Comparative analysis of the composition of bacterial communities from two constructed wetlands for municipal and swine wastewater treatment

Paula Arroyo, Gemma Ansola, Ivan Blanco, Patricia Molleda, Estanislao de Luis Calabuig and Luis E. Sáenz de Miera

ABSTRACT

This work provides information about bacterial community structure in natural wastewater treatment systems treating different types of wastewater. The diversity and composition of bacterial communities associated with the rhizosphere of *Typha latifolia* and *Salix atrocinerea* were studied and compared among two different natural wastewater treatment systems, using the direct sequencing of the 16S ribosomal RNA codifying genes. Phylogenetic affiliations of the bacteria detected allowed us to define the main groups present in these particular ecosystems. Moreover, bacterial community structure was studied through two diversity indices. Ten identified and five non-identified phyla were found in the samples; the phylum Proteobacteria was the predominant group in the four ecosystems. The results showed a bacterial community dominated by beta-proteobacteria and a lower diversity value in the swine wastewater treatment system. The municipal wastewater treatment system presented a high diverse community in both macrophytes (*Typha latifolia* and *Salix atrocinerea*), with gamma-proteobacteria and alpha-proteobacteria, respectively, as the most abundant groups.

Key words | bacterial diversity, municipal wastewater treatment, swine wastewater treatment, 16S rRNA gene

Paula Arroyo
Ivan Blanco
Patricia Molleda
IMARENABIO,
Universidad de León,
León 24071,
Spain

Gemma Ansola (corresponding author)
Estanislao de Luis Calabuig
Área de Ecología,
Universidad de León,
Campus de Vegazana,
24071 León,
Spain
E-mail: *gemma.ansola@unileon.es*

Luis E. Sáenz de Miera
Área de Genética,
Universidad de León,
León 24071,
Spain

ABBREVIATIONS

BOD_5	biochemical oxygen demand
HMAE®	Hierarchical Mosaic of Artificial Ecosystems
HRT	hydraulic retention time
MWWS	municipal wastewater system
PCR	polymerase chain reaction
SWWS	swine wastewater system

INTRODUCTION

The operation of wastewater treatment technologies relies on a combination of physical, chemical and biological factors. For many decades, these systems have been based mainly on information about chemical and physical parameters, having been quite successful in using the advantages of the amazing metabolic potential of microbial communities without detailed available knowledge about the organisms involved (Gilbride *et al.* 2006). The development of more stringent disposal limits to protect the environment demands an in-depth acquaintance with the parameters involved in wastewater treatment systems in order to improve and optimize their performance.

Secondary treatment is one of the key components of a wastewater treatment plant. It involves the biological reduction of organic matter, suspended solids and toxicity of industrial wastewaters, together with the production of

doi: 10.2166/wh.2009.123

low-nutrient effluents. These functions are carried out by the resident microbial (mainly bacterial) community, which is considered the foundation of the secondary treatment process. These organisms present complex interactions among themselves and with the environment, which makes this stage a sensitive process that needs to be carefully studied.

In recent years, the application of molecular techniques has made it possible to broaden insights into the vast diversity and interactions of microorganisms present in complex environments. The advantage of these methods is that they do not rely on cultivation and they therefore can be extremely sensitive and capable of describing the diversity of the microbial communities in much fuller detail (Gilbride *et al.* 2006).

Amplification of the 16S rRNA gene through the PCR technique using universal primers and its subsequent sequencing has been extensively used to study bacterial diversity, as it allows identification as well as an indication of the phylogenetic relationship of the samples (Kapley *et al.* 2007). This technique has been applied to different conventional wastewater treatment systems such as anaerobic digesters (Sawayama *et al.* 2006), activated sludge (Liu *et al.* 2006; Kapley *et al.* 2007), or membrane bioreactors (Miura *et al.* 2007). However, the study of these communities in non-conventional treatment systems such as constructed wetlands or lagoons is not so widespread (Moura *et al.* 2007).

Constructed wetlands (CWs) are man-made 'ecosystems' that mimic their natural counterparts. They are a cost-effective alternative to conventional wastewater treatment (Knox *et al.* 2006). They are designed to take advantage of many of the same processes that occur in natural wetlands, but do so within a more controlled environment (Vymazal 2005). In addition to bacteria, planted macrophytes constitute a further component participating in the biological treatment. The important role of these plants derives from the availability of a supply of oxygen emerging through the rhizosphere (Brix 2003), allowing the creation of an area where aerobic organisms are able to develop. This capacity of the two groups of organisms to interact is described by Brix (1997) as an indispensable component for the correct operation of these treatment systems. However, the role of this interaction still remains unclear, as other authors have reported that macrophytes do not play an active role improving water quality, but act only as a structural support for bacteria (Collins *et al.* 2004).

Various researchers have studied the bacterial communities of these low-cost systems. Some studies have been based on specific bacterial groups, such as the family Enterobacteriaceae (Vacca *et al.* 2005), or methanotrophic bacterial populations (DeJournett *et al.* 2007). Other projects have focused on communities from constructed wetlands that treat acid coal mine drainage (Nicomrat *et al.* 2008) or wastewater with differing phosphorus loadings (Ahn *et al.* 2007). Nevertheless, it remains difficult to find works that make reference to the bacterial communities of macrophyte rhizospheres. Thus, it is of considerable interest to increase the available knowledge with respect to these ecological niches.

