Bacterial community composition of recycled irrigation water of a NFT-experimental system, with and without a slow sand filter

Giovanni Cafà [1,2, #,*], Richard Thwaites[2] and Matthew J Dickinson[1]

[1] University of Nottingham, Plant Sciences Division, Sutton Bonington Campus, LE12 5RD Loughborough, UK

[2] The Food and Environment Research Agency (FERA), Sand Hutton, YO41 1LZ, York, UK

[#] Present address: CABI, Bakeham Lane, TW20 9TY

* Corresponding author: Giovanni Cafà g.cafa@cabi.org

ABSTRACT

The bacterial community composition (BCC) of recycled irrigation freshwater was monitored on a Nutrient Film Technique (NFT)-type experimental hydroponic system. Two identical NFT systems in a greenhouse were used to grow tomato plants. One was connected to a slow sand filter (SSF), and one was used as control. DNA was isolated from irrigation freshwater, and molecular methods were carried out to characterise the BCC. These included terminal-restriction fragment length polymorphism (T-RFLP), cloning and 454 pyrosequencing. A total number of 291 442 trimmed sequences, of 211 bp average length. A strong differentiation of the bacterial community composition in recycled irrigation water was triggered by the activity of the SSF. Phylogenetic affiliation revealed that *Bacilli*, *Alpha*- and *Gammaproteobacteria*, and *Nitrospira* were differentially abundant during filtration with the SSF. This study showed that SSF modified the relative amount of a set of bacterial genera. These included *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Burkholderia* and *Azospirillum*. These bacteria have been previously described as plant growth promoting rhizobacteria (PGPR). The presence, increased or exclusive, in Water *ssf* of bacteria such as *Bacteroidetes*, *Gemmatimonadetes*, *Nitrospira*, *Firmicutes*, *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*, was associated with an increased biomass in plants. Such findings are promising for future applications of a combined system NFT-SSF: NFT guarantees a controlled closed environment for the growth of plants, while the SSF secures the microbiological balance of recycled irrigation water.

INTRODUCTION

Recycled irrigation freshwater is an emerging agroecosystem of growing interest. In recent years, the possibility of growing plants using re-circulated and replenished nutrient solution has been increasingly investigated worldwide (Garland, 1994; Zhang and Tu, 2000; Alsanius *et al.*, 2001; Postma *et al.*, 2001; Frenkel *et al.*, 2010). It has been demonstrated that the same yields can be obtained in closed systems (where surplus solution is recovered, replenished, and recycled) as compared to open systems (once the nutrient solution is delivered to the plant roots, it is not reused) (Jensen, 1997), as long as good hygienic and environmental conditions are maintained (Gertsson *et al.*, 1994). The use of recycled irrigation freshwater can effectively reduce water usage and mitigate nutrient runoff from nursery production sites. However, serious concerns exist regarding the spread of phytopathogenic microorganisms via the recycled water (Waechter-Kristensen *et al.*, 1997; Pagliaccia *et al.*, 2008).

Irrigation freshwater can be recycled with nutrient film technique (NFT). NFT is a simple and cost effective method for growing plants, where roots are emerged in a continuous flow of re-circulating water containing all the nutrients that plants need. A root mat develops partly in the shallow stream of re-circulating water and partly above it. Thus the stream is very shallow, and the upper surface of the root mat that develops is above the water (Cooper, 1979). NFT can save water and reduce pollution associated with the need to discharge used solutions into the environment (Calvo-Bado *et al.*, 2006).

Recycling the water supply used for irrigation can generate contamination with plant pathogens from several sources. The pathogen may be a natural inhabitant of the water source, or reside in the soil, or in infected resident plants near the water and

only be a transient inhabitant of the irrigation water (Hong and Moorman, 2005). Any infectious propagule has the potential to make contact with the irrigation water and, upon entry into the nutrient solution, can ultimately make contact with a root (Stanghellini and Rasmussen, 1994). This contact, which is possible at several points along the distribution path (Hong and Moorman, 2005), can create a high risk of contamination for the entire crop. NFT systems are particularly exposed to the interaction between water and plant roots because of the density of roots exposed in the air (Stanghellini and Rasmussen, 1994; Clematis *et al.*, 2009). The layout of the NFT method, in which the plants are produced with bare roots, puts the crop in danger and, despite the many advantages introduced by the use of recycled irrigation freshwater, can create the condition for contamination by pathogens (Hong and Moorman, 2005).

Fungi are not the only problem related to recycled irrigation freshwater. The establishment of bacterial pathogens also represents a concern when producing minimally processed ready-to-eat vegetables such as tomatoes and lettuce. *Enterobacteriaceae* such as *Escherichia coli* and *Salmonella* spp. are common in greenhouse production, and the use of soil-less technologies may increase apprehension. Viruses can also spread via irrigation water after being released by plant roots (Büttner *et al.*, 1995): Pelargonium flower break virus can spread in recirculating nutrient solutions in greenhouses (Berkelmann *et al.*, 1995) as can tomato mosaic virus (Pares *et al.*, 1992).

Risk of contamination of irrigation freshwater can, however, be reduced by the use of disinfection methods such as slow sand filtration (SSF). SSF is a disinfection method available since 1974 (Huisman and Wood, 1974, Calvo-Bado et al., 2003). It is a technique that involves the slow passage of water or liquid through a porous

74 medium, which for horticultural uses is commonly constituted of sand. This
75 technology is employed to reduce contaminants from freshwater, as a result of a
76 complex consortium of microorganisms that develops and interacts in the top layer of
77 the sand column, called *schmutzdecke*. The schmutzdecke – a biologically active
78 layer, can generally be considered as a gelatinous biofilm containing a consortium of
79 bacteria, fungi, algae, protozoa, rotifers, and a range of aquatic insect larvae. The
80 activity of the microbiota of the schmutzdecke is directly responsible for much of the
81 treatment function, although it should be stressed that the underlying sand or other
82 bed material is also responsible for the removal of various fractions from the water. A
83 ripening period of 3-6 weeks is required for this layer to form, during which the filter
84 performance is sub-optimal (Joupert and Pillay, 2008).
85 Bacteria are an important component of the schmutzdecke in SSF. Bacterial
86 communities in the schmutzdecke have been studied since 1952 (Calaway *et al.*,
87 1952), where dominant organisms were identified with traditional culture dependent
88 techniques. One of the first studies that applied modern molecular techniques to
89 understand the microbiology of SSF was conducted by Petri-Hansen *et al.* (2006).
90 The bacterial population in the filter was found to be more diverse in distribution
91 between taxonomic groups and of different physiological functions than previously
92 recognised. A large component of the community was comprised of *Proteobacteria*
93 (25%), while the remaining 75% was of less commonly encountered bacterial taxa.
94 Petri-Hansen *et al*. (2006) showed that the bacterial composition of the
95 schmutzdecke was affected by influent temperature, and mainly determined by the
96 autochthonous bacteria from the aquatic environment. However, a more detailed
97 understanding of the bacterial community in SSFs is still needed (Page *et al.*, 2006),
98 as well as the effect of the SSF on the microflora of recycled irrigation freshwater.

The objectives of this study were therefore to investigate the bacterial community composition (BCC) of the recycled irrigation freshwater developing in a NFT-Type experimental system, focusing on the microflora of the water matrix. This study also describes the differences between the BCC of the recycled irrigation freshwater with and without slow sand filtration, focusing on the bacterial taxa that have a higher relative abundance caused by the activity of the SSF.

MATERIAL AND METHODS

Tomato plants on rockwool were grown on NFT-Type experimental systems, with recycled nutrient water fully recycled in the process. DNA was isolated from the water tomato pfor the investigation of the differences of the bacterial community composition (BCC) developing in the recycled irrigation freshwater. Each experiment was carried out in a 28 days cycle with 4 time points (7,14, 21, and 28 days). All data are presented as the average of three replicates of the 28 days experiments (NFT1, NFT2, and NFT3), with the exception of the pyrosequencing analysis, which was performed on one of the three replicates (NFT1).

Nutrient Film Technique (NFT) system. Two identical replicates of a NFT-Type system were established in a glasshouse (Sutton Bonington Campus, University of Nottingham, UK). One was connected to a slow sand filter (SSF, Fig. 1), and the other one was used as the control. Fifty ml samples of recycled irrigation freshwater were collected at 4 time points (7,14, 21, and 28 days) from the two water tanks of the NFT systems, in which tomato plants were grown (*Solanum lycopersicum* cv. Alicante).

