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**Assessing the Viability of Bacterial Species in Drinking
Water by Combined Cellular and Molecular Analyses
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2 **Assessing the viability of bacterial species in drinking water by combined cellular and**
3 **molecular analyses**
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1 ABSTRACT

2

3 The question which bacterial species are present in water and if they are viable is essential
4 for drinking water safety but also of general relevance in aquatic ecology. To approach this
5 question we combined Propidium iodide/SYTO9 staining (“live/dead staining” indicating
6 membrane integrity), Fluorescence Activated Cell Sorting (FACS) and community
7 fingerprinting for the analysis of a set of tap water samples. Live/dead staining revealed that
8 about half of the bacteria in the tap water had intact membranes. Molecular analysis using
9 16S rRNA and 16S rRNA gene-based single strand conformation polymorphism (SSCP)
10 fingerprints and sequencing of drinking water bacteria before and after FACS sorting
11 revealed: i) the DNA- and RNA-based overall community structure differed substantially,
12 ii) the community retrieved from RNA and DNA reflected different bacterial species,
13 classified as 53 phylotypes (with only two common phylotypes), iii) the percentage of
14 phylotypes with intact membranes or damaged cells were comparable for RNA and DNA
15 based analyses, and iv) the retrieved species were primarily of aquatic origin. The
16 pronounced difference between phylotypes obtained from DNA extracts (dominated by
17 *Betaproteobacteria*, *Bacteroidetes* and *Actinobacteria*) and from RNA extracts (dominated
18 by *Alpha*-, *Beta*-, *Gammaproteobacteria*, *Bacteroidetes* and *Cyanobacteria*) demonstrate
19 the relevance of concomitant RNA and DNA analyses for drinking water studies.
20 Unexpected was that a comparable fraction (about 21%) of phylotypes with membrane
21 injured cells was observed for DNA- and RNA-based analyses, contradicting the current
22 understanding that RNA-based analyses represent the actively growing fraction of the
23 bacterial community. Overall, we think that this combined approach provides an interesting
24 tool for a concomitant phylogenetic and viability analysis of bacterial species of drinking
25 water.

Key words: Live/Dead staining, SSCP fingerprints, Fluorescence Activated Cell Sorting (FACS), DNA and RNA based 16S rRNA gene analyses

INTRODUCTION

Drinking water commonly provides a diverse microflora to the end user despite the fact that water processing eliminates a large fraction of microorganisms present in raw water, as shown by detailed molecular studies [14, 34]. Bacteria originating from source water, regrowth in bulk water and biofilms of the distribution network contribute to the generation of a diverse bacterial community in drinking water [17] .

Molecular methods, such as 16S rRNA-based and 16S rRNA gene-based fingerprints, can provide an overview on the bacterial community and thus can overcome the restriction of cultivation based methods that detect only the few bacteria growing under the respective cultivation conditions [9]. These molecular methods allow overcoming the problem of non-culturability for viable-but-non-culturable (VNBC) bacteria, i.e. even under adequate cultivation conditions these bacteria do not grow due to physiological constraints [21] . However, molecular methods based on extracted nucleic acids cannot distinguish between live and dead bacteria [6, 31] . During the last years, a broad set of fluorescent stains was developed allowing insight into the physiological state of bacteria [22]. Stains assessing membrane integrity, such as Propidium Iodide (PI) and SYTO9, are considered to distinguish between membrane intact and membrane injured cells [7]. This staining procedure has been evaluated and compared by a set of studies to other staining procedures for assessment of the physiological state of the bacteria [11, 22, 4] . Membrane injury was evaluated as a reliable criterion for cell death where recovery is highly unlikely.

Bacterial community fingerprints and subsequent sequencing of the single fingerprint bands followed by phylogenetic analysis can provide an overview on the structure and composition of bacterial drinking water communities [14]. Besides providing an overview, fingerprints allow the study of any bacterial taxon in a community if specific primers are used to better understand its ecology [19] that is of special relevance for pathogenic taxa. 16S rRNA-based fingerprint analyses can be based on the analysis of environmental DNA or RNA. In general, it is assumed that RNA-based fingerprints represent the active part, especially the actively growing part, of the bacterial community whereas DNA-based analyses provide insight into the bacterial members present in the community [14, 29]. Since viability is a major issue for drinking water bacteria, the comparison of DNA- and RNA-based analyses is of great interest. Combining these DNA- and RNA-based fingerprint analyses with the distinction for membrane integrity was intended to provide new insights in the bacterial microflora and its viability.

Today's drinking water quality assessment is still based on the culture-based detection of indicator bacteria, i. e. *Escherichia coli* or fecal enterococci. Though molecular methods could provide better insights into the bacterial community, it is crucial to include the aspect of viability in the molecular methods used. To this end, we developed a procedure that combined the advantages of culture- independent molecular methods and the discrimination of membrane intact and membrane injured cells provided by the viability stains. Using Fluorescence Activated Cell Sorting (FACS), the membrane intact ("live") and membrane injured cells ("dead") were separated and afterwards analyzed by community fingerprinting. The aim of our study was to elucidate by this approach which bacterial taxa are alive in finished drinking water. Both nucleic acids, DNA and RNA, were extracted from three fractions, i.e. total, "live" and "dead", and analyzed by 16S rRNA-based and 16S rRNA gene-based SSCP fingerprinting followed by sequencing of the

fingerprint bands to provide insight into the taxonomic composition of the bacterial community. The study was encouraged by a previous analysis of the RNA based bacterial community structure of drinking water that showed the proof of principle of the technical approach [24]. This previous study indicated the need of a direct comparison of DNA and RNA community structure and a detailed phylogenetic analysis that are now provided. In the present study, differences between DNA- and RNA-based fingerprints were analyzed to gain information about the active vs. present part of the bacterial drinking water microflora in the light of membrane integrity. To our knowledge, this is the first study that applies both, DNA- and RNA- based community analysis up to the species level combined with FACS sorting based on live/dead staining. This allowed a comparison of present, “live” and “dead” bacterial species for RNA and DNA extracts.

MATERIAL AND METHODS

Study site and sampling.

Drinking water samples were obtained on 3 days, i.e. 25 March 2008 (sampling A), 31 March 2008 (sampling B) and 5 May 2008 (sampling C) from the tap in lab D0.04 of the Helmholtz Centre for Infection Research (HZI), Braunschweig-Stöckheim, Germany. Sampling A and B were taken as samples where a high similarity was expected due to the short time interval, sampling C was considered to display a distinct community due to the previously observed seasonal changes [19]. The drinking water originated from two surface water reservoirs (oligotrophic, and dystrophic water) situated in a mountain range 40 km south of Braunschweig. Water processing included flocculation/coagulation, sand filtration and chlorination (0.2 - 0.7 mg l⁻¹). In 2008 and 2009 no chlorine was detected at the nearest sampling point upstream to the HZI by the local water supplier by the colorimetric test “Aquaquant Chlor” from Merck for detection of free and total chlorine (detection limit

0.01mg/l). More details on the respective drinking water supply system are given elsewhere [14].

For live/dead staining and fluorescence activated cell sorting (FACS), drinking water microorganisms were concentrated 100-400 fold. 18 liter of drinking water were filtered onto a 0.2 μ m pore size polycarbonate filter (90-mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom), scraped and washed off from the filter carefully with 25 ml of 0.9% NaCl in sterile water (Fig. 1). A part of the biomass was either immediately used for the staining procedure as indicated below, and an aliquot was immediately frozen for later molecular analysis (-70°C).

For comparing the impact of concentration on the drinking water microflora, the drinking water microorganisms were additionally harvested by our routine procedure, i.e. filtration of 5 liters of drinking water on a filter sandwich consisting of a 0.2 μ m pore size polycarbonate filter (90 mm diameter; Nucleopore; Whatman, Maidstone, United Kingdom) with a precombusted glass fiber filter on top (90 mm diameter; GF/F; Whatman) [13]. Filter sandwiches were stored at -70°C until further analysis. Per sampling date, 5 sandwich filters were obtained.

Staining and enumeration of drinking water bacteria.

Total bacteria from formaldehyde-fixed samples (2% final concentration) were stained with Sybr Green I dye (1:10000 final dilution; Molecular Probes, Invitrogen) for 15min at room temperature in the dark. Five ml portions were filtered onto 0.2 μ m pore size Anodisc filters (Whatman) and mounted with Citifluor on microscopic glass slides [35]. Slides were either analyzed directly with epifluorescence microscopy or stored frozen (-20°C) until examination. For epifluorescence microscopy, a microscope (Axioplan, Zeiss)

with suitable fluorescence filters was used and the slides were examined using 100fold magnification. For each filter, either 10 photographs were taken and image sections of defined size (0.642mm x 0.483mm) were analyzed using the Image J software from MacBiophotonics ([http://www. macbiophotonics.ca/](http://www.macbiophotonics.ca/)) and/or 30 fields (0.125mm x 0.125mm) were counted by eye.

Heterotrophic plate counts (HPC).

HPCs were done in triplicate using an aliquot of the drinking water concentrate and the spread plate technique on either R2A agar (Oxoid) or tryptone soy agar (TSA; Oxoid) plates. Incubation was carried out at two different temperatures according to the German drinking water ordinance (36°C for 48h and 22°C for 72h) (Verordnung über die Qualität von Wasser für den menschlichen Gebrauch (Trinkwasserverordnung - TrinkwV 2001) Geändert durch Art. 363 V v. 31.10.2006 I 2407, 2001).

Concentrating, live/dead staining and FACS analysis of drinking water bacteria.

For fluorescence activated cell sorting (FACS), the concentrated biomass of the drinking water samples was stained for subsequent FACS analysis with SYTO 9 and propidium iodide (PI, final concentrations 5µM and 30µM, respectively; BacLight Kit, Molecular Probes [18] according to the prescription of the manufacturer. After an incubation time of 20min in the dark, cells were subjected to FACS sorting using a MOFLO cytometer (Beckman Coulter, Krefeld, Germany) with a 488nm laser. The band pass filters used were 530/40nm and 616/26nm for SYTO 9 and PI, respectively.

