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

Laura Sala-Comorera[1,2]*, Anicet R. Blanch[1,2], Arnau Casanovas-Massana[1,2#], Antonio Monleón-Getino[1] and Cristina García-Aljaro[1,2]

[1]Department of Genetics, Microbiology and Statistics, University of Barcelona, Avinguda Diagonal, 643 - 08028 Barcelona, Spain

[2]The Institute of Water Research, C/ Montalegre, 6 - 08001 Barcelona, Spain

[*]Corresponding author

Mailing address:

Avinguda Diagonal, 643, 08028 Barcelona, Catalonia, Spain

Phone: (+34) 934029012

Fax: (+34) 934039047

E-mail: laura.sala-comorera@ucd.ie

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

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 $\log_{10}$ 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.

Keywords: Natural mineral water, metagenomics, DGGE, bottled water, microbial diversity, bottling effect, *Polaromonas*

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 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 (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

98 reused, residual cleaning agents may interfere with bacterial populations, yielding a
99 bacteriostatic effect (Bischofberger et al., 1990). Moreover, the colour of the bottle material
100 affects microorganism content: lower colony counts have been found in transparent PVC bottles
101 than in dark glass bottles, the colour of which protects bacteria from daylight (Mavridou, 1992).
102 Currently, polyethylene terephthalate (PET) is the most widely used bottle material for mineral
103 waters due to its properties: low weight, colourlessness and transparency, resistance to
104 chemicals, strength, flexibility, impact-resistance and ease of recycling (Spangenberg and
105 Vennemann, 2008; Welle, 2011). PET bottles have replaced glass bottles, which are now mainly
106 used only in the hotel and catering sector (personal communication from water companies).

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

114

115 **2. Materials and methods**

116 **2.1. Sampling and storage of natural mineral waters**

117 Three commercial brands of bottled natural mineral water from three geographically
118 independent Spanish springs (A, B and C) were selected for this study. Each mineral water was
119 bottled on the same day in glass and PET bottles (1.0 to 1.5 litre bottles). For each water and
120 material, 35 bottles were collected at the end of the filling line and transported to the laboratory
121 within 24 h. Bottles were stored under dark conditions at 20±2˚C and processed on days 1, 7,
122 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 ± 2˚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

147 negative control using a polycarbonate membrane filter was performed for each DNA extraction
148 batch.

149

150 **2.5. DGGE and sequence analysis**

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

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

170

171 **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 x 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 normalization, 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-

198 Wilcoxon test with Statgraphics software (Statgraphics.net, Spain). For 16S rRNA massive
199 sequencing analysis, the Benjamini-Hochberg correction was used to adjust the p-value ("fdr"
200 method) for multiple hypothesis testing. The Kruskal-Wallis test was used to assess differences
201 between OTUs and taxonomic classifications of the different mineral water groups, because the
202 data did not present a normal distribution. Discriminant analysis was performed using linear
203 discriminant analysis in order to discriminate between mineral water groups using the R
204 functions lda() and svm() and artificial neural networks. Finally, an analysis of differential
205 proportions was performed to detect the most and least differentially expressed OTUs using a
206 binomial test of proportions in R, adjusting the p-value ("fdr" method) for multiple hypothesis
207 testing. Differences in the group's communities retrieved from Illumina experiment were
208 assessed by anosim using weighted UniFrac distance (vegan package, R), and amova using Bray
209 Curtis distance (Mothur).

210

211

212 **3. Results**

213 **3.1. Total, viable and culturable heterotrophic bacteria counts**

214 Changes in the number of total, viable and culturable cells in the 3 mineral water brands
215 examined are shown in Fig. 1. Total, viable and culturable cell counts were similar one day after
216 bottling regardless of the water brand and material, ranging from 10^3 to 10^4, 10^2 to 10^3 and
217 <0.02 to $0.5x10^0$, respectively. From this point onwards, all counts increased, showing a
218 maximum increase between one and two weeks after bottling in both materials. Subsequently,
219 total and viable cell counts remained stable in all waters and materials except in B PET-bottled
220 water, where a slight increase was observed from day 21 onwards. In the case of culturable
221 counts, no general trend was observed. A and B PET-bottled water presented a decrease in
222 culturable counts as the water bottles aged, whereas in their glass-bottled counterparts, the
223 number of culturable cells remained constant. It should be noted that culturable bacteria
224 represented less than 1% of the population 1 day after bottling and reached the highest counts at
225 15 or 21 days. From day 21 onwards, after the initial increase in all counts analysed, no
226 significant differences were observed between materials in viable and cultivable counts in A and
227 C bottled water ($p>0.05$, Mann-Whitney-Wilcoxon test). In B bottled water, no differences were
228 detected in viable counts ($p=0.67$, Mann-Whitney-Wilcoxon test), whereas culturable counts in
229 B glass-bottled water were higher ($p=0.012$, Mann-Whitney-Wilcoxon test). Regarding total
230 counts, there were no statistically significant differences in B and C water, but total counts after
231 21 days were different between materials in A water ($p=0.010$, Mann-Whitney-Wilcoxon test).

232

233 **3.2. DGGE analysis**

234 **3.2.1. Analysis of DGGE fingerprints**

235 An analysis of DGGE fingerprints showed that each brand of water had a distinct fingerprint
236 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 radicis* 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 **2f**) (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 (**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 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%.

315 The DGGE bands for random sample fingerprints matching the previous DGGE fingerprints are
316 shown in **Table S1**.

317

318 **3.3 Analysis of Illumina MiSeq reads**

319 **3.3.1. Microbiome structure**

320 The bacterial communities in the three mineral water brands bottled in PET and glass were
321 analysed in samples from the same batch acquired directly from water companies at 1, 30 and
322 90 days after bottling, and in the random sample from a different batch bought directly from a
323 retailer. After sequence quality, chimera filtering and low abundance OTU removal, a total of
324 3,390,286 reads were obtained for 24 samples, with a mean of 141261 ± 103535 (Mean ± SD),
325 which were grouped into 2971 OTUs.

326 Each water brand contained a distinct community structure at phylum, class, genus and OTU
327 level at 1 day after bottling and for different bottle materials from the same water company **(Fig**
328 **5**). The most striking differences at class level were observed in A water: glass-bottled samples
329 1 day after bottling were dominated by *γ-Proteobacteria* (76%) whereas PET-bottled samples
330 were dominated by *β-Proteobacteria* (95%) (**Fig. 5b**). *β-Proteobacteria* remained the dominant
331 class in the time-course experiment (over 80% in all cases) and even in the random bottle from
332 an independent batch purchased one year later. In contrast, in glass-bottled samples, α- and *β-*
333 *Proteobacteria* together represented 75% and 60% of the reads 30 and 90 days after bottling,
334 respectively, in detriment of *γ-Proteobacteria*. B water samples were dominated by *γ-*
335 *Proteobacteria* in both materials 1 day after bottling, but after 30 days' storage, α-
336 *Proteobacteria* predominated in both materials, that was replaced by α and *β-Proteobacteria*
337 until the end of the experiment. In C water, 1 day after bottling the community was mainly
338 composed of an unidentified class affiliated to the phyla *Parcubacteria* and a novel unidentified
339 phylum. These communities shifted and 30 days after bottling there was a higher proportion of

340 *β-Proteobacteria* followed by *α-Proteobacteria*, which was reversed in the glass bottles by the
341 end of the experiment. At genus level, the dominant genera were *Acinetobacter* (65%) and
342 *Polaromonas* (78%) in A glass- and PET-bottled water samples, respectively, which had been
343 replaced by several unclassified bacteria by the end of the experiment, although more than 50%
344 of the reads were still identified as *Polaromonas* in the PET bottles. In B glass-bottled samples,
345 the most abundant genus was *Acinetobacter* (28%), followed by an unclassified genus of the
346 *Enterobacteriaceae* family (15%), *Stenotrophomonas* (14%) and *Pseudomonas* (11%).
347 Diversity decreased dramatically 30 days after bottling, with only 4 genera accounting for
348 almost 100% of the reads (the unclassified genus affiliated to *Caulobacteraceae* (70%) followed
349 by *Roseateles* (16%) being the most abundant). In B PET-bottled samples, *Acinetobacter* was
350 the most abundant (28%) genus classified at the beginning of the experiment followed by
351 *Stenotrophomonas* (14%) and *Pseudomonas* (13%), resembling B glass-bottled samples;
352 however, by the end of the experiment, *Hirschia* (44%) and an unclassified genus affiliated to
353 the *Comamonadaceae* family (24%) dominated the community, whereas in B-glass-bottled
354 dominated *Roseateles* (35%) and an unclassified *Caulobacteraceae*. In C water, 1 day after
355 bottling the communities in both materials resembled each other. As with the other mineral
356 water brands and materials, the high number of genera found 1 day after bottling decreased 30
357 days after bottling, with *Polaromonas* (48%) and unclassified genera becoming the dominant
358 genera in glass bottles, although *Polaromonas* diminished to less than 1% of the reads in the
359 sample 90 days after bottling. In the case of C PET-bottled water, *Hydrogenophaga* (50%) and
360 *Acidovorax* (26%) dominated the bacterial community 30 days after bottling and remained in
361 similar proportions 90 days after bottling.