Irrigation water was obtained from nutrient stock solution (1:200 VITAFEED-214, VITAX, Leicester, UK) added to tap water. In all the experiments, an initial volume of 100 L of irrigation water was placed in the main tank; three rows of polyvinyl chloride channels (PVC) (Geberit, Aylesford, UK) containing 9 plants each were connected to the 100 L tank, and the pump was activated to allow the continuous flow of water from the top to the bottom of the PVC channels. The PVC channels were kept at an inclination of 1.5° to allow the water to return under gravity before being recirculated by the pump.

Plants were grown from seeds of *Solanum lycopersicum* cv. Alicante (Wilkinson, Worksop, UK) in incubators for 14 days before being transferred to rockwool cubes and relocated to the NFT system. The NFT system allowed roots to develop outside rockwool cubes, and establish a thick net of roots in the PVC channel, with water continuously surrounding the roots of the plants.

The flow of irrigation freshwater through the channels was regulated at 2 L/min, and maintained at a constant rate throughout the experiments. After completion of each replicate of the experiment, the NFT-Type system was thoroughly washed before placing new plants and fresh irrigation freshwater. Each experimental replicate was carried out for 28 days. Samples of 50 ml of recycled irrigation freshwater were collected every 7 days from the tank of the control system (Water *co*) and the tank of the NFT connected to the SSF (Water *ssf*). An equal volume of irrigation freshwater was added at the time of sampling to the two tanks. This volume was variable, and depended on the consumption of water of the system, e.g. a higher volume was added in warmer months of the season.

Characteristics of the Slow Sand Filter (SSF) connected to the NFT system. A SSF was linked to one of the two experimental NFT-Type systems (Fig. 1). The filter

was prepared with a 2 m Terrain PVC pipe of 20 cm diameter mounted vertically. The bottom of the filter was filled with a 30 cm depth of gravel. The column consisted of a sand bed of 1 m, headed with 60 cm of empty space. The latter space was occupied by excess water during runs. The water flow through the column was gravity assisted (speed of water through the column of sand was 0.15 meters per hour) with an outflow of water regulated at a speed of 4 L/h by a valve. An overflow pipe was used to maintain a constant water level above the sand column. The sand ratio consisted of different grain diameters of which 10% was > 1mm, 10% < 0.2mm, and 80% between 0.2mm and 1mm. This composition is considered the most effective to encourage the interaction between microorganisms and water, and allow the water to flow through the sand without being mechanically stopped (Pettitt, 2005). This experimental design determined half of the irrigation freshwater to be pumped to the top of the channels of the NFT-Type system, and the other half sent to the top of the column of the sand filter for biofiltration. Additional samples of sand were collected for molecular tests. These were collected via 'sampling ports' installed in the column (Fig.1) at different layers of the SSF column: the top (1 cm), the middle (50 cm) and the bottom layer (80 cm).

Dry weight of plants. Analysis of variance (ANOVA) was carried out on the dry weight of tomato plants. This allowed the comparison between the dry weight of the plants that were grown with recycled irrigation freshwater filtered with the slow sand filter (treatment plants, ssf-plants), and those grown with non-filtered recycled irrigation freshwater (control plants, co-plants).

DNA isolation. For each DNA extraction three technical replicates were produced. When extracting from water, a negative control of sterile water was processed in parallel with samples of recycled irrigation freshwater to check for contamination.

Genomic DNA was extracted from 50 ml of irrigation freshwater filtered onto a 0.2 µm Whatman nylon filter of 25 mm diameter (Horakova *et al.*, 2008). After filtration, the 0.2 µm Whatman filter was placed in a microtube, with 0.5 g of glass beads (Sigma-Aldrich, Gillingham, UK) and 700 µl of extraction buffer. Each tube was shaken for 3 min in a Fastprep (QBiogene, Cambridge, UK) for cells disruption, for 4 cycles of 45 seconds at 6.5 m s^{-1}. After fastprep, the supernatant was recovered. When extracting DNA from sand, 500 mg of sand were placed directly into the microtube with glass beads and extraction buffer, skipping the step of filtration with 0.2 µm Whatman filter.

To the recovered supernatant, 0.1% (w/v) of sodium dodecyl sulphate (SDS) were added, and the sample was homogenised and placed on ice for 10 min. Seven hundred µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added, followed by centrifugation at 5 000 x g for 15 min. The aqueous phase was recovered, and the DNA was precipitated with 1.5 volume of isopropanol, and 0.1 volume of 0.5 M NaCl. The mixture was incubated overnight at -20°C. After incubation, a centrifugation step was carried out at 10 000 x g for 10 min, and the pellet was washed twice with 300 µl of 70% ethanol. DNA was finally eluted in 30 µl of sterile water. The DNA was further purified by polyvinylpyrrolidone (PVPP) cleanup method (Menking *et al.*, 1999). One hundred mg of PVPP were added to a sterile spin column in a 1.5 ml tube. The column was washed twice with 300 µl of sterile water and centrifuged at 1 000 x g for 2 min. After washing, eluted DNA was added to the column and centrifuged at 1 000 x g for 2 min. DNA concentration was evaluated by 1.5% agarose gel electrophoresis, and by NanoDrop (ThermoScientific, Wilmington, USA).

PCR amplification of ribosomal DNA. Isolated DNA was PCR amplified with the primer pairs for the portions 16S (Muyzer *et al.*, 1993; Muyzer *et al.*, 1995), and the

23S (Anthony *et al.*, 2000) of the rRNA (Table 1). Amplifications were performed in a PTC200 thermocycler (MJ Research, St. Bruno, Quebec, Canada) in 25 µl reactions containing 1 µl of genomic DNA, 12.5 µl of 2X PCR MangoMix (Bioline, London, UK), 0.5 pmol of each primer and 10.5 µl of sterile distilled water. The following cycles were used for the DNA amplification: 1 cycle at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, annealing (Table 1) for 1 min and 72°C for 2 min and 30 s, and a final extension step of 72°C for 10 min. Amplified DNA was verified by gel electrophoresis of aliquots of PCR mixtures (4 µl) in 1.5% of agarose in 1X TBE buffer and ethidium bromide (0.5 µg/ml).

When PCR reactions were performed for T-RFLP, the reverse primer was fluorescently labelled with a Beckman dye at the 5'-end: for 16S rRNA, the primer 926rev was labelled with D3 (D3-926rev), and for 23S rRNA the primer 23Srev was labelled with D4 (D4-23Srev). All the fluorescent dyes are Beckman dyes provided by Sigma Proligo (Sigma-Aldrich). PCR products for T-RFLP were digested with two restriction enzymes (described below) to produce Terminal-Restriction Fragments (T-RFs) for each amplicon generated by PCR amplification. The combination of pairs of T-RFs was used for the identification of microorganisms.

Restriction digestion. Ten µl of PCR product were digested in two separate reactions for each of the two restriction enzymes *Mse*I and *Hae*III (New England Biolabs, Hitchin, UK) in a 20 µl reaction volume containing 1U of restriction enzyme according to the manufacturer's protocols. The digestion mix was incubated at 37°C for 2 hours, followed by enzyme denaturation by heating at 80°C for 20 min. Digestion products were verified by gel electrophoresis of aliquots of the digestion mixture (4 µl) in 2.5% of agarose in 1X TBE buffer and ethidium bromide (0.5 µg/ml).