Nucleic acid extraction from drinking water and sorted fractions.

DNA- and RNA- were extracted from the filter sandwiches and the concentrates of the drinking water samples; the latter were analyzed before and after staining and FACS-

1 sorting as described above. For extraction of DNA and RNA, a modified DNeasy/RNeasy
2 protocol (Qiagen, Hilden, Germany) was used. In this procedure, sandwich filters were cut
3 into pieces, incubated with lysis buffer containing 10mg/ml lysozym (Sigma) for 30 min
4 (DNA) or 20 min (RNA) in a 37°C water bath. After a mechanical homogenization by
5 shaking with glass beads the samples were heated to 70°C in a water bath for 20min (DNA)
6 or 15min (RNA). After filtration through a polyamide mesh with 250µm pore size, absolute
7 ethanol was added to the filtrate (ratio filtrate/ethanol 2:1) and the mixture was applied to
8 the adequate spin-column of the kit. After this step, the protocol was applied according to
9 the manufacturer's instructions. For the RNA, a subsequent on-column DNase digestion
10 was applied. Nucleic acids were eluted from the columns with DNase/RNase free water and
11 stored at -20°C. The nucleic acids were quantified using Ribogreen (RNA or ssDNA
12 quantification, Molecular Probes; Invitrogen) or Picogreen (dsDNA quantification,
13 Molecular Probes; Invitrogen) according to [36].
14

15 For extraction of the nucleic acids from the concentrated or the sorted fractions of
16 microorganisms (considered as dead or alive), 1-2 ml portions of the concentrates before
17 and after sorting were harvested by centrifugation for 15min at 15.000xg. The pellets were
18 either frozen or directly used for nucleic acid extraction using the DNeasy/RNeasy protocol
19 (Qiagen, Hilden; Germany). Pellet supernatant was checked by epifluorescence microscopy
20 for microorganisms; in no case cells were observed. DNase digestion for the RNA was
21 applied as described above.
22

23 **16S rRNA and 16S rRNA gene based community fingerprints.**

24 PCR amplification of 16S rRNA and of its respective genes from the extracted
25 nucleic acids were performed using the previously described primers COM1 (5'-
26 CAGCAGCCGCGGTAATAC-3') and COM2 (5'-CCGTCAATTCCTTTGAGTTT-3'),

1 amplifying positions 519 to 926 of the *Escherichia coli* numbering of the 16S rRNA gene
2 [33]. For single strand separation a 5'-biotin-labeled forward primer was used according to
3 [14]. From RNA, a reverse transcription was carried out before PCR using the First strand
4 cDNA synthesis Kit (Fermentas) following the manufacturer's recommendations. Each
5 amplification was carried out using 2 ng DNA/cDNA template in a final volume of 50 µl,
6 starting with an initial denaturation for 15 min at 95°C. A total of 30 cycles (30s at 95°C,
7 30s at 55°C, and 1 min at 72°C) was followed by a final elongation for 10 min at 72°C.
8 Amplification was achieved using HotStarTaq DNA polymerase (QIAGEN, Hilden,
9 Germany).

10
11 For the preparation of ssDNA and community fingerprints, a variant of the protocol
12 described by Eichler et al. [14] was applied. Briefly, magnetic streptavidin coated beads
13 (Promega, Madison, Wis.) were applied to obtain ssDNA from the PCR amplicons.
14 Quantification of the obtained ssDNA was performed on a 1.5% agarose gel by comparison
15 with a low-molecular-weight marker (Invitrogen low-DNA-mass ladder). For SSCP
16 fingerprinting analysis, 25 ng of the obtained ssDNA was mixed with gel loading buffer
17 (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol) in a final
18 volume of 7 µl. After incubation for 3 min at 95°C, the ssDNA samples were stored on ice,
19 loaded onto a nondenaturing polyacrylamide-like gel (0.6x MDE gel solution; Cambrex
20 BioScience, Rockland, Maine) and electrophoretically separated at 20°C at 400 V for 18 h
21 on a MacroPhor sequencing apparatus (Pharmacia Biotech, Germany). The gel was silver
22 stained according to the method described by [2]. Dried SSCP gels were digitized using an
23 Epson Expression 1600 Pro scanner, bands with an intensity of >0.1% of the total lane were
24 considered for further statistical analysis. Similarity coefficients were calculated using
25 Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair

Group Method with Arithmetic mean (UPGMA) using the GelCompare II software (Applied Maths, Kortrijk, Belgium).

Reamplification and sequencing of ssDNA bands from SSCP fingerprints.

Sequence information was obtained following the protocol of Eichler et al. [14]. Briefly, ssDNA bands were excised from the SSCP acrylamide gels, and boiled in Tris buffer (10 mM Tris-HCl, 5 mM MgCl₂, 5 mM KCl, 0.1% Triton X-100, pH 9). 7µl of the solution was used in a reamplification PCR with the unbiotinylated COM primers described above. After checking the PCR-amplicons on a 2% agarose gel, the amplicons were purified and subsequently sequenced by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, Calif.). Before analysis on an ABI Prism 3100 Genetic Analyzer, the products were purified using the BigDye Terminator purification kit (QIAGEN). Phylogenetic identification of the sequences was done either by the NCBI Tool BLAST/blastn [1] for comparison with the closest 16S rRNA gene sequence or the Ribosomal Data Base Project Seqmatch Tool [10] for the identification of the closest described relative (Gene Bank Data base until September 9, 2009). To define a phylotype we chose two definite sequence differences on a mean stretch of 300bp sequence length as criterion. The partial 16S rRNA gene sequences retrieved from the fingerprints are accessible at the GenBank/EMBL/DDBJ accession numbers GQ 917122-GQ 9171174.

RESULTS

Bacterial cell counts and heterotrophic plate counts.

The results on the bacterial counts are detailed in Figure 2. For drinking water samples obtained from the tap at the three sampling dates, the total bacterial cell numbers

were in the range of 3 to 4×10^5 cells ml^{-1} ; in the concentrates (100 to 400 fold) of the drinking water bacteria used for viability staining the cell numbers ranged from 5.1×10^7 to 1.2×10^8 cells ml^{-1} . After staining with PI and SYTO9, the fraction of membrane intact cells determined microscopically accounted for $53\% \pm 6\%$ of the total bacteria while the membrane injured fraction accounted for $47\% \pm 6\%$. Heterotrophic plate counts (HPC) made from the concentrates were on average substantially less than the total bacterial counts, i.e. four to five orders of magnitude depending on medium and incubation time. Heterotrophic plate counts on R2A agar at 22°C and after 72h exceeded all plate counts on the other media and temperatures, and ranged from 2.0 to 4.1×10^3 CFU ml^{-1} in the concentrate. For the not concentrated tap water between 3 and 31 CFU ml^{-1} were detected. (Preferred position for Figure 2)

FACS results of PI/SYTO stained drinking water bacteria.

After PI/SYTO staining, drinking water bacteria were analyzed based on two scatter parameters (forward and side scatter) and the fluorescence signal. For the analysis, some bacteria were excluded due to a lower forward scatter signal indicating cell debris with little or no DNA content (Fig. 3a). After staining, the majority (around 70-80%) of all cells could be sorted into two fractions, i.e. non membrane-injured SYTO9 positive cells and membrane-injured PI positive cells (Fig. 3b). Subsequent purity control as well as a check by epifluorescence microscopy demonstrated the effectiveness of the sorting (Fig. 3c and d). Flow cytometric analysis of the drinking water bacteria, based on comparison with reference beads of defined sizes, indicated that all fractions of microorganisms (total, SYTO9 positive, PI positive) had a narrow size distribution and a rather small diameter, i.e. on the average $0.69\mu\text{m}$ ($c_v:1.3\%$) (data not shown). In the three sorting experiments, total cell numbers recovered from FACS ranged around 10^6 cells per fraction (membrane intact,

membrane injured) that were subsequently subjected to nucleic acid extraction and fingerprinting. (Preferred position for Figure 3)

Structure of the bacterial community of drinking water before and after sorting.

DNA- and RNA-based 16S rRNA SSCP fingerprints were used to analyze the bacterial community structure and composition of the drinking water before and after the cells were sorted by FACS as membrane intact and membrane injured cell fractions, and to assess the effect of the concentration procedure on the bacterial community (Fig. 4). A general observation was that DNA- and RNA- based fingerprints from the same samples showed always very different banding patterns, a feature that was confirmed (see 3.4.) by the analysis of the species composition by sequencing of the fingerprint bands. DNA- and RNA-based SSCP fingerprints of the drinking water community with and without concentration (the latter sampled on filter sandwiches) were highly comparable (see Supplementary Material Fig. 1). Fingerprints of the unsorted drinking water concentrates generated on the three sampling dates clustered closely together indicating a high similarity for the structure of the drinking water bacterial community on the three sampling dates (Fig. 4, 5). As shown in Fig. 5, the highest similarity was observed among sampling A and B for the DNA-based fingerprints (95%); the similarity among the drinking water concentrates was always higher than 76% irrespective of DNA- or RNA-based analyses or the sampling date (Fig. 5a,b, respectively) (Preferred position for Figure 4 and 5)

DNA-based fingerprints of the membrane intact and membrane injured sorted fractions showed a very distinct pattern for each sampling day (Fig. 4a, 5a). Comparative cluster analysis of the DNA-based fingerprints showed that for each sampling date the fingerprints from each fraction clustered more closely together than the different sampling dates, indicating that the community structure became more dissimilar among the sampling

1 dates due to the live/dead sorting (Fig. 5a). Remarkably, after sorting the live and dead
2 fractions of all three samplings were most closely related to each other indicating that the
3 DNA-based fingerprints reflected the same live and dead bacteria (phylotypes). In contrast,
4 the RNA-based fingerprints of the sorted cell fractions showed a similar pattern among the
5 membrane intact fractions irrespective of the sampling date (Fig. 4b, 5b) as indicated by a
6 tight clustering (similarity >70%, Fig. 4b). The membrane injured sorted fractions showed a
7 more diverse pattern for the three sampling dates, mainly caused by the large discrepancy
8 for sampling C.

