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# Evaluation of bacterial diversity in traditional drinking water reservoirs of rural areas: a molecular approach

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**ABSTRACT:** In Morocco, storage reservoirs are particular systems of water supply in rural areas (a case study in Assif El Mal Valley). These reservoirs are fed by rainwater and/or directly from the river through opened channels, and are used without any treatment as a drinking water by the surrounding population.

This study was conducted to evaluate the bacterial contamination of drinking water reservoirs in this rural area using a molecular approach studying the 16S-rDNA bacterial diversity in water, via Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique.

The application of PCR-DGGE techniques on stored water in the reservoirs showed a high bacterial diversity, including pathogens, namely *Salmonella sp., E.coli, Sphingomonas spp*. and *Aeromonas sp.,* which indicated a high risk of infection for the user population.

Comparative cluster analyses of the DNA based fingerprints revealed the six studied reservoirs according a gradient accumulation of bacterial contaminants from upstream to downstream.

The molecular approach in this study gives a very helpful tool to confirm without any doubt the bacterial contamination of drinking water. Otherwise, this study provides an overview of the dominant bacterial groups in the traditional storage reservoirs of water in Moroccan rural area and the impact of environmental changes on bacterial diversity.

**KEYWORDS:** bacterial diversity, water reservoir, 16S-rDNA, DGGE-PCR technique.

## 1 INTRODUCTION

One third of the world's population live in countries with some level of water stress and water scarcity is expected to increase in the next few years due to increases in human population, per capita consumption and the resulting impacts of human activity on the environment [1]. Water is a pertinent component of life and its main sources include groundwater, surface and rain water.

The contamination of water resources has important repercussions for the environment and human health [2]. Irregular water supply, insufficient chlorination and sewage flooding seem to be associated with self-reported diseases [3]. More than that, an estimated of 2 million children die each year from diarrheal disease. Almost all of them are living in developing countries and are aged<5 years [4]. Infants younger than 1 year account for more than half of these deaths, and the risk can be 2–3 times higher among infants who are not exclusively breastfed [5], [6]. Many of these deaths are attributed to the use of unsafe drinking-water [4].

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The most common and widespread health risk associated with drinking water is contamination, either directly or indirectly, by human or animal excreta and the microorganisms contained in feces [7]. Detection of bacterial indicators in drinking water means the presence of pathogenic organisms that are the source of waterborne diseases.

Drinking water in many countries is routinely monitored for recent fecal contamination by testing for fecal indicator organisms *Escherichia coli*, *thermotolerant coliforms*, and/or *intestinal enterococci* to demonstrate microbial safety [8]. Although these indicator organisms have been used for many decades, they have some limitations: the number of *E. coli/coliform/enterococcus* bacteria in feces is relatively low [9], [10], and they sometimes might be able to grow in the environment [11]. Moreover, these tests don't give an idea about bacterial diversity.

Ensuring public water quality therefore requires that we develop improved methods, more accurately to identify human fecal pollution. Consequently, scientists have been searching for alternative methods rapid, very sensible to detect all bacterial diversity in environmental samples. Therefore, a molecular detection method is needed, since such methods are highly specific and sensitive. The methods used are typically based on the detection and quantification of specific segments of the pathogen's genome (DNA or RNA). These methods allow researchers to rapidly and specifically detect microorganisms of public health concern. Additionally, recent improvements have allowed simultaneous detection of several microorganisms in a single assay [12], [13], [14].

Molecular techniques for the specific detection and quantification of bacterial pathogens also offer several advantages over conventional methods: high sensitivity and specificity, speed, ease of standardization and automation. There are also quantitative analytical tools for studying specific pathogens, including new emergent strains and indicators [15]. Molecular approaches have become popular and efficient methods for characterizing and tracking changes in the community structures of microbial populations [16]. With the introduction and implementation of molecular techniques in clinical microbiological diagnostic laboratories, the identification of bacteria present in fecal samples has increased dramatically [17], [18]. During recent years, the polymerase chain reaction (PCR) technique has allowed rapid and reliable diagnosis of microbial infections due to its unique specificity and sensitivity [19]. Previous studies have subjected 16S-ribosomal DNA (rDNA) amplicons derived from water to PCR and denaturing gradient gel electrophoresis (DGGE) to identify the bacterial diversity [20], [21]. It is therefore expected that this approach will contribute to the understanding of the genetic diversity of complex microbial populations. Also, extracting total DNA from bacteria in drinking water is more rapid and accurate than traditional separation and identification methods, and can reflect the diversity of bacteria in drinking water more directly [21], [22].