222 **Terminal Restriction Fragment Length Polymorphism (T-RFLP).** T-RFLPs were
223 determined by electrophoresis with a CEQ™ 8000, Genetic Analyzer System
224 (Beckman Coulter, High Wycombe, UK). The product of restriction analysis was
225 loaded into a 96 well plate with each well containing 38.5 µl of sample loading
226 solution (GenomeLab, Beckman Coulter, High Wycombe, UK) and 0.5 µl of size
227 standard-600 (GenomeLab, Beckman Coulter). Samples were covered with mineral
228 oil and separated on the CEQ™ 8000. Digestions of 16S and 23S rRNA PCR
229 amplicons were run in the same reaction because they were tagged with different
230 dyes, D3 and D4 respectively, each of which is read at a different wavelength; to
231 each well, 1.5 µl of each digestion product was loaded.
232 A quartic polynomial model was run for size standard calibration to improve
233 correlation between expected and actual sizes (McEniry *et al.*, 2008), particularly for
234 fragments in the range 400-600bp (Brodie *et al.*, 2002). T-RFs that differed by <0.5
235 bp in size between replicated profiles were considered identical and only T-RFs that
236 occurred in at least two of the three replicates were included in the analyses (Dunbar
237 *et al.*, 2001). T-RFLP datasets were normalised dividing each peak height value by
238 the sum of all peak height values in the correspondent profile (Hartmann and
239 Widmer, 2008). Analysis of similarities (ANOSIM) was carried out on quality filtered
240 T-RFLP datasets, with Bray-Curtis dissimilarity used to compute distance matrices of
241 correspondence between samples. The ordination method non-metric
242 multidimensional scaling (nMDS) was carried out on distance matrices with the
243 software PAST (http://folk.uio.no/ohammer/past/), with a Shepard plot and a stress
244 value reported for each plot.
245 Putative phylogenetic identities of T-RFs were assigned using a database obtained
246 from the collection of known sequences from NCBI (http://www.ncbi.nlm.nih.gov).

The DNA sequences were digested *in silico* with the software pDRAW32 (AcaClone Software, http://www.acaclone.com/), which identified all the restriction sites that were present in the DNA sequences. The database provided pairs of expected gene-enzyme combinations specific for each group of microorganisms, which were ultimately compared with experimental T-RFLP datasets.

Purification of PCR products. PCR products, when used as inserts for cloning reactions, were purified with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's protocol. After elution in water, the concentration of PCR product was calculated with NanoDrop.

Ligation and cloning. Purified ribosomal DNA amplicons were used as inserts for the ligation into pGEM®-T Easy Vector (Promega, Madison, WI USA). The ligation reaction was carried out in 10 µl volumes with 3U of T4 DNA ligase, 5 µl of 2X ligation buffer, 1µl of pGEM®-T Easy (50 ng/µl) and 22.5 ng of purified PCR product, and incubated overnight at 4°C. Promega *Escherichia coli* JM109 cells were transformed according to the manufacturer's protocol.

Transformed cells were incubated at 37°C in Petri dishes with Luria Bertani (LB) medium, containing agar 15 g/L, IPTG (Isopropyl β-D-1-thiogalactopyranoside) 0.05 mM, X-GAL (5-bromo-4-chloro-3-indolyl-betagalactoside) 80 µg/ml and Ampicillin 100 µg/ml (Sambrook *et al.*, 1989). White colonies (potential positive clones) were selected and screened by colony PCR with vector-targeted PCR: M13for (5'-GTAAAACGACGGCCAGT-3') and M13rev (5'-CAGGAAACAGCTATGAC-3') were used in 25 µl of PCR reaction in the same conditions as above, with an annealing temperature of 56°C. Amplified DNA was verified by 1.5% agarose electrophoresis. Positive clones from the colony PCR were checked by restriction analysis with the two restriction enzymes *Mse*I and *Hae*III. Clones displaying different banding

patterns were selected for sequencing with a Beckman CEQ™8000 automated sequencer.

Pyrosequencing of the V6 region of 16S rRNA. Pyrosequencing reads were obtained from PCR amplicons of the V6 hyper variable region of the 16S rRNA (Table 1) using a Roche 454™ pyrosequencer (Roche, Basel, Switzerland) available at FERA (Food and Environment Research Agency, York, UK).

Amplifications were performed in a GeneAmp PCR system 9700 (Life Technologies Ltd, Paisley, UK) in 25 µl reaction volume containing 1 µl of genomic DNA, 5 µl of 5X KAPAHiFi fidelity buffer (KAPABIOSYSTEMS, Boston, MA USA), 0.75 µl of a mix of dNTPs 10 mM each, 0.5 pmol of each primer, 0.5 µl KAPAHiFi™ HotStart DNA polymerase and sterile distilled water. The following cycling conditions were used: 1 cycle at 95°C for 4 min followed by 35 cycles of 98°C for 20 sec, annealing at 57°C for 15 sec and 72°C for 30 sec, with a final extension step of 72°C for 5 min. Amplified DNA was verified by gel electrophoresis of aliquots of the PCR mixtures (4 µl) in 1.5% of agarose in 1X TBE buffer and ethidium bromide (0.5 µg/ml). The forward primer contained the adapter forward (5'-CGTATCGCCTCCCTCGCGCCATCAG-3'), and the reverse primer the adapter reverse (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3'). These two adapters were used in the library preparation step of pyrosequencing to create a bond between single stranded amplicons and glass beads. Furthermore, the forward primer was provided of a specific 10 nucleotide (-nt) multiple identifier (MID) that was added to the 3'-end of the adapter and to the 5'-end of the forward primer. A set of 4 different MIDs (MID1 to 4 according to manufacturer's indications) was used to combine different samples in the same reaction mixture, and retrieve the original sample composition at the end of the pyrosequencing reaction. PCR products of an

297 approximate length of 280-nt were quantified with PicoGreen (Life Technologies)
298 followed by quality analyses performed according to the manufacturer's instructions.
299 Equal amounts of samples were mixed in groups of 4 (containing the 4 different
300 MIDs) and run overnight for the pyrosequencing reaction.
301 **Pyrosequencing data analysis.** Raw pyrosequencing data were further analysed
302 after the quality filtering provided by the 454 pyrosequencer. Samples composition
303 was investigated through diversity measures, while the phylogenetic identity of the
304 DNA reads was evaluated with a) RDP classifier (Cole *et al.*, 2009) and b) BLAST
305 searches, followed by MEGAN (Huson et al., 2007).
306 The software mothur (Schloss *et al.*, 2009) and the RDP pipeline (Cole *et al.*, 2009)
307 were used in combination for the trimming, alignment and clustering of the
308 pyrosequencing reads. The statistical and graphics software package R (www.r-
309 project.org) was used to generate rarefaction curves, and the software EstimateS
310 (Colwell, 2006) to produce diversity indices. In addition, diversity measures were
311 used to compare the two datasets obtained with T-RFLP and pyrosequencing (Table
312 5).
313 Quality filtered sequencing reads were trimmed according to published
314 recommendations (Huse *et al.*, 2007) using the RDP pyrosequencing pipeline (Cole
315 *et al.*, 2009), with reads shorter than 50bp and containing ambiguous bases
316 excluded from the analysis. Pyrosequencing reads were aligned with Infernal
317 (Nawrocki and Eddy, 2007), followed by complete linkage clustering of the RDP
318 pipeline.
319 The Naïve Bayesian classifier RDP-classifier (Wang *et al.*, 2007) provided rapid
320 phylogenetic assignment for taxonomic classification. This method was used to
321 compare the DNA reads obtained from filtered recycled irrigation freshwater (Water

322 *sff*), the control recycled irrigation freshwater (Water *co*), and the sand of the top
323 layer of the SSF (Sand *top*) throughout the 4 time points.
324 The analysis of the time point '28 days' was implemented with a BLASTn analysis
325 followed by the phylogenetic analysis with the software MEGAN (Huson *et al.*, 2007).
326 MEGAN provides hierarchical tree construction based on BLAST searches (Altschul
327 *et al.*, 1990), assigning the 16S rRNA gene reads to NCBI taxonomy. MEGAN also
328 calculates relative abundances of pyrosequencing reads among samples. BLAST
329 searches give a more accurate analysis of query sequences as compared to the
330 RDP classifier; however, the production of the BLASTn file (input file for MEGAN) is
331 highly time consuming and requires powerful processing units. For this reason,
332 BLASTn files were produced for the '28 days' time point only, with the assumption
333 that this time point would be the most informative of the 4.
334 **Diversity indices and richness estimators.** The diversity of bacteria in recycled
335 irrigation freshwater was estimated to compare the molecular methods T-RFLP and
336 pyrosequencing (Table 5). The richness estimators Chao1 (Chao and Bunge, 2002)
337 and ACE (Chao *et al.*, 2006) were used for the analysis of pyrosequencing data.
338
339 **RESULTS**
340 **Plant growth.** The dry weight of the plants *Solanum lycopersicum* cv. Alicante,
341 grown in the experimental NFT-Type systems were used to assess the effect of slow
342 sand filtration on plant growth. ANOVA (Figure 2) showed that plant growth was
343 significantly affected by the filtration with the slow sand filter ($F_{1,18} = 4.78$, $p = 0.036$).
344 Plants grown with filtered recycled irrigation freshwater (*ssf*-plants) had a larger
345 biomass than those grown with control recycled irrigation freshwater (*co*-plants).

