGGE bands, B PET-bottled water and C glass-bottled water presented higher diversity than their respective counterparts, unlike A water, whose diversity was similar in both materials and did not decrease during the period analysed. In this mineral water (A), the number of DGGE bands

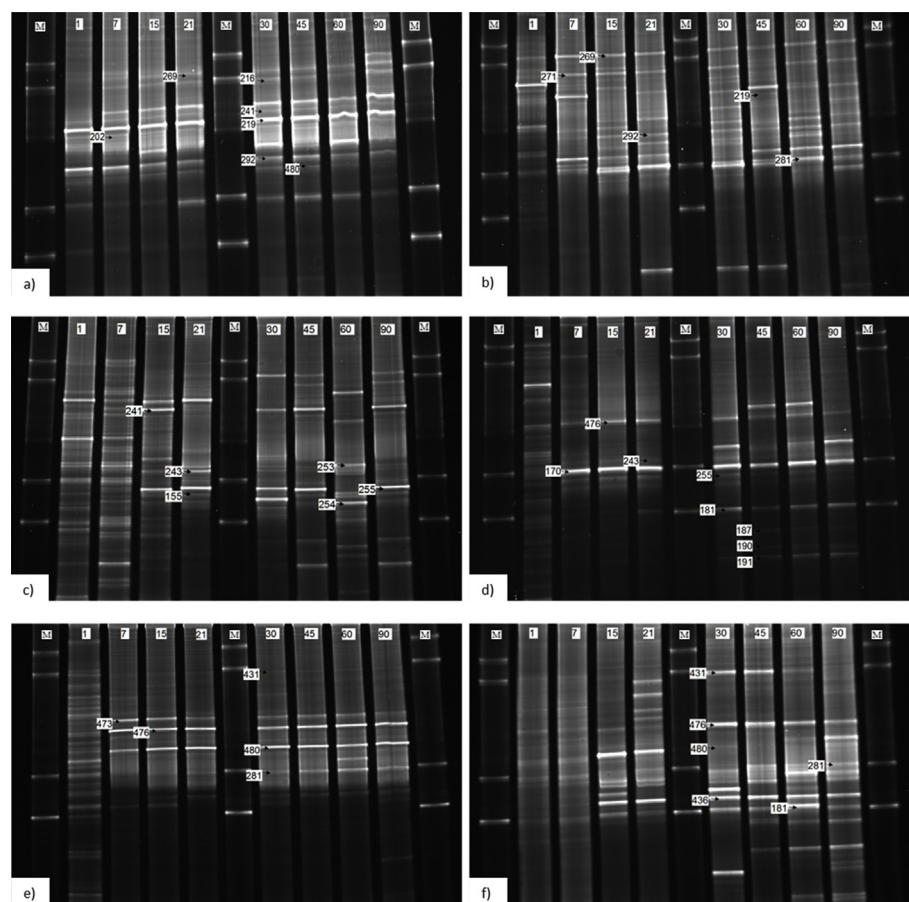


Fig. 2. DGGE fingerprints of the 3 water mineral brands analysed: a) A PET-bottled water, b) A glass-bottled water, c) B PET-bottled water, d) B glass-bottled water, e) C PET-bottled water and f) C glass-bottled water) over 90 days after bottling (1, 7, 15, 21, 30, 45, 60 and 90 days). Marker (M) was added to normalize DGGE gels. Sequenced bands are given numbers.

was constant over time.

3.2.3. DGGE analysis of bottles from a different batch

Dice coefficient similarities between the DGGE fingerprints of bottled waters selected randomly and the DGGE fingerprints of the batch studied (data not shown) varied between 36 and 56%. The DGGE bands

for random sample fingerprints matching the previous DGGE fingerprints are shown in Table S1.

Table 1

Frequency of detection and similarity with the closest relatives of the DGGE bands. Frequency*: number of samples with the band detected/total number of samples in the batch study.

