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Research Article

Microbial Characteristics through Drinking Water Aquifer Sand Material*

Public health concerns about pathogens present in animal manure are emerging constraints to water supplies in many areas of the world. The objective of this study was to examine the microbial community composition in aquifer material impacted by contaminants from different sources. Total microbial community profiles were compared by using eubacterial primers to amplify 16S rRNA genes from total bacterial DNA and RNA. PCR and reverse transcriptase (RT) PCR were used to amplify 16S ribosomal RNA, and the products were subjected to denaturing gradient gel electrophoresis (DGGE). DGGE analysis of RT-PCR products detected a subset of bands visible in the DNA-based analysis, indicating that some dominantly detected bacterial populations did not have high levels of metabolic activity. The sequences detected by the RT-PCR approach were, however, derived from a wide taxonomic range, suggesting that the activity in the aquifer sand material was not determined at broad taxonomic levels but rather was a strain- or species-specific phenomenon. Comparative analysis of DGGE profiles grouped all DNA-derived aquifer samples together in a cluster. At the end of the experimental period, the aquifer material entered a stable population state, which was characterized by a greater diversity of DNA-based fingerprints compared to viable bacteria. Our data showed that the active members of the community are a sub-population of that community that performs certain biological functions during water filtration through aquifer material. Therefore, recharging surface water through aquifer sand material may produce a microbial population quite different from the input source due to the availability of nutrients for bacterial growth.

Keywords: Bacteria, Contaminated water, DNA, Pollutants

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1 Introduction

The analysis of bacterial abundance using culture-dependent methods cannot determine what kinds of bacteria are responsible for changes in the bacterial community, and which type of organism could be used as a pollution indicator, because most of the bacteria in the environment have not been cultivated [1–2]. The analysis of bacterial communities, including non-cultivated bacteria, can therefore provide more precise information on the bacterial populations responsible for rapid changes in the bacterial community and the pollution indicators of fecal contamination in groundwater ecosystems. A variety of molecular methods have been used to determine the species composition of bacterial communities without enrichment culture [3–4]. These methods rely on 16S ribosomal RNA (rRNA) sequences, including in situ hybridization [5–6], direct amplification of 16S rRNA, and further analysis using community fingerprinting such as denaturing gradient gel electrophoresis (DGGE) [7–8], temperature gradient gel electrophoresis (TGGE) [9–10], terminal RFLP [11], and 16S rRNA cloning-sequencing [12].

Molecular ecological studies in soils have been based on both direct and indirect soil DNA extraction protocols [13], and such studies have permitted the assessment of the fate of both introduced microorganisms [13–14] and functional genes [15], and of indigenous bacterial communities [16–17]. As DNA-based studies of indigenous bacterial communities in soil revealed a remarkable stability of molecular profiles over

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time versus treatments [17], it was surmised that a RNA-based analysis might more readily reveal shifts in the community structure or the activity (reflecting adaptational processes) than a DNA-based one. The use of 16S rRNA as a basis for assessing microbial diversity offers the potential advantage of the natural amplification of target numbers, as one cell can contain thousands of ribosomes. Moreover, many bacterial species are known to vary their ribosome numbers in accordance with their cellular activity [18], and hence RNA-based approaches can provide a means to analyze the diversity of the active fractions of microbial communities in soil.

The aim of this study was therefore to determine possible alterations in the bacterioplankton community composition of aquifer sand material impacted by Santa Ana River water which is dominated by secondary effluent from different municipalities within the Santa Ana River watershed. Using DGGE, the community structure was determined on the DNA and RNA levels. Due to the high number of ribosomes in active cells, RNA is an indicator of metabolically active cells, whereas DNA reflects the general presence of a phylogenetic unit [19]. Analysis of both DNA and RNA should therefore lead to a higher resolution of possible secondary effluent-induced alterations in the composition of the bacterioplankton community in aquifer sand material. The subsurface aquifer material collected from a recharged zone was studied and packed in a 1.2 × 1.2 × 1.8 m filtration tank with secondary treated water running through the tanks for 15 d. The water passes through Liquid Separation units that may act as a solar disinfection system for the inactivation of enteric bacteria. The inactivation mechanism of solar disinfection is complex and not yet fully understood. The central hypothesis is that UVA light produces reactive oxygen species which can damage nucleic acids, proteins or other life-supporting cell structures [20]. It was also found that broad-spectrum UVA light blocks the electron transport chain, inactivates transport systems, interferes with metabolic energy production and can cause a general increase in the permeability of the membrane [21]. Furthermore, direct inhibition of certain enzymes (e.g., catalase) has also been observed [22]. Running of secondary effluent water through the units was repeated three times in order to evaluate the reliability of data. Total DNA and RNA were extracted from aquifer material as well as total DNA from influent and effluent water.