346 **Bacterial Community Composition (BCC) with T-RFLP.** ANOSIM was performed
347 on T-RFLP datasets (Table 2) to test the effect of the slow sand filtration (H_0 ssf) and
348 time (H_0 time) on the BCC of recycled irrigation freshwater. Slow sand filtration and
349 time had a significant effect on the BCC of recycled irrigation freshwater. Datasets
350 were analysed to test the null hypothesis that i) there were no differences between
351 datasets over the 4 time points (H_0 time); and ii) there were no differences in the
352 BCC between filtered recycled irrigation freshwater (Water *ssf*) and control recycled
353 irrigation freshwater (Water *co*) (H_0 ssf). The results from 16S and 23S rRNA genes
354 showed R-values between 0.8 and 1 with all gene-enzyme combinations, and were
355 supported by low probability values (all <0.0001).
356 Multivariate statistical tests of T-RFLP datasets with nMDS showed that samples of
357 Water *ssf* occupied the same part of the plot starting at 14 days of filtration (Fig. 3).
358 This was particularly clear with the gene-enzyme combination 23S-*Mse*I: the nMDS
359 plot shows that after 7 days, Water *ssf* and Water *co* (black spots and red crosses
360 respectively) grouped together on the left hand side of the plot. For the other time
361 points, samples of Water *co* plotted in the top right hand side, while Water *ssf*
362 grouped together and seemed to stabilise in the same position of the plot after 14, 21
363 and 28 days.
364 **Pyrosequencing of the V6 region of 16S rRNA gene.** Pyrosequencing analysis
365 was performed on the hypervariable V6 region of the 16S rRNA. A total of 332 608
366 DNA reads were obtained by 454 pyrosequencing (Table 3). Of those, 291 442
367 (87.6%) passed the quality filter check, with an average length of 211 bp. DNA reads
368 were further analysed with the RDP pyrosequencing pipeline and mothur.
369 For the description of the communities, trimmed sequences were clustered into
370 groups of defined sequence variation that ranged from unique sequences (no

371 variation) to 10% differences by mothur. These clusters were then used to plot OTUs
372 versus the number of tags for generating rarefaction curves (data not shown) and for
373 obtaining richness estimators such as the abundance-coverage estimator (ACE) and
374 Chao1. These indices showed that the species diversity estimation was of the third
375 order of magnitude. At 97% similarity, Chao1 predicted that the maximum number of
376 OTUs was between 11 016 and 15 100 for Water *ssf*, 4 530 and 17 369 for Water *co*
377 and 7 271 to 16 126 for sand of the top layer (Sand *top*) (Table 4).
378 ACE and Chao1 constantly estimated a number of OTUs higher than those that were
379 effectively obtained, suggesting that sampling represented a limitation for the
380 identification of the total number of species.
381 **Bayesian hierarchical identification of pyrosequencing reads.** Pyrosequencing
382 reads of recycled irrigation freshwater and the sand of the top layer of the SSF were
383 phylogenetically assigned with the Naïve Bayesian classifier of the RDP-classifier
384 (Fig. 4). Major changes were observed for the classes of *Bacilli*, *Alpha*- and
385 *Gammaproteobacteria* and *Nitrospira*. The latter taxon was more abundant in Water
386 *ssf* and Sand *top*, while only a minor number of reads were detected in Water *co*.
387 *Alphaproteobacteria* decreased in relative abundance throughout the 4 time points.
388 *Gammaproteobacteria* were less abundant in Water *ssf* after 28 days as compared
389 to Water *co*. *Bacilli* increased their relative abundance in three of the four time
390 points. Only after 21 days, *Bacilli* were more abundant in the Water *co* as compared
391 to Water *ssf*.
392 **Phylogenetic identification of pyrosequencing reads using MEGAN.** To provide
393 a higher resolution of phylogenetic assignment beyond the class level, recycled
394 irrigation freshwater and sand of the top layer of the SSF were analysed with
395 BLASTn and MEGAN (Fig 5). Slow sand filtration increased the number of taxa in

396 the recycled irrigation freshwater from 26 of Water *co* to 45 of Water *ssf*. The three
397 investigated samples (Water *ssf* after 28 days-28 *ssf*; Water *co* after 28 days-28 *co*;
398 and Sand *top* after 28 days-28 *top*) shared 17 genera, with only 6 shared by Water
399 *ssf* and Water *co*. Overall, the most abundant phyla were *Actinobacteria*,
400 *Bacteroidetes*, *Firmicutes* and *Proteobacteria*.
401 Several groups of bacteria were exclusively present in Water *ssf (*28 *ssf*). These taxa
402 included *Fluviicola* and *Flavobacterium* of the class of *Bacteroidetes*, *Deinococcus* of
403 the class of *Deinococci*, *Exiguobacterium* and *Lactobacillus* of the class of *Bacilli*,
404 *Prosthecomicrobium*, *Methylobacterium*, *Rhizobium*, *Sinorhizobium*, *Stappia*,
405 *Magnetospirillum* and unclassified *Rickettsiales* of the class of *Alphaproteobacteria*,
406 *Burkholderia*, *Acidovorax*, *Delftia* and *Aquabacterium* of the class of
407 *Betaproteobacteria*, and finally *Acinetobacter* and unclassified
408 *Gammaproteobacteria*.
409 Other groups of bacteria increased their relative abundance after 28 days of filtration
410 with the SSF. These included unclassified *Gemmatimonadetes*, *Nitrospira* of the
411 phylum *Nitrospira*, *Azorhizobium*, *Rhodobacter*, *Roseobacter* and *Stella* of the class
412 of *Alphaproteobacteria*, *Nitrosomonas* and *Azoarcus* of the class of
413 *Betaproteobacteria*, *Bdellovibrio* and *Geobacter* of the class of *Deltaproteobacteria*,
414 and finally *Pseudomonas* of the class of *Gammaproteobacteria*. Interestingly, two
415 microorganisms responsible for two different step of the oxidation of nitrogen were
416 enhanced by the slow sand filtration, such as *Nitrosomonas* and *Nitrospira*. In
417 particular, the latter showed a large increase in number of reads between Water *ssf*
418 and Water *co*, with 1749 reads against 18.
419 Other microorganisms showed a reduction of relative abundance between Water *ssf*
420 and Water *co.* These included *Microbacterium* of the class of *Actinobacteria*,

421 *Sphingomonas* and *Azospirillum* of the class of *Alphaproteobacteria*, unclassified
422 *Alcaligenaceae* of the class of *Betaproteobacteria* and unclassified
423 *Enterobacteriaceae* of the class of *Gammaproteobacteria*.
424 Finally, some organisms did not appear in Water *ssf*, while did in Water *co*. These
425 included, *Leucobacter* and *Streptomyces* of the class of *Actinobacteria, Pedobacter*
426 of the class of *Sphingobacteria*, unidentified *Neisseriaceae* of the class of
427 *Betaproteobacteria,* and *Legionella* of the class of *Gammaproteobacteria*.
428 **Comparison of pyrosequencing and T-RFLP.** T-RFLP and pyrosequencing results
429 showed some degrees of similarity. T-RFLP provided a rapid overview of changes in
430 dominant members of the community and, when implemented with clone libraries,
431 their phylogenetic identification. However, pyrosequencing data provided in depth
432 information about changes in the relative amount of thousands of OTUs, which were,
433 at least, of 2 orders of magnitude higher than T-RFLP.
434 T-RFLP underestimated the diversity of the systems expressed by Shannon and
435 Simpson indices (Table 5). These two indicators were always lower for T-RFLP data.
436 Nonetheless, T-RFLP provided reliable information about major groups of bacteria
437 and their patterns of relative abundances. 16S rRNA T-RFLP detected the increase
438 of *Pseudomonas* sp., *Cellvibrio* sp. and *Nitrosospira* sp. and the decrease of
439 *Novosphingobium* sp. in Water *ssf* as compared to Water *co* (Table 6). When T-
440 RFLP was performed on 23S rRNA amplicons, an increase was detected for
441 *Azoarcus aromaticum*, *Paenibacillus* sp. and *Ochrobactrum intermedium*, while a
442 decrease was detected for *Sphingomonas* sp.
443
444

