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Changes of the bacterial assemblages throughout an urban drinking water distribution system

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Abstract We analyzed the bacterial 16S rRNA gene diversity throughout the major components of the drinking water distribution system of a ca. 52,000-inhabitants city (Trikala City, Greece) in order to describe the changes of the bacterial assemblages and to detect possible bacterial pathogens which are not included in the standard monitoring process. Bacterial DAPI counts and DNA extraction was performed in the water pumping wells, the water treatment tank and tap water from households. Approximately 920 bp of the bacterial 16S rDNA were PCR-amplified, cloned, and sequenced for a total of 191 clones, which belonged to 112 unique phylotypes. The water of the pumping wells harbored a typical subsurface bacterial assemblage, with no human pathogens, dominated by β-Proteobacteria. Cell abundance in the water treatment tank decreased significantly, close to detection limit, but bacterial diversity remained high. However, the dominance of β-Proteobacteria decreased considerably, indicating the sensitivity of this group to drinking water disinfection treatment. Tap water from the households hosted a much less diverse, low-cell bacterial assemblage, dominated by *Mycobacterium*-like phylotypes, related to biofilm bacterial communities.

Keywords Bacteria · 16S rRNA · Diversity · Drinking water

Introduction

The provision of safe drinking water is considered a top priority issue in any civilized society because safe drinking water is a basic need to human development, health, and well-being. Microbiologically and chemically contaminated drinking water has been linked with several health problems, e.g. cholera and typhoid fever are diseases associated with microbiologically contaminated drinking water (Hurst et al. 2002; WHO 2004). Several of the microbiological problems that render fresh water resources dangerous for human consumption are related with drinking water distribution systems (DWDS) after the disinfection stage, including biofilm growth, nitrification, microbially mediated corrosion, and the persistence of pathogens (Berry et al. 2006). Besides the health

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aspect of contaminated drinking water, aesthetically unacceptable drinking water undermines the confidence of consumers, thus, affecting the efficiency of water utility companies' practices.

Monitoring and regulation of the microbiological parameters relevant to public health in drinking water installations relies primarily on culture-based analyses that specifically target specific indicator organisms and most of these analyses are based on methodologies developed in the early 1900s. In addition, these methodologies do not include the investigation of neither the possible threats from pathogenic bacteria which form biofilms in the pipes of the DWDS nor the disinfectant-resistant microorganisms. Maintaining a disinfectant (usually chlorine) residual is the usual practice to microbiological control in DWDS. However, it is often ineffective at controlling microbial growth (LeChevallier et al. 1996). On the other hand, little is known on the in situ growth of DWDS dwellers and the broader microbial ecology (e.g. competition with other species, biofilm formation) of DWDS. Such information can prove valuable in controlling microbiological risks in these engineered systems. Although the standard, cultured-based procedures for the microbiological risks of drinking water are considered well accepted by governmental authorities and the private sector, several studies based on sequence of genes related to phylogeny reveal that standard enrichment techniques significantly underestimate the actual abundance and diversity of microorganisms in a wide variety of environments (Pace 1997). In addition, some waterborne pathogens (e.g., *Vibrio cholera* and *Legionella pneumophila*) are documented to remain viable for extended periods of time, but are unrecoverable by otherwise successful culture protocols (Colwell et al. 1985; Hussong et al. 1987).

Another important benefit from the application of molecular approaches is the effective and fast detection of scarce, waterborne pathogens—simultaneously with the non-pathogenic species—that are able to persist and reproduce in the DWDS and, eventually, can cause infections to humans (Szewzyk et al. 2000). For example, the DWDS pathogens comprising the *Mycobacterium avium* complex are the most common bacterial infection in AIDS patients (Aronson et al. 1999).

Finally, drinking water disinfection is not sterilization and, thus, viruses and some pathogenic bacteria are not destroyed by chlorination, although we need to know which are these microorganisms. Although the advent of molecular approaches in the last 20 years, with which the identification on microorganisms is feasible down to the species level even from samples with very low abundance, has contributed dramatically to overcome such limitations (Stackebrandt 2006), such practices are still not very frequent in the field of drinking water quality monitoring (Call 2005; Berry et al. 2006).

The aim of this study was to analyze the 16S rRNA gene diversity of Bacteria, as a supplementary tool to standard practices of microbiological quality in the whole DWDS of the Trikala City, Greece. We aimed at (a) the detection of possible microbiological risks that are not included in the standard monitoring process and (b) the investigation of the changes of the bacterial cell numbers and community structure from the water pumping wells to the water disinfection tank and from there to representative households in order to monitor possible persistent pathogens throughout the DWDS.

Materials and methods

Water samples were collected from the pumping wells, the water collection and treatment tank and from two households in Trikala City, central Greece, on 29 January 2007. This DWDS serves ca. 52,000 inhabitants. For the purpose of our study, representative water samples of the three major parts of the Trikala City DWDS were investigated (information provided by permission of the Trikala Municipality Water Utility Company, TMWUC): (1) there are 13 drilling wells located inside and outside the city, with drilling depth 107–205 m. We analyzed a pooled sample (TA) of the pumped water from all the wells, consisting of 2 l from each well, after discarding at least 5 l. (2) There are seven water collection tanks with a total capacity of 9,600 m^3 of water from the pumping wells. Disinfection takes place in the last one before the water is supplied to the supply pipes. Water disinfection is achieved by chlorine.

Residual chlorine is always <0.2 mg l^{-1}. From the treatment tank one sample of 20 l was collected ca. 1 m below surface. (3) The water supply distribution system brings the disinfected water to the end users and it consists of plastic (PVC), amiantus, and iron pipes. The two individual randomly chosen household samples (8 l each) were also pooled and sampling was performed after sterilizing the water tap with ethanol 70% and after discarding at least 3–5 l.

The water samples were transferred to the laboratory in less than 8 h after sampling, under cool conditions and in darkness. All three samples were transferred in clean, pre-sterilized with ethanol 70% polyethylene 30 l carboys. In all sampling sites temperature, conductivity, pH, and total dissolved salts (TDS) were measured in situ by automatic probes (Combo HI 98129, HANNA) (Table 1).

To enumerate bacterial cells, 10–15 ml of water samples from each of the carboys were preserved with formaldehyde at a final concentration of 2%. Samples were kept at 4°C in the dark, filtered on black Nuclepore filters (pore size of 0.2 μm), and stained with DAPI (Porter and Feig 1980) within a few hours of sampling and stored at −20°C until counting on an Axiostar (Zeiss) epifluorescence microscope at ×1,000.

Upon retrieval to the laboratory, 8 l from each of the three carboys were filtered on a 0.2-μm pore size polycarbonate filters of 47 mm diameter (Millipore, USA) under low vacuum (≤150 mm Hg) and the filters were stored at −80°C. DNA was extracted using the UltraClean Soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's protocol after slicing the filters with a sterile scalpel. Bacterial 16S rDNA was amplified using the bacterial primers BAC-8F (5′-AGAGTTTGATCCTGGC TCAG-3′) and BAC-907R (5′-CCCGTCAATT CCTTTGAGTTT-3′) (Lane 1991). PCR included an initial denaturation step at 94°C for 3 min was followed by 40 cycles consisting of denaturation at 95°C for 45 sec, annealing at 52.5°C for 45 sec and elongation at 72°C for 1 min and 30 sec; a final 10 min elongation step at 72°C was added. Each PCR from the three samples was repeated with different cycle numbers (between 16 and 38). The lowest number of cycles that gave a positive signal was then used (28 cycles for TA and 36 for TB and TC samples) for cloning and sequencing in order to eliminate PCR innate limitations (Spiegelman et al. 2005) and to avoid differential representation of 16S rDNA genes with low and high copy numbers.