Mineral water sample	Band	Frequency*	Closest relative (% sequence similarity)	Accession number
A PET, A glass	219	8/8, 2/8	<i>Hyphomicrobium</i> sp. (99%)	LN876552.1
A PET, A glass, C PET, C glass	480	8/8, 4/8, 8/8, 5/8	<i>Hydrogenophaga palleronii</i> (100%)	NR_114132.1
A PET, A glass	292	8/8, 8/8	<i>Rhodococcus</i> sp. (99%)	KX064755.1
A glass	271	8/8	<i>Acinetobacter johnsonii</i> (100%)	NR_117624.1
A PET, A glass, C PET, C glass	281	3/8, 8/8, 7/8, 6/8	<i>Novosphingobium</i> sp. (100%)	LC133664.1
			<i>Sphingomonas</i> sp. (100%)	KJ654775.1
A PET, A glass, B PET	241	8/8, 3/8, 6/8	Uncultured bacterium (100%)	KT714231.1
B PET, B glass	255	6/8, 8/8	Uncultured bacterium (100%)	GU742462.1
B PET, B glass	155	6/8, 4/8	<i>Porphyrobacter sanguineus</i> (100%)	NR_113808.1
B PET	254	6/8	<i>Leptospira</i> sp. (99%)	KX245334.1
B PET, B glass	170	3/8, 6/8	<i>Ramlibacter henchirensis</i> (99%)	NR_025203.1
B PET, B glass	243	5/8, 6/8	Uncultured <i>Schlegelella</i> sp. (99%)	GQ243114.1
B PET, C PET, C glass	253	6/8, 1/8, 2/8	<i>Leptospira</i> sp. (100%)	NR_044042.1
B PET, B glass, C PET, C glass	476	2/8, 8/8, 8/8, 8/8	<i>Acidovorax radialis</i> (99%)	NR_117776.1
C PET, C glass	473	8/8, 1/8	Uncultured bacterium (100%)	KX670409.1
C glass	436	7/8	Uncultured bacterium (94%)	LC023390.1
B glass, C glass	181	6/8, 6/8	<i>Oligotropha carboxidovorans</i> (100%)	NR_074142.1
			<i>Rhodopseudomonas pseudopalustris</i> (100%)	NR_036771.1
			<i>Afipia massiliensis</i> (100%)	NR_122099.1

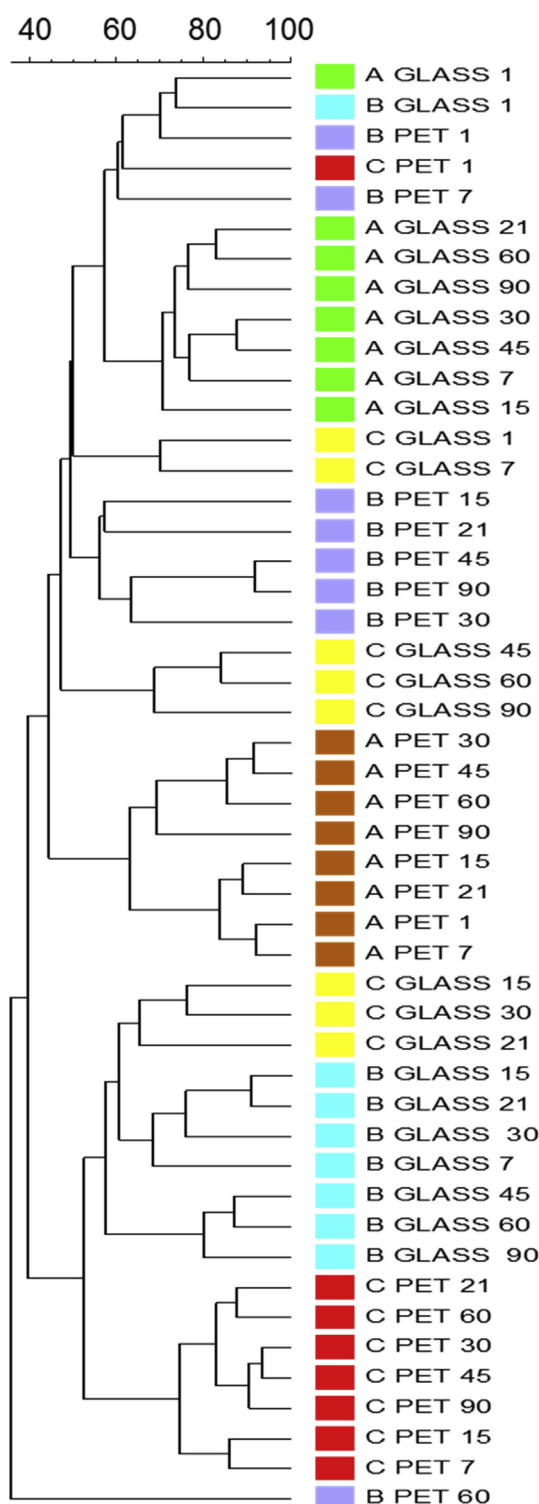


Fig. 3. Cluster analysis of all the mineral water brands using Dice's coefficient and UPGMA. The number indicates the number of days after bottling. (COLOUR PRINTING). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Analysis of Illumina MiSeq reads