DISCUSSION

A strong differentiation of the bacterial community composition (BCC) in recycled irrigation freshwater was triggered by the activity of the slow sand filter (SSF). The bioactive layer of the SSF, the schmutzdecke, affects the BCC of recycled irrigation freshwater. Specifically, phylogenetic affiliation of DNA reads revealed that the most affected classes of bacteria were *Bacilli*, *Alpha*- and *Gammaproteobacteria*, and *Nitrospira*. In addition, a larger diversity of bacteria was detected in recycled irrigation freshwater filtered with the SSF (Water *ssf*), as compared to control recycled irrigation freshwater (Water *co*): after 28 days of a NFT-type experiment, pyrosequencing data detected 45 taxa in Water *ssf*, against 26 in Water *co*. In addition, plant growth was enhanced when recycled irrigation freshwater was filtered with the SSF (Fig. 2). The activity of the schmutzdecke has been previously reported to be able to affect the BBC (Joupert and Pillay, 2008), but also to affect the abundance of common plant pathogens such as zoosporic fungi (Calvo-Bado *et al.*, 2003; Calvo-Bado *et al.*, 2006; Deniel *et al.*, 2006). Further experiments were carried out to estimate whether the schmutzdecke needs adaption to be able to carry out its function in the SSF. Such tests confirmed that maturity is reached after 8 weeks (data not shown). The qualities of a SSF are promising for future applications of a combined system NFT-SSF: NFT guarantees a controlled closed environment for the growth of plants, while the SSF secures a microbiological balance of the system, which, in our experiments, has shown to enhance plant growth (Fig. 2).

Four main phyla of bacteria were detected as major components of the microflora of recycled irrigation freshwater. These included *Actinobacteria*, *Proteobacteria*, *Firmicutes Bacteroidetes*. High diversity was detected within these groups of bacteria, indicating that the nutrient solution of a hydroponic system consists of a rich

470 microbiological environment (Berkelmann *et al.*, 1994). Major taxa within the above
471 phyla that were identified in this study have been previously described as common
472 inhabitant of nutrient solutions. These included *Pseudomonadaceae*,
473 *Xanthomonadaceae*, *Rhizobiaceae* and *Bacillaceae* (Berkelmann *et al.*, 1994;
474 Koohakan *et al.*, 2004; Calvo-Bado *et al.*, 2006). In addition, several species
475 detected in this study have been previously reported as efficient biocontrol agent
476 against the common plant pathogen *Fusarium oxysporum*. These included *Bacillus*
477 *subtilis* (Baysal *et al.*, 2008), *Bacillus megaterium* and *Burkolderia cepacia* (Omar *et*
478 *al.*, 2006),
479 The activity of the SSF determined a change in the BCC of recycled irrigation
480 freshwater. This study showed that SSF influenced the relative abundance of a set of
481 bacterial genera such as *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Burkholderia* and
482 *Azospirillum*. These bacteria have been previously described as plant growth
483 promoting rhizobacteria (PGPR) (Glick, 1995; Lucy *et al.*, 2004). Several reports
484 have suggested that PGPR stimulate plant growth by facilitating the uptake of
485 minerals into the plant (Kloepper *et al.*, 1988). However, there is some controversy
486 regarding the mechanisms that PGPR employ in the uptake of minerals (Bashan *et*
487 *al.*, 1990). Increased plant growth was observed when the nutrient solution was
488 filtered with the SSF, after the period of ripening. However, no evidence has been
489 previously reported of plant growth promotion by slow sand filtration. To support
490 such findings, further work should be addressed at the isolation and deployment of
491 specific consortia of microorganisms, in order to test their efficiency alone. Ideally,
492 this should facilitate the formulation of recipes of consortia of microorganisms with
493 which recycled nutrient solution in soilless hydroponic systems should be enriched.

494 The presence, increased or exclusive, in Water *ssf* of bacteria such as
495 *Bacteroidetes*, *Gemmatimonadetes*, *Nitrospira*, *Firmicutes*, *Alpha*-, *Beta*-, *Gamma*-
496 and *Deltaproteobacteria*, was associated with an increased biomass in plants.
497 However, it remains unclear whether physical-chemical interactions in the sand bed
498 of the SSF actively contribute to the plant growth promoting effect. For instance,
499 small scale sand filters could be used to prime nutrient solutions of recycled irrigation
500 water, enabling the enrichment of the bacterial population, followed by an incubation
501 period for the multiplication of the bacteria before employing the solution in
502 hydroponic systems. The use of a slow sand filter represents a natural and
503 inexpensive biological solution to enrich the bacterial population of recycled irrigation
504 water, in a system where higher diversity reduces risks of colonization of single
505 species (Stecher *et al.*, 2010). Furthermore, these results increase scepticism on the
506 use of disinfection methods that greatly reduce the microbial content such as UV
507 disinfection (Zhang and Tu, 2000) and heat treatments (Runia *et al.*, 1988). These
508 findings suggest that a higher biodiversity of the BCC in the recycled irrigation
509 freshwater has positive effects for the ecosystem, in which multiple interactions
510 among species of several different phyla contribute to the efficiency of the NFT-Type
511 experimental system.
512 The integration of fingerprinting and next generation sequencing provided the
513 identification of microorganisms in recycled irrigation water. In this work, T-RFLP
514 was developed for detecting microorganisms in water, using experimental tests to
515 estimate the reliability and sensitivity of the method. These procedures showed that
516 bacteria inoculated in sterile water are detected when their concentration is above
517 the threshold of 10^3 cells ml^{-1}, and that an identical T-RFLP profile is obtained from
518 overlaid T-RFLP profiles of single bacterial colonies, after DNA isolation/PCR/T-

519 RFLP procedures, only when the concentration of cells in freshwater is at least of 10^6
520 ml^{-1} (data not shown). At this concentration, T-RFLP is reliable and able to estimate
521 relative amount of single species. T-RFLP has the advantage of being a rapid and
522 reliable method that, at affordable prices, provides an overview of the system
523 nonetheless. Another advantage of T-RFLP is that it provides datasets that can used
524 to test complex hypotheses with multivariate statistics, which remains extremely
525 useful when comparing different environments. In addition, this method showed
526 complementarity with pyrosequencing results. Other authors (Jakobsson *et al.*, 2010)
527 have reported congruence between T-RFLP and pyrosequencing results. Our data
528 supported such findings, showing that T-RFLP provides accurate information about
529 dominant groups of microorganisms. Pyrosequencing, on the other hand, provides in
530 depth information on thousands of operational taxonomic units (OTUs).
531 The analysis of pyrosequencing data produced useful species richness estimators
532 that facilitated the ecosystem characterization. For example, Chao1 (Chao *et al.*,
533 2006) was used to predict the species diversity of recycled irrigation water. This
534 estimation, on average, showed that 50 ml contained around 10 000 OTUs at 97%
535 similarity, going down to around 5 000 OTUs at 90% similarity. Other authors have
536 previously estimated this diversity: Berkelmann *et al.* (1994) showed that the nutrient
537 solution of a hydroponic system with rockwool as substrate, can contain up to 10^5-
538 10^6 colony-forming units (CFU) ml^{-1} after 20 hours from planting tomato plants. Other
539 attempts to estimate richness have mainly focused on other matrices rather than
540 water. For example, using 1 gram of soil as the unit, the estimation of OTUs is
541 considered to be between 2 000 and 5 000 (Schloss and Handelsman, 2005), and
542 the number of distinct genomes, based on DNA reassociation kinetics, between 2
543 000 and 18 000 (Torsvik *et al.*, 1990; Torsvik *et al.*, 1996; Sandaa *et al.*, 1999;