362 Overall, taking all the water brands and materials together the proportions of the different OTUs
363 present in the samples, the proportion of OTUs varied more between day 1 and 30 than between
364 day 30 and 90 day indicating a more stable community along the time (adj. p-value <0.001,
365 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.

391 An analysis of β-diversity using weighted UniFrac, which is a phylogenetic-based non-metric
392 multidimensional scaling analysis, clustered with statistical significance the samples according
393 to brand, supporting previous DGGE results (anosim, p=0.0089, 1000 permutations and amova,
394 (A-B-C) p <0.001; (A-B), p= 0.012; (A-C), p= 0.004, (C-B), p= 0.008) (**Fig. S4**)

395

396 **4. Discussion**

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

401 In this study, three different mineral water brands were selected to study changes in the bacterial
402 communities present in mineral waters bottled in two commonly used materials (PET and glass)
403 and obtain a more global understanding of the microbiology of mineral water supplied to
404 consumers. Total bacterial counts one day after bottling were about $1x10^3$ cell/ml in all the
405 water brands analysed, but more than 90% were damaged cells. Thereafter, between one and
406 two weeks after bottling, the total number of cells increased up to 1-2 $\log_{10}$ units. This finding is
407 in agreement with previous studies which concluded that total cell counts remained constant in
408 the initial months post-bottling, after reaching a value of about $1x10^4$ cell/ml - $1x10^5$ cell/ml
409 (Defives et al., 1999; Lesaulnier et al., 2017).

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

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

489

490 **5. Conclusions**

491 In conclusion, no differences in total, viable and culturable bacteria counts were observed
492 between PET- and glass-bottled mineral water samples during long-term storage. Moreover, a
493 succession of microorganisms was observed in both materials during shelf storage of the
494 different mineral water samples. Nevertheless, the bottle material exerted an effect on

495 community structure development since the communities detected in samples of the same water
496 were different for PET- and glass-bottled samples despite the fact that the bottling process had
497 occurred simultaneously for both materials. The use of molecular techniques enabled
498 differentiation of these three mineral waters during a shelf life of 90 days in spite of the
499 succession of microorganisms. Nonetheless, it should be noted that although molecular methods
500 constitute a powerful technique for the study of microorganisms in aquatic environments, in
501 some of the analysed waters the microorganisms that succeeded in adapting to the glass-bottled
502 mineral waters were culturable bacteria, accounting in some cases for more than 50% of the
503 viable counts. Further research is required to explore the culturable bacteria fraction, since this
504 could provide a simpler and more cost-effective technology for water companies.

505

References

Allen, M.J., Edberg, S.C., Reasoner, D.J., 2004. Heterotrophic plate count bacteria--what is their significance in drinking water? Int. J. Food Microbiol. 92, 265–274.

Anonymous, 2009. Directive 2009/54/EC of the European Parliament and of the Council of 18 June 2009 on the exploitation and marketing of natural mineral water.

Ballesté, E., Blanch, A.R., 2011. Bifidobacterial diversity and the development of new microbial source tracking indicators. Appl. Environ. Microbiol. 77, 3518–25.

Bischofberger, T., Cha, S.K., Schmitt, R., Konig, B., Schmidt-Lorenz, W., 1990. The bacterial flora of non-carbonated, natural mineral water from the springs to reservoir and glass and plastic bottles. Int. J. Food Microbiol. 11, 51–71.

Burtscher, M.M., Zibuschka, F., Mach, R.L., Lindner, G., Farnleitner, A.H., 2009. Heterotrophic plate count vs. in situ bacterial 16S rRNA gene amplicon profiles from drinking water reveal completely different communities with distinct spatial and temporal allocations in a distribution net. Water SA.

Casanovas-Massana, A., Blanch, A.R., 2012. Diversity of the heterotrophic microbial populations for distinguishing natural mineral waters. Int. J. Food Microbiol. 153, 38–44.

Defives, C., Guyard, S., Oulare, M.M., Mary, P., Hornez, J.P., 1999. Total counts, culturable and viable, and non-culturable microflora of a French mineral water: a case study. J. Appl. Microbiol. 86, 1033–1038.

Diduch, M., Polkowska, Ż., Namieśnik, J., 2016. The role of heterotrophic plate count bacteria in bottled water quality assessment. Food Control 61, 188–195.

Falcone-Dias, M.F., Farache Filho, A., 2013. Quantitative variations in heterotrophic plate count and in the presence of indicator microorganisms in bottled mineral water. Food Control 31, 90–96.

Falcone-Dias, M.F., Vaz-Moreira, I., Manaia, C.M., 2012. Bottled mineral water as a potential source of antibiotic resistant bacteria. Water Res. 46, 3612–22.

França, L., Lopéz-Lopéz, A., Rosselló-Móra, R., da Costa, M.S., 2014. Microbial diversity and dynamics of a groundwater and a still bottled natural mineral water. Environ. Microbiol.

Gobet, A., Boetius, A., Ramette, A., 2014. Ecological coherence of diversity patterns derived from classical fingerprinting and Next Generation Sequencing techniques. Environ. Microbiol. 16, 2672–2681.

Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66, 5488–91.

Hammes, F., Vital, M., Egli, T., 2010. Critical evaluation of the volumetric "bottle effect" on microbial batch growth. Appl. Environ. Microbiol. 76, 1278–81.

Haugland, R.P., 1996. Assays for cells viability, porliferation and function. In Handbook of Fluorescent Probes & Research Chemicals, in: Spence, M.T. (Ed.), . Molecular Probes Inc, pp. 365–398.

Hunter, P.R., 1993. The microbiology of bottled natural mineral waters. J. Appl. Microbiol. 74, 345–352.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41.

551 Kraková, L., Šoltys, K., Budiš, J., Grivalský, T., Ďuriš, F., Pangallo, D., & Szemes, T. (2016).
552 Investigation of bacterial and archaeal communities: novel protocols using modern
553 sequencing by Illumina MiSeq and traditional DGGE-cloning. Extremophiles, 20(5), 795-
554 808.

555 Leclerc, H., Moreau, A., 2002. Microbiological safety of natural mineral water. FEMS
556 Microbiol. Rev. 26, 207–222.

557 Lesaulnier, C.C., Herbold, C.W., Pelikan, C., Berry, D., Gérard, C., Le Coz, X., Gagnot, S.,
558 Niggemann, J., Dittmar, T., Singer, G.A., others, 2017. Bottled aqua incognita: microbiota
559 assembly and dissolved organic matter diversity in natural mineral waters. Microbiome 5,
560 126.

561 Loy, A., Beisker, W., Meier, H., 2005. Diversity of bacteria growing in natural mineral water
562 after bottling. Appl. Environ. Microbiol. 71, 3624–3632.

563 Mavridou, A., 1992. Study of the bacterial flora of a non-carbonated natural mineral water. J.
564 Appl. Microbiol. 73, 355–361.

565 McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive
566 Analysis and Graphics of Microbiome Census Data. PLoS One 8.

567 Muyzer, G., Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE)
568 and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van
569 Leeuwenhoek 73, 127–141.

570 Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P.R.,
571 O 'hara, R.B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner,
572 H., 2017. vegan: Community Ecology Package. R Packag. version 2.4-4 https://CRAN.R–
573 project.org/package=vegan.

574 R Development Core Team, 2016. R: A Language and Environment for Statistical Computing.
575 R Found. Stat. Comput. Vienna Austria 0, {ISBN} 3–900051–07–0.

576 Rosenberg, F.A., 2003. The microbiology of bottled water. Clin. Microbiol. Newsl. 25, 41–44.

577 Samarajeewa, A.D., Hammad, A., Masson, L., Khan, I.U.H., Scroggins, R., Beaudette, L.A.,
578 2015. Comparative assessment of next-generation sequencing, denaturing gradient gel
579 electrophoresis, clonal restriction fragment length polymorphism and cloning-sequencing
580 as methods for characterizing commercial microbial consortia. J. Microbiol. Methods 108,
581 103–111.

