Post-print of: Sánchez O, Ferrera I, Garrido L, Gómez-Ramos MM, Rodríguez Fernández-Alba A, Mas J (2014). Arch Microbiol 196:219-226

Prevalence of potentially thermophilic microorganisms in biofilms from greenhouse-enclosed drip irrigation systems

Olga Sánchez[1]*†, Isabel Ferrera[1,2†], Laura Garrido[1], Maria del Mar Gómez-Ramos[3], Amadeo Rodríguez Fernández-Alba[3], Jordi Mas[1]

[1]*Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Spain*

[2]*Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar, ICM-CSIC, Barcelona, Spain*

[3] *Departamento de Hidrogeología y Química Analítica, Universidad Almería, 04120-La Cañada de San Urbano, Almería, Spain*

A manuscript re-submitted to: Archives of Microbiology (AOMI-D-13-00080).

*Corresponding author: Mailing address: Departament de Genètica i Microbiologia, Facultat de Biociències, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Phone: 34-93 586 8022. Fax: 34-93 581 2387. E-mail: Olga.Sánchez@uab.es

†Equal contributors to this article

Abstract

Drip irrigation systems using reclaimed water often present clogging events of biological origin. Microbial communities in biofilms from microirrigation systems of an experimental greenhouse in Almería, SE Spain, which used two different qualities of water (treated wastewater and reclaimed water), were analyzed by DGGE (Denaturing Gradient Gel Electrophoresis) and subsequent sequencing of amplified 16S rRNA gene bands. The most remarkable feature of all biofilms was that regardless of water origin, sequences belonging to *Firmicutes* were prevalent (53.5% of total mean band intensity) and that almost all sequences recovered had some similarity (between 80.2 and 97%) to thermophilic microorganisms. Mainly, sequences were closely related to potentially spore-forming organisms, suggesting that microbial communities able to grow at high temperatures were selected from the microbiota present in the incoming water. These pioneer results may contribute to improve management strategies to minimize the problems associated to biofouling in irrigation systems.

*Keywords**:** DGGE;* 16S rRNA gene; Thermophilic bacteria; Biofilms; Wastewater reuse

Introduction

Agricultural practices in Mediterranean countries with low rainfall have progressively shifted towards the use of drip irrigation, also called trickle or microirrigation, as a tool to optimize water utilization as well as to improve control of water delivery to the crops due to its high irrigation efficiency (up to 90%). Drip irrigation is particularly suitable for wastewater reuse because it minimizes the health risks to farmers and consumers due to the little contact with wastewater. However, the usage of reclaimed water for drip irrigation is often associated to clogging events that impair proper operation of the system. When clogging occurs, more than 90% of the accumulated material has a biological origin. In most cases, the clogging process is usually initiated by bacterial biofilms (biofouling) (Gilbert et al. 1981; Taylor et al. 1995).

Previous studies have focused on biofilm formation and clogging during reutilization of wastewater for irrigation purposes (Capra et al. 2004; Lubello et al. 2004; Yan et al. 2009; Tarchitzky et al. 2013). Among them, Yan et al. (2009) analyzed the matrix structure in biofilms from drip irrigation emitters distributing reclaimed water using scanning electron microscopy (SEM). However, despite their importance in agriculture, no information describing the composition of the microbial communities present in biofilms from drip irrigation systems is available so far. Nevertheless, assessing the composition of these microbial communities constitutes a critical step to develop good management strategies for this type of facilities.

In this work, we describe the microbial communities forming biofilms in the irrigation systems (drippers and pipes) of an experimental greenhouse in Almería, SE Spain, used to grow a Virginia tobacco crop using two different qualities of water (a treated wastewater and a reclaimed water). Previous studies in this experimental greenhouse showed that the irrigation system was prone to develop biofouling and clogging of the drippers (Muñoz et al. 2010). In order to gain a better understanding of this issue we characterized the composition of these biofilms by using Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al. 1998), a molecular tool useful to obtain fingerprints of natural microbial communities and to identify the more abundant members of these communities. As a consequence of the lack of information on the type of organisms developing biofilms in this particular kind of installations, these pioneer results provide

useful information to assess the risk that they might suppose for the crops involved as well as contribute to improve management strategies to minimize the problems associated to biofouling in microirrigation.

Materials and Methods

Description of the experimental greenhouse

The study took place in an experimental greenhouse located at the agricultural research farm belonging to the University of Almería, in Southeastern Spain (36°510N, 2°160W and 87 m elevation). The total greenhouse area was 1935 m^2 (43 x45 m), with an available area for cultivation of 1784 m^2.

Virginia tobacco (*Nicotiana tabacum L.*) was grown from October 2007 to June 2008. The crop can be used to obtain products with high industry value. Tobacco seedlings were grown over two months by a professional grower (Ejidoplant S.L., Almeria, Spain), and transplanted at the beginning of October into the experimental greenhouse. Plants were grown in the greenhouse for a period of 9 months until harvested. Experimental details are described in Muñoz et al. (2010). At the end of this period the facility was dismantled and we took the opportunity to dissect the irrigation system, taking samples from both drippers and pipes for biofilm analysis.

Plants were irrigated with two qualities of water, which from now on we refer to treated wastewater as **SEW** (Secondary Effluent Water) and to reclaimed water as **TEW** (Tertiary Effluent Water). SEW was the secondary effluent from the local wastewater treatment plant of El Ejido (Almería, Spain). This plant treats 13.800 m^3 day^{-1} of urban wastewater. Input wastewater undergoes a physical pre-treatment to remove coarse solids and greases, along with primary settling of particulates. Secondary treatment then takes place by activated sludge, after which the treated wastewater is discharged. The treated wastewater to be reused in the greenhouse was collected after passing through the secondary settling tank, without any further treatment. TEW consisted of the tertiary effluent from the El Toyo Wastewater Treatment Plant (El Toyo, Almería, Spain). In this plant, primary and secondary treatments are identical to those of El Ejido plus ozone treatment and the addition of approximately a proportion of 30% of rainwater and desalinated water

from the Carboneras desalination plant. TEW is the water commonly used in this area for agriculture. Standard chemical parameters of these irrigation waters were measured following standard procedures and are listed in Supplementary Table S1.