The use of fresh water storage tanks is common in rural areas of arid region with limited or diminishing water resources. Homeowners living in remote areas with low-yield and seasonally dry wells or no wells must depend on water that is tracked in with tankers and stored on site to cover basic living necessities. Due to the scarcity of water in semi-arid regions, the idea of storing some rainwater in proper water systems at home in rural areas has been suggested. During rainy seasons, rain water can serve as a good source of water for domestic purpose if properly stored [23]. Several factors affect the microbial flora of stored waters. These are sedimentation; activities of other organisms, light ray, temperature, and food supply [24].

Water related disease is a common problem in developing countries, mainly in rural communities that consume rainwater without a proper microbiological quality. Poor rural communities in Morocco, like those in other developing countries that do not have access to piped water, have mainly been reliant on others water resources harvesting systems as part of low cost strategies for improving water supply and sanitation. Typically, rainwater and surface water is collected and stored in traditional reservoirs and then conserved for drinking and cooking in the dry season. It's the case of ASSIF EL MAL valley; our study site that has the characteristics mentioned previously.

Global studies have identified and analyzed the pathogenic bacteria of water that cause diarrhea, but there have been few studies in Morocco, and any of them are used a molecular technique to identified this bacteria and to evaluate their potential risk for human. Until now, our study site, the valley of Assif El Mal, had not been an area of any work characterizing the epidemiological aspect of drinking water. It was therefore interesting to undertake a study in this direction in order to identify some data on the risk of waterborne infection in humans.

The aim of this study is to evaluate the bacterial contamination of drinking water reservoirs in a rural area. This is done by studying the 16S-rDNA using Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique.

## 2 MATERIALS & METHODS

### 2.1 STUDY AREA

The basin of Assif El Mal is located in the north side of the High Atlas to a hundred kilometers southwest of Marrakech. In the valley Assif El Mal, the population living in the plains suffers from drinking water shortage. In terms of hygiene conditions, these local populations don't have a sewer system, and none had garbage collection. The poor socioeconomic status of the locals does not enable them to dig wells. As a consequence, they are using an archaic method as the only source of water for any kind of use (consumption, watering of livestock ...etc), water is stored in a kind of traditional cisterns buried in the ground, called "*Matfya*" with no prior treatment. They are supplied by river and / or rain water through channels called "*Seguia*". (Fig.1)