PCR products from the minimum number of cycles were visualized on a 1.2% agarose gel under UV light, purified with the Montage Purification Kit (Millipore, USA). The purified PCR products were cloned using the TOPO XL PCR

Table 1 Physical and chemical characteristics of the sampling points in the Trikala City, Greece, drinking water system, January 2007

Sample	Temperature (°C)	pH	E_c(μS cm^{-1})	TDS (ppm)	Water flow (m^3h^{-1})
TA1	19.1	7.9	326	196	102
TA2	19.5	7.8	350	208	96
TA3	19.0	7.7	399	235	264
TA4	19.7	7.7	399	233	264
TA5	18.6	7.8	391	214	156
TA6	20.3	7.7	401	224	144
TA7	19.1	7.9	414	235	192
TA8	20.3	7.9	431	265	72
TA9	17.2	7.5	575	331	–[a]
TA10	18.3	7.8	397	224	216
TA11	17.6	7.7	419	236	84
TA12	16.9	7.7	414	237	156
TA13	23.0	8.0	307	177	44
TB	22.0	7.6	478	233	–
TC1	22.1	7.8	395	230	–
TC2	22.5	7.8	401	232	–

TA pumping well, *TB* water treatment tank, *TC* household, *TDS* total dissolved salts
[a]Occasional usage

cloning kit (Invitrogen, USA) using chemically competent cells according to the manufacturer's specifications. For each clone library, ca. 30–60 clones were grown in liquid LB medium with kanamycin and their plasmids were purified using the Nucleospin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co. KG, Germany) for DNA sequencing.

Sequence data were obtained by capillary electrophoresis (Macrogen Inc., Korea) using the BigDye Terminator kit (Applied Biosystems Inc., USA) with the primers M13F (5′-GTAAAA CGACGGCCAG-3′) and M13R (5′-CAGGAAA CAGCTATGAC-3′). Each sequence read was approximately 850 bp. For each individual clone, forward and reverse reads were assembled, and then the assembled sequences were checked for chimeras using the CHIMERA-CHECK function of the Ribosomal Database Project II (Maidak et al. 2001). All sequences were compared with the BLAST function (http://www.ncbi.nlm.nih.gov/BLAST/) for the detection of closest relatives. Sequence data were compiled using the MEGA4 software (Tamura et al. 2007) and aligned with sequences obtained from the GenBank (www.(ncbi.nlm.nih.gov)) databases, using the ClustalX aligning utility. Phylogenetic analyses were performed using minimum evolution and parsimony methods implemented in MEGA4 (Tamura et al. 2007). Heuristic searches under minimum evolution criteria used 1,000 random-addition replicates per data set, each followed by tree bisection-reconnection topological rearrangements. The topology of the tree was based on neighbor-joining according to Jukes-Cantor. Bootstrapping under parsimony criteria was performed with 1,000 replicates. Sequences of unique phylotypes found in this study have GenBank accession numbers EU746687–EU746738, EU746739–EU746775, and EU746776–EU746798 for the pumping wells (TA3), treatment tank (TB3), and households (TC3), respectively.

Library clone coverage was calculated by the formula $[1 - (n_i/N)]$ (Good 1953), where n_i is the number of operational taxonomic units (OTU) represented by only one clone and N is the total number of the clones examined in each library.

Results

Abiotic parameters and bacterial abundance Temperature in the pumping wells (TA) varied between 16.9 and 23.0°C (coefficient of variation, CV = 8.3%) while in the water treatment tank (TB) and the households (TC) varied between 22.0 and 22.5°C (Table 1). pH showed low variation between 7.5 and 8.0 (CV = 1.7%) in all samples (Table 1). Conductivity ranged between 307 and 575 μS cm^{-1} (CV = 15.9% in TA) (Table 1). Total dissolved salts varied between 177 and 331 (CV = 15.9% in TA), while in TB and TC TDS concentration was very similar (230–233 ppm) (Table 1). No statistically significant correlations were found between temperature, pH, E_c, TDS of the pumping wells and pumping depth. Bacterial abundance was 5,714 ± 187, 400 ± 98, and 532 cells ml^{-1} in TA, TB, and TC, respectively (Fig. 1). There were no seasonal differences in the bacterial cell counts for each of the samples.

Bacterial diversity In total, 191 16S rDNA clones were analysed, excluding chimeric sequences (Table S1). Of these sequences, 52, 37, and 23 unique phylotypes belonged to TA, TB, and TC, respectively. Clone coverage analysis curves based on Good's C estimator (Good 1953; Kemp and Aller 2004), suggests that the full extent of the existing diversity of Bacteria was not achieved

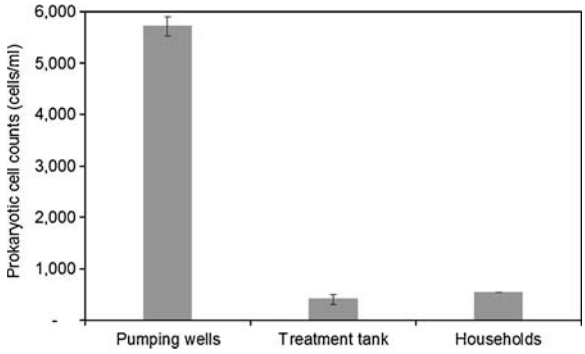


Fig. 1 Bacterial cell counts in the drinking water distribution system of the Trikala City, Greece, January 2007. *Error bars* standard deviation of three to five replicates for the pumping wells (13 pooled samples), treatment tank, and households (two pooled samples)

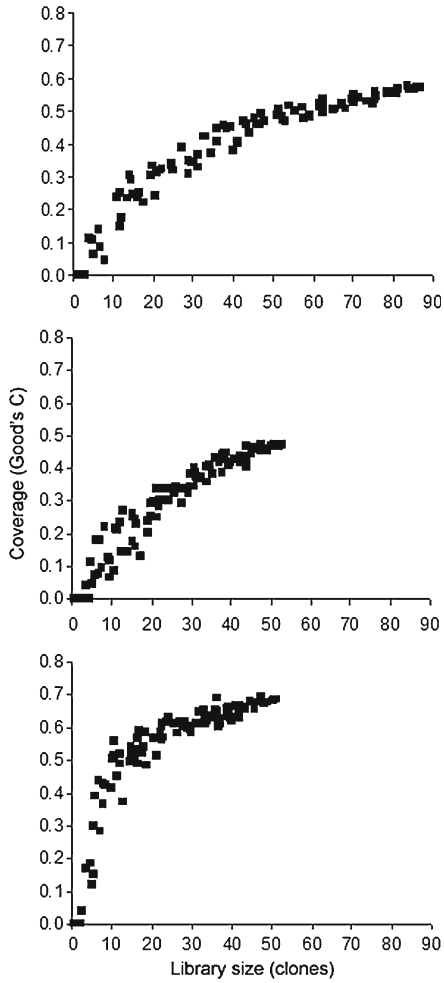


Fig. 2 Clone library coverage based on Good's C estimator of the bacterial clone libraries from the water pumping wells (TA), the water collection and treatment tank (TB), and households water (TC) of the drinking water distribution system of the Trikala City, Greece, January 2007

(Fig. 2). Based on the S_{Chao1} estimator, 43.6%, 35.0%, and 37.7% of the estimated phylotypes were revealed for TA, TB, and TC, respectively.