544 Dunbar *et al.*, 2001). Clearly, traditional microbiological methods for the isolation of
545 microorganisms have the potential to determine only dominant populations, and tend
546 to mask the detection of low-abundance species (Sogin *et al.*, 2006). The large
547 number of highly diverse, low-abundance species in an ecosystem constitutes a rare
548 'biosphere' that is largely unexplored. Recent developments in high throughput
549 molecular methods are beginning to provide deep insights into a wide range of
550 ecosystems, supplying information on microorganisms and their roles in the
551 environment.
552 In conclusion, this study shows that the BCC of recycled irrigation freshwater is a
553 diverse ecosystem, and supports the finding of Berkelmann *et al.* (1994), for which
554 irrigation freshwater should be carefully preserved in order to avoid the colonisation
555 of available ecological niches by single species and/or dangerous pathogens. In
556 addition, in this study the use of a SSF showed that the BCC of irrigation freshwater
557 can be altered by biofiltration, and that this had positive effect on plant growth. The
558 use of fingerprinting methods such as T-RFLP provided useful information on the
559 dynamics of bacterial populations in freshwater, and allowed rapid and inexpensive
560 monitoring of the microflora. A consistant monitoring of recycled irrigation freshwater,
561 however, remains of extremely importance, in order to avoid the intrusion of
562 allochthonous microorganisms that can occur anytime in the distribution path of a
563 hydroponic system (Hong and Moorman, 2005). Also, when dealing with closed
564 hydroponic systems, this aspect becomes even more important: the invasion of
565 external species has the potential of spreading to the entire crop, assuming that the
566 environmental conditions are favourable for the pathogen.
567
568

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746
747

FIGURE LEGENDS

Figure 1 Schematic representation of the experimental NFT system connected to the slow sand filter. Recycled irrigation water was directed to the top of the column of the sand filter and to the channels of the NFT with the aid of a pump (*). Channels of PVC had an inclination of 1.5° for allowing the water to flow down the channels and returning to the tank and restart the cycle. Arrows show the direction of recycled irrigation water. The figure is not to scale.

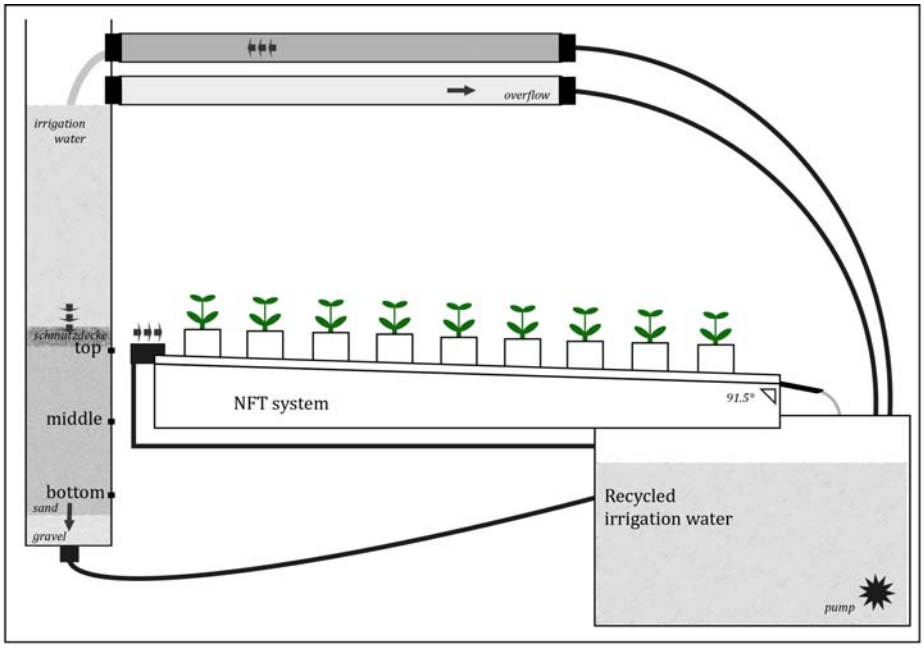
Figure 2 Dry weight of tomato plants grown in the NFT-type experimental system. Error bars indicate S.E.M.

Figure 3 Non-metric multidimensional scaling of microorganisms inhabiting the recycled irrigation water of the experimental NFT system, using T-RFLP profiles of 16S rRNA (a, c) and 23S rRNA (b, d). Each shape represents a different time point and a different water sample of filtered (Water *ssf*) and non filtered (Water *co*) recycled irrigation water: black dots 7ssf; red crosses are 7co; blue squares 14ssf; pink filled squares 14co; green crosses 21ssf; purple circles 21co; green diamonds 28ssf; and blue stars 28co. Elliptic shapes group samples of filtered (*ssf*) recycled irrigation water after 14, 21 and 28 days. Each rRNA gene is represented by two profiles obtained with two different restriction enzymes: 16S *Mse*I (a) and *Hae*III(c), 23S *Mse*I (b) and *Hae*III(d). Each nMDS plot is provided with correspondent stress value and regression analysis.

Figure 4 Major classes of bacteria identified with the pyrosequencing of the V6 region of bacterial 16S rRNA. Plots show relative abundance (percentage) of the

773 reads obtained at the 4 time points (7, 14, 21 and 28 days) for a) filtered recycled
774 irrigation water (Water *ssf*), b) control recycled irrigation water (Water *co*) and c) top
775 layer of the slow sand filter (Sand *top*).
776
777 **Figure 5** V6 amplicon sequences from the three groups of samples assigned with
778 BLAST and MEGAN. Coloured bars display the relative abundance for each taxon of
779 bacteria.
780