3.3.1. Microbiome structure

The bacterial communities in the three mineral water brands bottled in PET and glass were analysed in samples from the same batch acquired directly from water companies at 1, 30 and 90 days after

Table 2

Jackknife analysis results. Numbers represent the percentage of DGGE fingerprints of bottled water assigned to each water and material group. The number of misidentifications for members of each group is given in the columns. Note that the values in the matrix are not reciprocal, and the matrix is not symmetrical.

	A PET	A glass	B PET	B glass	C PET	C glass
A PET	100	12	12			12
A glass		88	50	12	12	
B PET			25			
B glass				88		12
C PET					88	13
C glass			13			63
	100	100	100	100	100	100

bottling, and in the random sample from a different batch bought directly from a retailer. After sequence quality, chimera filtering and low abundance OTU removal, a total of 3,390,286 reads were obtained for 24 samples, with a mean of 141261 ± 103535 (Mean \pm SD), which were grouped into 2971 OTUs.

Each water brand contained a distinct community structure at phylum, class, genus and OTU level at 1 day after bottling and for different bottle materials from the same water company (Fig. 5). The most striking differences at class level were observed in A water: glass-bottled samples 1 day after bottling were dominated by γ -Proteobacteria (76%) whereas PET-bottled samples were dominated by β -Proteobacteria (95%) (Fig. 5b). β -Proteobacteria remained the dominant class in the time-course experiment (over 80% in all cases) and even in the random bottle from an independent batch purchased one year later. In contrast, in glass-bottled samples, α - and β -Proteobacteria together represented 75% and 60% of the reads 30 and 90 days after bottling, respectively, in detriment of γ -Proteobacteria. B water samples were dominated by γ -Proteobacteria in both materials 1 day after bottling, but after 30 days' storage, α -Proteobacteria predominated in both materials, that was replaced by α and β -Proteobacteria until the end of the experiment. In C water, 1 day after bottling the community was mainly composed of an unidentified class affiliated to the phyla *Parcubacteria* and a novel unidentified phylum. These communities shifted and 30 days after bottling there was a higher proportion of β -Proteobacteria followed by α -Proteobacteria, which was reversed in the glass bottles by the end of the experiment. At genus level, the dominant genera were *Acinetobacter* (65%) and *Polaromonas* (78%) in A glass- and PET-bottled water samples, respectively, which had been replaced by several unclassified bacteria by the end of the experiment, although more than 50% of the reads were still identified as *Polaromonas* in the PET bottles. In B glass-bottled samples, the most abundant genus was *Acinetobacter* (28%), followed by an unclassified genus of the *Enterobacteriaceae* family (15%), *Stenotrophomonas* (14%) and *Pseudomonas* (11%). Diversity decreased dramatically 30 days after bottling, with only 4 genera accounting for almost 100% of the reads (the unclassified genus affiliated to *Caulobacteraceae* (70%) followed by *Roseateles* (16%) being the most abundant). In B PET-bottled samples, *Acinetobacter* was the most abundant (28%) genus classified at the beginning of the experiment followed by *Stenotrophomonas* (14%) and *Pseudomonas* (13%), resembling B glass-bottled samples; however, by the end of the experiment, *Hirschia* (44%) and an unclassified genus affiliated to the *Comamonadaceae* family (24%) dominated the community, whereas in B-glass-bottled dominated *Roseateles* (35%) and an unclassified *Caulobacteraceae*. In C water, 1 day after bottling the communities in both materials resembled each other. As with the other mineral water brands and materials, the high number of genera found 1 day after bottling decreased 30 days after bottling, with *Polaromonas* (48%) and unclassified genera becoming the dominant genera in glass bottles, although *Polaromonas* diminished to less than 1% of the reads in the sample 90 days