2 Materials and Methods

2.1 Sampling Collection

Santa Ana River water at the Orange County Water District Field Station was collected during day 1, 3, 6, 10 after initiating the experiment. The water consists of source water (water from the river), processed water (water from Liquid Separation Inc. processor (LSI)), and filtrate water (water from aquifer material after passing through the processor). This process was repeated three times to determine the reliability of data. The experiment was conducted from April to June, 2004 in a 1.2 × 1.2 × 1.8 m filtration tank built with stainless steel outside the Field station. Aquifer material consisted of heterogeneous native lake sediment that had been processed through a sand washing plant to remove the majority of silt and clay particles. The material was trucked to the station and packed into the tanks. Samples from aquifer material were collected on day 15 at the end of each experiment.

Two processors were used for this study: the active processor with a magnet and the passive or control processor without a magnet. Source water was obtained from the Santa Ana River and run through a 1 inch PVC pipe into the processors. From the processor, the water then runs another 1 m into the filtration tank containing aquifer materials. The active LSI unit works by releasing ionic bonds of water and making the water less likely to hold onto particulates and dissolved solids. This in turn allows the micro-filtration or settling tanks to become more efficient and faster in reducing contaminants from water by filtration. The technology was evaluated by the Orange County Water District (Anaheim, CA) to determine the rate of water percolation through aquifer material.

2.2 DNA and RNA Preparation and cDNA Synthesis

UltraClean soil and water DNA kits (MO BIO, Inc., Solana Beach, CA) were used for the extraction of DNA from soil and water. FastRNA Pro Soil-Direct kit for the rapid isolation of total RNA from soil (BIO101, Vista, CA) was applied for the extraction of total RNA from aquifer material. During RNA extraction, several precautions were taken. The various stages involved in the RT-PCR assay were physically separated into designated areas. For each area, dedicated pipettes, aerosol barrier tips and protective clothing were used. In addition, RNase free reagents and plastics were used where possible. All steps involved in the RNA extraction protocol were performed on ice and centrifugations steps were performed in a refrigerated microcentrifuge at 4 °C. Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA) were employed for PCR amplification of 16S rRNA genes. For cDNA synthesis**,** RNA was reverse transcribed into first-strand cDNA using Ready-To-Go-You-Prime First-Strand Beads (Catalog No. 27-9264-01; Pharmacia Biotech) as recommended by the manufacturer. The primers used were $pd(N)_6$ (Catalog No. 27-2166; Pharmacia Biotech) random hexamers at a final concentration of 200 ng per reaction. Immediately after the reactions were completed, RNA was digested by adding 2 μL of RNase I A (stock 200 ng/L) to each reaction mixture and incubating the mixtures at 55 °C for 30 min. First-strand cDNA was cleaned using QIAquick spin columns (Qiagen). The final volume was 20 μL in elution buffer (Qiagen). PCR and RT-PCR primers used for this study were primers 388F and 518R [23].

2.3 DGGE and Image Analysis

DGGE was performed with 8 % [wt/vol] acrylamide gels containing a linear chemical gradient ranging from 30 to 70 % denaturant with 100 % defined as 7 M urea containing 40 % formamide. Gels were run for 3 h at 200 V with the Dcode™ Universal Mutation System (Bio-Rad Laboratories, Hercules, CA). DNA was visualized after ethidium bromide staining by

UV transillumination and photographed with a Polaroid camera. Major bands were excised for the identification of bacterial species. Bands were placed into sterilized vials with 20 μL of sterilized, distilled water and stored overnight at 4 °C to allow the DNA to passively diffuse out of the gel strips. Ten μL of eluted DNA was used as the DNA template with the Eubacteria primers. DNA cloning and sequencing of bands were carried out as described above. Sequence analyses were done using the BLAST database (National Center for Biotechnology Information: www.ncbi.nlm.nih.gov).

DNA fingerprints obtained from the 16S rRNA banding patterns on the DGGE gels were photographed and digitized using ImageMaster Labscan (Amersham-Pharmacia Biotech, Uppsala. Sweden). The lanes were normalized to contain the same amount of total signal after background subtraction. The gel images were straightened and aligned using ImageMaster 1D Elite 3.01 (Amersham-Pharmacia Biotech, Uppsala, Sweden) and analyzed to give a densitometric curve for each gel. Band positions were converted to R_f values between 0 and 1 and profile similarity was calculated by determining Pearson's coefficient for the total number of lane patterns from two gels [7]. Dendrograms were constructed by using the unweighted pair group method with mathematical averages (UPGMA). Pearson's similarity coefficients generated from all the sampling points were integrated and analyzed using ImageMaster 1D database 2.01 (Amersham-Pharmacia Biotech, Uppsala, Sweden). The data obtained were used for the construction of a library to determine the best-fit profile and to integrate the area under each peak for every gel and for the construction of a dendrogram from both the DNA and the RNA generated gels.