582 Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski,
583 R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G.,
584 Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: Open-source, platform-
585 independent, community-supported software for describing and comparing microbial
586 communities. Appl. Environ. Microbiol. 75, 7537–7541.

587 Spangenberg, J.E., Vennemann, T.W., 2008. The stable hydrogen and oxygen isotope variation
588 of water stored in polyethylene terephthalate (PET) bottles. Rapid Commun. Mass
589 Spectrom. 22, 672–676.

590 Tiodjio, R.E., Sakatoku, A., Nakamura, A., Tanaka, D., Fantong, W.Y., Tchakam, K.B.,
591 Tanyileke, G., Ohba, T., Hell, V.J., Kusakabe, M., Nakamura, S., Ueda, A., 2014.
592 Bacterial and archaeal communities in Lake Nyos (Cameroon, Central Africa). Sci. Rep. 4,
593 6151.

594 Urmeneta, J., Navarrete, A., Sancho, J., 2000. Isolation and identification of autochthonous
595 microbiota from a granitic aquifer and its variation after the bottling process. Curr.
596 Microbiol. 41, 379–383.

597 Vaz-Moreira, I., Egas, C., Nunes, O.C., Manaia, C.M., 2011. Culture-dependent and culture-
598 independent diversity surveys target different bacteria: a case study in a freshwater sample.
599 Antonie Van Leeuwenhoek 100, 245–57.

Venieri, D., Vantarakis, A., Komninou, G., Papapetropoulou, M., 2006. Microbiological evaluation of bottled non-carbonated ("still") water from domestic brands in Greece. Int. J. Food Microbiol. 107, 68–72.

Welle, F., 2011. Twenty years of PET bottle to bottle recycling—An overview. Resour. Conserv. Recycl. 55, 865–875.

Zobell, C.E., Anderson, Q., 1936. Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces. Biol. Bull. 71, 324–342.

615 **Tables and figures**

616 **Table 1.** Frequency of detection and similarity with the closest relatives of the DGGE bands.
617 Frequency*: number of samples with the band detected/ total number of samples in the batch
618 study.

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

619

620

621 **Table 2.** Jackknife analysis results. Numbers represent the percentage of DGGE fingerprints of
622 bottled water assigned to each water and material group. The number of misidentifications for
623 members of each group is given in the columns. Note that the values in the matrix are not
624 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

625

626 **Table 3.** Sensitivity and specificity obtained with the 10 OTUs selected in the discriminant
627 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

628

629

630

631

632

633

634

635

636

637

Figure 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.

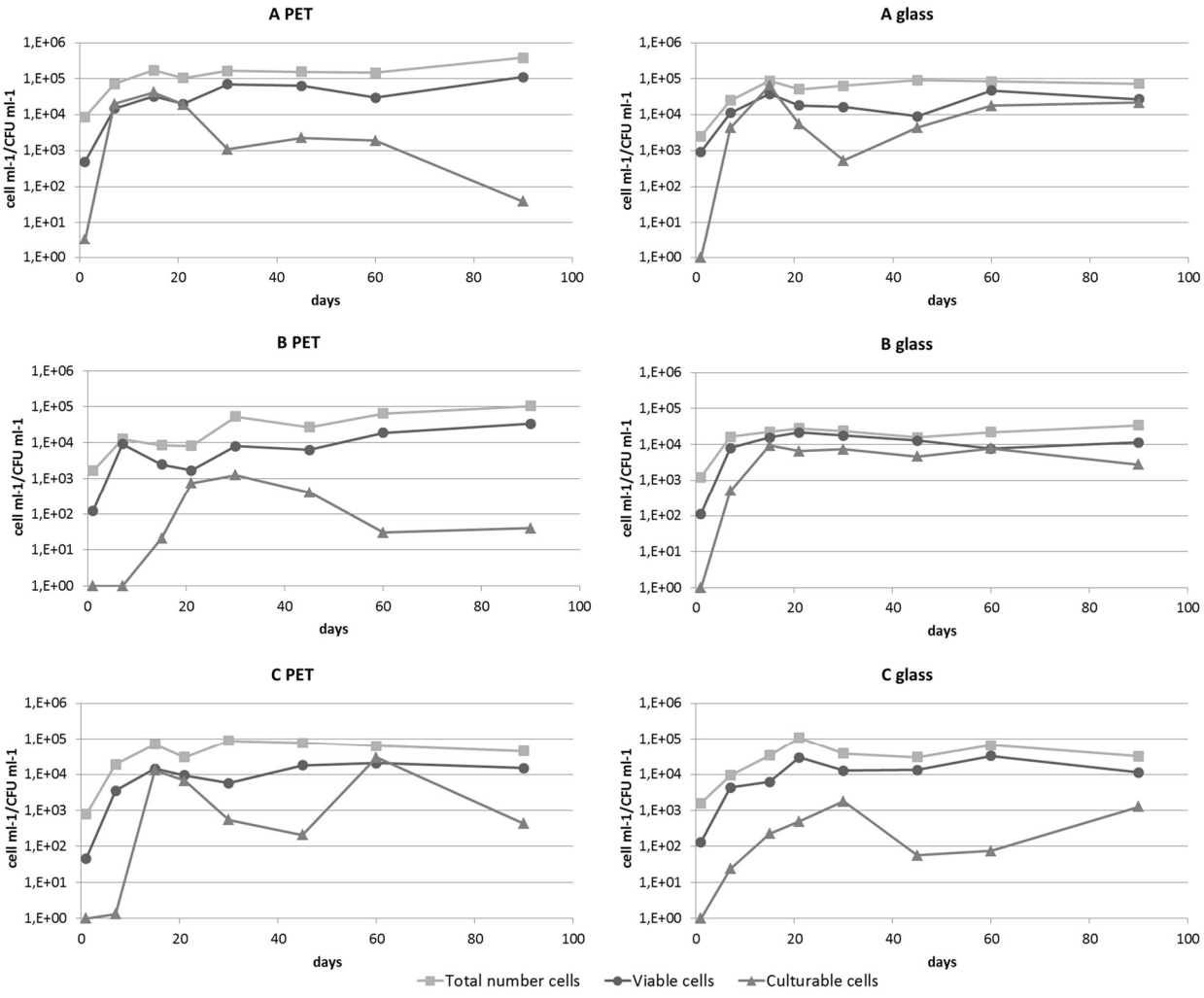


Figure 2. DGGE fingerprints of the 3 water mineral water brands analysed: a) A PET-bottled water, b) A glass-bottled water, c) B PET-bottled water, d) B glass-bottled water, e) C PET-bottled water and f) C glass-bottled water) over 90 days after bottling (1, 7, 15, 21, 30, 45, 60 and 90 days). Marker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