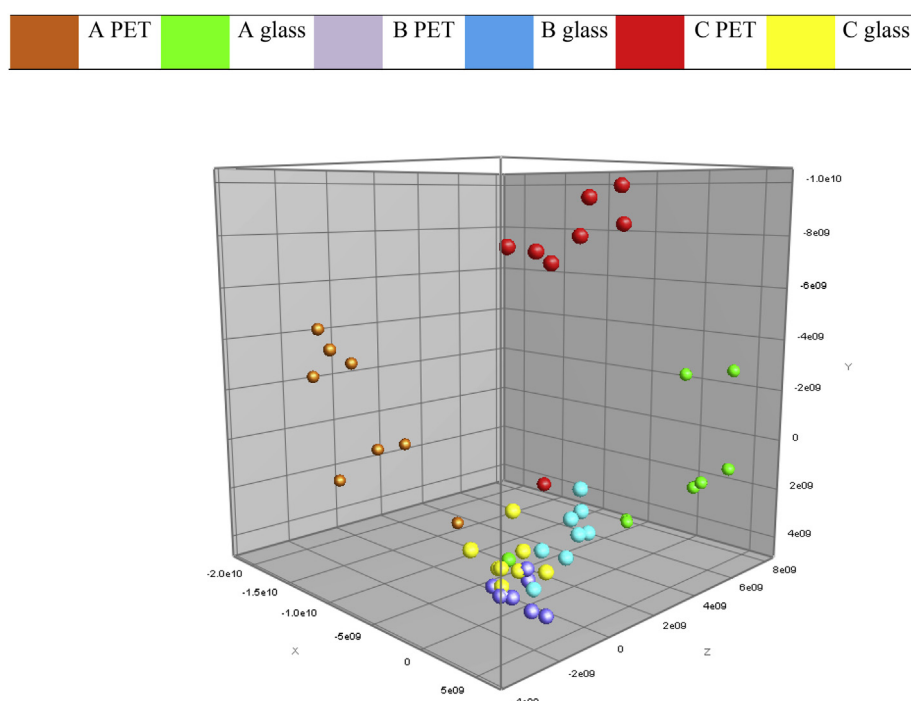


Fig. 4. Principal components analysis (PCA) with the DGGE fingerprints. PC1, PC2 and PC3 are shown on x, y, z axes, respectively. (COLOUR PRINTING). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

after bottling. In the case of C PET-bottled water, *Hydrogenophaga* (50%) and *Acidovorax* (26%) dominated the bacterial community 30 days after bottling and remained in similar proportions 90 days after bottling.

Overall, taking all the water brands and materials together the proportions of the different OTUs present in the samples, the proportion of OTUs varied more between day 1 and 30 than between day 30 and 90 day indicating a more stable community along the time (adj. p -value < 0.001 , binomial test of proportions for the 25 most abundant OTUs).

Taking into consideration OTUs with counts higher than 10, three genus were present in more than 50% of the samples, although in different proportions. These ubiquitous OTUs were identified as *Polaromonas*, an unclassified *Comamonadaceae* and an unclassified *Bradyrhizobiaceae*. On the other hand, very few OTUs were common to all samples from the same brand and material (Fig. S3). The sample from the random bottle analysed for each material and brand shared at least 50% of OTUs with the closest sample in terms of material and time, except in the case of the random C glass-bottled sample taken 11 days after bottling, which had 119 unique OTUs and the maximum number of shared OTUs was 27 with the rest of the samples.

In spite of the observed differences between brands and bottle material during shelf storage, a discriminant analysis enabled differentiation of the water brands without taking into consideration the bottle material, with an accuracy of 0.9583 (95% confidence interval, 0.7888–0.9989, $p = 0.0001$). This indicates that the method accurately separated the groups of samples into A, B and C in around 96% of cases. Table 3 lists the sensitivity and specificity for each water brand. The 10 most frequent OTUs for each group selected in the discriminant analysis are shown in Fig. S5.