The purpose of this work was to characterize and compare the bacterial communities associated with the rhizospheres of two macrophyte species (*Typha latifolia* and *Salix atrocinerea*) found in two different systems. One had been designed as a full-scale HMAE® (Hierarchical Mosaic of Artificial Ecosystems) for the treatment of municipal wastewater (MWWS), the other as a pilot-scale HMAE® to treat swine wastewater (SWWS).

MATERIALS AND METHODS

Description of the pilot systems

A HMAE® was built to treat the municipal wastewater of Bustillo de Cea, a village located in the Province of León, in the northwest of Spain, with an equivalent population of over 1,400 inhabitants in winter and 1,600 in summer. The MWWS, in operation since 1998, is made up of four stages. After pre-treatment, wastewater flows to the first stage, a free flow 1.5–2 m depth lagoon with a microphyte aquatic ecosystem and a total surface of 230 m^2. Both the second (210 m^2) and the third (87.5 m^2) compartments are surface flow, semi-aquatic ecosystems planted with *Typha latifolia* and *Iris pseudacorus* macrophyte species, respectively. *Salix atrocinerea* was selected as the main species for the fourth stage (362.5 m^2) which acts as a gravel filter (terrestrial ecosystem).

The mean wastewater flow is 4.28 $m^3 h^{-1}$ with a mean BOD_5 inlet value of 88.58 ± 12.22 O_2 mg l^{-1} and a HRT (hydraulic retention time) of 13 days. Further details of the process are related elsewhere (Ansola *et al.* 2003).

The SWWS was constructed to treat wastewater from CENTROTEC, a swine reproduction technology centre. This system is also located in the northwest of Spain, at Campo de Villavidel in the Province of Leon, about 40 km away from Bustillo de Cea. Raw wastewater is pumped into a tank and diluted with fresh water. The system is composed of three basins that comprise a total area of 13.62 m^2. The first basin to receive the mixture is a surface flow constructed wetland with *Typha latifolia*. The next two basins are both subsurface flow constructed wetlands planted with *Salix atrocinerea*.

The average flow is 7.1 $dm^3 h^{-1}$ with a mean BOD_5 inlet value of 122 ± 35.1 O_2 mg l^{-1} and a HRT of 16 days. The dimensions are based on the model proposed by Kadlec & Knight (1996).

Both pilot systems belong to an area with a Mediterranean type of climate (Papadakis 1961) with approximately 546.7 mm annual rainfall and 10.2°C mean annual temperature.

Sampling of bacteria

Plant roots were sampled in autumn, after the life cycle of the macrophytes had been completed. Several individual specimens of *Typha latifolia* and *Salix atrocinerea* were collected randomly from their respective basins in both treatment systems. A total of four samples were collected and studied: *Typha*-MWWS, *Salix*-MWWS, *Typha*-SWWS and *Salix*-SWWS.

For each sample, 45 g of a mixture of plant roots and the surrounding planting medium was placed in polypropylene tubes. Saline solution (0.9% NaCl) was added up to 30 ml, and this mixture was stirred energetically in order to transfer the biofilm to the saline solution. After filtration, 16 ml were centrifuged to precipitate bacteria. Genomic DNA was obtained in accordance with the procedure of Zhu *et al.* (1993).

16S rRNA gene PCR amplification

16S rRNA genes were amplified through a PCR using universal bacterial primers 27f and 907r. Amplification was carried out in an Applied Biosystems thermal cycler (Model Gene Amp PCR System 9700) programmed for 28 cycles. Denaturation in each cycle was performed at 94°C for 45 seconds, the annealing temperature was 53°C for 60 seconds and extension was carried out at 72°C for 90 seconds. After the 28 amplification cycles, samples were allowed to remain for 7 minutes at 72°C to complete the extension process.

A mass of PCR belonging to different species was obtained and then amplified DNA was ligated into pGEM-T (Promega), following the manufacturer's recommendations, and transformed into *Escherichia coli* DH5α competent cells prepared by the Inoue *et al.* (1990) method.

Fifty positive clones from each bacterial community were selected using two criteria: a colour criterion, as the manufacturer recommends; and a size criterion, estimating the recombinant plasmid lengths through electrophoresis.

16S rRNA gene sequencing and phylogenetic analysis

Two hundred selected clones were sequenced in both directions, using double-stranded DNA universal primers, the Thermo Sequenase™ Fluorescent Labelled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions, automatic sequencing, and MEGABACE 500 (Amersham Bioscience). No more than ten clones from each PCR were selected.

Each sequence was used to identify the species present in the sample by a comparative search in the GenBank database using the BLASTN method (Altschul *et al.* 1997). The most similar sequences to the clones in the database were retained for inclusion in subsequent phylogenetic analysis. Other methods, such as the software from The Ribosomal Database Project (Cole *et al.* 2007), including the program RDP Classifier (Wang *et al.* 2007), offer similar results. Sequence alignment was carried out with the CLUSTAL-W method (Thomson *et al.* 1994), using the standard parameters suggested by the program and then refined by eye.