Drip irrigation with fertigation was used to supply both water and fertilizers to the crop. Two independent fertigation systems, corresponding to SEW and TEW water, were installed. SEW wastewater was collected on a bimonthly basis and transported by truck to the greenhouse, where it was stored in two 5000 L tanks. An immersed pump was used to drive it through two consecutive ring filters with a pore size of 120 and 105 μm. In the greenhouse, SEW and TEW distribution and application were separated in two independent irrigation lines. The length of the laterals was 20 meters. Water was distributed by means of pressure-compensating on line Autotwin® drippers (Mondragón Soluciones, Albuixech, Spain) located on the soil surface, with a nominal flow of 3 L h^{-1}, placed at a density of 4 units m^{-2}. Drippers were made of polypropylene whereas pipes were made of polyethylene. The crop was typically irrigated once a day, 3-4 times a week, with an average amount of 9 mm water each time. A total of 1.25 m^3 m^{-2} was applied for each treatment.

Sampling

After 9 months of continuous operation of the greenhouse, biofilms from the inner plastic surfaces of both SEW and TEW irrigation lines were collected. Five pipe sections from the end of irrigation lines distributing each type of water were cut under sterile conditions and the inner biofilms were scraped by using a sterilized metal spatula and stored in an eppendorf tube at -20ºC until further analysis. Drippers were dismantled and the inner filter was collected in 15 mL of saline solution (0.9% sodium chloride). In total, five drippers from each irrigation line (TEW and SEW) were sampled and pooled together. By pooling the samples we could not compare inter sample variability but as a trade-off we ensured that we collected enough biomass to obtain representative information of the entire line. Dripper samples were placed in an ultrasonic water bath (Bransonic 5, Branson) for 3 minutes. After sonication, the liquid was centrifuged for 5 minutes at 10.000*g* (Eppendorf centrifuge 5804 R) and the resulting pellet was stored at -

20ºC for further DNA extraction (Sánchez et al., 2006). About 300 mL of each type of water were also filtered through polycarbonate filters of 0.22 μm of pore size and the filters were stored at –20ºC for further DNA extraction.

DNA Extraction

The DNA Power Soil kit MO BIO ref. 12888-50 (MO BIO Laboratories, Inc., USA) was used for DNA extraction from biofilms, while the UltraClean water kit MOBIO ref. 14880-25 (MO BIO Laboratories, Inc., USA) was utilized for water filters. DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder as a standard (Invitrogen, Life Technologies, USA) using the Quantity One software package (Bio-Rad, Spain) for gel documentation and analysis.

PCR-DGGE fingerprinting

Fragments of the bacterial 16S rRNA gene suitable for DGGE analysis were obtained by using the specific primer 358F with a 40-bp GC clamp, and the universal primer 907RM as described in Sánchez et al. (2007). DGGEs were run in a DCode system (Bio-Rad, Spain) as described by Muyzer et al. (1998) using a 6% polyacrylamide gel with a gradient of 30-70% DNA-denaturant agent. Seven hundred ng of PCR product were loaded for each sample and the gels were run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes, Life Technologies, USA) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate, placed in a UV-transparent gel scoop, and visualized with UV in a Gel Doc XRS (Bio-Rad, Spain). Digitized DGGE images were analyzed using Quantity One from (Bio-Rad, Spain). Visible bands were excised from the gels, resuspended in milli-Q water overnight and reamplified for sequencing.

16S rRNA gene sequencing and analyses

Purification of PCR products from DGGE bands and sequencing reactions was performed by Macrogen (South Korea) with primer 907RM. They utilized the Big Dye Terminator version 3.1 sequencing kit and

reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type. For accurate taxonomic classification, sequences were submitted to the Ribosomal Data Project (RDP) (Cole et al. 2009). Hierarchical taxonomy based on a naïve Bayesian rRNA classifier (release 10) was used to classify sequences at higher taxonomic rank (Wang et al. 2007) and Seq-Match (RDP) was performed in order to obtain an identity for each sequence. Gene sequences were deposited in GenBank under accession numbers HE573183-HE573228.

In order to obtain direct descriptors of the diversity of bacterial assemblages, we calculated the Shannon index (*H'*), as explained by Magurran (1988). For biofilm community composition analyses, a dissimilarity matrix (Bray-Curtis) was constructed based on the relative abundance (square root transformed) of each band and visualized using non-metric multidimensional scaling (NMDS) and hierarchical clustering analysis in R Vegan (Oksanen et al. 2013). Differences between samples were tested by analysis of similarities (anosim) in R Vegan.

Results and discussion

We analyzed the composition of the bacterial community forming biofilms in the irrigation system (drippers and pipes) of an experimental greenhouse in Almería, SE Spain, as well as the communities in the incoming water corresponding to two different water qualities used for irrigation, treated wastewater (SEW) and reclaimed water (TEW), by using Denaturing Gradient Gel Electrophoresis (DGGE). This technique results in a fingerprint of bands, each band corresponding to an OTU (Operational Taxonomic Unit). Figs. 1A and 1B show the DGGE fingerprints from the two different incoming waters, revealing that the community composition was different, as expected from waters of different quality. The DGGE analysis from the two water samples yielded a total of 25 bands from TEW (Fig. 1A) and 31 from SEW (Fig. 1B). Shannon diversity index, which provides information about the diversity of a sample, was calculated from the DGGE fingerprints, showing a relatively high value (Fig. 2). SEW resulted in numbers

of 3.0 and TEW of 2.9, which are within the upper range reported for other wastewater types (Boon et al. 2002; Calheiros et al. 2009).