10 **Taxonomic composition of the different cell fractions.**

11 A total of 111 bands from the DNA- and RNA-based SSCP fingerprints were
12 sequenced to determine the taxonomic composition of the different fractions. Using a limit
13 of $\geq 99\%$ 16S rRNA gene sequence similarity as discrimination criterion, we retrieved 53
14 unique phylotypes for these bands (Supplementary Material Table 1). For identification, the
15 obtained sequences were compared to all databank entries in the GenBank. Out of the 53
16 unique phylotypes, 31 were retrieved from the RNA-based fingerprints, and 24 from the
17 DNA-based fingerprints with only two phylotypes that were retrieved from both RNA and
18 DNA. RNA-phylotype 1 and DNA-phylotype 52 were affiliated with the same species but
19 were distinct by 8 nt and therefore assigned to different phylotypes. Thus, the bacterial
20 community reflected by both fingerprint types differed to a large extent.

22 Comparing the major taxonomic groups, the analysis of the DNA-based fingerprints
23 (Table 1, Fig. 6a,) showed that the drinking water samples were dominated by members of
24 the *Betaproteobacteria* (8 phylotypes, with an average abundance of 14.9%), *Bacteroidetes*
25 (7 phylotypes, 17.8%), and *Actinobacteria* (2 phylotypes, 15.3%). All other classes and
26 phyla, i.e. *Alpha-* and *Gammaproteobacteria*, *Planctomycetes* and *Cyanobacteria*, had a

low diversity (1-2 phylotypes) and a low abundance (0.2 - 3.3%). The RNA-based fingerprints (Fig. 6b) of the drinking water samples were dominated by members of the *Betaproteobacteria* (4 phylotypes, 20.8%), *Cyanobacteria* (6 phylotypes, 15.6%), *Alphaproteobacteria* (5 phylotypes, 15.5%), *Gammaproteobacteria* (8 phylotypes, 9.5%), and *Bacteroidetes* (3 phylotypes, 8.3%). The remaining 4 phyla, i. e. *Nitrospira*, *Firmicutes*, *Planctomycetes*, *Chloroflexi*, had a low diversity (1-2 phylotypes) and a low abundance (0.1-4.6%). While most phyla occurred in both the RNA- and DNA-based analyses, *Actinobacteria* were never observed in the RNA-based analyses, whereas *Chloroflexi* (with a high abundance of 16% in the membrane intact fraction of the RNA-based analyses) were never observed in the DNA-based analyses (Table 1, Supplementary Material Table 1). The single phylotypes of *Nitrospira* and *Firmicutes* also occurred only in the RNA-based analyses but had low and variable abundances (below 2.3%).

An overview on the phylogenetic diversity is shown by Supplementary Figure 3a by a tree based on the phylogenetic analysis of the retrieved phylotypes together with the nearest cultured species. Supplementary Figure 3a shows all occurring phyla and Fig. 3b shows the phylum *Proteobacteria* in more detail. Details of the phylogenetic analyses are listed in Supplementary Table 1. Overall, the bacterial drinking water community retrieved from RNA and DNA analyses was mostly composed of bacteria that were not related to any described species. For the DNA-based analyses 46% of the phylotypes were not related to any described genus, 42% were affiliated with a described genus, and 38% were affiliated with a described species. For RNA-based analyses 58% of the phylotypes were not related to any described genus, 32% were affiliated with a described genus, and 23% were affiliated with a described species. The phylotypes affiliated with a described genus were mostly members of the *Bacteroidetes*, *Alpha*-, *Beta*- and *Gammaproteobacteria*.

From the 24 phylotypes of the DNA analyses, three phylotypes contributed to more than 5% (up to 12%) of the total (unsorted) drinking water community (Supplementary Material Table 1 & 2). Two of these three dominating phylotypes were related to uncultured *Actinobacteria* (phylotype 48, 49). The bacterium with the highest abundance of 12.4% showed 98% similarity to the freshwater bacterium *Sediminibacterium salmoneum*, a cultured *Bacteroidetes* (phylotype 35). From the 31 phylotypes of the RNA analyses, five phylotypes contributed to more than 5% (up to 18%) of the total (unsorted) drinking water community. These five dominating phylotypes were composed of one cyanobacterium (phylotype 46; affiliated with the genus *Synechococcus*), one gammaproteobacterium with related only to uncultured bacteria (phylotype 19), one betaproteobacterium related to the species *Acidovorax facilis* (phylotype 1), one alphaproteobacterium related to the species *Bosea vestrii* (phylotype 14), and one member of the *Bacteroidetes* (phylotype 23) not related to any described genus.

All 24 DNA-based phylotypes were recovered after cell sorting in the membrane intact and/or membrane injured fractions indicating a recovery of 100% of the phylotypes in the sorted fractions. 38% of the DNA-phylotypes occurred only in the membrane intact fraction, 21% occurred only in the membrane injured fraction, and 42% occurred in both fractions. Phylotypes of the major taxa *Betaproteobacteria* and *Bacteroidetes* contributed to all three fractions, i.e. membrane intact, membrane injured and total. The two phylotypes of the *Actinobacteria* were always retrieved from the membrane intact and membrane injured fractions. Based on the RNA analyses, 28 of the 31 phylotypes (90%) were retrieved after sorting in the membrane intact and/or membrane injured fraction. From the retrieved 28 phylotypes, 32% of the RNA-phylotypes occurred only in the membrane intact fraction, 21% occurred only in the membrane injured fraction, 46% occurred in both fractions. Phylotypes of the classes *Gammaproteobacteria*, *Cyanobacteria* and the phylum

1 *Bacteroidetes* contributed to all three fractions, i.e. membrane intact, membrane injured and
2 total. All phylotypes of the *Alphaproteobacteria* were always retrieved from membrane
3 intact and membrane injured fractions. Thus, the phylotypes obtained from RNA- and
4 DNA-based analyses showed a similar ratio with respect to retrieval of their cells from the
5 membrane intact and injured fractions: 32-38% had cells only in the membrane intact
6 fractions, 21% only in the membrane injured fractions, and 42%-46% in both fractions.

7
8 After FACS-sorting, major changes of the abundances of the phylotypes occurred that
9 were far more pronounced for the DNA-based analyses than for the RNA-based analyses.
10 Supplementary Table 1 and Supplementary Fig. 2 are providing the details on the changes
11 of abundances with respect to the phylotypes before and after sorting, while Table 1.
12 provides an overview on the phyla/class level. These changes of abundances through sorting
13 were most pronounced in the membrane intact sorted fraction for the *Chloroflexi* (PT 24) in
14 the RNA- based analyses and the *Planctomyces* (PT 62) in the DNA-based analyses.
15 Overall, we observed only few phylotypes with a high abundance in the sorted cell fractions
16 of the DNA-based electropherograms (Supplementary Material Fig. 2a) while in the RNA-
17 based electropherograms (Supplementary Material Fig. 2b) phylotypes with a high
18 abundance were present in the non-sorted as well as in the sorted fractions.

19
20 For an estimate of the origin of the phylotypes, the habitat of the most similar
21 bacterial sequence from the public data bases is given in Supplementary Table 1. Provided
22 that the most similar sequence i) had a similarity of higher or equal to 91% 16S rRNA gene
23 similarity and ii) was of aquatic origin, the phylotype was rated as “of aquatic origin”.
24 Below 91% 16S rRNA gene sequence similarity the relatedness was regarded as too low to
25 give information on the potential habitat of the phylotype. Based on these criteria, 76% of
26 the DNA and the RNA-based phylotypes were considered as of aquatic origin which most

of them from freshwater habitats. Six out of the RNA phylotypes and three out of the DNA phylotypes were not used for this assignment due to too low 16S rRNA gene sequence similarity (all these sequences had a similarity below 88% to the next sequence in the public data bases).

DISCUSSION

Community structure and composition of drinking water bacteria using DNA- and RNA-based fingerprints.

DNA- and RNA-based molecular analyses provided a very different picture of the drinking water microflora. This comprised the overall fingerprint patterns, their changes due to sorting and the retrieved phylotypes. However, this overview does not precisely reflect the quantitative composition of the bacterial community. Since the amplification of 16S rRNA genes is based on PCR, a PCR bias has to be taken into account [16, 38] . According to our experience with aquatic community analysis by SSCP, the technique provides highly reproducible fingerprints of the community with high reproducibility in terms of the relative abundances of the single bands compared to the total community. Compared to real-time PCR detection of single phylotypes, low abundant phylotypes seem to be overestimated, while highly abundant phylotypes seem to be underestimated [8] . Thus, the fingerprint gives a biased but reproducible semi-quantitative picture of the bacterial community allowing comparison of different bacterial communities and observation of the dynamics of single community members.

The fingerprint analysis of the drinking water samples showed a highly consistent pattern among the three different sampling dates for both the RNA- and DNA-based analyses. A rather stable bacterial community of the investigated drinking water over time

1 had already been shown by the seasonal study of Henne et al. [19] using DNA-based
2 fingerprints. Though seasonal variation occurred for some members of the bacterial
3 community, the overall community structure was rather stable during the year. The SSCP
4 fingerprint patterns were completely different with respect to analysis of RNA and DNA of
5 the same samples. This different pattern was confirmed by sequencing and phylogenetic
6 analysis of the fingerprint bands. From the 24 phylotypes retrieved from the DNA-based
7 analysis, and 31 phylotypes retrieved from the RNA-based analysis only two phylotypes
8 (PT 4, 46) were identical, and two were affiliated with the same species (PT 1, 52). Though
9 the same phyla with a few exceptions were detected in RNA- and DNA-based analysis,
10 from the genus level upwards there was a pronounced divergence at the species level. This
11 strong discrepancy between RNA and DNA-based analysis concerning the fingerprint
12 pattern and the members of the bacterial community had already been observed by Eichler
13 et al. [14].