A second approach was used to determine the community structure based on the peak height from the different bacterial groups (16S rRNA bands) and was analyzed to generate diversity indices (H). The peak height values generated from the sampling points were integrated and analyzed. The data obtained were used to integrate the area under each peak for each lane in every treatment. For this analysis, each band was presumed to represent the ability of that bacterial species to be amplified. The Shannon index of diversity (H) was chosen to compare changes in the diversity of microbial communities within the four treatments at each time [24] by using the following function:

$$H = -\sum P_i \log P_i \quad (1)$$

where $P_i = n_i/N$, n_i is the height of peak, and N is the sum of all peak heights in the curve.

3 Results

3.1 Structure of the Microbial Community of River Water before and after the Filtration through Aquifer Material

The analysis of the composition of the bacterial community of water samples by PCR-DGGE was performed for all samples collected in April, May, and June. The DGGE gels covered samples taken from stream water and from water that has passed through the processors and the aquifer materials during the month of May as an example (see Figs. 1 A–C). The Image Master ID was used to analyze and create a database set for these samples. The set contained a total of 38 unique bands. The bands were found in different combinations among the DGGE banding patterns. Occurrence of different bands from the gels showed that about 40 % of the bands occurred in >70 % of the samples. Pattern comparisons were performed among the gels by using Pearson's coefficient. From the database analysis, the Pearson values indicated that all samples shared a large portion of the band set (data not shown), while a few had unique bands as indicated by the banding patterns in Figs. 1A–C. All bands were detected when the relative intensities were set above 1 % of the total peak height in a lane. The mean DGGE bands (species richness) varied between 5 in Lanes 10 and 11 (Santa Ana River water samples, see Fig. 3A) to 51 per sample for water from the processor (Lanes 1 and 2 in Fig. 1C). Five bands (DW1 to DW5) were common in all samples and these bands were excised, reamplified, cloned, and sequenced for species identification.

Overall, species diversity and richness increased from the initial start of the experiment from day 1 to day 10 in both the processed and filtered water per lane of DGGE. Except during the first day of the experiment, the diversity index was very stable between the water that has gone through the processor and aquifer sand material. At the end, the number of bands (species richness) and the Shannon-Weaver index of diversity were slightly higher in the control treatment than in the treatment that has gone through the processor (see Tab. 1). This means that exposure of source water to the processor with LSI had a minor impact or effect on the bacterial population, and further studies need to be done before a conclusive statement can be made of the effects of the LSI on the microbial diversity of the water samples. Since there was a slight increase in diversity indices and the number of bands, thus, the preexposure incubation period (10 d) was long enough to allow the growth of diverse bacterial consortia. As seen in Tab. 1, the Shannon-Weaver index of diversity H and the number of bands (species richness) were slightly higher in the control process water after 10 d compared to the water with the active LSI.

Differences in the community structure from the samples were addressed by cluster analysis of DGGE patterns from surface water-associated microbial communities. Since few distinct banding patterns were observed within some of the treatments as well as sampling times, the analysis was performed using the UPGMA algorithm. Fig. 2 is an example of the cluster pattern obtained from water samples during the month of May. Cluster analysis was applied to the DGGE banding pattern to illustrate the similarity of all possible pairs of each gel track. Cluster analysis from stream water and water from processor and aquifer revealed continuous changes in the bacterial community structure (see Fig. 2). The DGGE profile comparison as seen in this figure showed a changing community from day 1 to day 10 when the water samples were taken (filtrate, source, and processed water). This is because no single cluster was formed that showed all the source water from day 1 to 10 or from any of the treatments clustering together.