In order to determine the phylogenetic affiliation of most abundant groups, bands were excised from the gels and sequenced. Informative sequences were obtained from 15 bands of TEW and 9 bands of SEW (Table 1) due to the presence of faint bands. These informative bands accounted for 86.6% of the total band intensity in the case of TEW and 42.2% for SEW. Most of the total retrieved sequences were closely related to 16S rRNA gene sequences from uncultured microorganisms with similarities ranging between 80.9 and 99.6% and only 4 bands were similar to sequences from cultured organisms in the RDP database (Table 1). The phylogenetic groups retrieved (Fig. 3) were those typically found in other aquatic environments, including *Bacteroidetes* (with a contribution of 31.6% to total band intensity in TEW and 15.3% in SEW), *Betaproteobacteria* (35.5% in TEW and 19.5% in SEW) and *Gammaproteobacteria* (9.6% in TEW and 7.5% in SEW). *Chlorobi* could only be retrieved from TEW (9.9% contribution).

Contrarily, the analyses of the bacterial diversity in the four biofilm samples from the drip irrigation systems (drippers and pipes) resulted in a lower diversity. A total of 54 bands were detected, ranging from 13 to 15 per lane (Fig. 1C). Calculation of Shannon indexes (Fig. 2) revealed that the diversity was lower than that in the incoming waters (2.1-2.3). Previous studies (Lymperopoulou et al. 2012; Liu et al. 2012; Schauer et al. 2000) have measured this index in aquatic systems and values typically range between 0.5 and 5. Despite much variability exists in freshwater environments, drinking water reservoirs or marine systems in general values from 0.5 to 2 indicate low diversity whereas values from 2 to 5 refer to high diversity. In our work, Shannon index showed intermediate values of diversity and were higher in the incoming water than in the biofilms. The lower diversity in the irrigation systems reflects the selection of certain populations able to attach to the surfaces of drippers and pipes. However, we must point out that slight differences could also be the result of using different DNA extraction protocols (Ferrera et al. 2010) or to the inherent biases of PCR, i.e. biases due to differences in DNA template (Polz and Cavanaugh 1998).

Among the irrigation systems, calculation of Bray-Curtis dissimilarity indices among biofilm samples revealed that community profiles were more dissimilar depending on incoming water type than on the kind of irrigation system where they developed (average 0.66 vs. 0.54). Representation of hierarchical clustering supported the grouping of samples depending on incoming water (Supplementary Fig. S1A). Furthermore, biofilms from drippers and pipes using TEW were more similar among them than those using SEW (0.41 Bray-Curtis dissimilarity index in TEW vs. 0.67 in SEW) (Supplementary Fig. S1B). Pipes and drippers were made of different materials (polyethylene and polypropylene respectively) and despite analysis of similarities was not significant ($p>0.05$, $n=4$), these results indicate that the type of material used had a stronger influence on biofilm composition in SEW than in TEW. However, the potential effect on biofilm communities requires further research.

Nevertheless, despite the differences observed between TEW and SEW profiles, the presence of common bands (bands 1 and 9, bands 4 and 10) suggests that some organisms were prevalent in both types of biofilm. Bands excised were sequenced and a total of 22 bands resulted in informative sequences (Table 2) accounting for 75.3% of the total mean band intensity. Taxonomy analysis revealed that the composition of the biofilm samples was significantly different from the incoming water (Fig. 3). Most of the sequences showed high similarity to sequences from cultured organisms in RDP database. A total of three different phyla could be retrieved; 17 of the 22 identified sequences belonged to the phylum *Firmicutes*, with a total contribution of 53.5% to total mean band intensity, and had a high similarity to cultured microorganisms of the genera *Geobacillus*, *Bacillus*, *Symbiobacterium*, *Brevibacillus*, and *Alicyclobacillus.* Four of the remaining sequences belonged to the *Gammaproteobacteria* (17.9% of total mean band intensity) and were similar to *Xanthomonas* and *Lysobacter,* and one sequence was similar to an uncultured member of the *Chlorobi* group.

Noteworthy, in all biofilms from these irrigation systems sequences potentially belonging to the *Firmicutes* were prevalent regardless of water origin. All members of this diverse phylum are Gram-Positive bacteria with a rigid cell wall (Desvaux et al. 2006). As an example, one of the sequences recovered had a high

similarity (97%) to *Geobacillus thermoleovorans*, a Gram-positive, chemo-organotrophic, aerobic or facultatively anaerobic, obligately thermophilic bacterium, with an optimum growth temperature of 55-65ºC (Nazina et al. 2001). Likewise, two sequences of *Gammaproteobacteria* recovered in this study were related to *Lysobacter*, a genus that has been described as ubiquitous inhabitant of soil and water, with clades that probably include sequences obtained from hydrothermal vents (Sullivan et al. 2003). On the other hand, two other sequences were similar to *Xanthomonas vesicatoria*, a microorganism which constitutes a large problem in greenhouses where very high humidity and warm temperatures provide a good environment for these bacteria to grow (Diab et al. 1982). Finally, another sequence related to *Chlorobi* was similar to an uncultured microorganism assigned to *Ignavibacterium*, a genus which also includes thermophilic members (Iino et al. 2009).

Overall, our results show that a microbial community potentially able to grow and survive at high temperatures develops and becomes predominant in the inner surfaces of this irrigation system. The fact that these organisms were not present in high amounts in the incoming water indicates that conditions within the irrigation infrastructure, specifically the high temperatures, selected for these populations. Although we have no data on the actual temperatures within the pipes and drippers, the temperatures recorded daily inside the greenhouse during the 9 months of operation indicate maximum temperatures between 40 and 46 ºC. The temperature in the tubes and drippers could be even higher due to their dark color, since the darker the object the more heat is absorbed. Moreover, the irrigation lines operated only once a day (3-4 times a week) and at very low flow (3 L h^{-1}). Therefore, during most of the time water did not circulate and refrigeration due to external cold water entering the system did not occur.

From a point of view of microbiological risk for the irrigated crops, the prevalence of potentially thermophilic microorganisms suggests that conditions inside the irrigation system are not optimal for usual pathogenic organisms that thrive well in the mesophilic range, therefore their presence in the biofilms is unlikely. On the other hand, the presence of hypothetically spore-forming thermophiles might constitute a

problem when attempting to control biofilm formation through the action of biocides since endospores are highly resistant to the action of disinfectants.