3.3.2. Analysis of community diversity

In A water, the Shannon diversity indices showed a slight increase over time in both PET and glass bottles (Fig. S2b). Meanwhile, for the B and C brands, samples 1 day after bottling showed higher diversity in both materials than aged samples of the same brand and material. In addition, the Shannon diversity indices were one unit higher in A and C

glass-bottled waters than in their PET-bottled counterparts at all time points analysed. In the case of B water, no differences in diversity between the two materials were observed when the water was stored for between 30 and 90 days.

An analysis of β -diversity using weighted UniFrac, which is a phylogenetic-based non-metric multidimensional scaling analysis, clustered with statistical significance the samples according to brand, supporting previous DGGE results (anosim, $p = 0.0089$, 1000 permutations and amova, (A-B-C) $p < 0.001$; (A-B), $p = 0.012$; (A-C), $p = 0.004$, (C-B), $p = 0.008$) (Fig. S4). The pair-wise error rate (Bonferroni) was 0.0167 for anosim and amova.

4. Discussion

The microbiology of bottled mineral waters is relatively unknown, especially as regards the changes that occur during shelf life. Understanding these changes in microbial communities could provide water companies with a tool that enables traceability of their product to assure quality and fight brand counterfeits.

In this study, three different mineral water brands were selected to study changes in the bacterial communities present in mineral waters bottled in two commonly used materials (PET and glass) and obtain a more global understanding of the microbiology of mineral water supplied to consumers. Total bacterial counts one day after bottling were about 1×10^3 cell/ml in all the water brands analysed, but more than 90% were damaged cells. Thereafter, between one and two weeks after bottling, the total number of cells increased up to 1–2 \log_{10} units. This finding is in agreement with previous studies which concluded that total cell counts remained constant in the initial months post-bottling, after reaching a value of about 1×10^4 cell/ml - 1×10^5 cell/ml (Defives et al., 1999; Lesaulnier et al., 2017).

Viable counts accounted for less than 10% of total cell counts immediately after bottling. Nevertheless, the community in mineral water bottles experienced a rapid transition from predominantly inactive to active cells a few days after bottling. The highest increase in viable counts occurred in glass-bottled samples at days 15 or 21, reaching values higher than their PET-bottled counterparts, although the