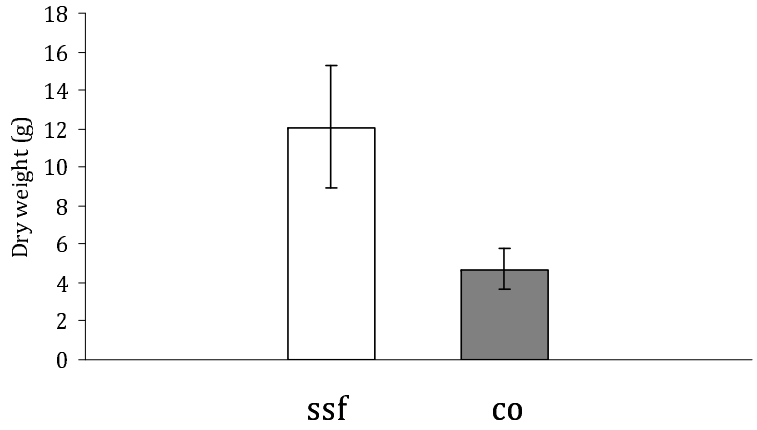
781 **FIGURES**



782

783 **Figure 1**

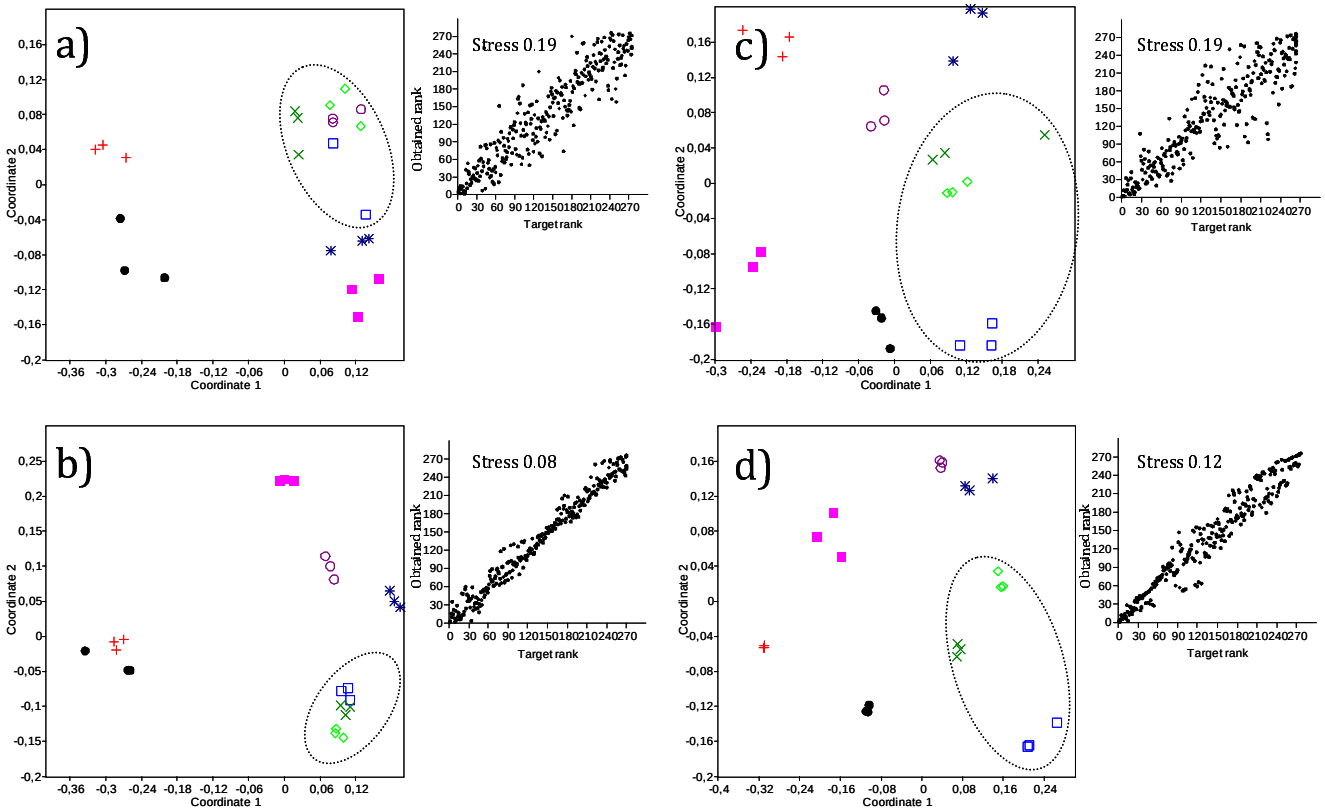
784



785

786 **Figure 2**

787



788

789 **Figure 3**

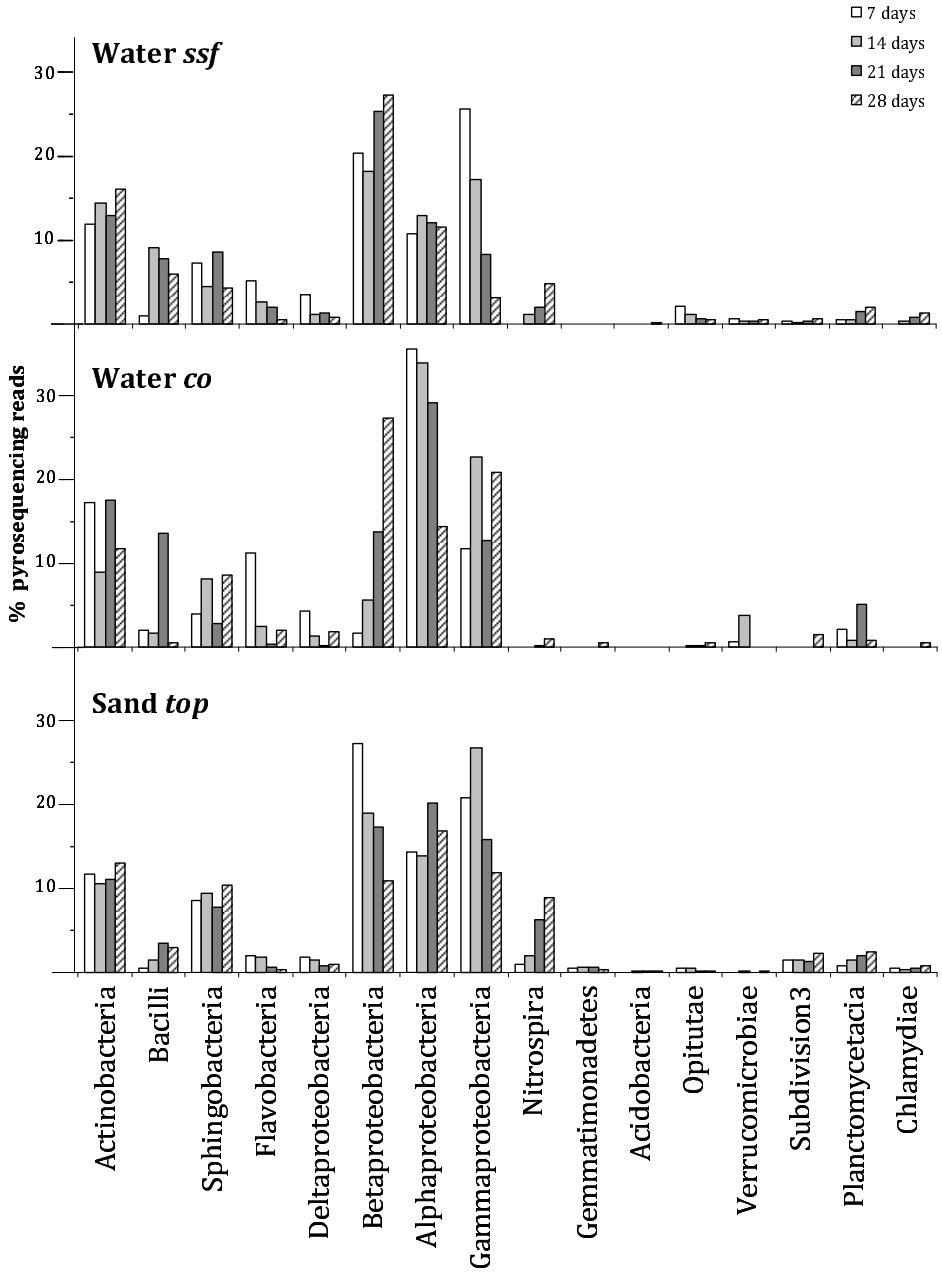


Figure 4

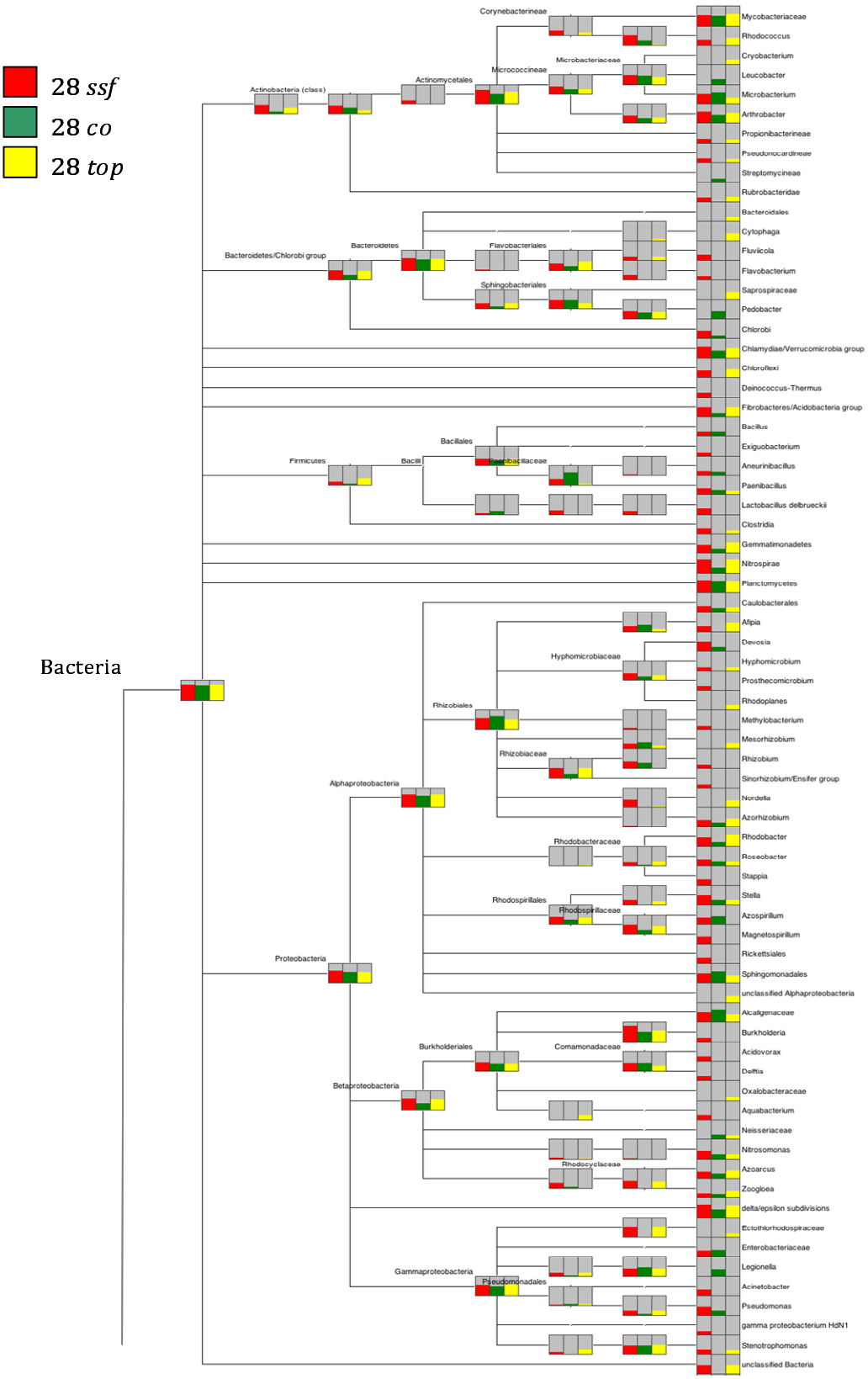


Figure 5

795 **TABLES**

Table 1 Primer pairs used in this study for the amplification of ribosomal DNA for T-RFLP and pyrosequencing