Although biofilm research has advanced in other areas, very little is known about those in the area of agriculture and particularly, in irrigation. We found that microbial communities in this drip irrigation system could be mainly composed of potentially thermophilic/thermoresistant microorganisms regardless of water origin (SEW or TEW). We are aware that the type of microbial community found might differ under other environmental conditions, since in other irrigation systems different factors could be involved in the selection of biofilm communities. However, this study provides the first insights into the microbial diversity that can be found in these systems and may provide useful information for future research on this topic that clearly needs to be expanded with the isolation and characterization of the microorganisms involved, which likely possess thermophilic capabilities. Moreover, future experiments should focus on the study of temperature patterns inside the tubing system in order to have a better understanding of the whole microbial community structure.

Acknowledgements

This work was supported by grants CSD2006-00044 TRAGUA (CONSOLIDER-INGENIO2010) and CTQ2009-14390-C02-02 from the Spanish Ministry of Education and Science to JM, and PET2008-0165-02 to OS.

References

Boon N, De Windt W, Verstraete W, Top EM (2002) Evaluation of nested PCR–DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol Ecol 39:101–112

Calheiros CSC, Duque AF, Moura A, Henriques IS, Correia A, Rangel AOSS, Castro PML (2009) Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment. Bioresour Technol 100:3228-3235

Capra A and Scicolone B (2004) Emitter and filter tests for wastewater reuse by drip irrigation. Ag Water Manage 68:135-149

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37: D141-145

Desvaux M, Dumas E, Chafsey I, Hébraud M (2006) Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. FEMS Microbiol Lett 256:1-15

Diab S, Bashan Y, Okon Y (1982) Studies of infection with *Xanthomonas campestris* pv. *Vesicatoria*, causal agent of bacterial scab of pepper in Israel. Phytoparasitica 10:183-191

Ferrera I, Massana R, Balagué V, Pedrós-Alió C, Sánchez O, Mas J (2010) Evaluation of DNA extraction methods from complex phototrophic biofilms. Biofouling 26:349-357

Gilbert RG, Nakayama FS, Bucks DA, French OF, Adamson KC (1981) Trickle irrigation, emitter clogging and flow problems. Ag Water Manage 3:159-178

Iino T, Mori K, Uchino Y, Nakagawa T, Harayama S, Suzuki K (2010) *Ignavibacterium album* gen. nov., sp. nov., a moderately thermophilic anaerobic bacterium isolated from microbial mats at a terrestrial hot spring in Japan and proposal of *Ignavibacteria* classis nov., for a novel lineage at the periphery of green sulfur bacteria. Int J Syst Evol Microbiol 60:1376-1382

Lymperopoulou DS, Kormas KA, Karagouni AD (2012) Variability of prokaryotic community structure in a drinking water reservoir (Marathonas, Greece). Microbes Environ 27:1-8

Liu Z, Huang S, Sun G, Xu Z, Xu M (2012) Phylogenetic diversity, composition and distribution of bacterioplankton community in the Dongjiang River, China. FEMS Microbiol Ecol 80:30–44

Lubello C, Gori R, Nicese FP, Ferrini F (2004) Municipal-treated wastewater reuse for plant nurseries irrigation. Water Res 38:2939-2947

Magurran AE (1988) Ecological Diversity and its Measurement. London, Princeton University Press

Muñoz I, Gómez MM, Férnandez-Alba AR (2010) Life cycle assessment of biomass production in a Mediterranean greenhouse using different water sources, Groundwater, treated wastewater and desalinated seawater. Agr Syst 10:31-39

Muyzer G, Brinkhoff T, Nübel U, Santegoeds C, Schäfer H, Wawer C (1998) Denaturing gradient gel electrophoresis DGGE in microbial ecology. In: Akkermans ADL, van Elsas JD, Bruijn FJ (eds) Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, pp 1-27

Nazina TN, Tourova TP, Poltaraus AB et al (2001) Taxonomic study of aerobic thermophilic bacilli, descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans.* Int J Syst Evol Microbiol 51:433-446

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., *et al*. (2013) *Vegan: community ecology package*. R package version 2.0-7.

Polz MF, Cavanaugh CM (2010) Bias in template-to-product ratios in multitemplate PCR. Appl Environ Microbiol 64:3724-3730

Sánchez O, Ferrera I, Vigués N, García de Oteyza T, Grimalt J, Mas J (2006) Role of cyanobacteria in oil biodegradation by microbial mats. Int Biodeter Biodegr 58:186-195

Sánchez O, Gasol JM, Massana R, Mas J, Pedrós-Alió C (2007) Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. Appl Environ Microbiol 73:5962-5967

Sullivan RF, Holtman MA, Zylstra GJ, White JF, Kobayashi DY (2003) Taxonomic positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes* based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic characteristics. J Appl Microbiol 94:1079-1086

Schauer M, Massana R, Pedrós-Alió C (2000) Spatial differences in bacterioplankton composition along the Catalan coast (NW Mediterranean) assessed by molecular fingerprinting. FEMS Microbiol Ecol 33:51-59

Tarchitzky J, Rimon A, Kenig E, Dosoretz CG, Chen Y (2013) Biological and chemical fouling in drip irrigation systems utilizing treated wastewater. Irrig Sci. doi: 10.1007/s00271-013-0406-0

Taylor HD, Bastos RK, Pearson HW, Mara DD (1995) Drip irrigation with waste stabilization pond effluents, solving the problem of emitter fouling. Water Sci Technol 31:417–424

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73: 5261-5267

Wimpenny J, Manz W, Szewzyk U (2000) Heterogeneity in biofilms. FEMS Microbiol Rev 24:661–671

Yan D, Bai Z, Mike R, Gu L, Ren S, Yang P (2009) Biofilm structure and its influence on clogging in drip irrigation emitters distributing reclaimed wastewater. J Environ Sci 21:834-841

Figure captions:

Fig. 1 DGGE images showing the fingerprints of the tertiary effluent water (A), the secondary effluent water (B) and the biofilm samples (C). TEW: tertiary effluent water; SEW: secondary effluent water; TEW_{DF}: biofilm from tertiary effluent water dripper filter; SEW_{DF}: biofilm from secondary effluent water dripper filter, TEW_P: biofilm from tertiary effluent water pipe, SEW_P: biofilm from secondary effluent water pipe

Fig. 2 Shannon indices (H') from the incoming and biofilm samples. TEW: tertiary effluent water; SEW: secondary effluent water; TEW_{DF}: biofilm from tertiary effluent water dripper filter; SEW_{DF}: biofilm from secondary effluent water dripper filter, TEW_P: biofilm from tertiary effluent water pipe, SEW_P: biofilm from secondary effluent water pipe

Fig. 3 Percentage of relative intensity of DGGE bands affiliated to different phylogenetic groups. TEW: tertiary effluent water; SEW: secondary effluent water; TEW_{DF}: biofilm from tertiary effluent water dripper filter; SEW_{DF}: biofilm from secondary effluent water dripper filter, TEW_P: biofilm from tertiary effluent water pipe, SEW_P: biofilm from secondary effluent water pipe, Firm: Firmicutes, Bact: Bacteroidetes, Beta: Betaproteobacteria, Gam: Gammaproteobacteria, Chlor: Chlorobi, Not id: Not identified

Supplementary Figure captions:

Fig. S1. Dendrogram resulting from hierarchical clustering (A) and non-metrical multidimensional (NMDS) plot (B) based on Bray–Curtis dissimilarities between biofilm samples calculated from the square root transformed relative abundance of each band from the DGGE fingerprint. In the NMDS plot, samples derived from SEW are represented in black and those from TEW in grey (stress value=0). Samples from pipes are represented as squares and those from drippers as circles. SEW: secondary effluent water; TEW: tertiary effluent water.

Fig. 1

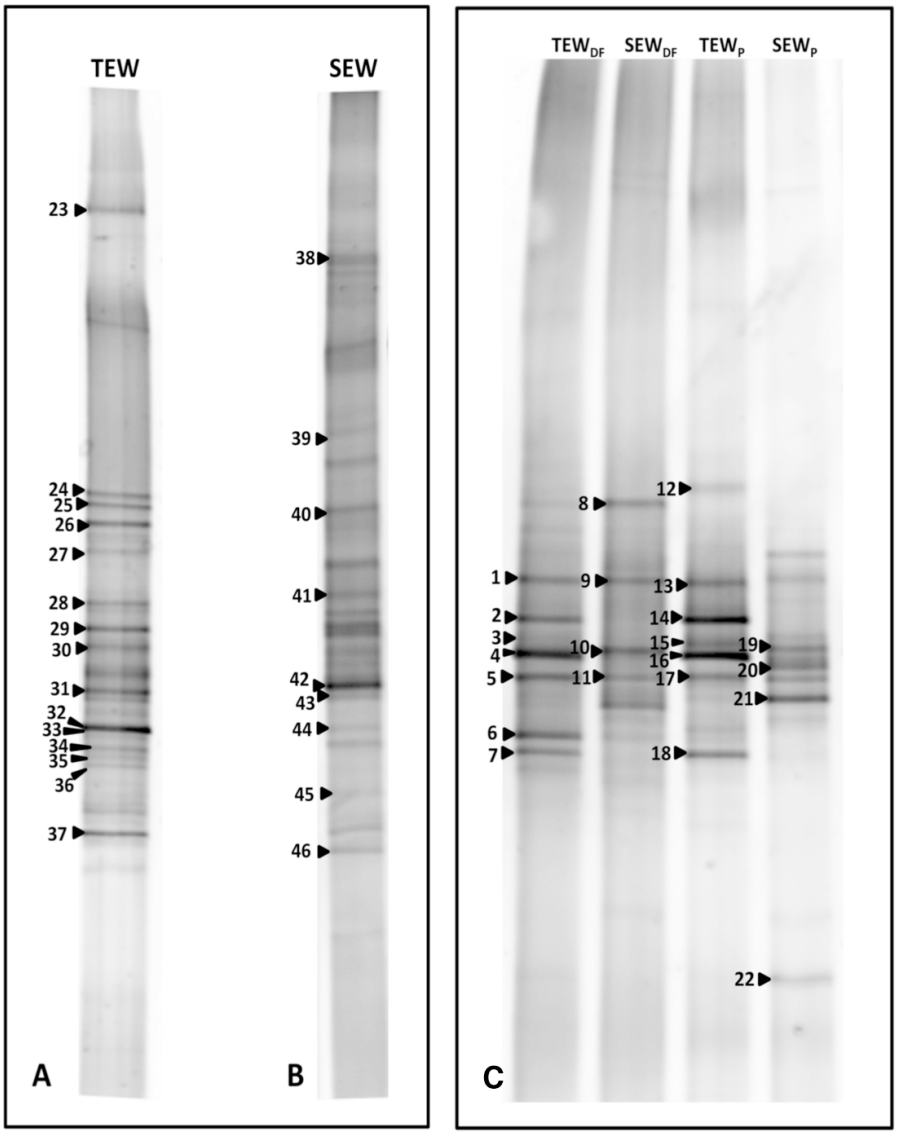

TEW
SEW
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
A
B
TEW$_{DF}$
SEW$_{DF}$
TEW$_{P}$
SEW$_{P}$
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
C