14
15 Our drinking water community was dominated by phyla and classes typical for
16 freshwater environments, i.e. *Bacteroidetes*, *Cyanobacteria*, *Betaproteobacteria* and
17 *Gammaproteobacteria*. This was also the case when looking at the higher level of
18 phylogenetic resolution, i.e. the phylotypes that were resolved approximately at the species
19 level. The majority of the phylotypes (76%) were most closely related to sequences
20 retrieved from aquatic habitats. This is consistent with findings of the study of the whole
21 drinking water supply system by Eichler et al. [14]. The phylotypes identified based on the
22 DNA-based analyses seemed to have a higher stability in the drinking water than the RNA
23 phylotypes. 55% of the DNA-phylotypes identified in this study were also detected in the
24 study of Eichler et al [14] in the same drinking water supply system 5 years ago. This was
25 different for the RNA-based phylotypes that had only a reoccurrence of 11%.

Assessment of live and dead bacterial cells using PI/SYTO9 staining.

In our study about half (53%) of the bacterial cells in the drinking water samples showed an intact membrane. This is in line with studies by Berney et al. [5] that reported a fraction of membrane intact cells of about 66% in tap water that was free of chlorine as it was the case in our study. For chlorine containing tap water, Hoefel et al. [20] reported 12% membrane intact cells for finished drinking water of an Australian water distribution system with a higher chlorination during treatment and transport, and a free chlorine residual level of 0.4 mg l⁻¹ at the tap.

The Propidium Iodide staining is considered to provide a good estimate for membrane injury of *Bacteria* and *Archaea* [27] . In a set of studies, this staining procedure has been evaluated and compared with other staining procedures for assessment of the physiological state of the bacteria [15, 22] . Besides the evaluation of methodological aspects, recently studies were done for drinking water with added bacteria and the indigenous microflora. Berney et al. [3] tested PI for *E. coli* in drinking water submitted to UV and sunlight irradiation using a set of different viability stains. The study showed that loss of membrane integrity as indicated by PI staining was the final signal after decrease of all other tested physiological functions. In a second study, Berney et al. [5] used PI staining for analyzing the microflora of a set of drinking water samples. The viability of the drinking water bacteria was higher for bottled water (about 90%) and drinking fountain water (about 85%) than for drinking water at the tap (about 66%). The high percentage of viable cells coincided with a high ATP content. The comparison of PI staining with other methods demonstrated PI staining was a valuable criterion for live-dead distinction for drinking water bacteria.

Autofluorescence is a feature that has to be taken into account as a potentially misleading signal for the analysis of aquatic bacterial communities by PI/SYTO9 staining [39]. According to our taxonomic analyses, two phylotypes were affiliated with the phylum *Chloroflexi* whose members are known to contain bacteriochlorophyll c and a in the chlorosomes and the cytoplasmic membrane resulting in green autofluorescence [28]. The *Chloroflexi* were detected in the membrane intact and membrane injured sorted fractions, but with a far higher detection in the membrane intact fractions (up to 23% for PT 24 in the RNA-based analyses). In the latter case a wrong “live” sorting due to the autofluorescence cannot be ruled out. On the other hand, a false “dead” sorting could have been caused by phylotypes affiliated with the genus *Synechococcus* due to the presence of red fluorescent phycoerythrin [37]. Phylotype 46 that was common in the RNA- and DNA-based analyses and closely related to *Synechococcus rubescens* had a high abundance in the “dead”- sorting of 10% for the DNA and of 15% for the RNA based analysis, respectively. Though autofluorescence may be misleading for the live-dead sorting of some bacteria with photosynthetic pigments, we do not consider this as a critical issue for the live/dead staining procedure as a distinction for drinking water bacteria. Autofluorescent bacteria are commonly not considered as pathogenic and therefore, autofluorescence does not seem a critical issue for our staining procedure in terms of public health.

Live and dead assessment of different phyla and phylotypes

All DNA-based phylotypes and 90% of the RNA-based phylotypes were retrieved after sorting in the membrane intact and/or membrane injured fraction. The three missing RNA phylotypes might have been missed due to their low abundance in the tap water. This close to complete recovery of the phylotypes after sorting allows a comparison of the sorting results between the DNA- and RNA-based analyses. Though the sequencing success was 77% for the RNA-based analyses, and only 57% for the DNA-based analyses, the

1 comparison can be done on the level of the retrieved phylotypes that indeed had a relatively
2 high abundance compared to the not retrieved phylotypes.

3
4 A comparison shows that the phylotypes of the DNA-based analyses had the same
5 size of the “dead fraction” as those reflected by the RNA-based analyses, i.e. 21%. Also, the
6 DNA- and RNA-phylotypes had a comparable percentage of only “live” sorted (DNA, 38%;
7 RNA, 32%) and of “mixed” sorted phylotypes (DNA: 42%, RNA, 46%). Phylotype 4
8 concomitantly retrieved from DNA- and RNA- analyses was recovered from membrane
9 intact and membrane injured fractions in the DNA- and RNA-based analysis, i.e. for the
10 only common phylotype comparable sorting results were obtained for the DNA and RNA-
11 based analysis. The second common phylotype (PT 46) cannot be compared due to the
12 potential interference with the pigments (see above). Based on our observation, we can say
13 that the fraction of phylotypes with only membrane injured cells is not higher for the
14 bacteria reflected by the DNA analyses than those of the RNA analyses. This is an essential
15 finding because it was often assumed that those reflected by the RNA are alive, and those
16 reflected by the DNA are dead [14] . Based on this observation, we assume that the reason
17 for the detection of a phylotype in the DNA- or RNA-based analyses might be the
18 phylotype-specific regulation of the DNA and the RNA pool and was obviously not related
19 to the viability of the respective phylotypes. This is consistent with analyses of [25]
20 showing a broad range of numbers of rRNA operons (1-13) specific for each bacterial
21 strain. On the other hand, we observed that all fingerprints of the membrane intact fractions
22 showed rather similar RNA-based fingerprints reflecting actively growing members of the
23 community. This tight clustering of the RNA-based fingerprints from live bacteria could
24 indicate that always the same actively growing members of the drinking water community
25 re-grew after chlorination had killed most of the bacteria in the waterworks. This is no
26 contradiction to the detection of a substantial amount of RNA-based phylotypes in the dead

fraction because several of the live RNA-phylotypes were different from the dead ones (phylotype 12, 24) or were abundant in different amounts (Fig. 6b, Supplementary Table 2b). These dead RNA-phylotypes could still be remnants of the highly active phylotypes before chlorination which have not re-grown. Overall, we think that the combination of FACS sorting and fingerprinting is a new way to obtain functional fingerprints – with the live RNA phylotypes representing the most actively growing members of the microbial community [30, 12] .

Taxonomic composition of the bacterial community of drinking water and human health

The bacterial community was composed of seven phyla (see Supplementary Material Table 1 and 2). The phyla as well as the phylotypes are primarily those typically present in aquatic ecosystems [14, 40] . However, one phylotype detected in the drinking water had the potential of being an opportunistic pathogen. The alphaproteobacterium PT 14 identified as closely related to *Bosea vestrii* in the RNA-based analysis was retrieved from the membrane intact and membrane injured sorted fraction, and was present in the drinking water at a high abundance of 13%. This species was occasionally associated with infections of immunocompromised people [26] .

However, the mere detection of a bacterium at a taxonomic resolution close to the species level is not sufficient as an indication of a health risk. Presence, viability and infectivity of pathogenic bacteria in drinking water are criteria that have to be fulfilled for assessing a threat to human health. Presence of bacteria can be assessed by the applied technology to the detection limit of the method which is about 0.1% of the total microflora. Viability was assessed by the live/dead staining. Infectivity asks first for the precise taxonomic identification of the pathogen and a separate, mostly experimental, assessment of

infectivity that has to be achieved in addition to molecular analyses. Concerning the precise assessment of the taxonomy, the about 400nt long sequences obtained from a SSCP gel can resolve, at best, the species level. Though this accuracy might be highly valuable for the study of environmental bacteria, for most pathogenic bacteria, a full (>1400nt) 16S rRNA sequence is needed or even a high resolution genotyping as exemplified for *Legionella pneumophila* in drinking water [23] or gene sequences associated with infectivity of the respective species (e.g. the *mip* gene for *L. pneumophila*). Thus, the proposed technology can provide a valuable monitoring tool that can show that a potentially harmful species is present - but it remains with the “potential” and the true risk has to be assessed consecutively by additional adequate measurements.

CONCLUSION

In summary, the approach used in this study is considered a valuable tool for analyses of aquatic bacterial communities. The applied PI/SYTO9 staining procedure indicating membrane injury of the bacterial cells is considered as a reliable criterion for damaged or dead bacterial cells. The combined approach of DNA- and RNA-based fingerprint analyses with live-dead staining and sorting was demonstrated as a straight forward monitoring tool. This tool still can be modified and extended with respect to sensitivity or methodological details. For example, in terms of methodology, PI/SYTO9 stain could be replaced by propidium monoazide application thereby avoiding the step of FACS sorting [32]. On the other hand, the sorted cells can be submitted to further labelling/staining and subsequent analyses. For increased sensitivity with respect to specific groups of pathogenic relevance, the general bacterial primers (COM1, 2) could be replaced by group specific primer reaching a lower detection limit and a better taxonomic resolution of the targeted group. Thus, the approach can be used for monitoring of bacteria relevant to

human health and can be applied as a valuable tool for drinking water monitoring with respect to the overall community or specific target pathogenic bacteria.