Phylogenetic analyses were carried out using the MEGA 3.1 software package (Kumar *et al.* 2004). LogDet/Paralinear distance model (Lake 1994), an estimator of divergence between nucleotide sequences devised to deal with unequal base frequencies, was chosen, and minimum

evolution criterion was used to obtain phylogenetic trees, the topology showing the smallest value of the sum of all branches is chosen as an estimate of the correct tree.

Study of bacterial community structures

Biological diversity can be quantified in many different ways. The two main ecological parameters taken into account when measuring diversity are richness and evenness. The Shannon–Weaver index (H′) has been used since 1949 to obtain a mathematical measurement of species diversity within a community (Shannon & Weaver 1949). Measurements of evenness have also been calculated for each sample. An evenness index is a measure of diversity used to quantify the numerical similarity of communities. The evenness of a community can be represented by Pielou's Evenness Index. These indexes were calculated for each sample, taking into account both phylum and order diversity and evenness.

Comparison between communities was achieved using various tools from the UniFrac interface (Lozupone & Knight 2005). The UniFrac interface provides a suite of tools for the comparison of microbial communities using phylogenetic information. It takes as input a single rooted phylogenetic tree that contains sequences derived from at least two different environmental samples and a file describing which sequences came from which sample. The UniFrac distance is calculated as the percentage of branch length leading to descendants from only one of the environments represented in the phylogenetic tree, and reflects differences in the phylogenetic lineages that are adapted to live in one environment verses the others. It makes pairwise comparisons between all environments represented in the input phylogenetic tree to determine whether they are significantly different and it compares sequences from many environments simultaneously using principal coordinates analysis (PCA) to determine whether there are environmental factors that group communities together.

RESULTS AND DISCUSSION

Analysis of bacterial communities

The study of the bacterial communities was carried out according to evolutionary criteria. The results show the phylogenetic relationship of the different bacteria, which allowed in-depth analysis of each community's composition.

A total of 200 clones from the rhizosphere communities of the municipal and swine wastewater treatment plants were sequenced. The characterization of the communities was based on 50 clones from each bacterial community.

Analysis of community composition and structure was based on the taxonomical report provided by the results from the BLAST analysis from the NCBI (National Center for Biotechnology Information). These results were complemented with the subsequent phylogenetic analysis performed by the authors. Phylogenetic trees were constructed using the studied sequences and the corresponding sequences with highest scoring hits in BLAST searches. More detailed sub-analyses of each taxonomic group were also performed. Three of these, covering alpha, beta and gamma-proteobacteria, are shown in Figures 1–3. These analyses allowed a more accurate classification of the amplified sequences. However, not all the clones determined presented a clear affiliation. The presence of different orders or phyla in the samples was assessed, to expand the analysis of diversity, as in some cases determination was possible only at the level of genus, family or order (the classes of Proteobacteria were considered as a phylum for this analysis).

From the great variety of lineages detected, ten different phyla were identified, and five other phyla were noted, but not clearly identified (Table 1). Of all the phyla, the highest abundance score was obtained by the group Proteobacteria. The relative abundance of this group involved 68.5% of all the sequences analysed. The range was from 64% for *Typha*-MWWS to 76% for *Typha*-SWWS. The phylum Bacteroidetes was the second most abundant, comprising 14.5% of the sequences. Other significant phyla were the Firmicutes, present only in SWWS, and the Acidobacteria, found only in MWWS.

The greatest richness of phyla and highest diversity values were found in the micro-ecosystem MWWS, in which richness reached values of 8 for *Typha* and 9 for *Salix* and the diversity figure was nearly 1.8. In the SWWS the richness levels for the phyla were 4 (*Typha*) and 5 (*Salix*) and the diversity value was below 1.45. These analyses treated alpha, beta, gamma and delta-proteobacteria