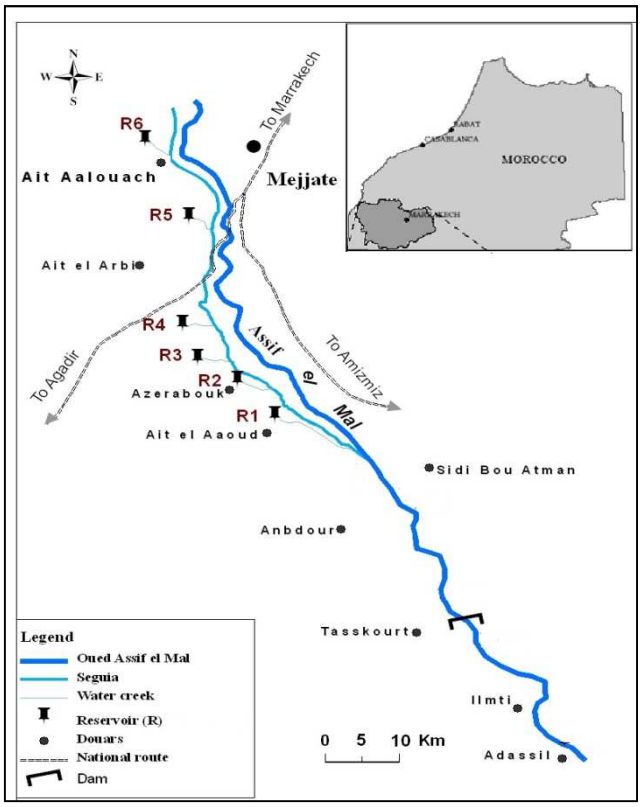


***Fig.1.The valley Assif El Mal; Map of situation and location of sampling sites (R: reservoir of sampling).***

### 2.2 WATER SAMPLES AND DNA EXTRACTION

Samples were collected from six storage reservoirs in Assif El Mal and well water from a control site (Fig.1). Water samples were collected in sterile 1-liter containers from surface waters and were stored on ice during transport to the lab. Upon return to the lab, we filtered water samples through 0.2-mm-pore-size Supor-200 filters. The membranes were immediately transferred into 15-ml screw cap tubes containing 10-ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA [pH 7.6]). The tubes were stirred in a vortex vigorously for 8–10 min to detach the bacteria from the membranes followed by centrifugation at 15,000 rpm for 30 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 2 ml of sterile distilled water. DNA was extracted using a special kit of extraction (Ultraclean$^{TM}$ Soil DNA isolation kit, MoBio Laboratories Inc., EE.UU), following the instructions of the manufacturer. DNA was finally suspended in TE buffer and stored at −20◦C.

### 2.3 PCR AMPLIFICATION OF TOTAL EXTRACTED DNA

For PCR purposes, the DNA concentration was measured by (Nano DropR ND- 1000 spectrophotometer, NanoDrop Technologies Inc.) and adjusted to a concentration of 10ng/µl.

The variable region V3 to V5 of the 16S rDNA was amplified using the universal primers 341F-GC and 907R as shown in table1. This set of primers was designed to be specific for most bacteria [25].The fecal microbiota is host-specific and relatively stable over time within each individual when onlyuniversal primers are used in 16S rRNA gene-DGGE population fingerprinting [26], [27]. PCRs were performed in a Thermal Cycler (TECHNE TC-5000) with 50 µL reaction mixtures. Each mixture was containing 3 mM MgCl2, 200 µM of each nucleotide, 1× PCR buffer with $(NH^4)_2SO_4$, 5% dimethylsulfoxide, 15 pmol of each primer, 1 U of Taq DNA polymerase, and 50–200 ng template DNA. The PCR program had an initial denaturation step at 94◦C for 5 min, followed by 30 cycles of 30 s at 92◦C, 30 s at 55◦C, and 30 s at 72◦C and a final extension step at 72◦C for 30 min. Negative control reactions without template DNA were performed simultaneously. The quality of the resulting PCR amplicons was confirmed by electrophoresis in 1.5% agarose gels using a molecular weight marker (GeneRuler™ 1 kb DNA ladder), after staining with ethidium bromide and visualization on a UV trans-illuminator.

***Table1. Oligonucleotide primers used for PCR.***

| Primer | Primer sequence (5' → 3') |
|---|---|
| **341F** | 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3' |
| **907R** | 5'-CCG TCAATT CCT TTG AGT TT-3' |

*F, forward; R, reverse; *GC clamp, CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.*

### 2.4 DENATURING GRADIENT GEL ELECTROPHOREIS (DGGE) ANALYZING

After optimization of experiments, PCR products were analyzed through DGGE, using a 45–65% denaturing gradient (100% denaturing gradient is 7 M urea and 40% deionized formamide) in 1 mm vertical polyacrylamide gels (8% (wt/vol) acrylamide in 0.5× TAE buffer). Electrophoresis was performed in a DCode™ universal mutation detection system (Bio-Rad ) using 0.5× TAE buffer containing 20 mM Tris, 10 mM acetic acid, and 0.5 mM EDTA (pH 8.0) during 16 h at 75 V, with an initial step at 120 V for 15 min. The gel was then stained for 5 min in an ethidiumbromide solution (5%) and then gently distained with agitation in distilled water for 15 min before image digitalization in a Molecular Imager FX™ system (Bio-Rad). The most intense bands from DGGE profiles were aseptically excised from the gel into 1.5 mL Eppendorf tubes and washed in 10 µL of sterile milli-Q-purified water, from which 5 µL of the eluted DNA was used for PCR amplification with the original primer pair. The isolation and identition of each DNA band was confirmed through DGGE, and if necessary, the extraction procedure was repeated until the targeted band was clearly isolated.