From an ecological perspective, the study provided comprehensive insights into the community composition and the viability of drinking water bacteria and shows that a very different spectrum of species was detected by DNA- and RNA-based analysis. A major finding in ecological terms is the fact that the viability of the phylotypes was comparable for RNA and DNA extracts. The viability of the phylotypes in addition to the very different spectrum of species detected (included pathogenic ones) demonstrate the value of adding RNA-based analyses to the commonly applied DNA-based analyses for drinking water studies or, in more general terms, for aquatic studies.

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REFERENCES

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-10
2. Bassam BJ, Caetano-Anoll G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 196: 80-83
3. Berney M, Weilenmann HU, Egli T (2006) Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). *Microbiology* 152: 1719-1729
4. Berney M, Hammes F, Bosshard F, Weilenmann H, Egli T (2007) Assessment and Interpretation of Bacterial Viability by Using the LIVE/DEAD BacLight Kit in Combination with Flow Cytometry. *Appl Environ Microbiol* 73: 3283-3290
5. Berney M, Vital M, Hülshoff I, Weilenmann H, Egli T, Hammes F (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Res* 42: 4010-4018
6. Birch L, Dawson C, Cornett J, Keer J (2001) A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Lett Appl Microbiol* 33: 296-301
7. Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R (1999) LIVE/DEAD BacLight , application of a new rapid staining method for direct enumeration of viable

- 1 and total bacteria in drinking water. J Microbiol Methods 37: 77-86
- 2
- 3 8. Brettar I, Labrenz M, Flavier S, Botel J, Kuosa H, Christen R, Höfle MG (2006)
- 4 Identification of a *Thiomicrospira denitrificans*-Like Epsilonproteobacterium as a
- 5 Catalyst for Autotrophic Denitrification in the Central Baltic Sea. Appl Environ
- 6 Microbiol 72: 1364-1372
- 7
- 8 9. Brettar I, Höfle MG (2008) Molecular assessment of bacterial pathogens - a
- 9 contribution to drinking water safety. Curr Opin Biotechnol 19: 274-280
- 10
- 11 10. Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM,
- 12 Bandela AM, Cardenas E, Garrity GM, Tiedje JM (2007) The ribosomal database
- 13 project (RDP-II), introducing myRDP space and quality controlled public data. Nucl
- 14 Acids Res 35: 169-172
- 15
- 16 11. Czechowska K, Johnson DR, Meer JRVD (2008) Use of flow cytometric methods for
- 17 single-cell analysis in environmental microbiology. Cur Opin Microbiol 11: 205-212
- 18
- 19 12. Dumont M, Harmand J, Rapaport A, Godon J (2009) Towards functional molecular
- 20 fingerprints. Environ Microbiol 11: 1717-1727
- 21
- 22 13. Eichler S, Weinbauer MG, Dominik K, Höfle MG (2004) Extraction of total RNA and
- 23 DNA from bacterioplankton, chapter 108 In: Kowalchuk GA, Bruijn FJD, Head IM,
- 24 Akkermans ADL, van ElsasJD (eds) Molecular microbial ecology manual, Kluwer
- 25 Academic Publishers, Dordrecht, The Netherlands, pp 103-120
- 26

14. Eichler S, Christen R, Höltje C, Westphal P, Bötel J, Brettar I, Mehling A, Höfle MG (2006) Composition and Dynamics of Bacterial Communities of a Drinking Water Supply System as Assessed by RNA- and DNA-Based 16S rRNA Gene Fingerprinting. *Appl Environ Microbiol* 72: 1858–1872
15. Falcioni T, Papa S, Gasol JM (2008) Evaluating the flow-cytometric nucleic acid double-staining protocol in realistic situations of planktonic bacterial death. *Appl Environ Microbiol* 74: 1767-1779
16. Farrelly V, Rainey F, Stackebrandt E (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol* 61: 2798-2801
17. Hammes F, Berney M, Wang Y, Vital M, Köster O, Egli T (2008) Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Res* 42: 269-277
18. Haugland R The Handbook—A Guide to Fluorescent Probes and Labeling Technologies (<http://www.invitrogen.com>)
19. Henne K, Kahlisch L, Draheim J, Brettar I, Höfle MG (2008) Polyvalent fingerprint based molecular surveillance methods for drinking water supply systems. *Water Science & Technology, Water Supply* 8: 527-532
20. Hoefel D, Monis P, Grooby W, Andrews S, Saint C (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. *J Appl*

Microbiol 99: 175-186

21. Huq A, Rivera I, Colwell RR (2000) Epidemiological significance of viable but non culturable microorganisms In Nonculturable Microorganisms in the Environment, Colwell RR, Grimes DJ (eds), American Society for Microbiology, pp 301-323

22. Joux F, Lebaron P (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes Infect 2: 1523-1535

23. Kahlisch L, Henne K, Draheim J, Brettar I, Höfle MG (2010) High-resolution in situ genotyping of *Legionella pneumophila* populations in drinking water by Multiple-Locus Variable-Number of Tandem Repeat Analysis (MLVA) using environmental DNA. Appl Environ Microbiol 76:6186-6195

24. Kahlisch L, Henne K, Groebe L, Draheim J, Höfle MG, Brettar I (2010) Molecular analysis of the bacterial drinking water community with respect to live/dead status. Water Science and Technology 61: 9-14

25. Klappenbach JA, Dunbar JM, Schmidt TM (2000) rRNA Operon Copy Number Reflects Ecological Strategies of Bacteria. Appl Environ Microbiol 66: 1328-1333

26. La Scola B, Mallet M, Grimont PAD, Raoult D (2003) *Bosea eneeae* sp nov, *Bosea massiliensis* sp nov and *Bosea vestrisii* sp nov, isolated from hospital water supplies, and emendation of the genus *Bosea* (Das et al 1996). Int J Syst Evol Microbiol 53: 15-20

- 1
- 2 27. Leuko S, Legat A, Fendrihan S, Stan-Lotter H (2004) Evaluation of the LIVE/DEAD
- 3 BacLight Kit for Detection of Extremophilic Archaea and Visualization of
- 4 Microorganisms in Environmental Hypersaline Samples. Appl Environ Microbiol 70:
- 5 6884-6886
- 6
- 7 28. Madigan MT, Martinko JM, Dunlap PV, Clark DP (2008) Brock Biology of
- 8 Microorganisms, 12th edn New York, USA, Pearson Higher Education
- 9
- 10 29. Mahmood S, Paton GI, Prosser JI (2005) Cultivation-independent *in situ* molecular
- 11 analysis of bacteria involved in degradation of pentachlorophenol in soil. Environ
- 12 Microbiol 7: 1349-1360
- 13
- 14 30. Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W (2008) How to get
- 15 more out of molecular fingerprints, practical tools for microbial ecology. Environ
- 16 Microbiol 10: 1571-1581
- 17
- 18 31. Moreno Y, Alonso JL, Botella S, Ferrús MA, Hernández J (2004) Survival and injury
- 19 of Arcobacter after artificial inoculation into drinking water. Res Microbiol 155: 726-
- 20 730
- 21
- 22 32. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK (2007) Use of Propidium
- 23 Monoazide for Live/Dead Distinction in Microbial Ecology. Appl Environ Microbiol
- 24 73: 5111-5117
- 25
- 26 33. Schwieger F, Tebbe CC (1998) A New Approach To Utilize PCR-Single-Strand-

Conformation Polymorphism for 16S rRNA Gene-Based Microbial Community
Analysis. Appl Environ Microbiol 64: 4870–4876

34. Steele HL, Streit WR (2005) Metagenomics, Advances in ecology and biotechnology.
FEMS Microbiol Lett 247: 105-111

35. Weinbauer MG, Beckmann C, Höfle MG (1998) Utility of Green Fluorescent Nucleic
Acid Dyes and Aluminum Oxide Membrane Filters for Rapid Epifluorescence
Enumeration of Soil and Sediment Bacteria. Appl Environ Microbiol 64: 5000–5003

36. Weinbauer MG, Fritz I, Wenderoth DF, Höfle MG (2002) Simultaneous Extraction
from Bacterioplankton of Total RNA and DNA Suitable for Quantitative Structure
and Function Analyses. Appl Environ Microbiol 68: 1082–1087

37. Wilbanks S, Glazer A (1993) Rod structure of a phycoerythrin II-containing
phycobilisome I Organization and sequence of the gene cluster encoding the major
phycobiliprotein rod components in the genome of marine *Synechococcus* sp
WH8020. J Biol Chem 268: 1226-1235

38. Wintzingerode FV, Göbel UB, Stackebrandt E (1997) Determination of microbial
diversity in environmental samples, pitfalls of PCR-based rRNA analysis FEMS
Microbiol Rev 21: 213-229.

39. Yentsch CM, Horan PK, Muirhead K, Dortch Q, Haugen E, Legendre L, Murphy LS,
Perry MJ, Phinney DA, Pomponi SA (1983) Flow Cytometry and Cell Sorting, A
Technique for Analysis and Sorting of Aquatic Particles. Limnol Oceanogr 28: 1275-

1 1280

2

3 40. Zwart G, Crump B, Kamst-van-Agterveld MP, Hagen F, Han S (2002) Typical
4 freshwater bacteria, an analysis of available 16S rRNA gene sequences from plankton
5 of lakes and rivers. *Aquat Microb Ecol* 28: 141-155

6

TABLES

Table 1. Abundances of the phylotypes (PT) summed up per Phyla/class displayed for the unsorted and sorted (“live/dead”) fractions. The abundances of the phylotypes are derived from the SSCP analyses of DNA and RNA extracts as shown in Fig. 4 (for details on the abundances of the single phylotypes see Supplementary Table 2). The mean of the three samplings A,B and C plus the standard deviation SD is given for the tap water sample before sorting (“All (unsorted)”), the “live” sorted fraction (cells with intact membranes), and the “dead” sorted fraction (cells with injured membranes).