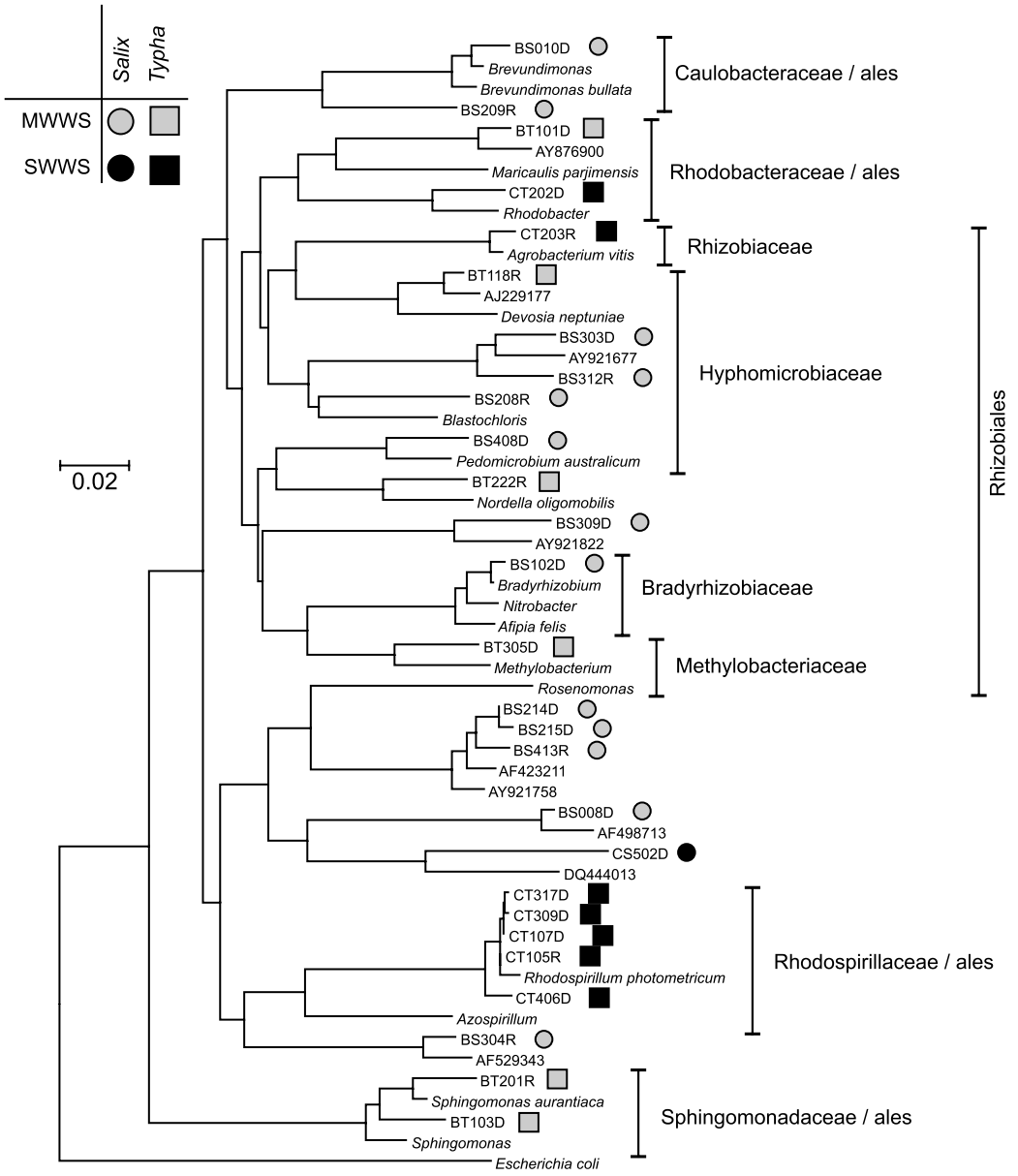

Figure 1 | Phylogenetic tree showing the relationship between partial 16S rRNA sequences of different taxa belonging to the class alpha-proteobacteria. *E. coli* was used as an out-group to root the tree. Clone prefixes: B, municipal wastewater treatment system (MWWS); C, swine wastewater treatment system (SWWS); S, rhizosphere of *Salix*; T, rhizosphere of *Typha*. LogDet distances were used to construct the tree. The topology was chosen on the basis of the minimum evolution criterion.

groupings as phyla. However, when the evenness of the samples was analysed, the four communities showed similar values (between 0.60 and 0.56). The diversity of orders was greater than that obtained for phyla, although the relationship found at the phylum level was maintained, with MWWS communities more diverse than those in the SWWS (Table 2).

To characterize the four communities properly, it was essential to look at the phylum Proteobacteria in detail, in the light of its prominence (Tables 1 and 3). The class alpha-proteobacteria was the most abundant in the rhizosphere of *Salix* (Figure 1, and Table 3) in the MWWS, with 13 sequences. Its presence in this ecosystem was quite normal, as many orders from this grouping