### 2.5 SEQUENCING AND NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequence in the bands of DGGE were excised and 20 µl sterile water was added, and kept at −80°C for 30 min and then transferred to room temperature. Freeze and dissolution was repeated three times. PCR was performed using 3µl mixtures of extracted DNA solutions as templates with the primers of 341f and 907r (Table 1) under the same PCR conditions described above and then sequenced.

The sequences determined were deposited in the GenBank database. A BLAST search (http://www.ncbi.nlm.nih.gov) was used to explore similarity against sequences deposited in the GenBank database. Most similar type species with 97% similarity <3% diversity) to the isolates sequences were designated as the same species.

### 2.6 STATISTICAL ANALYSIS OF DGGE FINGERPRINTS

The general biodiversity of the Shannon index (H9) and the concentration of the dominance or Simpson index (D or S) were calculated based on the number of bands and their relative intensities among the individual samples. A commonly adopted summary statistic is the Shannon-Weiner index: $H' = -\sum pi \ln pi$, where pi is the frequency of the ith species.

For DGGE data, the presence or absence of co-migration points was converted to a binary matrix (0/1), taking into account each band present in at least one sample as a single descriptor. The DGGE profiles were analyzed using the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories, Hercules, CA, USA). The bands with a relative intensity less than 0.5% in each lane were not considered for statistical analyses.

A dendrogram was created with the similarities calculated using the Pearson correlation coefficient (95% probability).

## 3 RESULTS AND DISCUSSION

### 3.1 BACTERIAL DIVERSITY OF WATER

It emerges from this study a bacterial contamination of stored water in traditional reservoirs in this valley compared to the control site.

#### 3.1.1 ANALYSIS OF DGGE PROFILES

Bacterial diversity and community structure was studied using DGGE of PCR-amplified 16S rRNA fragments of 7 samples (Fig.2).With exception of the control site that devoid of any bands. It is shown that reservoirs R1 to R6 all contained bands 4, 5 and 8, indicating that the predominant bacteria were the same in the water samples from the same area. At the same time, for other bands there were discrepancies between different site water samples from the same area. Each site had its special bands, for example, band 1 only existed in R5; band 2, 6, 7 and 10 exists in R4 and R6. It seems that R4 and R6 have more than five or six common bands indicating the great similarity in bacterial diversity and the high contamination level of both reservoirs. This results was confirmed by the highest microbial diversity (H'=3.17, P<0.05) and the lowest dominance (S=0.11, P<0.05) that were observed in the station R4 and R6. In the opposition, the band numbers and H' index of the tree upstream reservoirs (R1, R2 and R3) (H'=1.59, P<0.05) were significantly lower (Table 2).

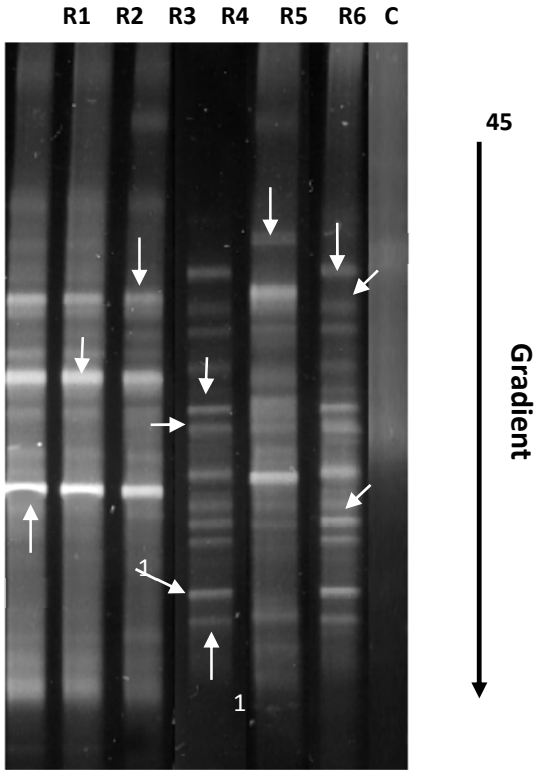


*Fig. 2. DGGE patterns from drinking water study (R: water reservoirs, C: control well).*