DNA based analysis		All (unsorted)		Live		Dead	
Phyla/Class	PT(n)*	mean A-C	SD	mean A-C	SD	mean A-C	SD
Alphaproteobacteria	1	2.83%	2.24%	1.56%	1.48%	0.92%	1.49%
Betaproteobacteria	8	14.93%	0.52%	16.82%	6.55%	9.92%	10.80%
Gammaproteobacteria	2	0.34%	0.59%	0.50%	0.86%	1.05%	1.81%
Actinobacteria	2	15.32%	1.12%	4.52%	3.71%	5.68%	6.11%
Bacteroidetes	7	17.75%	4.56%	15.45%	22.67%	8.49%	3.02%
Cyanobacteria	2	3.30%	0.97%	0.64%	1.10%	10.10%	17.49%
Planctomycetes	2	0.22%	0.38%	12.81%	22.19%	n.d.	
identified as PTs	24	54.69%	3.83%	52.29%	21.52%	36.16%	23.66%
% PTs with only “dead” cells		20.8%					

RNA based analysis		All (unsorted)		Live		Dead	
Phyla/ Class	PT(n)*	mean A-C	SD	mean A-C	SD	mean A-C	SD
Alphaproteobacteria	5	15.49%	11.41%	21.04%	20.67%	11.52%	8.19%
Betaproteobacteria	4	20.76%	5.66%	9.28%	3.14%	10.33%	4.27%
Gammaproteobacteria	8	9.50%	4.02%	17.58%	5.60%	14.27%	11.31%
Bacteroidetes	3	8.31%	2.64%	2.49%	2.31%	2.49%	3.96%
Chloroflexi	2	0.70%	0.47%	15.75%	9.10%	3.28%	2.13%
Cyanobacteria	6	15.59%	7.56%	7.85%	7.33%	16.23%	8.13%
Firmicutes	1	2.25%	1.95%	0.95%	1.64%	n.d.	n.d.
Nitrospira	1	0.10%	0.17%	n.d.		1.82%	1.84%
Planctomycetes	1	4.60%	7.96%	n.d.		n.d.	
identified as PTs	31	77.30%	3.38%	74.93%	20.17%	59.94%	5.68%
% PTs with only “dead” cells		21.4%					

Legend,
PT, phylotype
*, number of phylotypes per phyla/class
n.d., not detected

FIGURE LEGENDS

FIG. 1. Flow chart of the combined analysis of drinking water samples using FACS and SSCP fingerprinting. 18 liters of drinking water were filtered onto a 0.2µm Nucleopore filter, scraped and washed off the filter with 0.9 % saline solution. The drinking water bacteria were stained with the BACLight Kit™ for 20 min in the dark. After cell sorting, the differently stained fractions were analyzed by molecular methods (dashed lines), i.e. nucleic acids (DNA and RNA) were extracted and subjected to SSCP analysis. Sequence information was gained by reamplification and sequencing of single bands.

FIG. 2. Total bacterial cell numbers of the drinking water concentrate used in the three FACS sorting experiments sorting A (25.03.08, open bars) sorting B (31.03.08, black bars) and sorting C (05.05.2008, hatched bars). Total bacterial counts were determined by epifluorescence microscopy using Sybr Green I staining of formaldehyde fixed samples. Heterotrophic plate counts were determined using 1ml (or appropriate dilutions) concentrated drinking water and the spread plate technique on the media and temperatures indicated. Error bars represent standard deviation of at least 3 replicates.

FIG. 3. Results of the FACS analysis of the drinking water community. Microorganisms from 18 liters of drinking water were concentrated, stained with the BacLight Kit™ and analyzed by the flow cytometer. (a) Flow cytometric analysis of unstained cells. Cells in gate R1 are included in the analysis and cells outside the gate were considered cell debris. (b) Flow cytometric analysis of microorganisms stained with the BacLight Kit™. Cells in gate R4 are Syto 9 positive, cells in gate R5 are PI positive. Purity control of the sorted fractions, (c) Syto 9 positive cells (gate R3) but PI negative (gate R6) and in (d) PI positive cells (gate

R9) but negative for Syto 9 (gate R8). Fluorescence channel, FL 1, 530±40nm; FL3, 616±16nm; FSC, forward scatter; SSC, side scatter.

FIG. 4. (a) DNA-based 16S rRNA gene SSCP fingerprints of the different FACS sorting experiments, sorting A (25.03.08) sorting B (31.03.08) and sorting C (05.05.2008). Numbers represent single phylotypes of sequenced and identified bands. Phylogenetic information about these phylotypes is given in Supplementary Material Table 1. Designations of single samples, ST, species standard; DW Conc., concentrated, unsorted drinking water samples from the respective dates; membrane intact (“live”) sorted, SYTO9 positive fraction of the drinking water; membrane injured (“dead”) sorted, propidium iodide positive fraction of the drinking water. The asterisk indicates a lane from a different SSCP gel. **(b)** RNA-based 16S rRNA SSCP fingerprints of the different FACS sampling dates. Sample designations and numbering of sequenced phylotypes are as for panel a. Phylogenetic information about the numbered phylotypes is given in Supplementary Material Table 2.

FIG. 5. Cluster analysis of the two SSCP gels given in Figure 4. Similarity coefficients were calculated using Pearson correlation algorithm. Dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic mean. **(a)** DNA-based SSCP fingerprints of the different FACS sampling dates, sorting A (25.03.08) sorting B (31.03.08) and sorting C (05.05.2008). Sample designations are as in Fig. 4a. The lane labeled with an asterisk is from a different SSCP gel. **(b)** RNA-based SSCP fingerprints of the different FACS sampling dates. Species standards were taken as out-group for the cluster analysis. Sample designations are as in Fig. 4b.

FIG. 6. Comparison of relative abundances of the phylotypes found in the different cell fractions and the drinking water concentrate (DW) on the three different sampling dates. (a)

1 Phylotypes from the DNA-based SSCP fingerprints. (b) Phylotypes from the RNA-based
2 SSCP fingerprints. Numbers represent the single phylotypes given in Supplementary Material
3 Table 1a and 1b, respectively. The colors are corresponding to the major phylogenetic groups
4 of the phylotypes, Yellow – *Alphaproteobacteria*; Blue – *Betaproteobacteria*; Red –
5 *Gammaproteobacteria*; Green – *Cyanobacteria*; Violet – *Bacteroidetes*; Brown –
6 *Planctomycetes*; Orange – *Actinobacteria*; Grey – *Chloroflexi*. Hatched bars represent
7 unidentified bands.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

1 **SUPPLEMENTARY MATERIAL - Kahlisch et al. - Assessing viability of drinking water bacteria -**

2

3 **SUPPLEMENTARY TABLE 1.** Taxonomic identification of single phylotypes of the DNA-based (1a) and the RNA based (1b) SSCP fingerprints

4 (for phylotypes occurrence see Fig. 4 & 6).

5 Legend: Live/dead assignment of the phylotype: L, cells of the phylotype only present in “live”, membrane intact sorted fractions; D, only present in

6 “dead”, membrane injured sorted fractions; LD, present in both fractions.

7 N.A., not applicable (i.e., the closest described species has a similarity of < 75%).

8

9 **Supplementary Tab. 1a - Phylotypes of DNA based analyses**

Phylotype	Live/Dead assignment	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
4	LD	GQ917124	<i>Betaproteobacteria</i>	Uncultured beta proteobacterium clone A23YP01RM (FJ569567.1)	soil, snow melt site	100	<i>Ralstonia syzygii</i> T (U28237)	100
35	LD	GQ917152	<i>Bacterioidetes</i>	Uncultured bacterium clone Lc2yS22_ML_205 (FJ355014.1)	lake Charles	99	<i>Sediminibacterium salmoneum</i> strain NJ-44 (EF407879.1)	98
37	LD	GQ917153	<i>Bacterioidetes</i>	Uncultured Bacterioidetes bacterium from DGGE gel band S1 (AY184382.1)	lake Stor Sandsjon	100	<i>Sediminibacterium ginsengisoli</i> strain DCY13 (EF067860.1)	94
39	D	GQ917154	<i>Bacterioidetes</i>	Uncultured Pedobacter sp. clone RUGL1-94 (GQ421069.1)	soil	93	<i>Pedobacter composti</i> (AB267720.1)	93