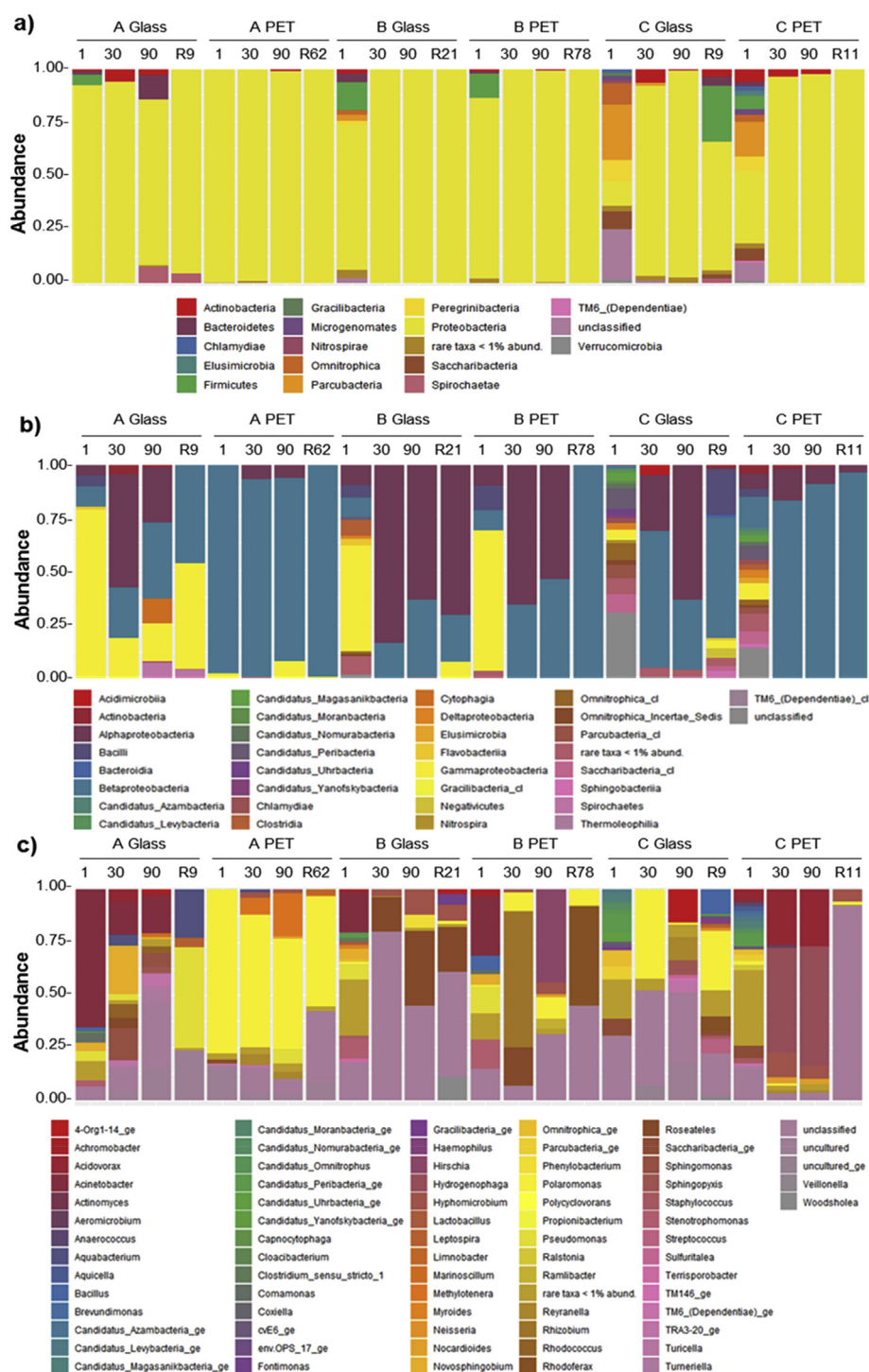


Fig. 5. Community structure of the mineral water brands at phylum (a), class (b) and genus (c) level according to 16S rRNA massive sequencing analysis. Results are expressed as relative abundance of reads. The number of days after bottling of each sample is indicated at the top of the columns (samples from the same batch). R indicates that the sample is a random sample purchased directly from a retailer. (COLOUR PRINTING). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3

Sensitivity and specificity obtained with the 10 OTUs selected in the discriminant analysis for A, B and C water brands.

	A	B	C
Sensitivity	1.0000	0.8750	1.0000
Specificity	1.0000	1.0000	0.9375

increase in glass-bottled waters occurred one week later than in PET-bottled waters. This increase after bottling may be due to the lysate of dead cells produced during the bottling process, which may have provided nutrients to support growth, or to community adaptation to an enclosed oligotrophic environment (Defives et al., 1999).

No culturable bacteria were detected in B water (LOD = 1/500 ml) and low counts were obtained for A and C waters after bottling. The initial increase in culturable counts occurred earlier in glass-bottled

samples. After this initial increase, culturable bacteria counts fluctuated over time, and in general, the periods of growth followed by declines observed over the study period showed no clear tendency. The culturable counts obtained were higher in glass-bottled than PET-bottled water from brands A and B, whereas C water showed higher counts in PET-bottled water. Nevertheless, culturable counts represented less than 10% of total cell counts in the majority of samples analysed. Therefore, these fluctuations in the number of culturable cells may have not been reflected in the DGGE fingerprints, since DGGE based on the 16S rRNA gene only enables detection of bacteria that constitute at least 10% of the bacterial community (Muyzer and Smalla, 1998). Regardless, bottle material did not affect total, viable or culturable counts after the initial increase observed around two weeks after bottling.

Concerning the presence of culturable bacteria in mineral waters, a previous study concluded that bacteria retrieved via cultivation methods did not constitute the dominant populations in the entire community (Burtcher et al., 2009). This was especially true immediately after bottling (mainly in glass-bottled waters). Nevertheless, in terms of seeking a traceability marker, the utility of culturable bacteria for traceability purposes cannot be ruled out because in some samples they accounted for more than 50% of the viable bacteria in aged samples. We were unable to ascertain if these culturable bacteria (and/or molecular markers) were already present in the wells or formed part of the bottling environment of the bottling plants. However, as traceability markers, their source is irrelevant provided that they are detected in all bottles from the same brand.