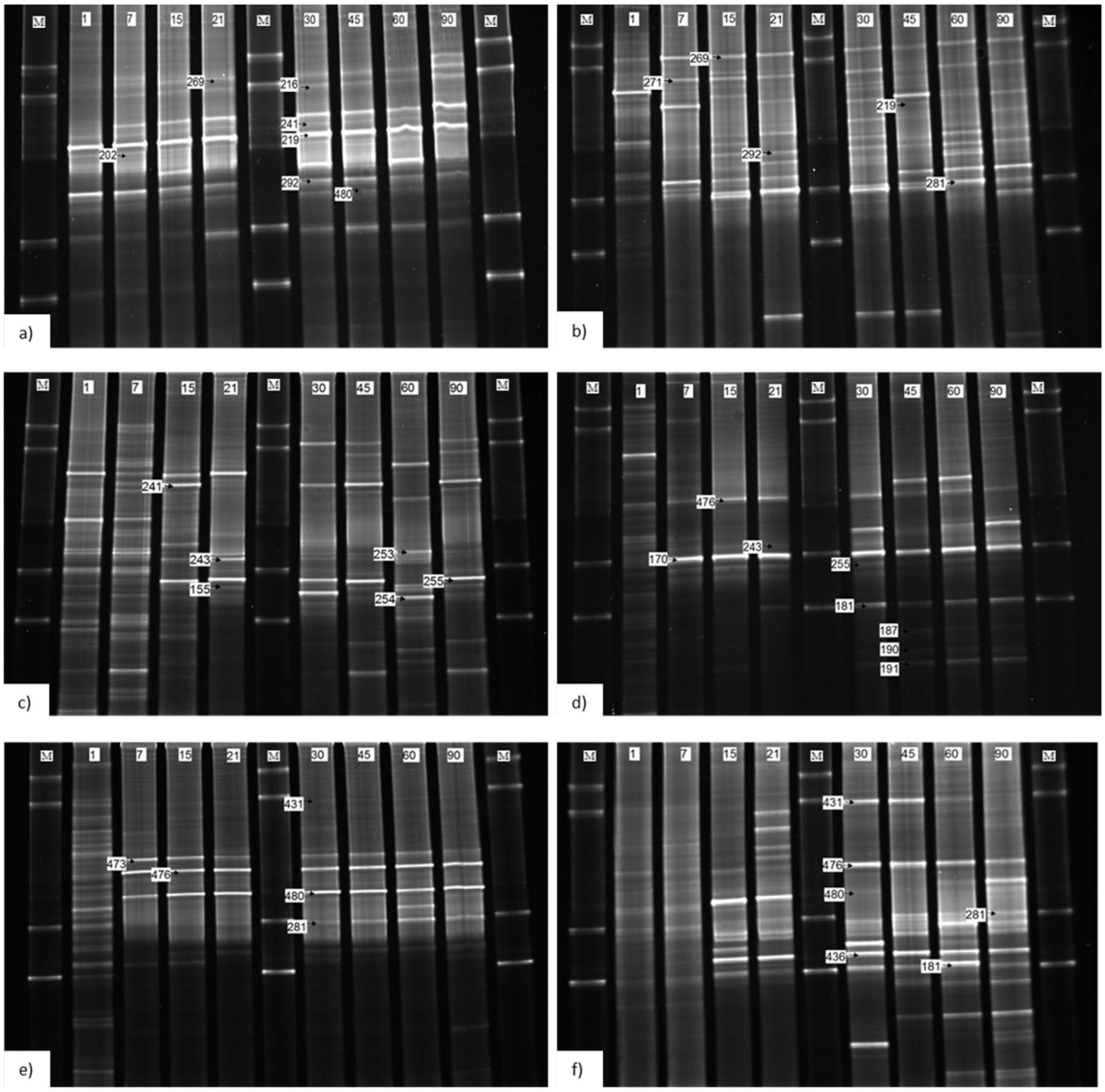


Figure 3. Cluster analysis of all the mineral water brands using Dice's coefficient and UPGMA. The number indicates the number of days after bottling. (COLOUR PRINTING)

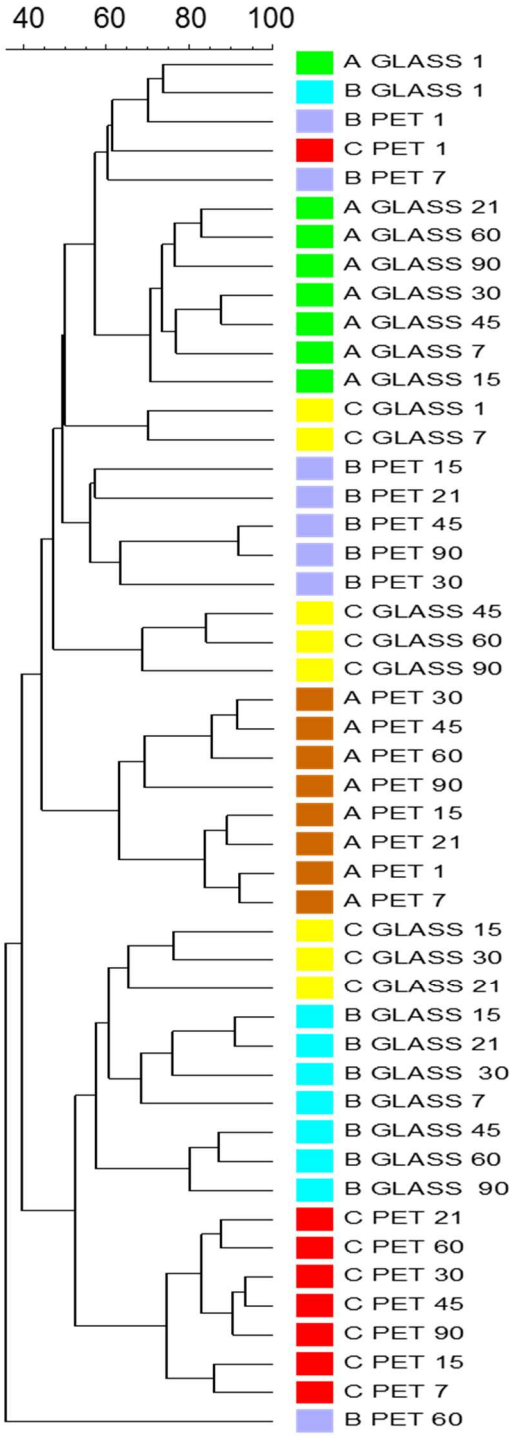
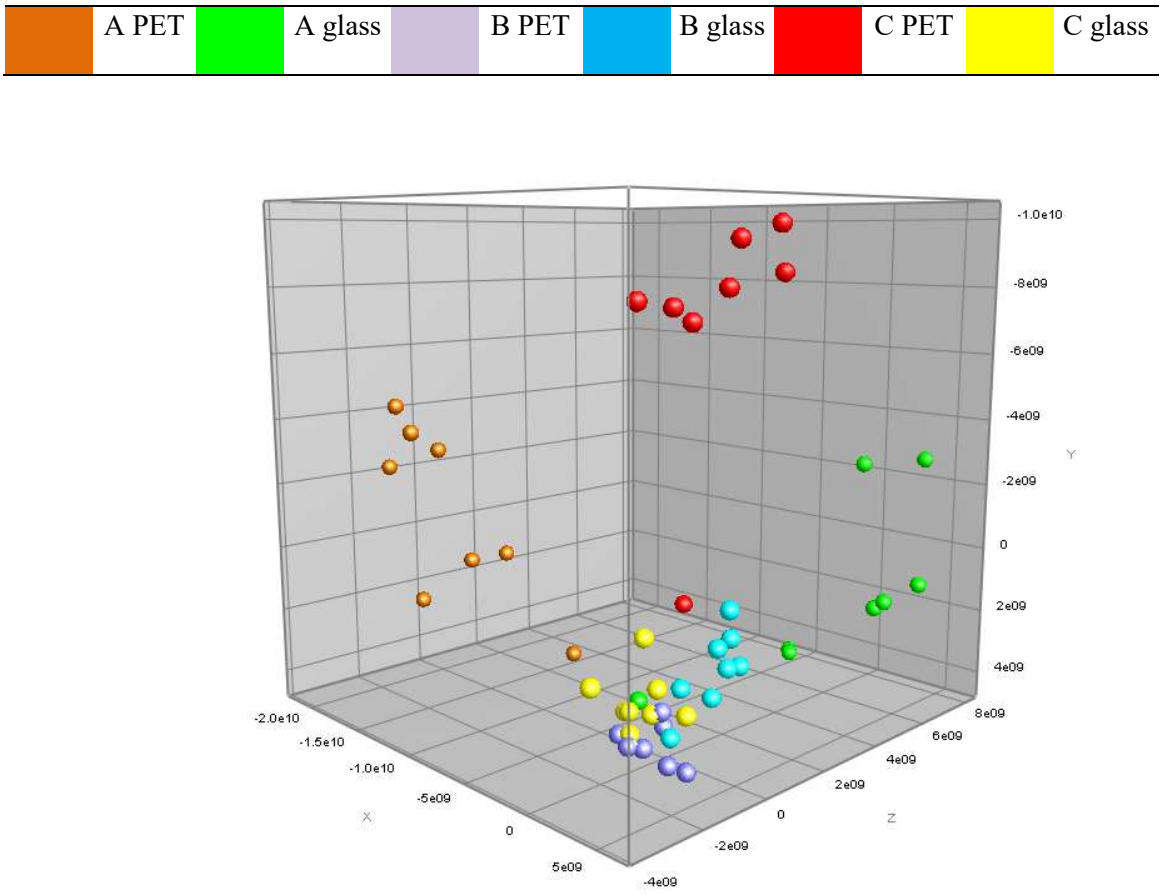
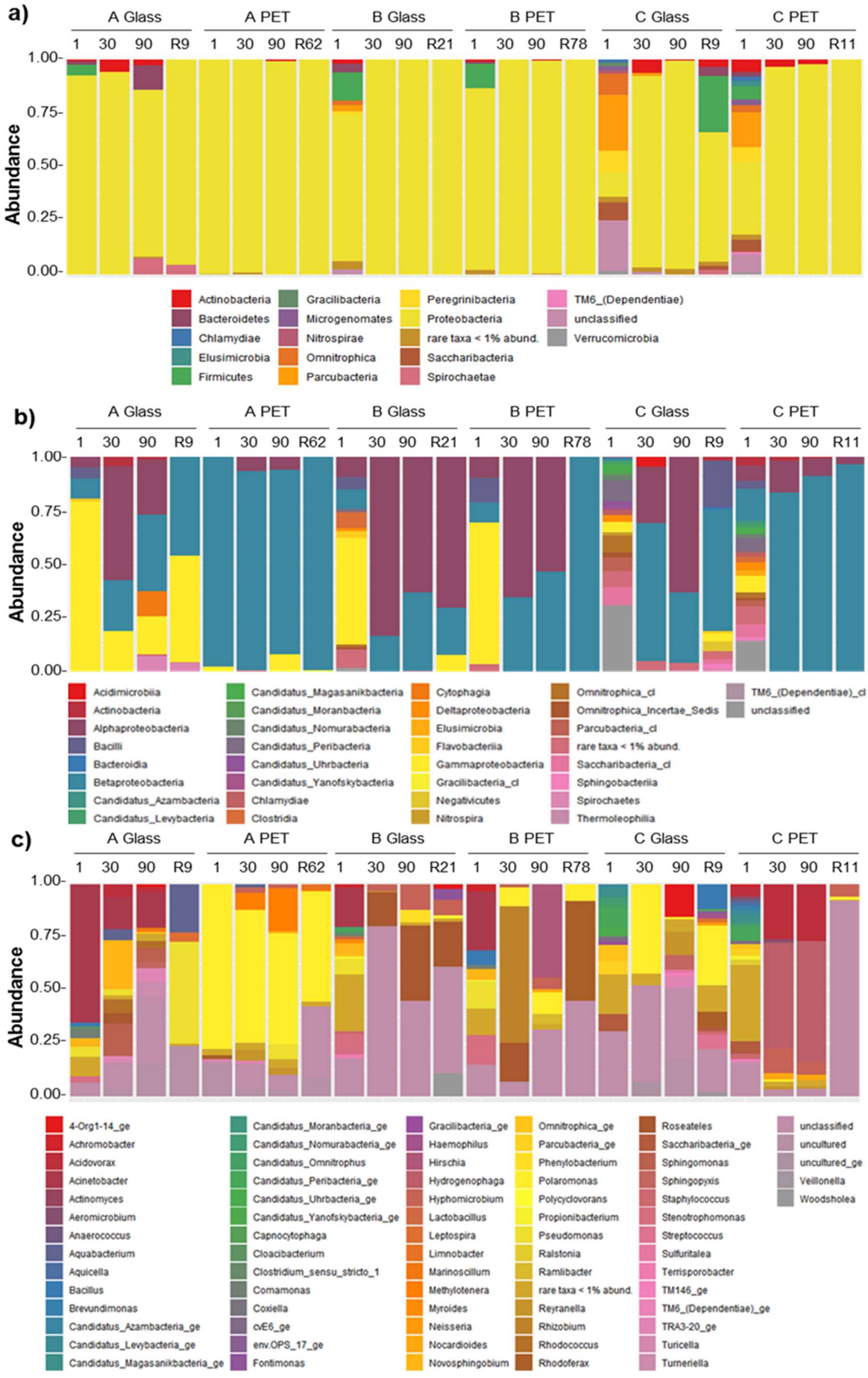