40	D	GQ917155	<i>Bacterioidetes</i>	Uncultured bacterium clone nbw601b12c1 (GQ115765.1)	skin	99	<i>Cloacibacterium normanense</i> T (AJ575430)	99
41	L	GQ917156	<i>Bacterioidetes</i>	Uncultured Bacteroidetes bacterium DGGE gel band FD 15 (DQ385020.1)	Baltic Sea water	99	<i>Polaribacter glomeratus</i> strain KOPRI_22229 (EU000227.1)	93
42	L	GQ917157	<i>Bacterioidetes</i>	Uncultured Bacteroidetes bacterium clone OU-3-1-1-L (EU626662.1)	sea urchin	98	<i>Lutibacter litoralis</i> T (AY962293)	98
43	L	GQ917158	<i>Bacterioidetes</i>	Uncultured Bacteroidetes bacterium clone NUD-17-1-1 (EU626712.1)	sea urchin	97	<i>Tenacibaculum mesophilum</i> (AB032504.1)	86
44	LD	GQ917159	<i>Alphaproteobacteria</i>	Uncultured bacterium clone LC10_L05A11 (FJ546770.1)	lake Cadagno	99	<i>candidatus Pelagibacter ubique</i> / <i>Wolbachia pipientis</i> (AJ548800)	83
45	L	GQ917160	<i>Planctomycetes</i>	Uncultured bacterium clone FFCH623 (EU135171.1)	soil	93	<i>Gemmata obscuriglobus</i> (X85248)	87
46	LD	GQ917161	<i>Cyanobacteria</i>	Uncultured Synechococcus sp. clone XZNC83 (EU703265.1)	oligosaline lake	100	<i>Synechococcus rubescens</i> SAG 3.81 (AM709629.1)	98
47	LD	GQ917162	<i>Cyanobacteria</i>	Uncultured cyanobacterium from DGGE band ESBAC-4 (AM261464.1)	lake Estanya	88	<i>Synechococcus rubescens</i> SAG 3.81 (AM709629.1)	86
48	LD	GQ917163	<i>Actinobacteria</i>	Uncultured bacterium clone metagen16S_cs_97 (FJ447619.1)	lake Bourget	99	<i>Iamibacter majanohamensis</i> (AB360448)	87
49	LD	GQ917164	<i>Actinobacteria</i>	Uncultured bacterium clone YU201C01 (FJ694627.1)	Yukon river	100	<i>Demequina aestuarii</i> (DQ010160)	91
50	L	GQ917165	<i>Gammaproteobacteria</i>	Uncultured bacterium clone FFCH895 (EU134767.1)	soil	93	<i>Methylobacter alcaliphilus</i> (EF495157)	87
51	D	GQ917166	<i>Gammaproteobacteria</i>	<i>Stenotrophomonas acidaminiphila</i> strain ST32 (FJ982935.1)	waste water sludge	100	<i>Stenotrophomonas acidaminiphila</i> strain ST32 (FJ982935.1)	100
52	LD	GQ917167	<i>Betaproteobacteria</i>	Uncultured bacterium clone 081127-Aspo-Fracture-Biofilm-KA1362A06 (GQ240219.1)	groundwater biofilm	100	<i>Acidovorax facilis</i> strain 228 (EU730927.1)	99

54	LD	GQ917168	<i>Betaproteobacteria</i>	Uncultured <i>Bordetella</i> sp. clone F3feb.47 (GQ417631.1)	biological degreasing system	87	<i>Kerstersia gyiorum</i> strain LMG 5906 (NR_025669.1)	87
55	L	GQ917169	<i>Betaproteobacteria</i>	Uncultured bacterium clone 3C003283 (EU801904.1)	Chesapeake Bay	84	<i>Polynucleobacter necessarius</i> (FN429668.1)	84
56	L	GQ917170	<i>Betaproteobacteria</i>	Uncultured bacterium clone LC10_L05C06 (FJ546788.1)	lake Cadagno	98	<i>Polynucleobacter necessarius</i> (FN429668.1)	98
57	L	GQ917171	<i>Betaproteobacteria</i>	Uncultured Burkholderiaceae bacterium clone LW18m-2-18 (EU642357.1)	lake Michigan	96	<i>Polynucleobacter necessarius</i> subsp. <i>asymbioticus</i> (FN429668.1)	86
58	LD	GQ917172	<i>Betaproteobacteria</i>	Uncultured beta proteobacterium clone LW18m-1-70 (EU642286.1)	lake Michigan	99	<i>Methylophilus methylotrophus</i> (GQ175365)	95
61	D	GQ917173	<i>Betaproteobacteria</i>	<i>Polynucleobacter necessarius</i> strain: USHIF010 (AB470464.1)	lake Ushikunuma	99	<i>Polynucleobacter necessarius</i> (FN429657)	99
62	L	GQ917174	<i>Planctomycetes</i>	Uncultured sludge bacterium A12 (AF234727)	wastewater sludge	94	<i>Zavarzinella formosa</i> T (AM162406)	87

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2 Supplementary Tab. 1b (Phylotypes of RNA based analyses)

Phylotype	Live/Dead assignment	GenBank accession no.	Taxonomic group	Closest 16S rRNA gene sequence (Accession no.)	source of closest sequence	% Similarity	Closest described species (Accession no.)	% Similarity
1	LD	GQ917122	<i>Betaproteobacteria</i>	Uncultured bacterium clone 1C227656 (EU799977.1)	Newport harbour	100	<i>Acidovorax facilis</i> strain TSWCSN46 (GQ284412.1)	99
2	LD	GQ917123	<i>Betaproteobacteria</i>	Uncultured beta proteobacterium clone 500M5_F3 (DQ514229.1)	deglaciated soil	87	<i>Thauera terpenica</i> strain 21Mol (AJ005818.1)	87
4	LD	GQ917124	<i>Betaproteobacteria</i>	Uncultured <i>Ralstonia</i> sp. from DGGE gel band C4 (GQ255450.1)	shellfish hemolymph	100	<i>Ralstonia insidiosa</i> (FJ772078)	100
5	LD	GQ917125	<i>Betaproteobacteria</i>	Uncultured anaerobic bacterium clone C-147 (DQ018816.1)	anaerobic swine lagoon	83	<i>Thauera mechernichensis</i> (Y17590)	83

6	L	GQ917126	<i>Gammaproteobacteria</i>	freshwater Bacterium A2(2009) (GQ398339.1)	river biofilm	98	<i>Moraxella osloensis</i> strain FR1_63 (EU373514.1)	98
7	L	GQ917127	<i>Gammaproteobacteria</i>	Uncultured bacterium clone 2B20 (EU835445.1)	Reverse osmosis membrane biofilm	98	<i>Legionella erythra</i> T (Z32638)	96
8	L	GQ917128	<i>Gammaproteobacteria</i>	Uncultured bacterium clone 1B17 (EU835422.1)	Reverse osmosis membrane biofilm	80	<i>Legionella erythra</i> T (Z32638)	81
9	D	GQ917129	<i>Gammaproteobacteria</i>	Uncultured bacterium clone YSK16S-15 (EF612978.1)	acid mine drainage	91	<i>Legionella pneumophila</i> ; <i>Alcoy</i> 2300/99 (EU054324)	88
10	D	GQ917130	<i>Gammaproteobacteria</i>	<i>Pseudomonas koreensis</i> strain JDM-2 (GQ368179.1)	farm soil	99	<i>Pseudomonas koreensis</i> strain JDM-2 (GQ368179.1)	99
11	D	GQ917131	<i>Gammaproteobacteria</i>	Uncultured bacterium clone nbw232g03c1 (GQ069759.1)	skin	98	<i>Pseudomonas putida</i> strain GNL8 (FJ768454.1)	98
12	L	GQ917132	<i>Gammaproteobacteria</i>	Uncultured bacterium from SSCP band RNA 2-8-7 (DQ077602.1)	drinking water supply system	100	<i>Methylocaldum gracile</i> (U89298)	92
13	D	GQ917133	<i>Nitrospira</i>	Uncultured bacterium clone 3BR-3AA (EU937879.1)	freshwater biofilm	88	<i>Nitrospira moscoviensis</i> T (X82558)	86
14	LD	GQ917134	<i>Alphaproteobacteria</i>	Uncultured bacterium clone W_0307_65 (GQ379456.1)	soil	97	<i>Bosea vestrisii</i> T (AF288306)	97
15	LD	GQ917135	<i>Alphaproteobacteria</i>	Uncultured alpha proteobacterium clone sw-xj62 (GQ302527.1)	cold spring	97	<i>Pedomicrobium americanum</i> (X97692)	94
16	LD	GQ917136	<i>Alphaproteobacteria</i>	Uncultured alpha proteobacterium clone GASP-KB3S3_H06 (EU298674.1)	soil	99	N.A.	
17	LD	GQ917137	<i>Alphaproteobacteria</i>	Uncultured bacterium clone 0MHA_A12 (GQ306092.1)	periglacial soil	93	<i>Belnapia moabensis</i> (AJ871428)	93
18	LD	GQ917138	<i>Alphaproteobacteria</i>	Uncultured bacterium clone P10-78 (EU375422.1)	lake Puma Yumco	98	<i>Roseococcus suduntuyensis</i> (EU012448)	96

19	LD	GQ917139	<i>Gammaproteobacteria</i>	Uncultured bacterium clone 5C231590 (EU803928.1)	lake Gatun	96	<i>Methylohalobium kenyense</i> (EU006088)	85
20	L	GQ917140	<i>Firmicutes</i>	Uncultured bacterium clone KO2_aai19h11 (EU776338.1)	Kangaroo feces	81	<i>Ruminococcus flavefaciens</i> strain AR72 (AF104841.1)	81
21	LD	GQ917141	<i>Planctomycetes</i>	Uncultured planctomycete, clone DSP41 (AJ290189.1)	river Spittelwasser biofilm	94	<i>Rhodopirellula baltica</i> (FJ624344)	85
22	L	GQ917142	<i>Bacterioidetes</i>	Uncultured bacterium clone HH1409 (FJ502249.1)	lake Cadagno	98	<i>Pedobacter</i> sp. Tianshan 221-3 (EU305635.1)	93
23	D	GQ917143	<i>Bacterioidetes</i>	Uncultured Bacteroidetes bacterium clone A21YG08RM (FJ568900.1)	soil at snow melt site	95	<i>Flexibacter canadensis</i> (AB078046)	89
24	LD	GQ917144	<i>Chloroflexi</i>	Uncultured bacterium clone 538.F4 (EU357588.1)	soil	99	N.A.	
25	LD	GQ917145	<i>Chloroflexi</i>	Uncultured bacterium clone: CMBR-4 (AB305032.1)	wastewater treatment plant	91	<i>Caldilinea aerophila</i> (AB067647)	83
26	L	GQ917146	<i>Cyanobacteria</i>	Uncultured bacterium from SSCP band Li-8R-10-2 (DQ077567.1)	drinking water supply system	95	<i>Glaucocystis nostochinearum</i> (X82496)	79
27	L	GQ917147	<i>Cyanobacteria</i>	Uncultured bacterium from SSCP band TW15-RNA1-14-2 (DQ077556.1)	drinking water supply system	94	<i>Glaucocystis wittrockiana</i> (X82495)	83
28	LD	GQ917148	<i>Bacterioidetes</i>	Uncultured bacterium clone F126 (FJ348594.1)	waste water sludge	99	<i>Thermolithobacter carboxydivorans</i> (DQ095862)	90
29	D	GQ917149	<i>Cyanobacteria</i>	Uncultured bacterium clone IFBC1H11 (EU592534.1)	freshwater lake	88	<i>Synechococcus</i> sp. KORDI-78 (FJ497748.1)	87
31	LD	GQ917150	<i>Cyanobacteria</i>	Uncultured bacterium clone N05Dec-74 (EU442941.1)	lake Nam Co	90	<i>Cyanobium</i> sp. JJM10A4 (AM710358.1)	90
32	L	GQ917151	<i>Cyanobacteria</i>	Uncultured bacterium clone LaP15L91 (EF667687.1)	river sediment	97	<i>Synechococcus</i> sp. MH305 (AY224198.1)	100
46	LD	GQ917161	<i>Cyanobacteria</i>	Uncultured <i>Synechococcus</i> sp. clone XZNM83 (EU703265.1)	lake Namucuo	100	<i>Synechococcus rubescens</i> SAG 3.81 (AM709629.1)	98