In TA, proteobacterial phylotypes dominated (71.3% of the total number of phylotypes and 65.4% of the total number of unique phylotypes) (Fig. 3). In particular, the β-Proteobacteria dominated both in abundance (47.1%) and number of unique phylotypes (44.2%). These phylotypes were closely related (97–100%) to uncultivated Bacteria from freshwater surfaces (sediments, biofilms) and the terrestrial subsurface (aquifers, springs). The affinity with known relatives was lower (91–99%) and included *Ralstonia solanacearum*, *Siderooxidans lithoautotrophicus*, *Thiobacillus aquaesulis*, and *Hydrogenophaga* spp.

In TB, the dominance of proteobacterial phylotypes was retained, both in abundance (73.6%) and in number of unique phylotypes (75.7%) (Fig. 4). However, the contribution of the β-Proteobacteria was reduced (18.9% of the total abundance and 24.3% of the unique phylotypes), while the δ-Proteobacteria and γ-Proteobacteria increased (22.6% and 15.1% of the total abundance, respectively; 21.6% and 16.2% of the unique phylotypes, respectively). The most abundant phylotypes in TB were closely related to any known Bacteria, except TB3–11 which was closely (99%) related to *Sphingomonas* sp. These phylotypes were related (78–99%) to phylotypes originating from aquatic sediments, human skin, and DWDS. Other abundant phylotypes belonged to the Planctomycetes (TB3–50), Chloroflexi (TB3–21), and were distantly related (80%) to phylotypes from soil and, more closely (96%), to brackish sediment.

In TC, the proteobacterial dominance dropped significantly (23.5% of the total abundance and 25.8% of the unique phylotypes) while the Actinobacteria, which did not appear in the TA and TB clone libraries, dominated (52.9% of the total abundance but only 19.4% of the unique phylotypes). All the actinobacterial phylotypes were closely (97–99%) relates to known species the genus *Mycobacterium* (Fig. 5). Other abundant phylotypes belonging to the Proteobacteria and Verrucomicrobia were related (86–99%) to Bacteria from lake and cave ecosystems.

A small number of phylotypes were detected in more than one of the three investigated clone libraries, based on the ≥98% similarity threshold. From the pumping wells, three proteobacterial phylotypes, namely TA3–10, TA3–13, and TA3–68, were found in the treatment tank as TB3–60, TB3–37, and TB3–14, respectively. In addition, the proteobacterial phylotype TA3–59 was found in the treatment tank (as TB3–17) and the households sample, as well, as TC3–22. Finally, the proteobacterial phylotype TA3–69 recurred

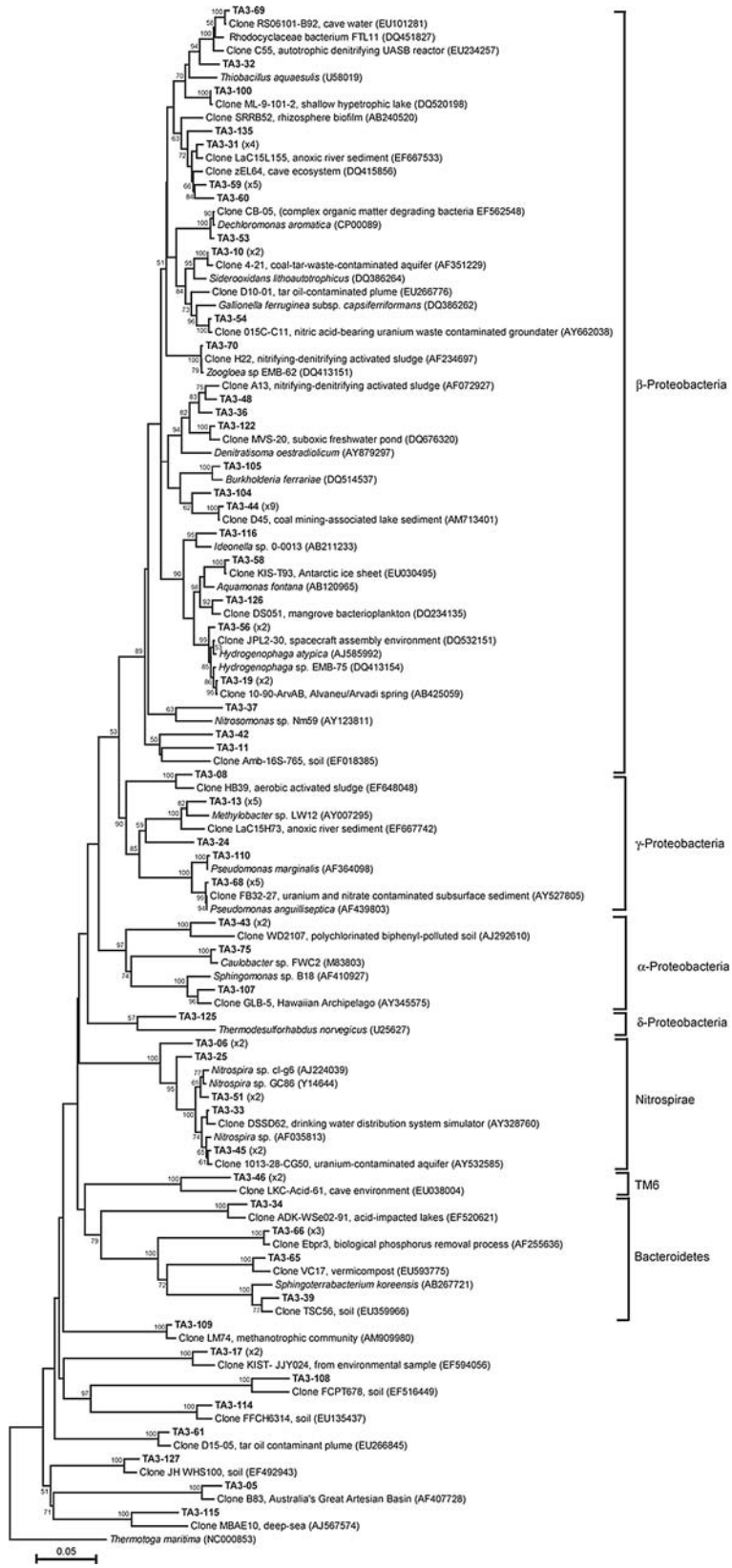


Fig. 3 Phylogenetic tree of relationships of 16S rDNA (ca. 920 bp) of the representative bacterial clones found in the water pumping wells, based on the neighbor-joining method as determined by distance Jukes–Cantor analysis. One thousand bootstrap analyses (distance) were conducted and percentages greater than 50% are indicated at the nodes. The tree was rooted by using *Thermotoga maritima* as an outgroup. Groups (*bold letters*) that have ≥98% similar nucleic acid sequences are represented by a single sequence, with the *number* of clones out of the total in *parentheses*. The *numbers in brackets* are GenBank accession numbers. *Scale bar* represents 5% estimated distance