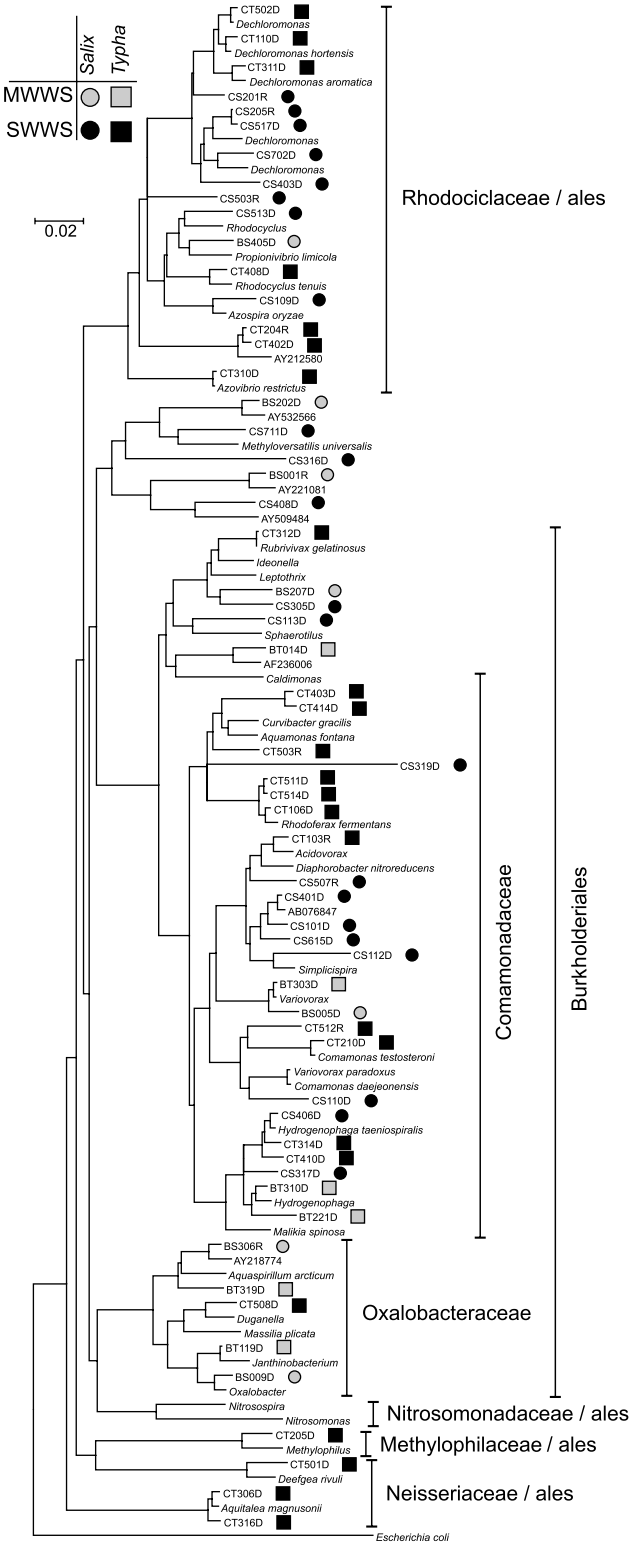

Figure 2 | Phylogenetic tree showing the relationship between partial 16S rRNA sequences of different taxa belonging to the class beta-proteobacteria. *E. coli* was used as an out-group to root the tree. See Figure 1 for further details.

(such as Rhizobiales) include plant endosymbionts, methane-oxidizing bacteria and in particular, a large number of nitrogen-fixing species (Wan-Taek *et al.* 2006). In fact, the largest association of individuals from alpha-proteobacteria belonged to, or was related to, Rhizobiales (six sequences in total). This situation was completely different from that of the same species in the SWWS, where their presence was the smallest in the phylum (one sequence).

The composition of the community in the rhizosphere of *Typha* for the two treatment systems was quite similar (6 and 7 sequences in MWWS and SWWS, respectively). The sequence distribution within the class showed variations according to the type of wastewater treated in the system. In the MWWS, the most frequent order was Rhizobiales, whereas in the SWWS, the order Rhodospirillales was the most frequent. However, lineage-specific analysis using UniFrac did not detect any significant differences in the four bacterial communities for the class alpha-proteobacteria ($P = 0.3712$).

The greatest contrast between the communities of the two HMAE® was found in the class beta-proteobacteria. The relative abundance of this taxon was not very great in the case of the MWWS, with similar results for *Typha* (6 sequences) and *Salix* (7 sequences). The sequence distribution was also similar in the two communities, the order Burkholderiales being the most abundant, although the affiliation of some sequences remained unknown in the case of *Salix*. On the other hand, this class was more widely represented in the rhizospheres of *Salix* (22 sequences) and *Typha* (24 sequences) in the SWWS (Figure 2 and Table 3). As happened with the municipal wastewater, Burkholderiales was the most abundant order, followed by Rhodocyclales. The remaining sequences did not show any clear relationship or, in the case of *Typha*, belonged to Neisseriales or Methylophilales. Many of the bacteria of this class play an important role in nitrogen fixation in several types of plant, which explains their predominance in a relatively nitrogen-loaded environment such as swine wastewater. It was only among the beta-proteobacteria that lineage-specific analysis using UniFrac detected any significant differences between the four bacterial communities ($P = 0.0016$).

Gamma-proteobacteria (Figure 3, Table 3) constituted the dominant group in the *Typha*-MWWS. This community