Supplementary Table 2. Abundances of phylotypes sorted according to the respective Phyla/class. The abundances of the phylotypes are derived from the SSCP analyses of DNA (Table 2a) and RNA (Table 2b) extracts as shown in Fig. 4. The mean of the three samplings A,B and C plus the standard deviation SD is given for the tap water sample before sorting (“All (unsorted)”), the “live” sorted fraction (cells with intact membranes), and the “dead” sorted fraction (cells with injured membranes). “Sum” indicates the sum of the abundances of all phylotypes of the same phyla/class, “SD” is the standard deviation of the sums per sampling day (A-C).

Legend:

PT, phylotype; phylotype signature corresponds to Supplementary Table 1.

n.d., not detected

*, phylotypes retrieved from both RNA and DNA extracts

1 **Supplementary Table 2a (DNA based analyses)**

DNA based analyses		All (unsorted)		Live		Dead	
Phyla/Class	PT signature	mean A-C	SD	mean A-C	SD	mean A-C	SD
Alphaproteobacteria	44	2.83%	2.24%	1.56%	1.48%	0.92%	1.49%
Betaproteobacteria	52	2.71%	1.28%	1.97%	3.12%	0.59%	1.03%
	4*	0.25%	0.22%	8.11%	8.43%	4.96%	8.60%
	54	0.20%	0.30%	0.18%	0.26%	1.83%	2.56%
	55	0.78%	1.35%	1.29%	1.27%	n.d.	
	56	4.81%	2.31%	1.78%	2.27%	n.d.	
	57	1.20%	0.87%	2.06%	1.51%	n.d.	
	58	4.56%	0.10%	1.42%	1.23%	1.19%	2.06%
	61	0.42%	0.72%	n.d.		1.35%	2.34%
	Sum	14.93%	0.52%	16.82%	6.55%	9.92%	10.80%
Gammaproteobacteria	50	0.34%	0.59%	0.50%	0.86%	n.d.	
	51	n.d.		n.d.		1.05%	1.81%
	Sum	0.34%	0.59%	0.50%	0.86%	1.05%	1.81%
Actinobacteria	48	6.87%	3.85%	2.35%	3.19%	2.01%	3.48%
	49	8.45%	3.86%	2.17%	0.52%	3.67%	3.24%
	Sum	15.32%	1.12%	4.52%	3.71%	5.68%	6.11%
Bacteroidetes	35	12.38%	0.28%	2.46%	1.80%	7.14%	3.74%
	37	1.34%	1.18%	0.57%	0.64%	0.97%	1.26%
	39	0.08%	0.13%	n.d.		0.23%	0.40%
	40	1.66%	0.45%	n.d.		0.15%	0.26%
	41	2.23%	3.87%	4.79%	8.30%	n.d.	
	42	n.d.		5.98%	10.35%	n.d.	
	43	0.06%	0.10%	1.65%	2.86%	n.d.	
	Sum	17.75%	4.56%	15.45%	22.67%	8.49%	3.02%
Cyanobacteria	46*	1.31%	1.24%	n.d.		9.50%	16.46%
	47	1.99%	0.29%	0.64%	1.10%	0.60%	1.03%
	Sum	3.30%	0.97%	0.64%	1.10%	10.10%	17.49%
Planctomycetes	62	n.d.		10.80%	18.71%	n.d.	
	45	0.22%	0.38%	2.01%	3.48%	n.d.	
	Sum	0.22%	0.38%	12.81%	22.19%	n.d.	
% identified as PTs		54.69%	3.83%	52.29%	21.52%	36.16%	23.66%

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1 **Supplementary Table 2b (RNA based analyses)**

RNA based analyses		All (unsorted)		Live		Dead	
Phyla/ Class	PT signature	mean A-C	SD	mean A-C	SD	mean A-C	SD
Alphaproteobacteria	14	13.03%	7.38%	5.05%	1.85%	7.50%	5.32%
	15	0.49%	0.85%	0.74%	1.29%	1.99%	1.92%
	16	1.21%	2.09%	7.12%	9.13%	0.09%	0.15%
	17	n.d.		4.38%	7.59%	0.92%	1.60%
	18	0.76%	1.31%	3.75%	3.64%	1.02%	1.76%
	Sum	15.49%	11.41%	21.04%	20.67%	11.52%	8.19%
Betaproteobacteria	1	18.14%	5.22%	5.22%	1.88%	5.89%	1.39%
	2	0.90%	1.01%	0.49%	0.49%	0.59%	1.03%
	4*	0.81%	1.26%	3.57%	2.81%	3.85%	2.48%
	5	0.91%	0.79%	n.d.		n.d.	
	Sum	20.76%	5.66%	9.28%	3.14%	10.33%	4.27%
Gammaproteobacteria	6	1.72%	2.98%	3.14%	1.61%	n.d.	
	7	0.14%	0.24%	1.84%	1.33%	n.d.	
	8	n.d.		1.86%	3.22%	n.d.	
	9	0.53%	0.92%	n.d.		3.01%	2.67%
	10	0.02%	0.03%	n.d.		2.36%	1.50%
	11	n.d.		n.d.		7.53%	8.81%
	12	1.38%	0.17%	9.10%	1.01%	n.d.	
	19	5.72%	1.01%	1.64%	1.42%	1.38%	1.39%
	Sum	9.50%	4.02%	17.58%	5.60%	14.27%	11.31%
Bacteroidetes	22	1.50%	2.60%	1.29%	1.32%	n.d.	
	23	6.63%	1.62%	n.d.		1.80%	3.12%
	28	0.18%	0.31%	1.20%	1.26%	0.69%	0.86%
	Sum	8.31%	2.64%	2.49%	2.31%	2.49%	3.96%
Chloroflexi	24	0.70%	0.47%	13.67%	8.14%	1.67%	1.78%
	25	n.d.		2.08%	1.38%	1.62%	1.98%
	Sum	0.70%	0.47%	15.75%	9.10%	3.28%	2.13%
Cyanobacteria	26	2.93%	0.47%	4.42%	7.66%	n.d.	
	27	n.d.		0.98%	0.68%	n.d.	
	29	0.48%	0.83%	n.d.		1.51%	2.61%
	46*	10.48%	7.38%	2.20%	1.72%	14.72%	5.59%
	31	0.88%	0.83%	n.d.		n.d.	
	32	0.82%	1.41%	0.25%	0.44%	n.d.	
	Sum	15.59%	7.56%	7.85%	7.33%	16.23%	8.13%
Firmicutes	20	2.25%	1.95%	0.95%	1.64%	n.d.	
Nitrospira	13	0.10%	0.17%	n.d.		1.82%	1.84%
Planctomycetes	21	4.60%	7.96%	n.d.		n.d.	
% identified as PTs		77.30%	3.39%	74.93%	20.17%	59.94%	5.68%

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Supplementary Fig. 1

Supplementary FIG 1. Comparison of SSCP electropherograms of concentrated drinking water samples directly extracted from concentrates and non-concentrated drinking water extracted from filter sandwiches using GelCompare II. (a) DNA-based SSCP electropherograms (b) RNA-based SSCP electropherograms.

1 **Supplementary Fig. 2**

2 a) DNA based SSCP

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1 b) RNA based SSCP

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SUPPLEMENTARY FIG. 2. (a) Detailed analysis of the electropherograms from the different sampling dates originating from the DNA-based SSCP gel given in Fig. 4a. Numbers correspond to the phylotypes given in Tab 1. Percentages in parentheses represent relative abundances of phylotypes. (b) Detailed analysis of the electropherograms from the different sampling dates originating from the RNA-based SSCP gel given in Fig. 4b.

1 **Supplementary Fig. 3**

2 **a) all phylotypes**

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1 b) Phylotypes affiliated with *Proteobacteria*

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SUPPLEMENTARY FIG 3.

Phylogenetic analysis of 16S rRNA gene sequences obtained from the bands of the SSCP fingerprints shown in Fig. 4 using the neighbor-Joining method. The optimal tree with the sum of branch length = 2.1515 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons (pair wise deletion option). Sequences are labeled with their origin plus the phylotype number (in parentheses) given in supplementary Table 1 and 2. Sequences are coded with different character types according to their origin in terms of nucleic acid type: DNA-based sequences are shown in bold, RNA-based sequences are shown in bold italic, and sequences occurring in DNA- and RNA-based fingerprints are shown in bold with an asterisk. (a) Phylogenetic tree of all detected phylotypes. (b) Phylogenetic tree of the detected phylotypes affiliated with the *Proteobacteria*.