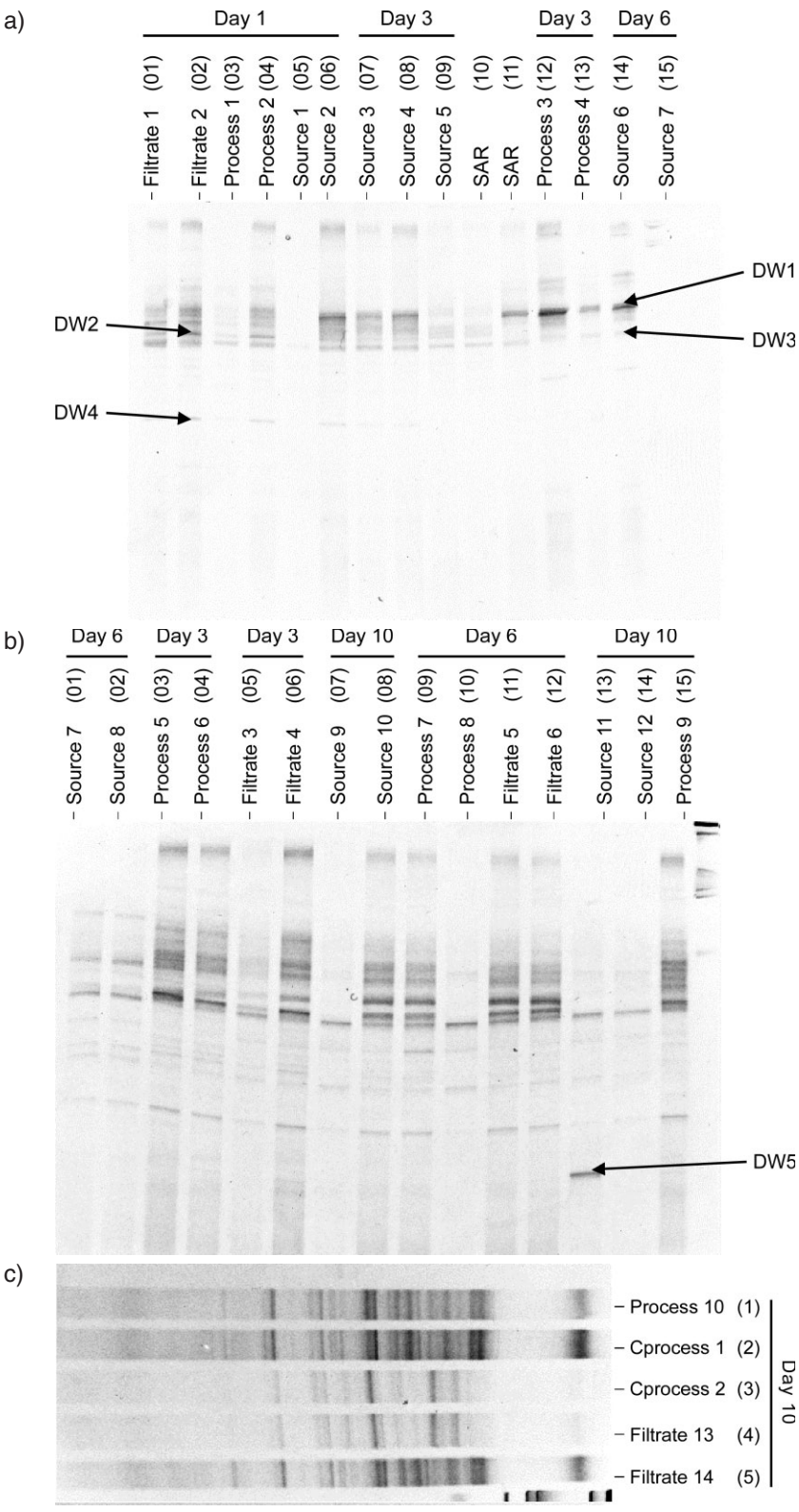

Figure 1. A–C. DGGE patterns produced from 16S rRNA of water samples collected before and after passing the aquifer system. Numbers following sample names indicate the day that the sample was taken (from day 1 to 10).

3.2 Structure of the Microbial Community of Aquifer Materials after 15 Days of Filtering River Water

The microbial community composition was determined from aquifer materials after running secondary effluent from Santa Ana River through it for 15 d. As noted above, for the water samples, DGGE analysis was performed on aquifer materials inside the 30 cm long columns. Two gels covering a total of 15 samples each were run with DNA and RNA samples, respectively (see Figs. 3 A and B). The Shannon-Weaver index of diversity was used to determine the complexities of both the DNA and RNA based gel profiles. The analysis of both gels showed that the microbial diversity and species richness at the DNA level was higher than at the RNA level (see Tab. 1). The bands were found in different combinations among the DGGE banding patterns. The mean highest DGGE band found in each lane was 50 (Lanes 5 and 7) and the mean lowest DGGE band was 32 (Lanes 12 and 14). On the other hand, the mean highest DGGE band for RNA was 39 (Lanes 1 and 4) and the lowest was 1 (Lanes 13 and 14). It should be noted that Lanes 13 and 14 were aquifer materials that were trucked in from the excavation site for the study. It also suggests the formation of a larger microbial community on the aquifer materials after Santa Ana River water has passed through it for 15 d.