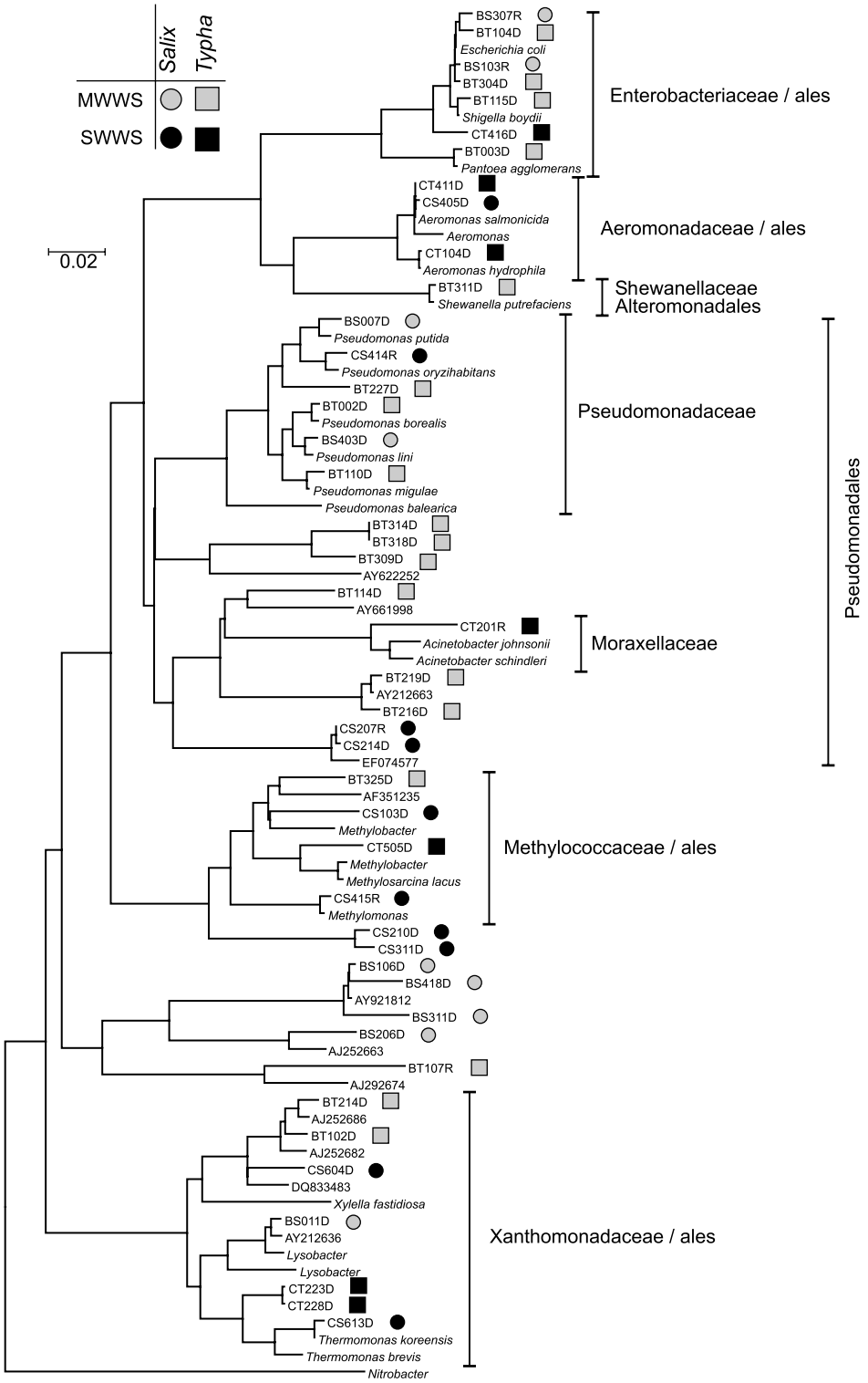

Figure 3 | Phylogenetic tree showing the relationship between partial 16S rRNA sequences of different taxa belonging to the class gamma-proteobacteria. *Nitrobacter* was used as an out-group to root the tree. See Figure 1 for further details.

Table 1 | Total numbers of clones found in this study; number of clones belonging to each determined phylum; those without a clear affiliation are included as an unknown phylum

	Municipal wastewater		Swine wastewater		
Taxon	***Typha***	***Salix***	***Typha***	***Salix***	**Total**
Alpha-proteobacteria	6	13	7	1	27
Beta-proteobacteria	6	7	24	22	59
Gamma-proteobacteria	18	9	7	10	44
Delta-proteobacteria	2	4	0	1	7
Acidobacteria	2	2	0	0	4
Actinobacteria	4	3	2	0	9
Bacteroidetes	7	7	5	10	29
Chloroflexi	0	1	0	0	1
Fibrobacteres	0	1	0	0	1
Firmicutes	0	0	5	1	6
Nitrospirae	1	0	0	0	1
Planctomycetes	0	0	0	1	1
Verrucomicrobia	2	1	0	0	3
Unknown phylum 1	0	0	0	2	2
Unknown phylum 2	0	0	0	2	2
Unknown phylum 3	0	1	0	0	1
Unknown phylum 4	1	0	0	0	1
Unknown phylum 5	1	1	0	0	2
Total	50	50	50	50	200

showed a heterogeneous composition, clustering principally in the Pseudomonadales, Enterobacteriales and Xanthomonadales orders, with just one individual for each of the orders Alteromonadales and Methylococcales. These groups were also present in *Typha*-SWWS. However, in this case their relative abundance was much lower with respect to the other classes of this phylum (seven sequences) and the distribution among them was more homogeneous. With regard to *Salix*, the number of sequences in both HMAE® was very similar (nine in MWWS and ten in SWWS). The clones were clustered in the orders Pseudomonadales, Enterobacteriales and Xanthomonadales in the first case, and Pseudomonadales, Xanthomonadales, Methylococcales and Aeromonadales in the second one. The presence of these bacteria may have been due to their role in decomposition processes, biodegradation and participation in the carbon and nitrogen cycles. Benhizia *et al.* (2004) found members of this class in legume nodules, occupying them together with forms of more usual nitrogen-fixing bacteria or in the absence of these more customary occupants. Thus, their presence in the rhizosphere of the macrophytes may have been linked to these other bacteria, which would be in agreement with the statements of the cited authors.