Gene	Primer	Method	Sequence (5'→3')[a]	Annealing Temperature (ºC)
23S rRNA	23Sfor 23Srev	T-RFLP	GCGATTTCYGAAYGGGGRAACCC TTCGCCTTTCCCTCACGGTACT	63
16S rRNA	341for 926rev	T-RFLP	CCTACGGGAGGCAGCAG CCGTCAATTCCTTTRAGTTT	52
16S rRNA	V6for V6rev	Pyrosequencing	AACAGGATTAGATACCCTGGTAGTC ACAYCCYTGCASCACCT	57

[a] R=A+G, Y=C+T, S=C+G

Table 2 Observed two-way ANOSIM test values and probabilities of null hypotheses (H_0) tests obtained comparing T-RFLP datasets of 16S and 23S rRNA genes with the enzymes *Mse*I and *Hae*III. H_0 was tested between time points (H_0 time) and water sample coming from recycled irrigation water treated with the slow sand filter and control water (H_0 ssf)

NFT		***Mse*I**		***Hae*III**	
	Gene	**R-value**	***Probability***	**R-value**	***Probability***
NFT1		H_0 time			
	16S	0.81778	< 0.0001	0.93519	< 0.0001
	23S	0.99537	< 0.0001	0.82469	< 0.0001
		H_0 ssf			
	16S	0.85417	< 0.0001	1	< 0.0001
	23S	0.87963	< 0.0001	1	< 0.0001
NFT2		H_0 time			
	16S	0.98611	< 0.0001	0.96759	< 0.0001
	23S	0.89198	< 0.0001	0.94599	< 0.0001
		H_0 ssf			
	16S	0.98148	< 0.0001	0.99074	< 0.0001
	23S	0.87037	< 0.0001	0.92593	< 0.0001
NFT3		H_0 time			
	16S	0.77006	< 0.0001	0.97840	< 0.0001
	23S	1	< 0.0001	1	< 0.0001
		H_0 ssf			
	16S	0.83333	< 0.0001	0.97222	< 0.0001
	23S	1	< 0.0001	1	< 0.0001

807 **Table 3** Data summary of total reads from pyrosequencing data. “Trimmed reads”
808 represent the number of DNA sequences longer than 50bp that were kept after
809 quality filtering. “Unique reads” represent the number of distinct sequences within a
810 set of Trimmed reads
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Sample ID	Total reads	Trimmed reads	Unique reads
7ssf	25005	22951	11237
14ssf	31852	28133	15083
21ssf	34518	29400	15282
28ssf	23580	21125	11525
7co	21080	16967	7367
14co	58995	52248	23295
21co	21107	19845	8872
28co	8808	6740	3803
7top	22842	21191	10697
14top	42472	38961	19473
21top	26581	22842	11723
28top	15768	11039	6131
∑	332608	291442	144488

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815 **Table 4** Similarity-based OTUs and species richness estimators

Sample ID	Trimmed reads	Cluster distance								
		0.03			0.05			0.1		
		OTUs	ACE	Chao1	OTUs	ACE	Chao1	OTUs	ACE	Chao1
7ssf	22951	4942	18053	11016	3848	11713	7819	2730	6831	4929
14ssf	28133	6344	22750	13970	4900	14660	9875	3434	8449	6115
21ssf	29400	6634	23351	15100	5126	16287	10828	3602	8582	6221
28ssf	21125	5684	22455	13288	4642	16133	9893	3385	9673	6549
7co	16967	3107	10495	6868	2378	7269	4936	1648	4469	3165
14co	52248	8468	26004	17369	6211	16225	11847	3954	8314	6801
21co	19845	3567	10942	7436	2661	6893	4953	1738	3729	3053
28co	6740	1883	7916	4530	1522	5340	3397	1102	3448	2258
7top	21191	4928	18547	11588	3768	12325	8001	2564	7089	4875
14top	38961	7507	24877	16126	5604	15738	10928	3624	7872	5976
21top	22842	5244	17773	11664	4087	12364	8425	2820	7378	5257
28top	11039	3147	11409	7271	2518	8312	5440	1825	5921	3757

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Table 5 Number of OTUs and diversity indices obtained from T-RFLP and pyrosequencing of the 16S region of rRNA

Sample ID	T-RFLP						Pyrosequencing cluster distance								
	16S-*Mse*I			16S-*Hae*III			0.03			0.05			0.1		
	OTUs	*H*' [a]	1-D	OTUs	*H*'	1-D	OTUs	*H*'	1-D	OTUs	*H*'	1-D	OTUs	*H*'	1-D
7ssf	4	1.26	0.66	4	1.42	0.71	4942	5.43	0.98	3848	5.21	0.98	2730	4.78	0.97
14ssf	5	0.96	0.42	3	1.06	0.64	6344	5.64	0.99	4900	5.41	0.99	3434	4.93	0.98
21ssf	16	2.54	0.90	17	2.54	0.90	6634	5.52	0.98	5126	5.28	0.98	3602	4.83	0.97
28ssf	13	2.20	0.84	13	2.12	0.82	5684	5.79	0.97	4642	5.56	0.97	3385	5.06	0.96
7co	11	2.07	0.84	5	1.43	0.72	3107	4.55	0.97	2378	4.34	0.97	1648	3.94	0.96
14co	6	1.63	0.77	24	2.89	0.93	8468	5.19	0.98	6211	5.00	0.98	3954	4.62	0.98
21co	15	2.53	0.91	6	1.56	0.76	3567	4.87	0.97	2661	4.70	0.97	1738	4.24	0.96
28co	16	2.50	0.90	18	2.61	0.91	1883	4.90	0.98	1522	4.67	0.98	1102	4.19	0.97
7top	14	2.24	0.86	13	2.04	0.80	4928	5.50	0.98	3768	5.28	0.98	2564	4.82	0.98
14top	11	2.06	0.83	21	2.65	0.90	7507	5.51	0.98	5604	5.27	0.97	3624	4.74	0.97
21top	22	2.78	0.92	18	2.51	0.89	5244	5.73	0.96	4087	5.50	0.96	2820	4.93	0.94
28top	28	3.10	0.95	18	2.62	0.91	3147	5.52	0.98	2518	5.30	0.98	1825	4.84	0.98

[a] *H*' = Shannon index; 1-D = Simpson's index

Table 6 Major microorganisms identified by T-RFLP and clone libraries. The column Relative abundance indicates the increase (arrows going upwards), or the decrease (arrows going downwards) of the relative amount of fluorescence in filtered water (Water *ssf*) as compared to control water (Water *co*)

Gene	Genus/Species	T-RFs		Relative abundance
		***Mse*I**	***Hae*III**	
16S rRNA	*Cellvibrio* sp.	589	509	↑*
	Nitrosospira sp.	588	588	↑*
	Novosphingobium sp.	564	484	↓
	Pseudomonas sp.	374	31 (585)	↑*
23S rRNA	*Azoarcus aromaticum*	372	312	↑*
	Paenibacillus sp.	422	169	↑
	Ochrobactrum intermedium	515	201	↑
	Sphingomonas sp.	337	202	↓

* indicates that the difference of relative abundance between filtered water (Water *ssf*) and control water (Water *co*) was statistically significant