To gain insight into the identities of major bacterial populations, prominent DGGE bands from both the DNA- and RNA-derived aquifer profiles and bands derived from aquifer materials before they were packed into the filtrations (see Fig. 3A, Lanes 13 and 14) were excised and

Table 1. Sequence analysis of bands excised from DGGE gels derived from bacterial 16S rRNA extracted from aquifer materials at different depths.

Bands/sample source	Related bacterial sequences	Similarity [%]	Samples present	Accession number
DW1/source6	Uncultured bacterium	99	All samples	AY221056
DW2/filtrate	Pseudomonadaceae bacterium E7	99	All samples	AF539761
DW3/source6	Uncultured bacterium	98	All samples	AF255644
DW4/filtrate	Uncultured bacterium clone m43	95	Some samples	AY444984
DW5/source11	Uncultured *Verrucomicrobium* sp.	95	Source 11	AY157106
DS1process 1–10 cm	*Thermococcales archaeon* T30a-18	98	All samples	AJ585958
DS2process 1–10 cm	Uncultured bacterium	100	All samples	AF339858
DS3process 1–20 cm	Uncultured *Chloroflexi* bacterium	91	All samples	AY921826
DS4process 1–30 cm	Uncultured Alpha-proteobacterium	95	All samples	AY690293
DS5process 2–10 cm	Uncultured bacterium clone fcr171	93	All samples	AY438817
DS6 process 2–20 cm	*Methylobacillus* sp. KT1	92	All samples	U31280
RS1/process 1–10 cm	Uncultured Gamma-proteobacterium SUR-ATT- 21	100	All samples	AF114621
RS2/process 1–10 cm	Uncultured bacterium	95	All samples	AF255644
RS3/process 1–10 cm	*Pseudomonas* sp. 4	98	All samples	AY269867
RS4/process 1–10 cm	Unidentified Eubacterium	100	All samples	AF010082

used for nucleotide sequence analysis. Most of the excised bands produced legible DNA sequences and were used to determine species compositions in the aquifer samples. DGGE bands in a single gel that appeared to be identical based on mobility did indeed produce identical nucleotide sequences (data not shown). Furthermore, DGGE bands that appeared to be identical in the DNA- and RNA-derived profiles produced different sequence profiles (see Tab. 1). The majority of the DGGE bands showed the highest levels of identity to clones recovered from soil environments or sequences obtained from strains isolated from soils. The highest level of identity between a DGGE band and a previously defined sequence was the level of identity observed for bands DS1, DS2, RS1, and RS4 (see Tab. 1).

The banding pattern revealed some differences between the control treatment and the treatment with the LSI processor. On the DNA level (compare Fig. 3A), bands DS1 and SD2 were present in much lower intensities in the control samples and were completely missing at the 20 and 30 cm depths. The effect was seen in band DS5 and DS6 where DNA could not be extracted from the control samples for comparison due to low intensity. However, bands DS3 and DS4 were present in both the control processor and the active processor with LSI. These two bands were clearly visible in the original aquifer material used for filtration (see Fig. 3A, Lanes 13 and 14). At the RNA level, the differences between the control and the aquifer material impacted by water that passed through the active processor were more pronounced (see Fig. 3B). Bands with the control treatment were less intensive than bands with the active processor. The four major bands RS1, RS2, RS3, and RS4 were present in all the samples, and Lanes 13 and 14 did not show any detectable band. The two lanes were from original aquifer material that was used for filtration. Most of the bands examined for the RNA-derived community profiles were also detected almost in the same position in the DNA-derived DGGE patterns, except band DS3 and DS6, but sequence identification showed that most of them were different bacterial species.

In the DNA-derived profiles, approximately 6 dominant bands were excised from all samples. Most of the bands detected were observed for more than a single month experimental treatment. In order to compare DGGE patterns, UPGMA was used to create a dendrogram describing pattern similarities (see Fig. 4). This analysis clearly distinguished between the DNA- and RNA-derived DGGE patterns. Most of the samples collected from the first 20 cm, produced DGGE patterns that grouped together as most similar to each other. The grouping based on DNA-derived patterns that did not go through the filtration process clustered together. The RNA-derived profiles showed a different pattern of clustering. In this case, almost all the samples from the control aquifer materials grouped together and there was no clear separation from the other samples.