Fig. 2

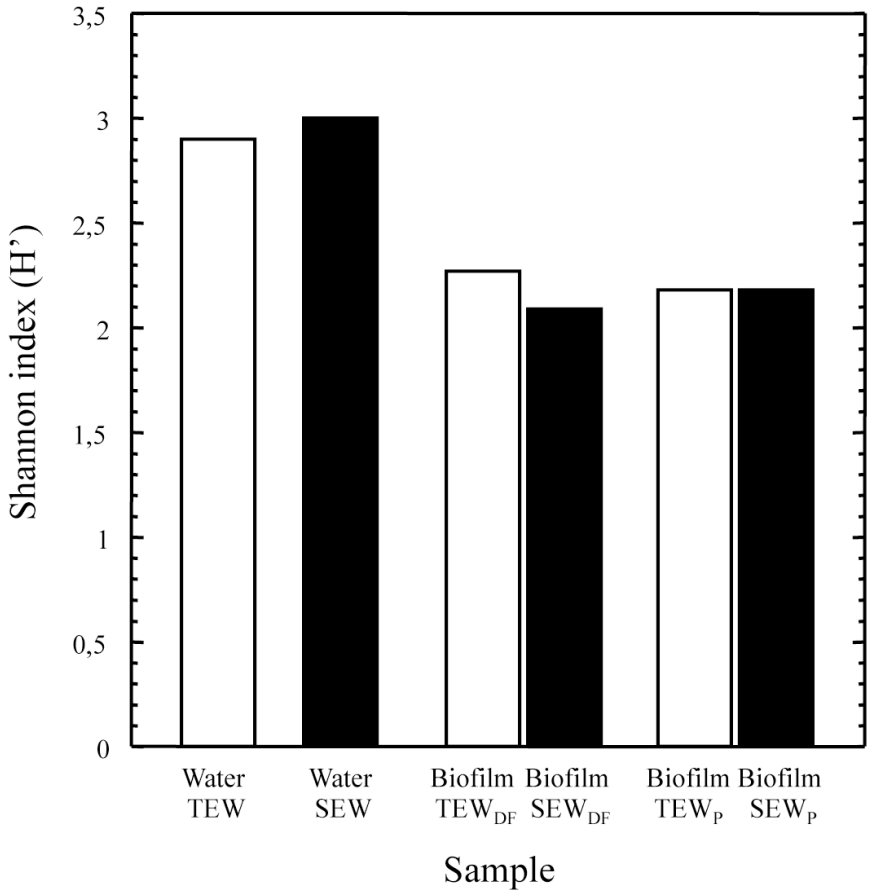
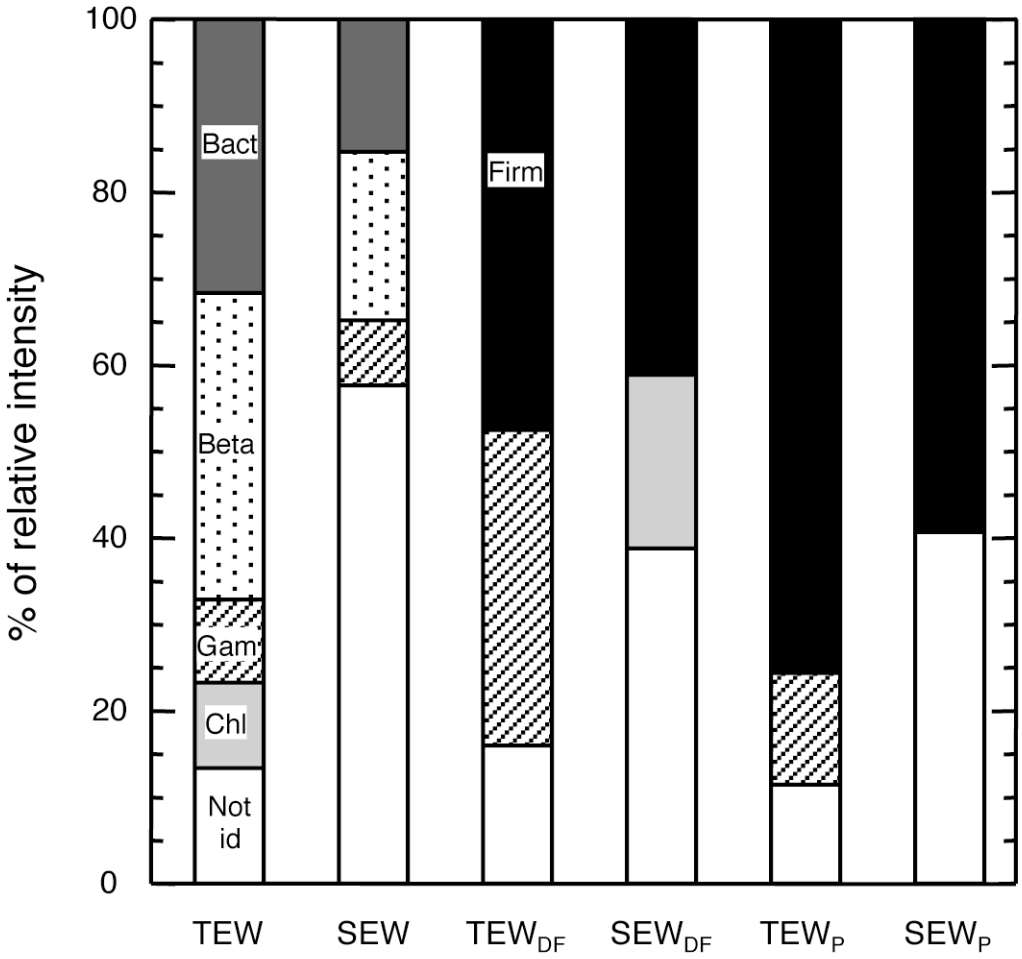

3,5
3
2,5
2
1,5
1
0,5
0
Shannon index (H')
Water TEW
Water SEW
Biofilm TEW$_{DF}$
Biofilm SEW$_{DF}$
Biofilm TEW$_{P}$
Biofilm SEW$_{P}$
Sample

Fig. 3


100
80
60
40
20
0
% of relative intensity
Bact
Beta
Gam
Chl
Not id
Firm
TEW
SEW
TEW$_{DF}$
SEW$_{DF}$
TEW$_{P}$
SEW$_{P}$