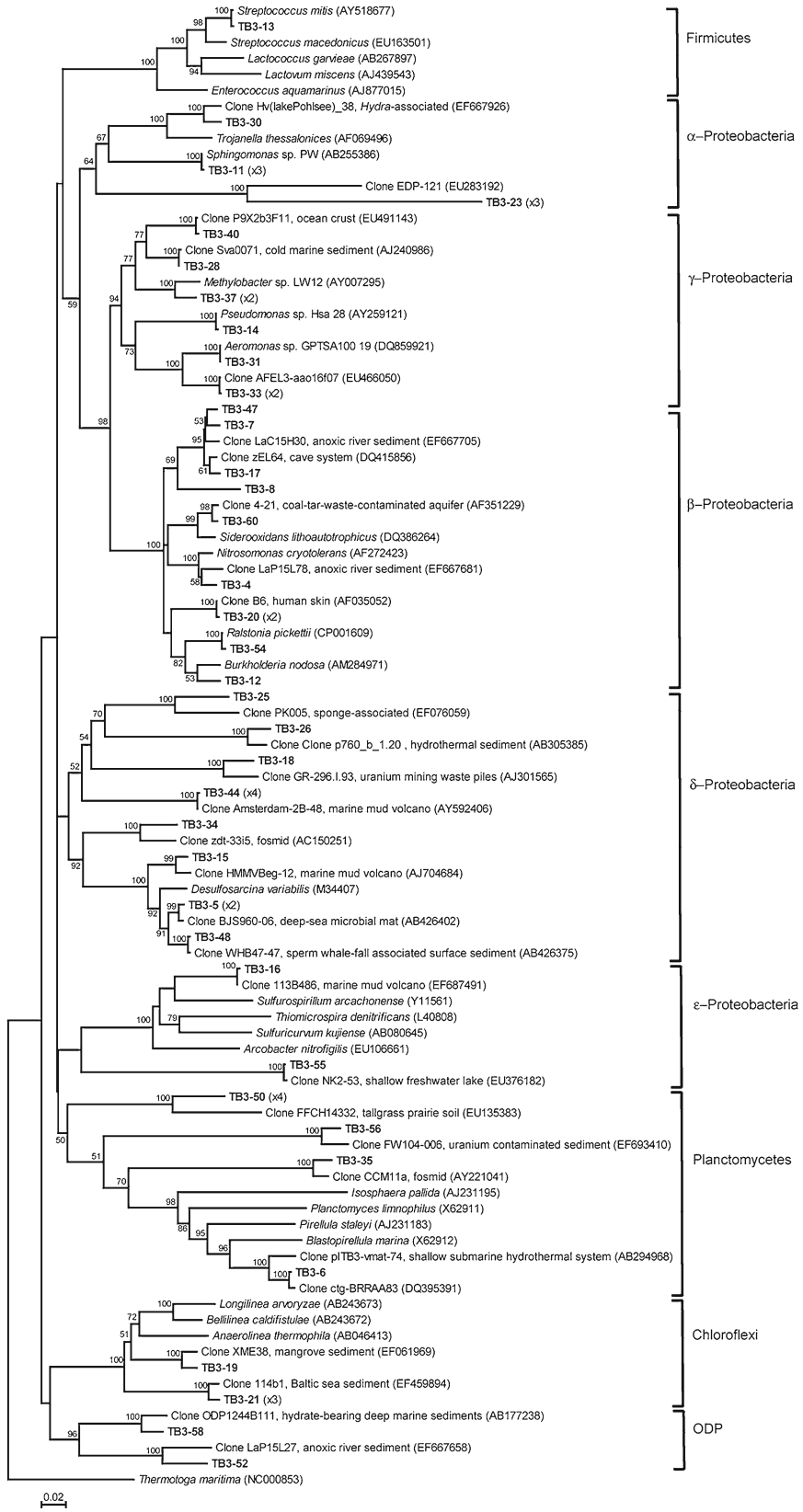
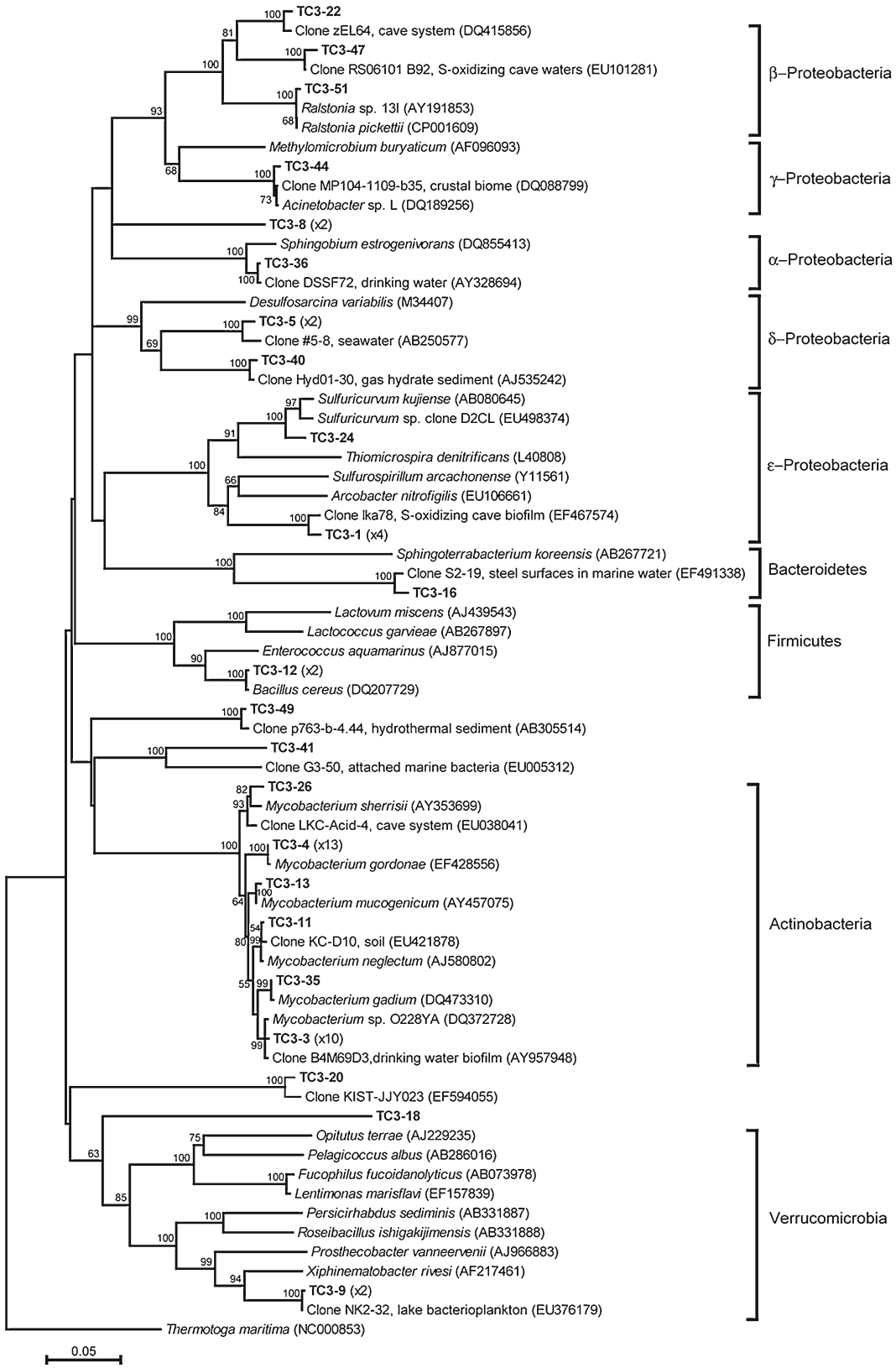