Figure 4. Principal components analysis (PCA) with the DGGE fingerprints. PC1, PC2 and PC3 are shown on x, y, z axes, respectively. (COLOUR PRINTING)



668 **Figure 5.** Community structure of the mineral water brands at phylum (a), class (b) and genus
669 (b) level according to 16s rRNA massive sequencing analysis. Results are expressed as relative
670 abundance of reads. The number of days after bottling of each sample is indicated at the top of
671 the columns (samples from the same batch). R indicates that the sample is a random sample
672 purchased directly from a retailer. (COLOUR PRINTING)

673


a)
A Glass
A PET
B Glass
B PET
C Glass
C PET
1 30 90 R9 1 30 90 R62 1 30 90 R21 1 30 90 R78 1 30 90 R9 1 30 90 R11
1.00
0.75
0.50
0.25
0.00
Abundance
Actinobacteria
Bacteroidetes
Chlamydiae
Elusimicrobia
Firmicutes
Gracilibacteria
Microgenomates
Nitrospirae
Omnitrophica
Parcubacteria
Peregrinibacteria
Proteobacteria
rare taxa < 1% abund.
Saccharibacteria
Spirochaetae
TM6_(Dependentiae)
unclassified
Verrucomicrobia
b)
A Glass
A PET
B Glass
B PET
C Glass
C PET
1 30 90 R9 1 30 90 R62 1 30 90 R21 1 30 90 R78 1 30 90 R9 1 30 90 R11
1.00
0.75
0.50
0.25
0.00
Abundance
Acidimicrobiia
Actinobacteria
Alphaproteobacteria
Bacilli
Bacteroidia
Betaproteobacteria
Candidatus_Azambacteria
Candidatus_Levybacteria
Candidatus_Magasanikbacteria
Candidatus_Moranbacteria
Candidatus_Nomurabacteria
Candidatus_Peribacteria
Candidatus_Uhrbacteria
Candidatus_Yanofskybacteria
Chlamydiae
Clostridia
Cytophagia
Deltaproteobacteria
Elusimicrobia
Flavobacteriia
Gammaproteobacteria
Gracilibacteria_cl
Negativicutes
Nitrospira
Omnitrophica_cl
Omnitrophica_Incertae_Sedis
Parcubacteria_cl
rare taxa < 1% abund.
Saccharibacteria_cl
Sphingobacteriia
Spirochaetes
Thermoleophilia
TM6_(Dependentiae)_cl
unclassified
c)
A Glass
A PET
B Glass
B PET
C Glass
C PET
1 30 90 R9 1 30 90 R62 1 30 90 R21 1 30 90 R78 1 30 90 R9 1 30 90 R11
1.00
0.75
0.50
0.25
0.00
Abundance
4-Org1-14_ge
Achromobacter
Acidovorax
Acinetobacter
Actinomyces
Aeromicrobium
Anaerococcus
Aquabacterium
Aquicella
Bacillus
Brevundimonas
Candidatus_Azambacteria_ge
Candidatus_Levybacteria_ge
Candidatus_Magasanikbacteria_ge
Candidatus_Moranbacteria_ge
Candidatus_Nomurabacteria_ge
Candidatus_Omnitrophus
Candidatus_Peribacteria_ge
Candidatus_Uhrbacteria_ge
Candidatus_Yanofskybacteria_ge
Capnocytophaga
Cloacibacterium
Clostridium_sensu_stricto_1
Comamonas
Coxiella
cvE6_ge
env.OPS_17_ge
Fontimonas
Gracilibacteria_ge
Haemophilus
Hirschia
Hydrogenophaga
Hyphomicrobium
Lactobacillus
Leptospira
Limnobacter
Marinoscillum
Methylotenera
Myroides
Neisseria
Nocardioides
Novosphingobium
Omnitrophica_ge
Parcubacteria_ge
Phenylobacterium
Polaromonas
Polycyclovorans
Propionibacterium
Pseudomonas
Ralstonia
Ramlibacter
rare taxa < 1% abund.
Reyranella
Rhizobium
Rhodococcus
Rhodoferax
Roseateles
Saccharibacteria_ge
Sphingomonas
Sphingopyxis
Staphylococcus
Stenotrophomonas
Streptococcus
Sulfuritalea
Terrisporobacter
TM146_ge
TM6_(Dependentiae)_ge
TRA3-20_ge
Turicella
Turneriella
unclassified
uncultured
uncultured_ge
Veillonella
Woodsholea

675 **Supplementary Material**

676

677 Traceability of different brands of bottled mineral water during shelf life, using next generation
678 sequencing techniques

679

680

681 Laura Sala-Comorera[1,2]*, Anicet R. Blanch[1,2], Arnau Casanovas-Massana[1,2#], Antonio Monleón-
682 Getino[1] and Cristina García-Aljaro[1,2]

683

684 [1]Department of Genetics, Microbiology and Statistics, University of Barcelona, Avinguda
685 Diagonal, 643 - 08028 Barcelona, Spain

686 [2]The Institute of Water Research. C/ Montalegre, 6 - 08001 Barcelona, Spain
687

688

689 *Corresponding author

690 Mailing address:

691 Avinguda Diagonal, 643, 08028 Barcelona, Catalonia, Spain

692 Phone: (+34) 934029012

693 Fax: (+34) 934039047

694 E-mail: laura.sala-comorera@ucd.ie

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

Materials and Methods

DNA extraction

Extraction was performed adding 0.37 g (≤106 µm) acid-washed glass beads, 0.25 g (1 mm) acid-washed glass beads, 1 piece (3 mm) acid-washed glass beads, 400 µl chloroform/isoamyl alcohol (24:1 v/v), 400 µl phenol and 400 µl CTAB-buffer (100 mM TRIS HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB. Samples were incubated for 30 min at 75℃ and then 0.2% (v/v) mercaptoethanol. Samples were subsequently lysed for 15 min, speed 10 with Vortex Genie® 2 (Scientific Industries Inc, USA), and immediately chilled on ice (1 min). The aqueous phase was separated by centrifugation (16,000 × g) for 5 min and mixed with 500 µl chloroform/isoamyl alcohol (24:1 v/v) followed by a centrifugation (16,000 × g) for 5 min. Supernatants were transferred to a 1.5-mL microtube with 0.6 volumes of isopropanol and incubated at room temperature overnight. After the incubation samples were centrifuged at 16,000 × g for 10 min at 4℃ and pelleted nucleic acids were washed twice in ice cold 70% ethanol and air dried prior to resuspension in 20 µl in Tris 10 mM pH=8.0.