*Table 2. Dominance Index (S) and Biodiversity Index (H') calculated from DGGE pattern for total bacterial community for the studied sites (C: control; R: reservoir).*

| | C | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|---|
| **Dominance (S )** | 0 | 0,33 | 0,33 | 0,33 | 0,11 | 0,25 | 0,11 |
| **Shanon (H')** | 0 | 1,59 | 1,59 | 1,59 | 3,17 | 2 | 3,17 |

Otherwise, the reservoirs that had higher microbial diversity (H'=3.17) were associated with lower concentration of dominance (S=0.11). Conversely, reservoirs exhibited low diversity indexes (H'=1.59) associated with high concentration of dominance (S =0.33) (an increase in S indicates a decrease in diversity).

The Bacterial communities in the studied water resources, analyzed by DGGE band patterns were clearly different and separated into distinct clusters, who's a clear gradient upstream-downstream of water storage reservoirs are showed (spatial gradient of the station R1 to R6); with a remarkable reconciliation between the R4 and R6 sites (Fig.3). Thus, we can say that microbial pollution has increased mainly in the R4 reservoir situated in the center of the agglomeration and R6 located downstream of the largest city in the valley.

This indicated that there is an accumulative effect of pollution from upstream-downstream and also at the proximity of most important human agglomeration (R4 & R6) that influence the bacteria density.

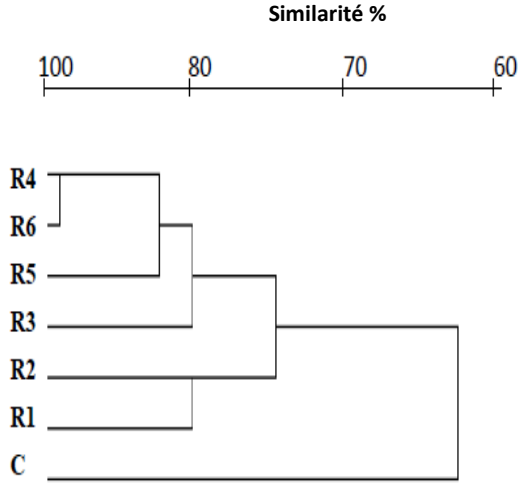


*Fig. 3 : Dendrogram tree of DGGE bands (water DGGE profile).*

### 3.1.2 SEQUENCING RESULTS ANALYSIS OF DOMINANT BAND

We sequenced a total of 26 bands from the RNA based community fingerprints for the assessment of the taxonomic composition of the reservoirs drinking water communities. Just eleven different DGGE bands were detected across all samples that are subsequently excised and sequenced using a limit of 98% sequence similarity and phylogenetic uniqueness as discrimination criteria. The sequences corresponding to each band are shown in table 3.

DNA-based fingerprints revealed several members of taxonomic groups typical for freshwater according to [28] and [29], such as Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes, and particular freshwater member of the Firmicutes (Table 3). These groups were represented by a pathogen's bacteria like as Salmonella sp., Sphingomonas spp. and Aeromonas sp in some sites; wish some of them may be harmful to human beings.

Many previous studies showed the pathogenic power of these bacteria. In facts; *Sphingomonas spp*. is widely distributed in nature and are resistant to many disinfecting and toxic chemicals [30]. The genus includes two potentially pathogenic species, *Sphingomonas paucimobilis* and *S. parapaucimobilis* [31].

Indeed, Salmonella is the most frequent agents of bacterial gastroenteritis [32]. *Salmonella* is isolated from water in lower numbers than indicator bacteria such as *faecal coliforms*, *faecal streptococci* and *enterococci*, which are several orders of magnitude higher [33]. However, low numbers of Salmonella in water may pose a public health risk [34]. A survey of recent studies showed an increasing interest over the last two years on the role of non-host habitats, such as surface water environments as natural reservoir in the transmission of *Salmonella* and other enteric pathogens [35],[36],[37],[38].

*Aeromonas spp*. have also been found in drinking water [39], it can be considerated as a sole enteropathogen responsible for diarrhea [40]. Furthermore, gastrointestinal infections of *Aeromonas* species are generally considered waterborne. Some strains of *Aeromonas* isolated from water have been shown to possess virulence traits, such as adhesions, hemolysins, and cytotonic enterotoxins, presumably involved with human pathogenicity [41], [42].