Supplementary Fig. S1

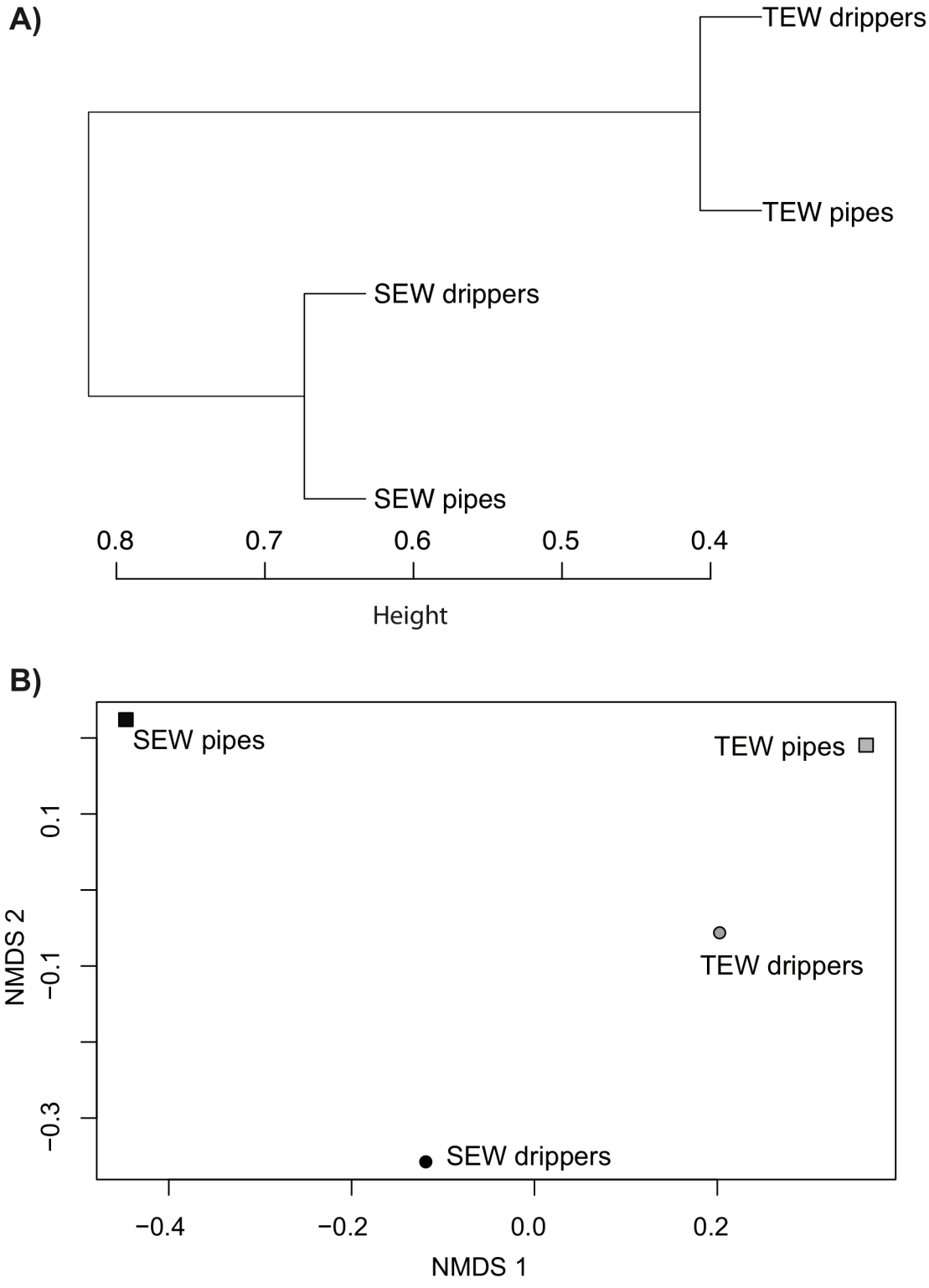


Table 1. Phylogenetic identification of sequences obtained from effluent water (TEW) and secondary effluent water (SEW) DGGE bands

Band	Closest RDP Sequence match[a]	%similarity (nº bases)[b]	Hierarchical RDP assigned taxonomy[c]	Relative intensity (%)
23	uncultured bacterium (EU037363)	99.6 (522)	Bacteroidetes;Flavobacteria;Flavobacteriales;Flavobacteriaceae;*Flavobacterium*	11.2
24	uncultured bacterium (FJ820373)	98.6 (518)	Bacteroidetes;Sphingobacteria;Sphingobacteriales;Sphingobacteriaceae;*Solitalea*	3.4
25	uncultured bacterium (FJ382687)	99.4 (518)	Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;*Anaerorhabdus*	3.8
26	uncultured bacterium (FJ375427)	99.6 (525)	Bacteroidetes;unclassified Bacteroidetes	6.4
27	uncultured bacterium (HM445932)	97.7 (524)	Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;*Rikenella*	2.7
28	uncultured Sphingobacteriales (AJ697698)	90.3 (515)	Bacteroidetes;Sphingobacteria;Sphingobacteriales;Cyclobacteriaceae;*Algoriphagus*	4.1
29	uncultured bacterium (EU234274)	90.3 (534)	Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;*Azospira*	5.6
30	*Pseudomonas alcaligenes* (Z76653)	95.5 (532)	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;*Pseudomonas*	4.5
31	*Pseudomonas* sp. (AB180241)	96.4 (526)	Proteobacteria;Gammaproteobacteria;Pseudomonadales;Pseudomonadaceae;*Pseudomonas*	5.1
32	uncultured bacterium (AF331976)	98.3 (534)	Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;*Dechloromonas*	12.6
33	uncultured bacterium (AY945901)	97.2 (535)	Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;unclassified Rhodocyclaceae	12.6
34	uncultured bacterium (JF429341)	95.7 (532)	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;*Curvibacter*	2.4
35	uncultured bacterium (AF236005)	93.4 (534)	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;*Curvibacter*	1.1
36	uncultured bacterium (EU919778)	97.2 (528)	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;*Curvibacter*	1.2
37	uncultured bacterium (HQ856351)	98.9 (528)	Chlorobi;unclassified Chlorobi	9.9
38	uncultured bacterium (AY570561)	92.9 (533)	Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;*Bacteroides*	4.8
39	uncultured bacterium (DQ256318)	99.4 (532)	Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;*Parabacteroides*	0.9
40	uncultured bacterium (GQ480041)	98.9 (534)	Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;*Rikenella*	4.4
41	uncultured bacterium (EU104155)	80.9 (470)	Bacteroidetes;Sphingobacteria;Sphingobacteriales;Saprospiraceae;*Haliscomenobacter*	3.4
42	uncultured bacterium (GQ002140)	86.7 (540)	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;*Diaphorobacter*	17.2
43	*Acidovorax anthurii* (KC261873)	95.8 (544)	Proteobacteria;Betaproteobacteria;Burkholderiales;Comamonadaceae;*Acidovorax*	2.3
44	uncultured bacterium (AB270073)	85.2 (519)	Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;*Prevotella*	1.8
45	*Halochromatium glycolicum* (X93472)	92.2 (529)	Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae;*Halochromatium*	2.6
46	uncultured bacterium (AF361090)	86.4 (551)	Proteobacteria;Gammaproteobacteria;unclassified Gammaproteobacteria	4.9