PCR conditions

The V3 hypervariable region from 16S rRNA gene was amplified by nested PCR. The first step, primers 27f (5' -AGA GTT TGA TCM TGG CTC AG- 3') and 1492r (5' -TAC GGY TAC CTT GTT ACG ACT T- 3') (Weisburg et al., 1991) were used. The PCR was performed in a total volume of 50 µl including 25 µl of DreamTaq Green PCR Master Mix (2x) (Thermo Scientific, USA), 0.5 µM of each universal bacterial primers and 2 µl of DNA. PCR conditions were: 94℃ for 5 min, 35 cycles of 94℃ for 1 min, 55℃ for 1 min and 72℃ for 1.5 min. A final extension phase of 72℃ for 7 min was used. In a second step, the samples were reamplified using the pair of primers PRBA338f (Lane, 1991; Ovreas et al., 1997) (5' -ACT CCT ACG GGA GGC AGC AG- 3') with a GC clamp attached to the 5' end (5' -CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G- 3') and PRUN518r (Muyzer et al., 1993) (5'-ATT ACC

GCG GCT GCT GG- 3'). Each 100-µl PCR mixture contained 50 µl of DreamTaq Green PCR Master Mix (2x) (Thermo Scientific, USA), 0.1 µM of each universal bacterial primers and 5 µl of DNA of the first PCR product. The nested-PCR was performed under the following conditions: 94˚C for 4 min; 30 cycles of 94˚C for 1 min, 59˚C for 1 min, and 74˚C for 1 min; and 74˚C for 3 min.

DGGE

Electrophoresis was performed with 1-mm thick 8% (w/v) polyacrylamide gels (30% acrylamide/bis-acrylamide [37.5:1]). Approximately 800 ng of nested-PCR products were loaded into lanes of a gel, containing a linear 35-70% denaturing gradient (100% denaturant agent was defined as 7 mol/L urea and 40% [v/v] formamide). Nested-PCR products were quantified by electrophoresis in a 2% agarose gel using Low DNA Mass Ladder (Invitrogen, USA). To compare different DGGE gels, an in-house DGGE-marker was used, consisting in four strains previously isolated from natural mineral water or drinking water (T32.2-*Micrococcus* sp., V44.2-*Sphingopyxis* sp., 3B.B24-*Pseudomonas* sp. and TR11.2-*Bacillus* sp.). They were cultured on Tryptic Soy Broth (Pronadisa, Spain) at 20 ± 2˚C for 24 ± 2 h. DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Spain) following manufacturer's instructions. A total of 10 µl of a mixed of nested-PCR product from the reference strains were loaded into 3 lanes in each gel.

Gels were run for 15 min at 20 V followed by 5 h at 200 V in 1x Tris-acetate acid-EDTA (TAE) (40 mmol/L Tris, 20 mmol/L sodium acetate, 1 mmol/L EDTA; pH = 7.4) at 60˚C. Gels were visualized by 45 min staining in 1x sodium chloride-Tris-EDTA buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA; pH 7.4) with SYBRGold nucleic acid stain (Molecular Probes Inc.,USA), followed by a subsequent analysis under UV radiation with ChemiDoc™ MP Imaging System (BioRad, USA). Gels were scanned using the Quantity One 4.6.7 program (Bio-Rad, USA).

749 Common bands were excised and transferred to a microtube containing 30 µl of ultra-pure water
750 and stored at 4˚C overnight. Five µl were used for PCR reamplification with the primers
751 PRBA338f and PRUN518r with the same conditions mentioned above. PCR products were
752 cloned into p-GEM®-T Easy Vector Systems (Pronadisa, Spain) according to manufacturer's
753 instructions and Sanger sequenced (ABI Prism 3700; PerkinElmer, Thermo Fischer Scientific,
754 USA). The 16S rRNA gene sequences were submitted for similarity searches to the NCBI using
755 Blast search tool (http://www.ncbi.nlm.nih.gov/Blast/).

756

757 **Tables and Figures**

758 **Table S1**. Dice similarity of DGGE fingerprints between the randomly bottled waters and the
759 closest DGGE fingerprint of the batch study. Number of the common bands observed between
760 the DGGE fingerprints of bottles randomly selected and the DGGE fingerprints of the batch
761 study. Frequency*: number of samples with the band detected/ total number of samples of the
762 batch study.

Mineral water sample randomly selected (days after bottling)	Dice similarity coefficient (%)	Mineral water sample	Common bands	
			Band name	Frequency*
A PET R (9)	56	A PET 7	219	8/8
			216	7/8
			269	6/8
A glass R (62)	36	A glass 60	292	8/8
			269	7/8
B PET R (21)	48	B PET 21	253	6/8
			243	5/8
B glass R (78)	40	B glass 90	476	8/8
			181	6/8
C PET R (30)	50	C PET 30	480	8/8
C glass R (11)	38	C glass 15	431	6/8

763

764

Figure S1. Cluster analysis of DGGE fingerprints with similarity matrix for (a) A PET and A glass mineral water, (b) B PET and B glass mineral water and (c) C PET and C glass mineral water. Clustering was performed using Dice's coefficient and UPGMA.

a) b) c)

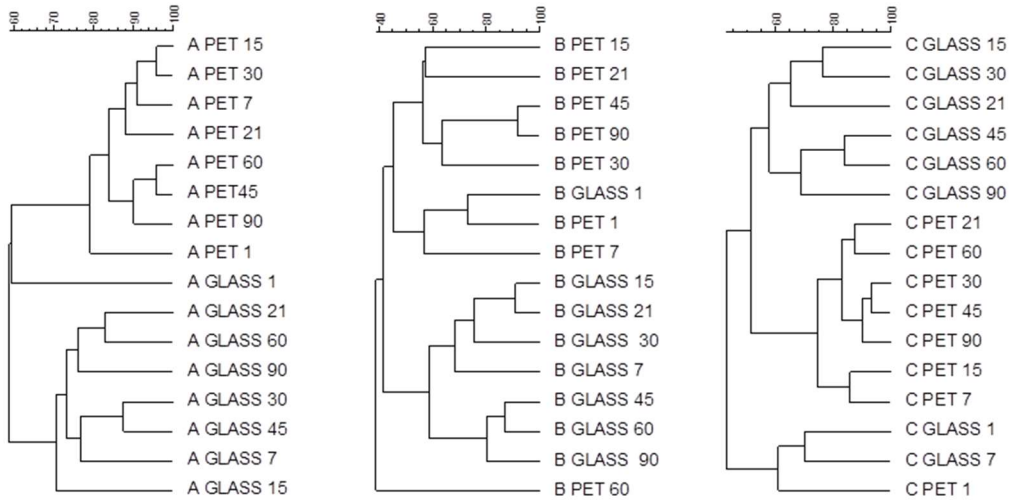
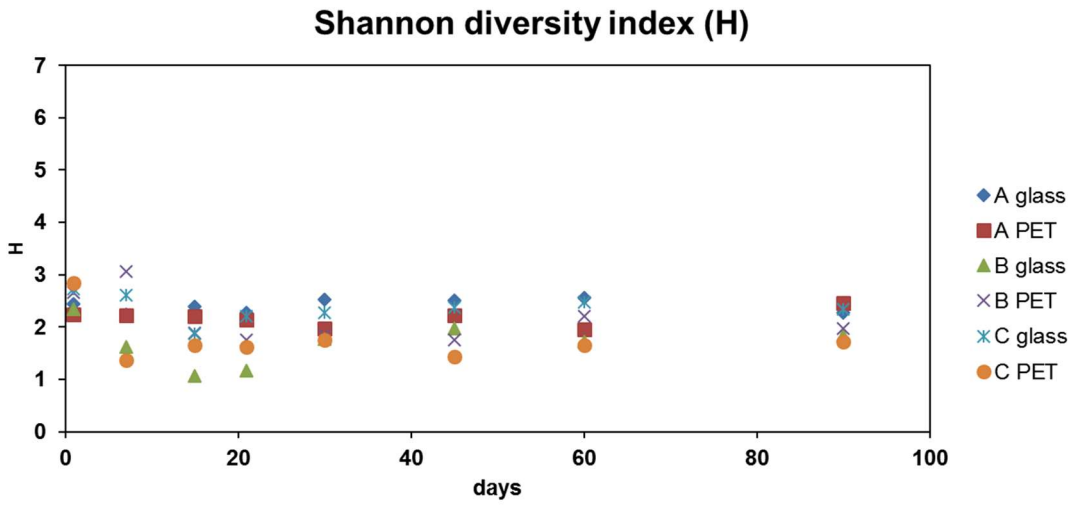