Sequencing of DGGE bands revealed that the majority of the dominant populations detected had 16S rRNA sequences that were most closely related to those of previously described bacteria originating from biofilm materials (Pseudomonadaceae bacterium E7, uncultured bacterium clone m43, uncultured Gamma-proteobacterium SUR-ATT-21) or from waste water/sediment (uncultured *Verrucomicrobium* sp, uncultured Alpha-proteobacterium, uncultured bacterium clone fcr171) or unidentified bacteria detected as environmental clones. For example, several Proteobacterium-like sequences were detected in the DNA-derived profiles, but only Gamma-proteobacteria sequences appeared to be derived from populations that were metabolically active in the column.

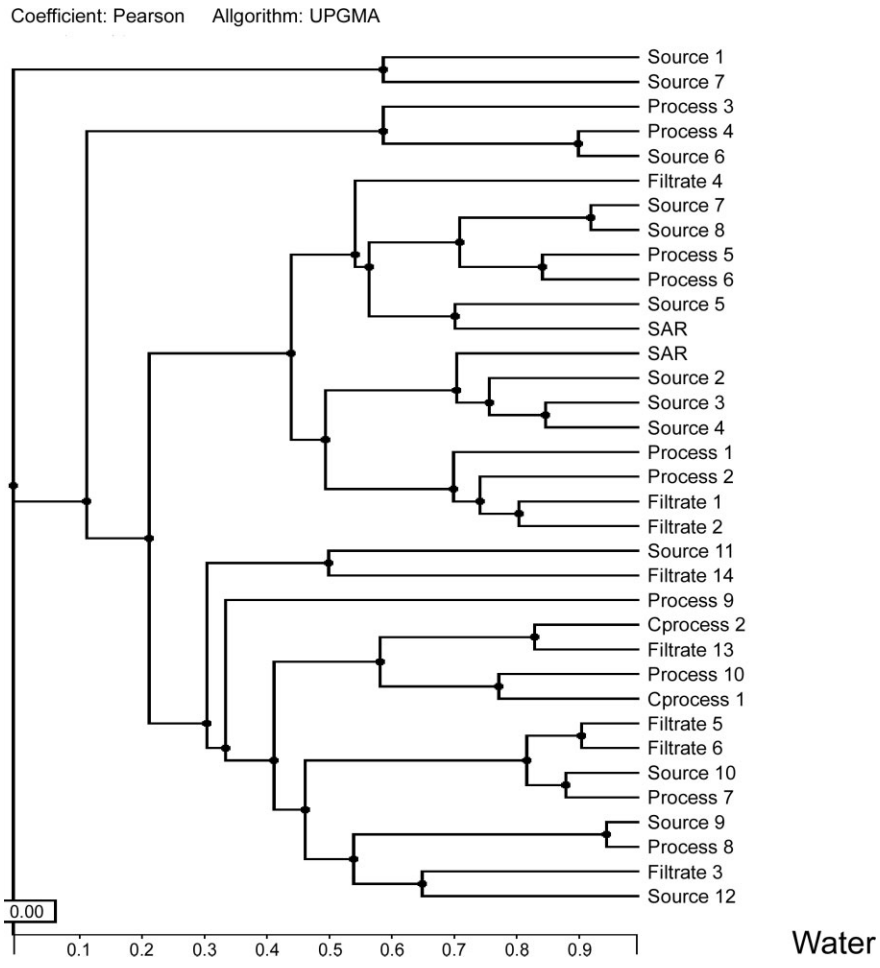

Figure 2. UPGMA tree representing the genetic similarity of the microbial community profiles obtained by PCR-DGGE from the water sample.
Numbers following sample names indicate the day that sample was taken (from day 1 to 10).

4 Discussion

The results presented in Tab. 1 show that the diversity index *H* and species richness patterns became more complex towards the end of the sampling period (10 days). This probably reflects an increased species richness within the bacterial community as the secondary effluent water with low carbon and nitrogen at the initial phase of the study was replaced by a more diverse bacterial community towards the end of the sampling period, leading also to a larger diversity of potentially utilizable substrate [25–26]. In each experiment, the banding patterns at the beginning of the preexposure of water to LSI incubation and in the subsequent filtration process through the microbial community of the aquifer sand material became more complex (see Figs. 1A–C), indicating highly reproducible experimental conditions and increase in microbial diversity.