The DGGE fingerprints of A glass-bottled water, B glass-bottled water and C PET-bottled water changed between one day after bottling and one week after bottling. During this week, total cell counts in these waters increased more than their respective counterparts (A PET-bottled water, B PET-bottled water and C glass-bottled water); therefore, the increase in total counts could have led to changes in the DGGE fingerprints. The microbial communities in water brands whose DGGE fingerprints changed during the first week subsequently remained constant, as deduced from their DGGE fingerprints. One explanation for this finding is that the microbial communities in these brands may have undergone rapid adaptation. In contrast, in the water brands that the DGGE fingerprint changed after the first week (B PET-bottled water and C glass-bottled water), these fingerprints changed in terms of band number, position and intensity over the 3 months analysed, with the exception of A PET-bottled water. The DGGE fingerprint of the latter remained constant over the 3 months studied. In addition, the bands that remained constant over the three months were different for the same water bottled in a different material.

The cluster analysis of all DGGE fingerprints revealed that the samples clustered mainly according to their origin and material in spite of the succession of communities during storage time, in accordance with previous studies, which have described a particular microbial community in each mineral water spring (Loy et al., 2005; Rosenberg, 2003). The DGGE fingerprint signatures appeared to be brand and material specific, although some common bands could be observed across different brands. Furthermore, the mineral water maintained a characteristic molecular signature even during long-term storage since the DGGE fingerprints did not converge. Consequently, as shown in PCA analysis, it may be possible to differentiate mineral waters according to their DGGE fingerprints. Although the DGGE fingerprints obtained from another batch showed lower Dice similarities than their corresponding sample, some of the most frequently observed bands were also present. Different storage conditions of these bottles (selected randomly) could have given rise to a different microbial community, since they were obtained from a retailer. In consequence, it would be possible to select certain bands so as to differentiate between brands and use these as a microbial marker.

Not surprisingly, all the genera detected by DGEE were also detected by 16S rRNA massive sequencing using the Illumina platform, with the exception of *Porphyrobacter*, which was not detected using next

generation sequencing techniques. Therefore, as suggested by Krakova et al. the PCR-DGGE method can still complement NGS techniques (Kraková et al., 2016). As has previously been reported, some of the differences between these two methods may be due to the use of different pairs of primers or the length of the amplicon generated. However, in spite of these differences, the overall results and the conclusions derived from our study were equivalent with both methods, as was also the case in other studies performed with other matrices (Gobet et al., 2014; Samarajeewa et al., 2015). As with DGGE fingerprinting, very few of the OTUs selected from the diverse seed microbiota observed in all samples 1 day after bottling dominated the samples 30 days after bottling. The genus *Polaromonas* was ubiquitous, being detected in more than 50% of samples, including all sample brands or materials. We cannot be certain if *Polaromonas* was present in the aquifers or in elements of the bottling plant environment such as pipelines and/or storage tanks, but another recent study also found it in mineral waters from other countries (Lesaulnier et al., 2017). Each water brand and material presented a unique community structure, with no more than 8 OTUs in common between all the analysed samples of a single brand and material, suggesting that the characteristic composition of the water depends not only on the seed community but also on the material used for storage. Nevertheless, considering all the samples of one particular mineral water together, it was possible to classify the water with an accuracy of nearly 96% based on the proportions of just 10 OTUs, which supports the possibility of developing a marker for mineral water brand traceability based on the detection and quantification of these selected OTUs.

5. Conclusions

In conclusion, no differences in total, viable and culturable bacteria counts were observed between PET- and glass-bottled mineral water samples during long-term storage. Moreover, a succession of microorganisms was observed in both materials during shelf storage of the different mineral water samples. Nevertheless, the bottle material exerted an effect on community structure development since the communities detected in samples of the same water were different for PET- and glass-bottled samples despite the fact that the bottling process had occurred simultaneously for both materials. The use of molecular techniques enabled differentiation of these three mineral waters during a shelf life of 90 days in spite of the succession of microorganisms. Nonetheless, it should be noted that although molecular methods constitute a powerful technique for the study of microorganisms in aquatic environments, in some of the analysed waters the microorganisms that succeeded in adapting to the glass-bottled mineral waters were culturable bacteria, accounting in some cases for more than 50% of the viable counts. Further research is required to explore the culturable bacteria fraction, since this could provide a simpler and more cost-effective technology for water companies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fm.2019.01.006>.

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