Figure S2. Changes in the Shannon-Weaver index of diversity based on the number and relative intensities of the bands of the DGGE profiles (a) and 16S rRNA Illumina reads (b).

a)



b)

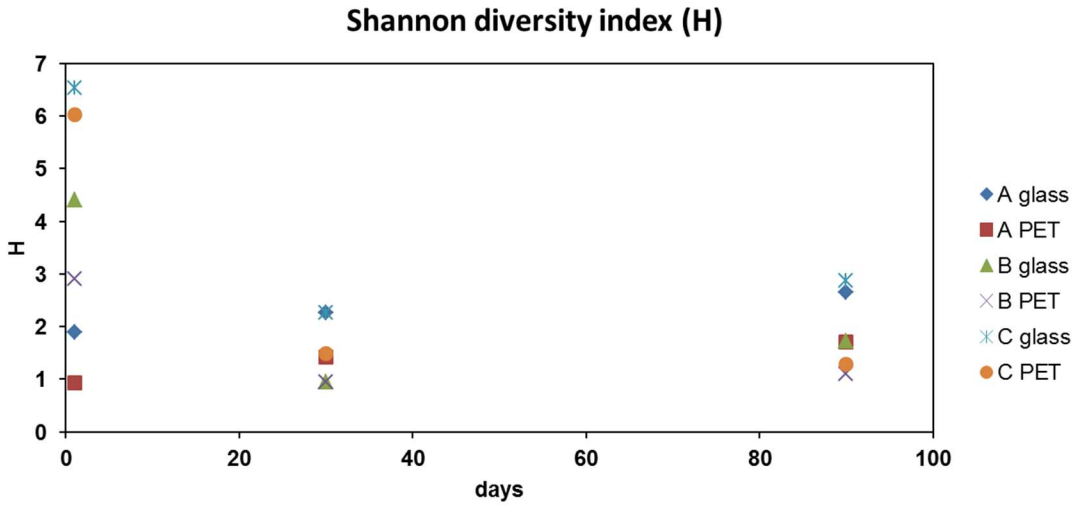


Fig S3. Venn diagrams showing the shared OTUs between the different water brands and materials. a) A water; b) B water; b) C water. * only detected in aged samples

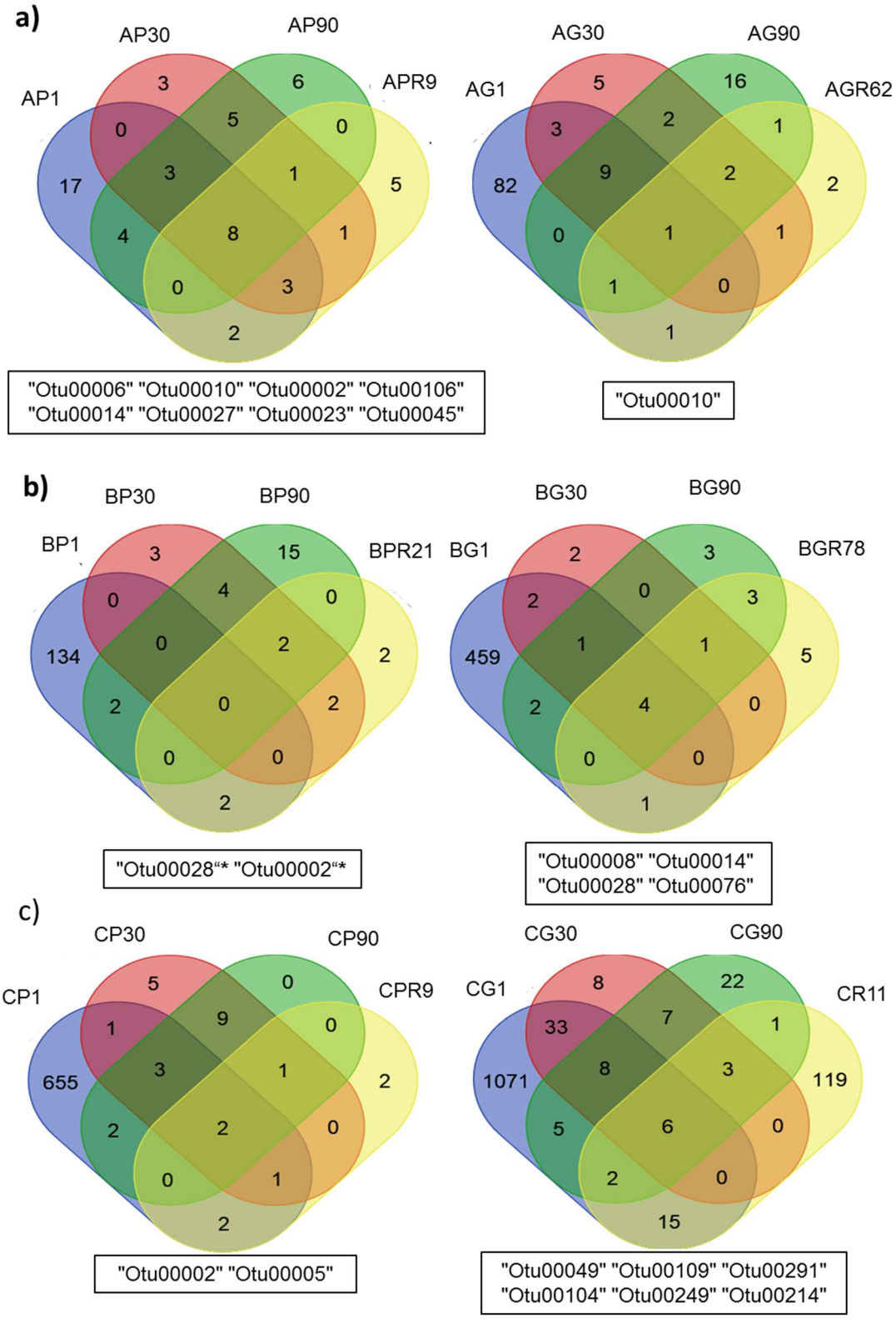
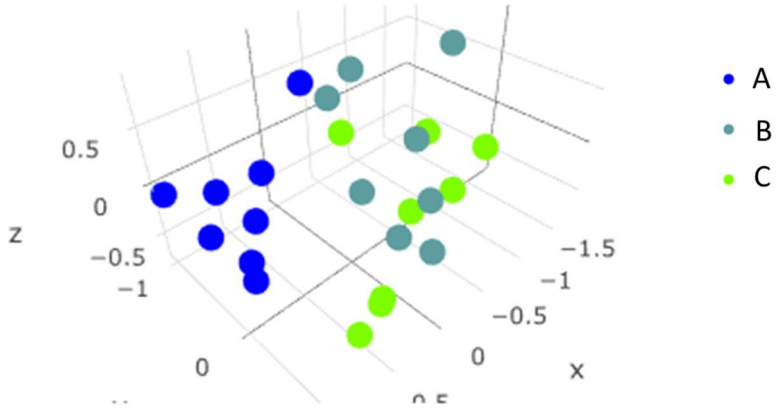


Fig S4. Analysis of β-diversity of the different water brands and materials using a) weighted UniFrac b) clustering of the different water brands. The gradient colour from red to yellow indicate higher to lower β-diversity.

a)



b)