At the end of the study, both the PCR-DGGE and RT-PCR-DGGE approaches showed a different microbial composition in the aquifer sand material. The RT-PCR-DGGE pattern exhibited an active microbial composition compared to the total microbial composition obtained by the PCR-DGGE method. This analysis clearly distinguished between the DNA- and RNA-derived DGGE patterns. Most of the duplicated samples, which were collected under the same conditions, produced DGGE patterns that grouped together as most similar to each other (see Fig. 4). The community fingerprints obtained by both extraction methods were essentially the same in most cases. Moreover, RT-PCR-DGGE and PCR-DGGE products showed similar band patterns as well. For example, intense bands at the same position in the lanes were generally found for DNA- and RNA-based methods. However, there were also some differences. Most notably, the number of bands was higher with the DNA-based method than in the RNA-based fingerprints. RNA-based fingerprints likely represent the community structure of active members, whereas DNA-based fingerprints aim at numerically abundant members.

The analysis of DNA and RNA bands from the same bacterial community leads to a characterization of bacterial species or phylotypes otherwise uncharacterized when the DNA or RNA bands would be analyzed alone. Tab. 1 indicates that nearly all the DNA bands identified were different at the species level from all the RNA derived bands. For example, bands DS1 to DS6 were only represented in the DNA library, while bands RS1 to RS4 were represented only in the RNA level (see Tab. 1). There are possibly three aspects in the distribution pattern of DNA and RNA patterns in our samples that are worth considering in an ecological context. First, DNA bands (DS2-DS6) might be representative for *bacteria* high in cellular abundance and/or with multiple operons within their cells. However, the much lower number or the absence of similar clones in the RNA library could indicate that these DNA clones are from *bacteria* with less ribosomes and therefore, probably representative for cells with reduced metabolic activity. Secondly, a high number of repetitive clones in the RNA library (RS1-RS4) could represent active members of the complex community with more ribosomes present in their cells. Thirdly, clones from *bacteria* with low cellular abundance and/or low operon numbers, which were not detected in the DNA library, might indicate members in the complex community that are not detectable on their DNA level (beyond the detection threshold of our approach), but on their RNA level. This observed mismatch between the DNA and RNA band suggests that these clones originate from *bacteria* low in cellular abundance but with a