Fig. 4 Phylogenetic tree of relationships of 16S rDNA (ca. 920 bp) of the representative bacterial clones found in the water collection and treatment tank. Tree construction details are as in Fig. 3. *Scale bar* represents 2% estimated distance


TC3-22
Clone zEL64, cave system (DQ415856)
TC3-47
Clone RS06101 B92, S-oxidizing cave waters (EU101281)
TC3-51
Ralstonia sp. 13I (AY191853)
Ralstonia pickettii (CP001609)
β–Proteobacteria
Methylomicrobium buryaticum (AF096093)
TC3-44
Clone MP104-1109-b35, crustal biome (DQ088799)
Acinetobacter sp. L (DQ189256)
γ–Proteobacteria
TC3-8 (x2)
Sphingobium estrogenivorans (DQ855413)
TC3-36
Clone DSSF72, drinking water (AY328694)
α–Proteobacteria
Desulfosarcina variabilis (M34407)
TC3-5 (x2)
Clone #5-8, seawater (AB250577)
TC3-40
Clone Hyd01-30, gas hydrate sediment (AJ535242)
δ–Proteobacteria
Sulfuricurvum kujiense (AB080645)
Sulfuricurvum sp. clone D2CL (EU498374)
TC3-24
Thiomicrospira denitrificans (L40808)
Sulfurospirillum arcachonense (Y11561)
Arcobacter nitrofigilis (EU106661)
Clone lka78, S-oxidizing cave biofilm (EF467574)
TC3-1 (x4)
ε–Proteobacteria
Sphingoterrabacterium koreensis (AB267721)
Clone S2-19, steel surfaces in marine water (EF491338)
TC3-16
Bacteroidetes
Lactovum miscens (AJ439543)
Lactococcus garvieae (AB267897)
Enterococcus aquamarinus (AJ877015)
TC3-12 (x2)
Bacillus cereus (DQ207729)
Firmicutes
TC3-49
Clone p763-b-4.44, hydrothermal sediment (AB305514)
TC3-41
Clone G3-50, attached marine bacteria (EU005312)
TC3-26
Mycobacterium sherrisii (AY353699)
Clone LKC-Acid-4, cave system (EU038041)
TC3-4 (x13)
Mycobacterium gordonae (EF428556)
TC3-13
Mycobacterium mucogenicum (AY457075)
TC3-11
Clone KC-D10, soil (EU421878)
Mycobacterium neglectum (AJ580802)
TC3-35
Mycobacterium gadium (DQ473310)
Mycobacterium sp. O228YA (DQ372728)
TC3-3 (x10)
Clone B4M69D3,drinking water biofilm (AY957948)
Actinobacteria
TC3-20
Clone KIST-JJY023 (EF594055)
TC3-18
Opitutus terrae (AJ229235)
Pelagicoccus albus (AB286016)
Fucophilus fucoidanolyticus (AB073978)
Lentimonas marisflavi (EF157839)
Persicirhabdus sediminis (AB331887)
Roseibacillus ishigakijimensis (AB331888)
Prosthecobacter vanneervenii (AJ966883)
Xiphinematobacter rivesi (AF217461)
TC3-9 (x2)
Clone NK2-32, lake bacterioplankton (EU376179)
Verrucomicrobia
Thermotoga maritima (NC000853)
0.05

◀ **Fig. 5** Phylogenetic tree of relationships of 16S rDNA (ca. 920 bp) of the representative bacterial clones found in the households' water. Tree construction details are as in Fig. 3. *Scale bar* represents 5% estimated distance

in the households sample as TC3–47. From the treatment tank, the *Ralstonia*-like phylotype TB3–54 was retrieved in the households sample as TC3–51, and the ODP-related phylotype TB3–58 resembled >99% with the phylotype TC3–49 of the household sample. Rare phylotypes (those that appeared once or twice in the clone library, Aller and Kemp 2008) consisted 88.5%, 86.5%, and 86.9% in TA, TB, and TC, respectively.

Discussion

We analysed the diversity of the bacterial 16S rRNA gene throughout the DWDS, including the water pumping wells, the water collection and treatment tank and two representative households at Trikala City, central Greece. We applied this approach in order to detect possible pathogenic microorganisms which are not included in the standard monitoring procedures and to reveal in which part of the DWDS such microorganisms occur. For these purposes we focused on: (a) the input sources of water as a whole (i.e. the pumped water) and for this reason we worked with pooled samples of the 13 wells, (b) the water collection and treatment tank in order to detect which microorganisms are more susceptible to disinfection, and (c) the end output of the DWDS (i.e. tap water from two arbitrary households). The household samples were also pooled as there are no differences in the pipe material and the water retention time in the pipes throughout the city (TMWUC, unpublished data).

Based on the S_{Chao1} clone library coverage estimator (Good 1953; Kemp and Aller 2004), our clone coverage was below 50%, making the calculation of any diversity index rather ambiguous. However, the high number of singletons and rare operational taxonomic units (OTU) in our clone libraries (Table S1) suggests that, at least, the most abundant phylotypes have been detected.

The bacterial assemblage of the water pumping wells (Fig. 3) was dominated by β- and α-Proteobacteria as is the case for natural waters used for drinking water purposes (Williams et al. 2004; Loy et al. 2005), but also for the majority of terrestrial subsurface waters (Staley and Reysenbach 2002). No phylotypes closely related to known human pathogens occurred, rendering the pumped water microbiologically suitable for drinking purposes.

Several phylotypes related to *Nitrospira* and *Nitrosomonas* occurred in the pumping wells (and only a few in the treatment tank, as well.) Such Bacteria have been identified in several studies as the dominant nitrifiers in bulk water and biofilms using 16S rRNA gene clone libraries (Regan et al. 2003; Lipponen et al. 2004; Martiny et al. 2005). The nitrification process mediated by these Bacteria could degrade the water drinking quality due to increasing the nitrate concentrations. In our samples, however, no risk from nitrate exists as the average concentrations during a simultaneous study in 2006–2007 varied between 1.8 and 12.4 mg l^{-1} (K. Skordas unpublished data) which are below the USEPA and EU limits of 25–50 mg l^{-1}. In Trikala City, the DWDS disinfection is achieved by chlorine. In the case of using chloramines, nitrification processes can be very important to DWDS management strategies, because they affect the chloramines residual (Berry et al. 2006). This suggests that the ammonia oxidizers that exist in the Trikala DWDS do not affect in this way the effectiveness of the disinfection process.

The number of bacterial cells in the water collection and treatment tank (disinfection) were significantly decreased compared to that of the pumping well, as a result of the disinfection procedure (Fig. 1). The number of OTUs in the tank, although less than the one in the pumping wells, was high, as well. However, there was a considerable reduction of the β-Proteobacteria OTUs (Fig. 4) and this is attributed to the water disinfection. It has been suggested that the β-Proteobacteria are very sensitive to disinfection agents compared to the α-Proteobacteria (Emtiazi et al. 2004; Williams et al. 2004). Our results also indicate that some of the δ- and

γ-Proteobacteria could be tolerant to disinfectant agents, unless they originate from biofilms in the DWDS where exposure to disinfectant can be less (see below). The fact that most of the phylotypes, which occurred in more than one of the three compartments of the DWDS we examined, belonged to the Proteobacteria, supports the concept that this phylum is of central importance in DWDS (Tokajian et al. 2005).