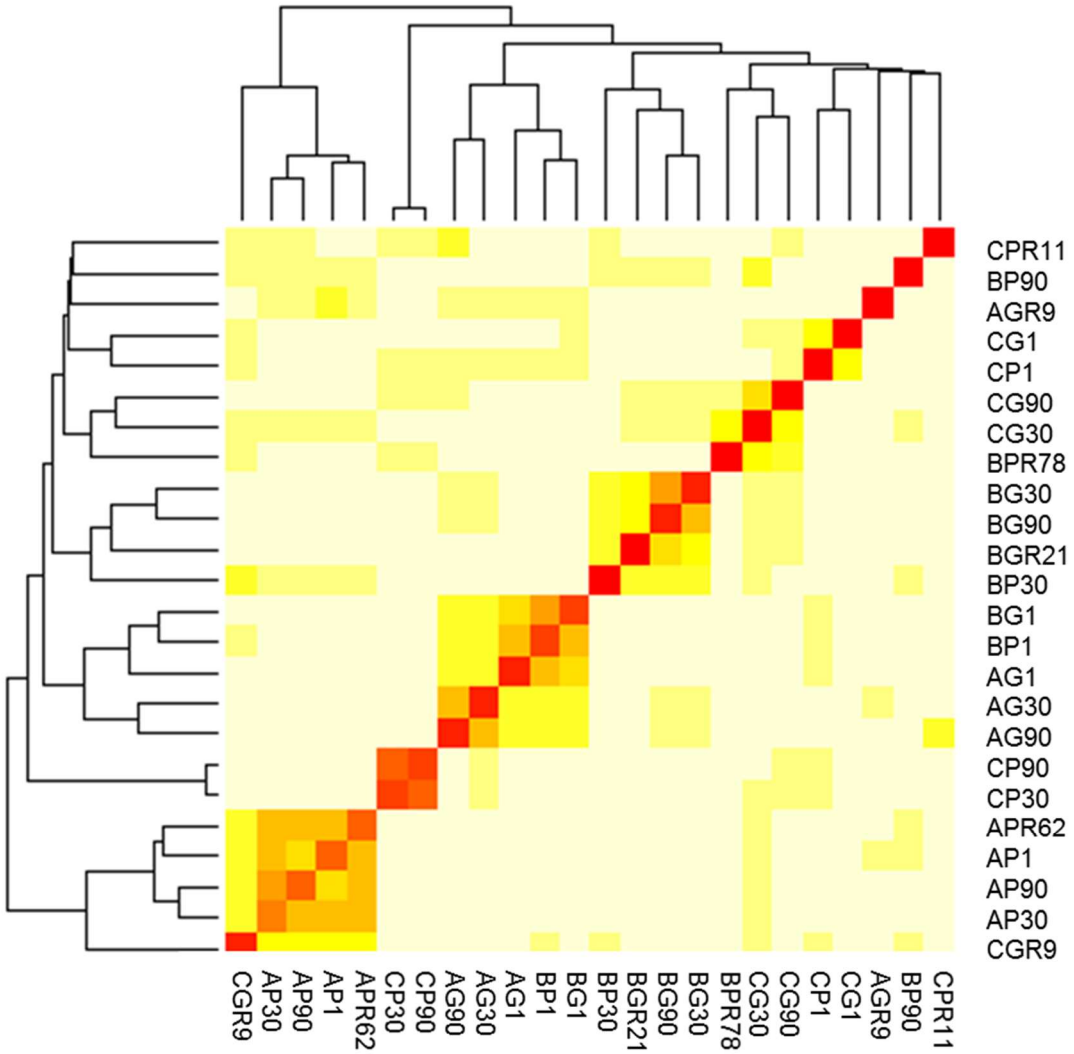
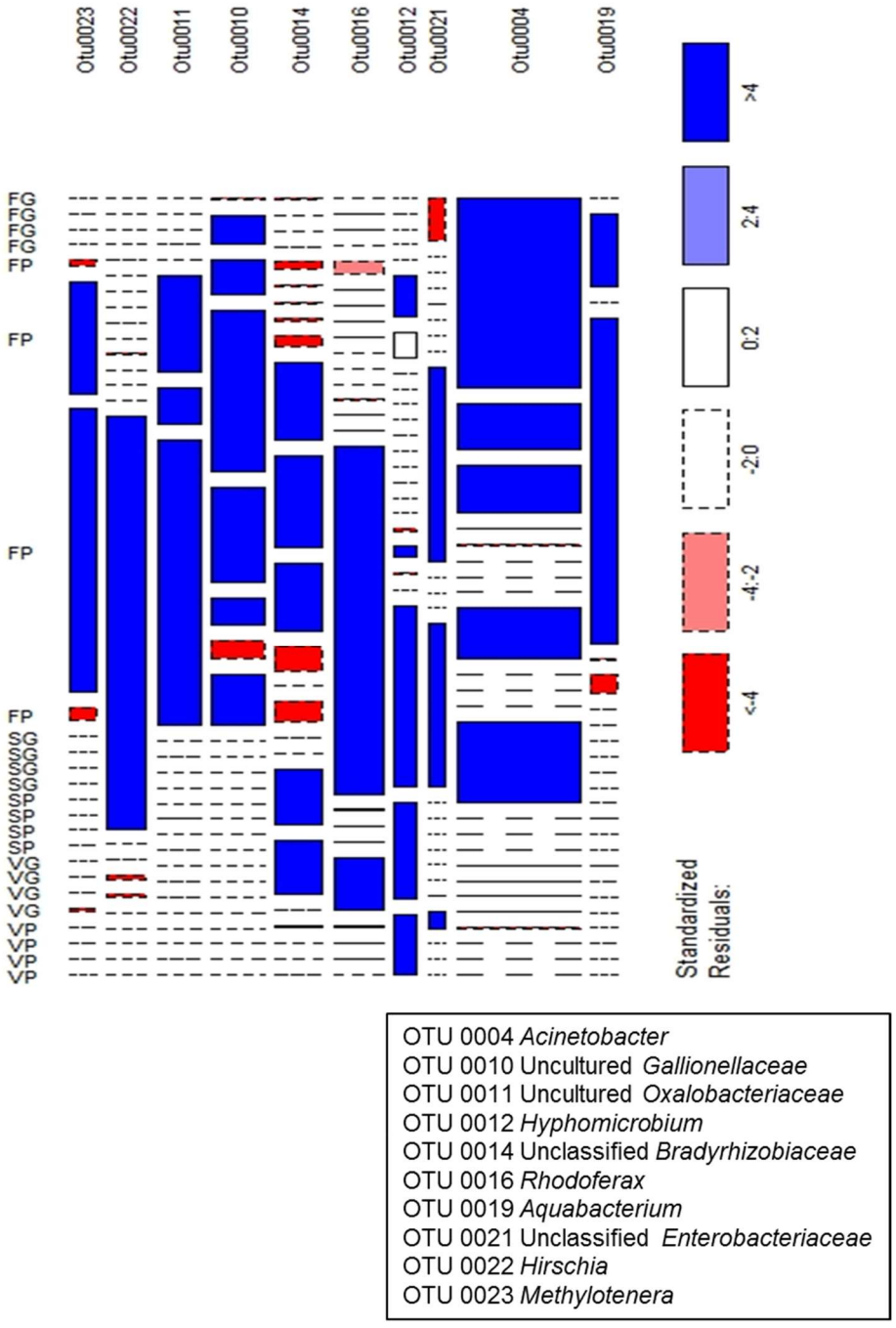

CPR11
BP90
AGR9
CG1
CP1
CG90
CG30
BPR78
BG30
BG90
BGR21
BP30
BG1
BP1
AG1
AG30
AG90
CP90
CP30
APR62
AP1
AP90
AP30
CGR9
CGR9
AP30
AP90
AP1
APR62
CP30
CP90
AG90
AG30
AG1
BP1
BG1
BP30
BGR21
BG90
BG30
BPR78
CG30
CG90
CP1
CG1
AGR9
BP90
CPR11

Fig S5. Mosaic plot of the OTUs selected in the discriminant analysis. Shadings are made based on the Pearson residuals of an independence model. The cutoffs are based on certain heuristics and are meant to bring out patterns in the Pearson residuals. The association plot shows the Pearson residuals directly, highlighting in which cells there are more or less observations than expected. In this case we can see in blue all the OTUS more present than expected, only in few cases there are OTUs fewer present than expected. Samples are coded as follows: water A, glass (AG); water A, PET (AP); water B, glass (BG); water B, PET (BP); water C, glass (CG); water C, PET (CP).



References

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. Chichester, United Kingdom.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

Ovreas, L., Forney, L., Daae, F.L., Torsvik, V., 1997. Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl. Environ. Microbiol. 63, 3367–3373.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.