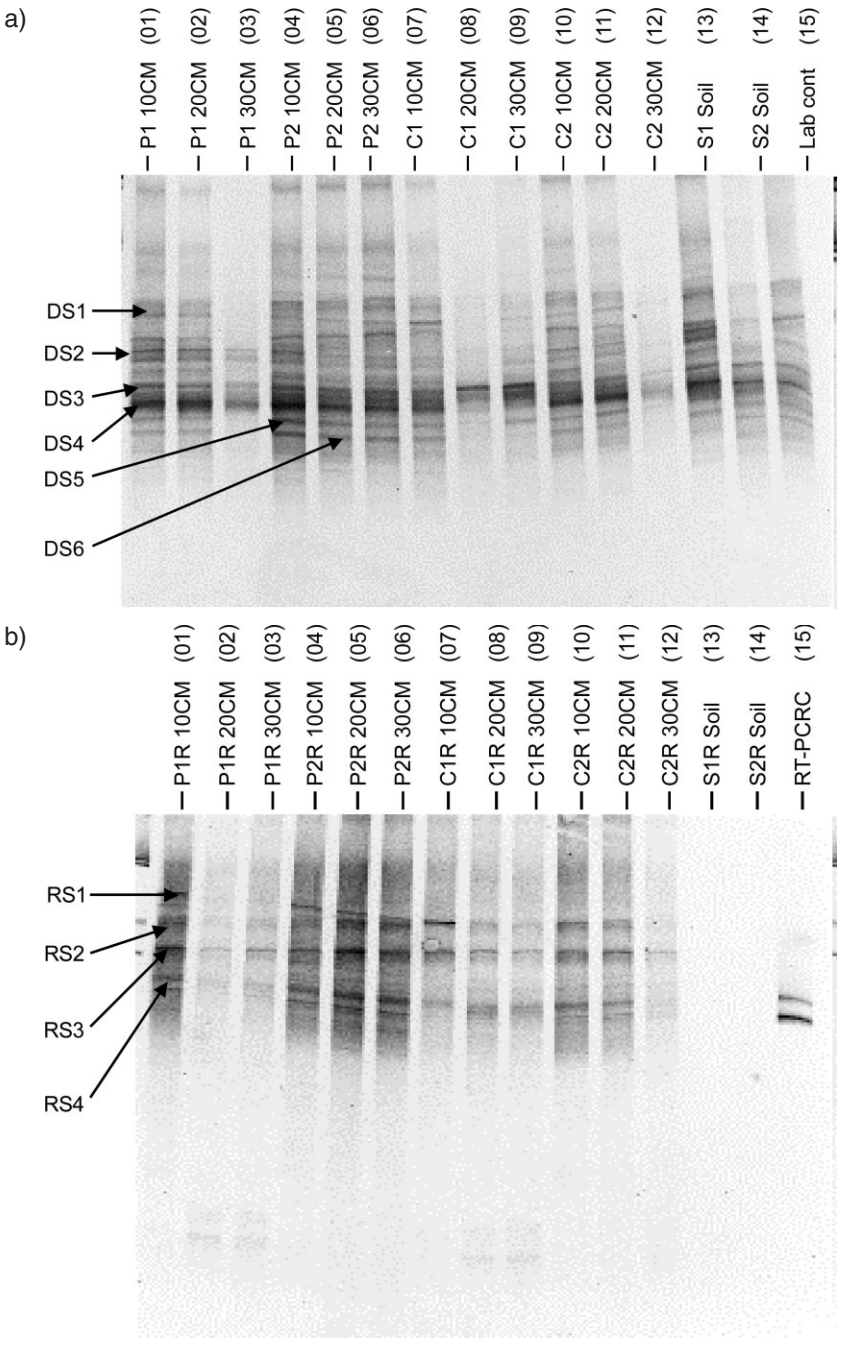

Figure 3. DGGE patterns produced from (A) 16S rRNA PCR and (B) 16S rRNA RT-PCR of aquifer materials after 15 d.
From Lanes 1 to 15 (P1 to lab cont) are 16S rRNA PCR samples and from Lanes 1 to 15 (P1R to RT-PCRC) are 16S rRNA RT-PCR samples of aquifer materials.

potentially high metabolic activity as indicated by their presence in RNA level [26–27].

Yamaghuchi et al. [28] reported a higher ratio of physiologic activity in bacterial populations in eutrophic than in oligotrophic rivers, and suggested that carbon availability is critical, because a lack of this element limits in situ respiratory activity [29]. For this reason, the higher ratios of cells associated with the aquifer materials (biofilms) would be influenced directly by the higher availability of carbon associated with the aquifer surface area that water has passed through for many days with different concentrations of nutrients resulting in the formation of biofilms for bacterial growth. The results of this study showed that bacterial populations in the aquifer material impacted by the active LSI may be associated with a significant higher ratio of microbial activity (39 bands with RNA samples) than bacterial cells directly from the sand that was trucked in for the study (1 RNA band, Fig. 3B, Lanes 13 and 14). It is also demonstrated that cluster analysis (considering the relative intensity of each band) from DGGE gels was able to detect, in more detail, subtle differences between complex

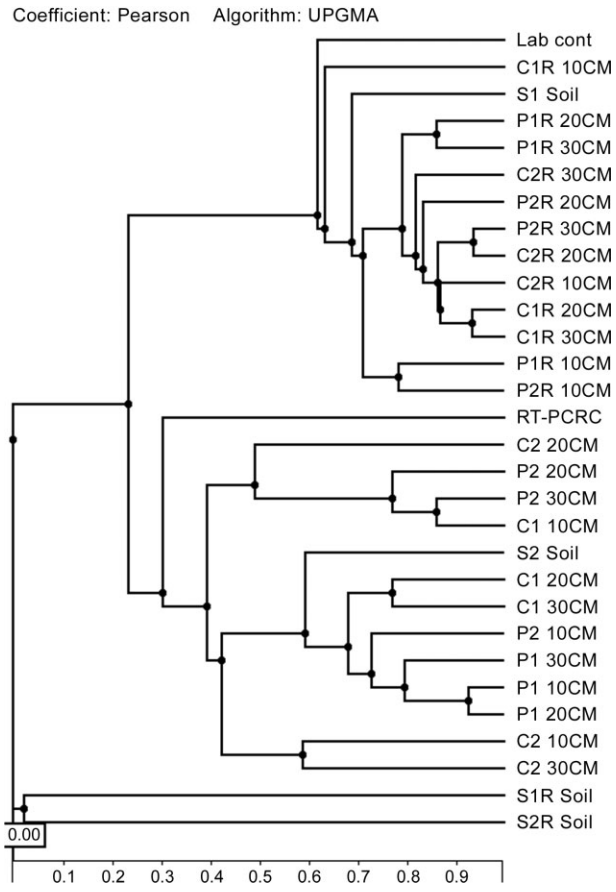

Figure 4. UPGMA tree representing the genetic similarity of the microbial community profiles obtained by PCR-DGGE and RT-PCR-DGGE.
Samples 1 through 15 refer to the lane numbers in the figure.

microbial assemblages from DNA and RNA based analysis. Thus, DGGE analysis supported with cluster proved to be a powerful tool to monitor changes in the community structure of aquifer material under continuous water flow.

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