Only very few putative pathogens were found in the water treatment tank. Phylotype TB3–31 was closely related to the genus *Aeromonas*. This is of special interest for public health issues as virulent *Aeromonas* spp. strains have been found in DWDS (Sen and Rodgers 2004). The presence of *Aeromonas* spp. in drinking water supplies is generally considered a nuisance. In fact, despite frequent isolation of *Aeromonas* spp. from drinking water, the body of evidence does not provide significant support for waterborne transmission. Aeromonads typically found in drinking water do not belong to the same DNA homology groups as those associated with cases of gastroenteritis (WHO 2006). Also, phylotype TB3–13 was closely related to a pathogenic strain of *Streptococcus mitis* (Lu et al. 2003). *S. mitis* is an important member of the viridans streptococci and a normal part of the oropharynx, skin, gastrointestinal system, and female genital system flora, is a bacterium with low pathogenicity and virulence although it can cause subacute bacterial endocarditis (Koneman et al. 2004).

A further decreased representation of the Proteobacteria took place in the households water (Fig. 5). On the other hand, Actinobacteria dominated in terms of abundance. In particular, *Mycobacterium*-like OTUs outcompeted others in abundance but not in the number of unique phylotypes. Mycobacteria are frequently found in several aquatic systems, including drinking water DWDS (Covert et al. 1999; Peters et al. 1995; Falkinham et al. 2001; Hilborn et al. 2006; Torvinen et al. 2004; Torvinen et al. 2007). Actinobacteria bear some features that allow them to prevail in environments such as DWDS: their unique morphology (i.e. cell wall), their ability to persist in biofilms and their tolerance in disinfectants (chlorine, ozone) (Le Dantec et al. 2002; Steed and Falkinham 2006; Yamazaki et al. 2006). In the current work, the viability of these *Mycobacterium*-like phylotypes was verified after growth on Löwenstein–Jensen medium Petri dish (Böttger 1991) (data not shown).

Apart from the Actinobacteria's potent physiological traits, their occurrence in the DWDS could be related to increased water flow velocities which at first provide higher transfer of nutrients to the biofilm and at higher flow velocities detach these biofilms (Torvinen et al. 2007). Maybe the most important abiotic factor for the prevalence of mycobacteria in DS is temperature (Torvinen et al. 2007). The prevailing temperature in the studied DWDS (ca. 22°C in the households and 17–23°C in the rest of the DS) favors their growth (Torvinen et al. 2004; Torvinen et al. 2007). Mycobacteria—which are not included in the routine drinking water microbiological analyses—and other biofilm forming microorganisms are potentially dangerous for public health because biofilms are protected from disinfection (Le Dantec et al. 2002; Steed and Falkinham 2006; Yamazaki et al. 2006), and eventually, can be released to the bulk water used for human consumption.

Other biofilm related, but not yet fully clear if they impose any danger to humans, are the Verrucomicrobia, which were found in the households sample. Verrucomicrobia are known to occur in DWDS and are believed to be involved in nitrogen cycling (Martiny et al. 2005).

The use of molecular tools to detect pathogens in drinking water systems offers a much more sensitive detection of pathogens than was previously possible with culture-based methods and it is not site- and organism-specific or pollution-source dependent. The application of such a molecular approach as a standard routine is still laborious and fairly expensive but this is changing fast due to technological advancements (Call 2005; Berry et al. 2006). This methodology could be applied as a monitoring practice by water quality bodies, at least on a seasonal or biannual time scale since having a specific network's phylotype database, the detection of persistent phylotypes or new invasive ones in the distribution system is feasible.

In this paper, using this approach we elucidated that a possible microbiological risk for the whole of the DWDS of Trikala City, Greece, is the occurrence of Mycobacteria-like Bacteria and

not from microorganisms which are monitored through the routine procedures. Also, we showed that the pumped water bears no threatening Bacteria and also that although the water treatment reduces significantly the total number of bacterial cells, the bacterial diversity still remains sizeable, mostly consisting of biofilm-related species, suggesting thus that extra attention in the monitoring procedure should be paid on the piping of the DWDS rather than the pumping wells and the treatment procedure. The knowledge of the ecology of the microbial communities in DWDS is important as recent investigations have found that pathogen resistance to chlorination is affected by the community biodiversity and interspecies relationships (Berry et al. 2006).

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Table S1. Occurring 16S rRNA gene phylotypes (~920 bp) and their relative abundance in the Trikala City drinking water distribution network, January 2007.

	Clone	*No. of similar (≥98%) clones*	*Putative affiliation*	*Closest sequence (similarity %) [GenBank accession No.]*	*Description*	*Closest organism (similarity %) [GenBank accession No.]*
PUMPING WELLS						
1	TA3-44	9	β-Proteobacteria	Clone D45 (99) [AM713401]	Coal mining-associated lake sediment	*Ralstonia solanacearum* (97) [EF585154]
2	TA3-13	6	γ-Proteobacteria	*Methylobacter* sp. LW12 (94) [AY007295]	Freshwater lake sediment	*Methylosarcina lacus* (94) [AY007296]
3	TA3-59	5	β-Proteobacteria	Clone zEL64 (97) [DQ415856]	Limestone-corroding stream biofilms, Frasassi Cave system, Italy	*Thiobacillus aquaesulis* (91) [U58019]
4	TA3-68	5	γ-Proteobacteria	Clone FB32-27 (98) [AY527805]	Uranium and nitrate contaminated subsurface sediment	*Pseudomonas anguilliseptica* (98) [AF439803]
5	TA3-31	4	β-Proteobacteria	Clone LaC15L155 (97) [EF667533]	Anoxic river sediment	*Thiobacillus aquaesulis* (92) [U58019]
6	TA3-66	3	Bacteroidetes	Clone Ebpr3 (98) [AF255636]	Biological phosphorus removal process	*Solibium soli* (94) [EF067860]
7	TA3-06	2	Nitrospirae	*Nitrospira* sp. clone g6 (90) [AJ224039]	Bed reactor	*Nitrospira* sp. GC86 (90) [Y14644]
8	TA3-10	2	β-Proteobacteria	Clone 4-21 (100) [AF351229]	Coal-tar-waste-contaminated aquifer	*Siderooxidans lithoautotrophicus* (96) [DQ386264]

9	TA3-17	2	Unidentified	Clone KIST-JJY024 (96) [EF594056]	Environmental sample	-
10	TA3-19	2	β-Proteobacteria	Clone 10-90-ArvAB (99) [AB425059]	Alvaneu / Arvadi spring	*Hydrogenophaga* sp. EMB 75 (99) [DQ413154]
11	TA3-43	2	α-Proteobacteria	Clone WD2107 (93) [AJ292610]	Polychlorinated biphenyl-polluted soil	*Micavibrio aeruginosavorus* (87) [DQ186612]
12	TA3-45	2	Nitrospirae	Clone 1013-28-CG50 (99) [AY532585]	Uranium-contaminated aquifer	*Nitrospira* sp. (98) [AF035813]
13	TA3-46	2	TM6	Clone LKC-Acid-61 (89) [EU038004]	Cave environment	-
14	TA3-51	2	Nitrospirae	*Nitrospira* sp. GC86 (98) [Y14644]	Nitrite-oxidizing bioreactor	-
15	TA3-56	2	β-Proteobacteria	Clone JPL2-30 (99) [DQ532151]	Spacecraft assembly environments	*Hydrogenophaga atypica* (99) [AJ585992]
16	TA3-05	1	Unidentified	Clone B83 (95) [AF407728]	Australia's Great Artesian Basin	-
17	TA3-08	1	γ-Proteobacteria	Clone HB39 (96) [EF648048]	Aerobic activated sludge	-
18	TA3-11	1	α-Proteobacteria	Clone Amb-16S-765 (88) [EF018385]	Soil	-
19	TA3-24	1	γ-Proteobacteria	Clone LaC15H73 (88) [EF667742]	Anoxic river sediment	-
20	TA3-25	1	Nitrospirae	*Nitrospira* sp. GC86 (96) [Y14644]	Nitrite-oxidizing bioreactor	-