RDP: Ribosomal Data Project

a. Genbank Accession Number shown in parentheses

b. Number of bases used to calculate the percentahe of sequence identity

c. Taxonomy rank shown as Phylum;Class;Order;Family;Genus

Table 2. Phylogenetic affiliation of sequences obtained from biofilm DGGE bands

Band	Closest RDP Sequence match[a]	%similarity (nº bases)[b]	Hierarchical RDP assigned taxonomy[c]	Relative intensity (%)
1, 9	*Geobacillus thermoglucosidasius* (AB021197)	93.4 (441)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	9.6, 10.6
2, 14	uncultured bactterium (EF636831)	92.0 (536)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Bacillus*	10.5, 16.8
3	*Geobacillus thermodenitrificans* (AF114425)	88.3 (409)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	2.8
4, 10	*Xanthomonas vesicatoria* (AF123088)	95.4 (525)	Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Xanthomonas*	24.5, 21.4
5, 17	*Lysobacter enzymogenes* (AJ298291)	94.6 (497)	Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;*Lysobacter*	12.0, 12.9
6	*Symbiobacterium thermophilum* (AB004913)	95.8 (480)	Firmicutes;Clostridia;Clostridiales;Clostridiales_Incertae Sedis XVIII;*Symbiobacterium*	16.0
7, 18	*Brevibacillus thermoruber* (Z26921)	91.4 (498)	Firmicutes;Bacilli;Bacillales;Paenibacillaceae 1;*Brevibacillus*	8.6, 16.0
8	uncultured bacterium (AJ412669)	91.1 (514)	Chlorobi;Ignavibacteria;Ignavibacteriales;Ignavibacteriaceae;*Ignavibacterium*	20.1
11	*Symbiobacterium* sp. (AB455239)	92.7 (534)	Firmicutes;Clostridia;Clostridiales;Clostridiales_Incertae Sedis XVIII;S*ymbiobacterium*	9.1
12	*Geobacillus subterraneus* (AY608956)	90.0 (538)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	7.7
13	*Geobacillus caldoxylosilyticus* (AJ564610)	88.7 (521)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	12.3
15	*Bacillus* sp (AB063312)	90.5 (411)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	4.2
16	*Geobacillus thermodenitrificans* (Z26927)	86.7 (405)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	18.6
19	*Geobacillus toebii* (DQ225186)	82.2 (409)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	5.4
20	*Alicyclobacillus macrosporangiidus* (AB264025)	80.2 (429)	Firmicutes;Bacilli;Bacillales;Alicyclobacillaceae;*Alicyclobacillus*	12.2
21	*Geobacillus thermoleovorans* (AY450926)	97.0 (536)	Firmicutes;Bacilli;Bacillales;Bacillaceae 1;*Geobacillus*	26.2
22	uncultured bacterium (DQ088766)	85.3 (407)	Firmicutes;Clostridia;Thermoanaerobacterales;Thermoanaerobacteraceae;unclassified_Thermoanaerobacteraceae	15.5

RDP: Ribosomal Data Project

a. Genbank Accession Number shown in parentheses

b. Number of bases used to calculate the percentahe of sequence identity

c. Taxonomy rank shown as Phylum;Class;Order;Family;Genus

Table S1. Standard quality parameters of the two types of water. SEW: secondary effluent water, TEW: tertiary effluent water

Parameters	SEW Average (min-max) (n)	TEW Average (min-max) (n)
pH	7.7 (7.3-8.0) (8)	7.8 (7.7-7.9) (2)
Electrical conductivity (µS.cm^{-1})	2300 (2060-2540) (8)	2026 (1472-25800) (2)
DBO (mg.L^{-1})	25,8 (11.5-33-5) (7)	ND
DQO (mg.L^{-1})	95.7 (54-144) (7)	ND
Carbonates (mg.L^{-1})	0 (0-0) (8)	<0.9 (<0.8-<0.10) (2)[b]
Bicarbonates (mg.L^{-1})	579.5 (409-726) (8)	459.6 (379.5-539.6) (2)
Sulfates (mg.L$^{-1)}$	137,8 (102-180) (8)	738 (1)
Nitrates (mg.L^{-1})	25,8 (5-50) (8)[a]	5.9 (0.10-11.7) (2)[a]
Chlorides (mg.L^{-1})	355.1 (275-390) (8)	322.28 (280.76-363.8) (2)
Sodium (mg.L^{-1})	253.3 (8-326) (8)	269.5 (221-318) (2)
Potassium (mg.L^{-1})	57.9 (24-260) (8)	20.9 (20.9-21) (2)
Calcium (mg.L^{-1})	74.8 (27-104) (8)	152.7 (122.2-183.2) (2)
Magnesium (mg.L^{-1})	60.1 (45-80) (8)	83.6 (38.9-128.4) (2)
Phosphorous (mg.L^{-1})	6 (1)	ND
Ammonium (mg.L^{-1})	55.5 (18-71) (8)	ND

ND: not determined

[a]: 1 sample below the detection limit

[b]: 2 samples below the detection limit