The group delta-proteobacteria had only a slight presence in the various environments. Absent from the *Typha*-SWWS, its representation in the *Salix*-SWWS was minimum, with just one clone. In the MWWS, Myxococcales was the only order found in association with the *Typha* sequences, whereas the *Salix* community included individuals belonging to several orders (Table 3).

Bacteroidetes was the second most important phylum in this analysis. The participation of members of Bacteroidetes in the decomposition of a variety of organic compounds is well known (Rincón *et al.* 2006). This means that its presence is not surprising in such environments.

Table 2 | Diversity of phyla and orders expressed by the Shannon-Weaver and evenness indices; these cover the bacterial communities belonging to the four micro-ecosystems: the rhizospheres of *Typha* and of *Salix* from the two treatment systems

	Wastewater treatment system	Micro-ecosystem	Richness	Shannon-Weaver index (H')	Max. diversity (H'_{max})	Evenness (E)
Phylum	Municipal wastewater	*Typha*	8	1.811	3.000	0.604
		Salix	9	1.786	3.170	0.564
	Swine wastewater	*Typha*	4	1.151	2.000	0.576
		Salix	5	1.440	2.585	0.557
Order	Municipal wastewater	*Typha*	20	3.948	4.322	0.913
		Salix	25	4.313	4.644	0.929
	Swine wastewater	*Typha*	18	3.626	4.170	0.870
		Salix	17	3.622	4.087	0.886

Table 3 | Number of proteobacteria clones obtained from each micro-ecosystem; the main divisions of the phylum proteobacteria and the orders detected

		Municipal wastewater		Swine wastewater		
Taxon	**Order**	***Typha***	***Salix***	***Typha***	**Salix**	**Total**
Alpha-proteobacteria	Rhizobiales	3	6	1	–	10
	Rhodospirillales	–	–	5	–	5
	CRT* Rhodospirillales	–	1	–	–	1
	Rhodobacterales	1	–	1	–	2
	Caulobacterales	–	2	–	–	2
	Sphingomonadales	2	–	–	–	2
	Undefined	–	4	–	1	5
Beta-proteobacteria	Burkholderiales	6	4	13	11	34
	Rhodocyclales	–	1	7	8	16
	Neisseriales	–	–	3	–	3
	Methylophilales	–	–	1	–	1
	Undefined	–	2	–	3	5
Gamma-proteobacteria	Pseudomonadales	9	2	1	3	15
	Enterobacteriales	4	2	1	–	7
	Xanthomonadales	2	1	2	2	7
	Aeromonadales	–	–	2	1	3
	Methylococcales	1	–	1	2	4
	CRT Methylococcales	–	–	–	2	2
	Alteromonadales	1	–	–	–	1
	Undefined 1	–	3	–	–	3
	Undefined 2	–	1	–	–	1
	Undefined 3	1	–	–	–	1
Delta-proteobacteria	Myxococcales	2	–	–	–	2
	CRT Bdellovibrionales	–	1	–	–	1
	CRT Syntrophobacterales	–	1	–	–	1
	Desulfuromonadales	–	–	–	1	1
	Desulfomonadales	–	1	–	–	1
	Undefined	–	1	–	–	1
Total number of taxa		32	33	38	34	137

*CRT = closely related to.

A UniFrac metric was used to perform a principal coordinate analysis on the communities (Table 4). The three components explained similar percentages of variation. In the case of the first component, the communities in the MWWS showed negative values. This was different from the SWWS communities, where values were positive. The second component divided the communities with respect to the macrophyte involved. In the case of the third component, the explanation was not so clear. In all cases, consideration of the significance of UniFrac results using the Bonferroni correction showed non-significant differences between all pairs of communities; although P values were lower than 0.06.

To summarize, the work presented here provided insights into the composition and structure of the bacterial communities from two different natural treatment systems, one used to treat swine wastewater with a high nitrogen load from a reproduction technology centre,

Table 4 | Results of principal coordinate analysis (PCA) showing which bacterial communities are the most closely related. The four environments are the bacterial communities from the *Typha* rhizosphere and *Salix* rhizosphere of the municipal wastewater treatment system (MWWS) and the *Typha* rhizosphere and *Salix* rhizosphere of the swine wastewater treatment system (SWWS)

pc vector number	1	2	3
Salix-MWWS	−0.352	−0.185	0.349
Typha-MWWS	−0.286	0.136	−0.414
Salix-SWWS	0.372	−0.369	−0.119
Typha-SWWS	0.266	0.417	0.184
Eigenvalues	0.416	0.362	0.342
Percentage variation explained	37.12	32.36	30.52

and the other used to treat municipal wastewater from a small village.

The study focused on the microbial populations associated with the rhizosphere of *Typha latifolia* and *Salix atrocinerea* in these two treatment plants. Ten identified and five non-identified phyla were detected in the samples, with the phylum Proteobacteria the predominant group in the four micro-ecosystems. The methodology allowed clone affiliations to be fixed with total certainty, although there were clones identified only at the level of genus, family or order.