21	TA3-32	1	β-Proteobacteria	Clone C55 (95) [EU234257]	Autotrophic denitrifying UASB reactor	Rhodocyclaceae bacterium FTL11 (94) [DQ451827]
22	TA3-33	1	Nitrospirae	Clone DSSD62 (99) [AY328760]	Drinking water distribution system simulator	*Nitrospira* sp. GC86 (98) [Y14644]
23	TA3-34	1	Bacteroidetes	Clone ADK-WSe02-91 (95) [EF520621]	Acid-impacted lakes	-
24	TA3-36	1	β-Proteobacteria	Clone A13 (95) [AF072927]	Nitrifying-denitrifying activated sludge	-
25	TA3-37	1	β-Proteobacteria	Clone 9R-22 (96) [EU224362]	Biofilm reactor for drinking water pretreatment	*Nitrosomonas* sp. Nm59 (95) [AY123811]
26	TA3-39	1	Bacteroidetes	Clone TSC56 (96) [EU359966]	Spruce, hemlock and grassland soils	*Sphingoterrabacterium koreensis* (95) [AB267721]
27	TA3-42	1	β-Proteobacteria	Clone SRRB52 (98) [AB240520]	Rhizosphere biofilm	-
28	TA3-48	1	β-Proteobacteria	Clone A13 (97) [AF072927]	Nitrifying-denitrifying activated sludge	-
29	TA3-53	1	β-Proteobacteria	Clone CB-05 (99) [EF562548]	Complex organic matter degrading bacteria	*Dechloromonas aromatica* (99) [CP000089]
30	TA3-54	1	β-Proteobacteria	Clone 015C-C11 (99) [AY662038]	Nitric acid-bearing uranium waste contaminated groundater	*Gallionella ferruginea* subsp. *Capsiferriformans* (95) [DQ386262]
31	TA3-58	1	β-Proteobacteria	Clone KIS.T93 (99) [EU030495]	Antarctic ice sheet	*Polaromonas* sp. JS666 (98) [CP000316]
32	TA3-60	1	β-Proteobacteria	Clone D0-01 (94)	Tar oil contaminant plume	*Siderooxidans lithoautotrophicus*

				[EU266776]		(93) [DQ386264]
33	TA3-61	1	Unidentified	Clone D15-05 (97) [EU266845]	Tar oil contaminant plume	-
34	TA3-65	1	Bacteroidetes	Clone VC17 (97) [EU593775]	Vermicompost	-
35	TA3-69	1	β-Proteobacteria	Clone RS06101-B92 (98) [EU101281]	Cave waters	Rhodocyclaceae bacterium FTL11 (97) [DQ451827]
36	TA3-70	1	β-Proteobacteria	Clone H22 (99) [AF234697]	Nitrifying-denitrifying activated sludge	*Zoogloea* sp. EMB 62 (99) [DQ413151]
37	TA3-75	1	α-Proteobacteria	*Caulobacter* sp. FWC2 (99) [M83803]	Marine/freshwater	-
38	TA3-100	1	β-Proteobacteria	Clone ML-9-101.2 (99) [DQ520198]	Shallow hypertrophic freshwater lake	*Thiobacillus sajanensis* (93) [DQ390445]
39	TA3-104	1	β-Proteobacteria	Clone D45 (95) [AM713401]	Coal-mining lake sediments	*Ralstonia* sp. OV225.1 (95) [AY216797]
40	TA3-105	1	β-Proteobacteria	*Burkholderia ferrariae* (97) [DQ514537]	Iron ore	-
41	TA3-107	1	α-Proteobacteria	Clone GLB-5 16S (97) [AY345575]	Hawaiian Archipelago	*Sphingomonas* sp. B18 (95) [AF410927]
42	TA3-108	1	Unidentified	Clone FCPT678 (92) [EF516449]	Soil	-
43	TA3-109	1	δ-Proteobacteria	Clone LM74 (98)	Methanotrophic community	-

				[AM909980]		
44	TA3-110	1	γ-Proteobacteria	*Pseudomonas* sp. J7 (99) [EU099375]	Herbicide resistant	*Pseudomonas marginalis* (99) [AF364098]
45	TA3-114	1	Unidentified	Clone FFCH6314 (96) [EU135437]	Soil	-
46	TA3-115	1	Unidentified	Clone MBAE10 (88) [AJ567574]	Deep-sea	-
47	TA3-116	1	β-Proteobacteria	*Ideonella* sp. 0-0013 (95) [AB211233]		-
48	TA3-122	1	β-Proteobacteria	Clone MVS-20 (98) [DQ676320]	Suboxic freshwater pond	*Denitratisoma oestradiolicum* (94) [AY879297]
49	TA3-125	1	δ-Proteobacteria	*Thermodesulforhabdus norvegicus* (89) [U25627]	Oil field water	
50	TA3-126	1	β-Proteobacteria	Clone DS051 (97) [DQ234135]	Mangrove bacterioplankton	*Aquamonas fontana* (94) [AB120965]
51	TA3-127	1	Unidentified	Clone JH-WHS100 (95) [EF492943]	Soil	-
52	TA3-135	1	β-Proteobacteria	Clone LaC15L155 (90) [EF667533]	Anoxic river sediment	*Thiobacillus aquaesulis* (92) [U58019]
TREATMENT TANK						
53	TB3-44	4	δ-Proteobacteria	Clone Amsterdam-2B-48 (99) [AY592406]	East Mediterranean deep-sea mud volcanoes	-
54	TB3-50	4	Planctomycetes	Clone FFCH14332 (88) [EU135383]	Tallgrass prairie soil	-

55	TB3-11	3	α-Proteobacteria	Clone HOClCi61 (99) [AY328610]	Drinking water distribution system	*Sphingomonas* sp. PW-1 (99) [AB255386]
56	TB3-21	3	Chloroflexi	Clone 114b1 (96) [EF459894]	Baltic Sea sediment	-
57	TB3-23	3	α-Proteobacteria	Clone EDP-121 (78) [EU283192]	-	-
58	TB3-5	2	δ-Proteobacteria	Clone BJS960-06 (98) [AB426402]	Deep-sea microbial mat	-
59	TB3-20	2	β-Proteobacteria	Clone nbt214g11 (99) [EU536498]	Human skin	
60	TB3-33	2	γ-Proteobacteria	Clone AFEL3-aao16f07 (99) [EU466050]	-	*Escherichia coli* W3110 (99) [AP009048]
61	TB3-37	2	γ-Proteobacteria	*Methylobacter* sp. LW12 (93) [AY007295]	Freshwater lake sediment	-
62	TB3-4	1	β-Proteobacteria	Clone LaP15L78 (96) [EF667681]	Anoxic river sediment	*Nitrosomonas cryotolerans* (95) [AF272423]
63	TB3-6	1	Planctomycetes	Clone ctg-BRRAA83 (98) [DQ395391]	Unknown	
64	TB3-7	1	β-Proteobacteria	Clone LaC15H30 (97) [EF667705]	Anoxic river sediment	-
65	TB3-8	1	β-Proteobacteria	Clone LaC15L155 (90) [EF667533]	Anoxic river sediment	-
66	TB3-12	1	β-Proteobacteria	*Burkholderia nodosa* (94) [AM284971]	-	-