***Table 3. Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands of the water DGGE profile.***

| Bande | Nearest species | Taxonomic group |
|---|---|---|
| **B1** | Uncultured bacterium AB174868 | uncultured bacterium |
| **B2** | *alpha proteobacterium clone LiUU-14-208* | AlphaProteobacteria (Gammaproteobacteria) |
| **B3** | *Escherichia sp. TX3* | Proteobacteria ›(Gammaproteobacteria) |
| **B4** | *Uncultured Bacteroidetes bacterium clone LiUU-9-210* | Bacteroidetes |
| **B5** | *Sphingomonas sp KIN163* | Alphaproteobacteria |
| **B6** | *Aeromonas sp. MM.2.5* | Proteobacteria (Gammaproteobacteria) |
| **B7** | Uncultured lake bacterium S10.17 | uncultured bacterium |
| **B8** | freshwater seep clone IS-83 GQ339169 | uncultured bacterium |
| **B9** | *Salmonella* sp. 'group B' (EU073022.1) | Proteobacteria (Gammaproteobacteria) |
| **B10** | *Aeromonas sp. 6A_18* | Proteobacteria (Gammaproteobacteria ) |
| **B11** | *Bacillus sp. OS-ac-18* | Firmicutes ( Bacillales) |

### 3.2 CORRELATION APPROACH

In the moving window about cluster analysis, it is apparent that obtained cluster attempts to differentiate between cohorts that contain large differences within the cohort itself. Cluster analysis, based on band patterns, showed a clear gradient upstream-downstream in water storage reservoirs (Fig. 3). So, this spatial gradient of the station R1 to R6, resulting from an accumulation of microbiological contaminants from upstream to downstream in water.

The proportions of the bacterial genera incidence were predominantly higher in the reservoirs R4 and R6. These two sets of samples clustered very tightly together in the dendrograms (Fig.3), with a similarity of more than 60% among each other. The individualization of these two stations (R4 and R6) is related to their exposition to the intensive local pollution sources [43].

It is obvious that the obtained results, using the molecular approach, in this study give a very helpful tool to confirm without any doubt the bacterial contamination of water .This study can be viewed as a model approach for (1) applying molecular techniques to be used as routine monitoring methods for many emerging pathogens. With such information, species of the band and their phylogenic state could be known. Even though that the bands obtained by 16SrDNA-PCR amplification are short and cannot be used to distinguish the exact taxonomic groups, they are useful to understand the distribution of bacterial population.

Longer 16SrDNA fragments can be obtained by improving the primers used in PCR and can be used to decide the taxonomic species effectively, and to study the structure of bacteria in drinking water. At the same time, conditions of un-culturable bacteria can be acquired, and the real environmental situation of water can be reflected. Such information is important for water supply enterprises to ensure the quality of drinking water.

In short, extracting total DNA from bacteria in drinking water, is more rapid and accurate than traditional separation and identification methods, and can reflect the diversity of bacteria in drinking water more directly. Other word, the use of the molecular approach is also a useful tool for very accurate and precise epidemiological studies. It could be seen as a preventive evaluation of the sanitary risk, for vulnerable population, before causing the diarrhea or other borne diseases.

## 4 CONCLUSION

In conclusion, the results of this study demonstrate that bacterial contamination is a common occurrence in traditionally water reservoirs used by rural communities in ASSIF EL MAL valley.

The application of Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique to samples from water reservoirs that are used as a drinking water sources by the surrounding population showed an accumulation of bacterial contaminants from upstream to downstream in water reservoirs especially in R4 and R6. This is due mainly to the increasing degree of the pollution impact caused from the activity of neighboring populations along the study area.

The DGGE banding profiles analysis, using the biodiversity index (H') that combines the relative abundance of species and the total species richness, showed that the higher number of different species are involved in the higher polluted reservoirs (R4 and R6). In other side, the DNA-based fingerprints analysis revealed that the studied water resources were contaminated with several pathogens, especially *E. coli*, *Salmonella* and *Aeromonas*, that may be harmful to the consumer people.

It is apparent that the obtained results using the molecular approach in this study give a very helpful tool to confirm without any doubt the bacterial contamination of water.

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