67	TB3-13	1	Firmicutes	Clone AYRV2-139 (99) [DQ990958]	Atacoma Desert	*Streptococcus mitis* (99) [AY518677]
68	TB3-14	1	γ-Proteobacteria	Clone FB32-27 (99) [AY527805]	Uranium-contaminated subsurface sediment	*Pseudomonas* sp. Hsa.28 (99) [AY259121]
69	TB3-15	1	δ-Proteobacteria	Clone HMMVBeg-12 (96) [AJ704684]	Håkon mosby mud volcano	*Desulfosarcina variabilis* (92) [M34407]
70	TB3-16	1	ε-Proteobacteria	Clone 113B486 (99) [EF687491]	Nile Deep Sea Fan, Eastern Mediterranean, mud volcano	-
71	TB3-17	1	β-Proteobacteria	Clone zEL64 (97) [DQ415856]	Frasassi cave system	-
72	TB3-18	1	δ-Proteobacteria	Isolate GR-296.I.93 (92) [AJ301565]	Uranium mining waste piles	-
73	TB3-19	1	Chloroflexi	Clone XME38 (96) [EF061969]	Mangrove sediment	-
74	TB3-25	1	δ-Proteobacteria	Clone PK005 (88) [EF076059]	Sponge-associated	-
75	TB3-26	1	δ-Proteobacteria	Clone p760-b-1.20 (94) [AB305385]	Hydrothermal sediments	-
76	TB3-28	1	γ-Proteobacteria	Clone Sva0071 (99) [AJ240986]	Cold marine sediments	-
77	TB3-30	1	α-Proteobacteria	Clone Hv(lakePohlsee)-38 (95) [EF667926]	Microbiota in the basal metazoan Hydra	Candidatus *Odyssella thessalonicensis* (90) [AF069496]

78	TB3-31	1	γ-Proteobacteria	*Aeromonas* sp. GPTSA100-19 (99) [DQ859921]	Warm spring	-
79	TB3-34	1	δ-Proteobacteria	Clone zdt-33i5 (93) [AC150251]	Fosmid clone	-
80	TB3-35	1	Planctomycetes	Clone CCM11a (95) [AY221041]	Cave system	-
81	TB3-40	1	γ-Proteobacteria	Clone P9X2b3F11 (99) [AY354134]	Ocean crust	-
82	TB3-47	1	β-Proteobacteria	Clone LaC15H30 (95) [EF667705]	Anoxic river sediment	-
83	TB3-48	1	δ-Proteobacteria	Clone WHB47-47 (99) [AB238972]	Sperm whale-fall surface sediment	-
84	TB3-52	1	ODP	Clone LaP15L27 (91) [EF667658]	Anoxic river sediment	-
85	TB3-54	1	β-Proteobacteria	*Ralstonia pickettii* (99) [CP001069]		-
86	TB3-55	1	ε-Proteobacteria	Clone NK2-53 (99) [EU376182]	Freshwater lake	-
87	TB3-56	1	Planctomycetes	Clone FW104-006 (96) [EF693410]	Uranium contaminated sediments	-
88	TB3-58	1	ODP	Clone ODP1244B11.11 (96) [AB177238]	Hydrate-bearing deep marine sediments	-
89	TB3-60	1	β-Proteobacteria	Clone 4-21 (97) [AF351229]	Coal-tar-waste-contaminated aquifer	*Siderooxidans lithoautotrophicus* (94) [DQ386264]

HOUSEHOLDS

90	TC3-4	13	Actinobacteria	*Mycobacterium gordonae* (99) [EF428556]	Frog pathogen	-
91	TC3-3	10	Actinobacteria	Clone B4M69D3 (99) [AY957948]	Drinking water biofilms	*Mycobacterium* sp. O228YA (99) [DQ372728]
92	TC3-1	4	ε-Proteobacteria	Clone lka78 (98) [EF467574]	Sulfur-oxidizing cave biofilms	-
93	TC3-5	2	δ-Proteobacteria	Clone #5-8 (97) [AB250577]	Grotta Azzurra of Palinuro Cape (Salerno, Italy)	-
94	TC3-8	2	γ-Proteobacteria	Clone pAMC466 (86) [AF150783]	Lake sediment	*Methylomicrobium buryaticum* (86) [AF096093]
95	TC3-9	2	Verrucomicrobia	Clone NK2-32 (99) [EU376182]	Freshwater lake	-
96	TC3-12	2	Firmicutes	*Clostridium* sp. AKG (99) [AM992116]	-	*Bacillus cereus* (99) [DQ207729]
97	TC3-11	1	Actinobacteria	Clone KC-D10 (99) [EU421878]	Himalayan soil	*Mycobacterium neglectum* (99) [AJ580802]
98	TC3-13	1	Actinobacteria	*Mycobacterium mucogenicum* (99) [AY457075]	-	-
99	TC3-16	1	Bacteroidetes	Clone S2-19 (96) [EF491338]	Submerged steel surfaces in marine water	-
100	TC3-18	1	Verrucomicrobia	Clone DA18 (89) [AY193187]	-	-
101	TC3-20	1	Unidentified	Clone KIST-JJY023 (97)	-	-

				[EF594055]		
102	TC3-22	1	β-Proteobacteria	Clone zEL64 (97) [DQ415856]	Limestone-corroding stream biofilms, Frasassi Cave system, Italy	-
103	TC3-24	1	□-Proteobacteria	Clone D2CL-Bac-16S-Clone8 (95) [EU498374]	Planktonic and biofloc-associated bacteria	-
104	TC3-26	1	Actinobacteria	Clone LKC-Acid-4 (98) [EU038041]	Cave-related system	*Mycobacterium sherrisii* (97) [AY353699]
105	TC3-35	1	Actinobacteria	*Mycobacterium gadium* (99) [DQ473310]	Associated with chronic pneumonia	-
106	TC3-36	1	α-Proteobacteria	Clone DSSF72 (99) [AY328694]	Drinking water	*Sphingobium estrogenivorans* (97) [DQ855413]
107	TC3-40	1	δ-Proteobacteria	Clone Hyd01-30 (98) [AJ535242]	Gas hydrate sediments	-
108	TC3-41	1	Unidentified	Clone G3-50 (86) [EU005312]	Marine attached bacteria	-
109	TC3-44	1	γ-Proteobacteria	Clone MP104-1109-b35 (99) [DQ088799]	Crustal biome	*Acinetobacter* sp. L (99) [DQ189256]
110	TC3-47	1	Unidentified	Clone RS06101-B92 (98) [EU101281]	Sulfur-oxidizing cave waters	-
111	TC3-49	1	Unidentified	Clone p763-b-4.44 (99) [AB305514]	Hydrothermal sediments, Southern Okinawa Trough	-
112	TC3-51	1	β-Proteobacteria	*Ralstonia pickettii* (99) [CP001069]		-