In autumn, the MWWS presented higher bacterial diversity associated with the rhizospheres of *Typha* and *Salix*. The richness and diversity found in these two communities were greater than those calculated for the swine wastewater system. Evenness was quite similar among communities at the phylum level. However, evenness values at order level were higher in the communities of the MWWS. This means that these two communities were not dominated by one or two groups, as happened in the SWWS, where communities in both micro-ecosystems were clearly dominated by beta-proteobacteria. This fact might be attributed to the more stressful wastewater characteristics in the swine system, which would favour the presence of this group. Even with the small samples sizes, differences among bacterial communities were observed, some of them statistically significant.

The study could be applied to establish dynamics over various seasons and correlate the bacterial community with wastewater characteristics, so as to gain greater insights into these natural systems and the efficiency of the treatments they provide.

ACKNOWLEDGEMENTS

This work was supported by the Diputación Provincial de León, and Junta de Castilla y León. It also benefited from the collaboration of CENTROTEC.

REFERENCES

Ahn, C., Gillevet, P. M. & Sikaroodi, M. 2007 Molecular characterization of microbial communities in treatment microcosm wetland as influenced by macrophytes and phosphorus loading. *Ecol. Indic.* **7**, 852–863.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Shang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997 Gappet BLAST and PSI-BLAST: a new generation of protein database search program. *Nucleic Acids Res.* **25**, 3389–3402.

Ansola, G., González, J. M., Cortijo, R. & de Luis, E. 2003 Experimental and full-scale pilot plant constructed wetlands for municipal wastewaters treatment. *Ecol. Eng.* **21**, 43–52.

Benhizia, Y., Benhizia, H., Benguedouar, A., Muresu, R., Giacomini, A. & Squartini, A. 2004 Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. *Syst. Appl. Microbiol.* **27**, 462–468.

Brix, H. 1997 Do macrophytes play a role in constructed treatment wetlands? *Water Sci. Technol.* **35**, 11–17.

Brix, H. 2003 Plants used in constructed wetlands and their functions. In *First International Seminar on the Use of Aquatic Macrophytes for Wastewater Treatment in Constructed Wetlands* (ed. V. Dias & J. Vymazal), pp. 81–109. ICN, INAG cop, Lisbon.

Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Bandela, A. M., Cardenas, E., Garrity, G. M. & Tiedj, J. M. 2007 The ribosomal database project (RDP-II): introducing my RDP space and quality controlled public data. *Nucleic Acids Res.* **35**, D169–D172.

Collins, B., Vaun McArthur, J. & Sharitz, R. R. 2004 Plant effects on microbial assemblages and remediation of acidic coal pile runoff in mesocosm treatment wetlands. *Ecol. Eng.* **23**, 107–115.

DeJournett, T. D., Arnold, W. A. & LaPara, T. M. 2007 The characterization and quantification of methanotrophic bacterial populations in constructed wetland sediments using PCR targeting 16S rRNA gene fragments. *Appl. Soil Ecol.* **35**, 648–659.

Gilbride, K. A., Lee, Y. & Beaudette, L. A. 2006 Molecular techniques in wastewater: understanding microbial communities, detecting pathogens, and real-time process control. *J. Microbiol. Meth.* **66**, 1–20.

Inoue, H., Nojima, H. & Okayama, H. 1990 High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**, 23–28.

Kadlec, R. H. & Knight, R. L. 1996 *Treatment Wetlands*. CRC Press, Boca Raton, FL.

Kapley, A., Thierry, D. B. & Hemant, J. P. 2007 Eubacterial diversity of active biomass from a common effluent treatment plant. *Res. Microbiol.* **158**, 494–500.

Knox, A. S., Paller, M. H., Nelson, E. A., Specht, W. L., Halverson, N. V. & Gladden, J. B. 2006 Metal distribution and stability in constructed wetland sediment. *J. Environ. Qual.* **35**, 1948–1959.

Kumar, S., Tamura, K. & Nei, M. 2004 MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**, 150–163.

Lake, J. A. 1994 Reconstructing evolutionary trees from DNA and protein sequences: paralinear distances. *Proc. Natl Acad. Sci. USA* **91**, 1455–1459.

Liu, X., Zhang, Y., Yang, M., Wang, Z. & Lv, W. 2006 Analysis of bacterial community structures in two sewage treatment plants with different sludge properties and treatment performance by nested PCR–DGGE method. *J. Environ. Sci.* **19**, 60–66.

Lozupone, C. & Knight, R. 2005 UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **71**, 8228–8235.

Miura, Y., Hiraiwa, M. N., Ito, T., Itonaga, T., Watanabe, Y. & Okabe, S. 2007 Bacterial community structures in MNRs treating municipal wastewater: relationship between community stability and reactor performance. *Water Res.* **41**, 627–637.

Moura, A., Tacão, M., Henriques, I., Dias, J., Ferreira, P. & Correia, A. 2007 Characterization of bacterial diversity in two aerated lagoons of a wastewater treatment plant using PCR–DGGE analysis. *Microbiol. Res* (doi:10.1016/j.micres.2007.06.005).

Nicomrat, D., Dick, W. A., Dopson, M. & Tuovinen, O. H. 2008 Bacterial phylogenetic diversity in a constructed wetland system treating acid coal mine drainage. *Soil Biol. Biochem.* **40**, 312–321.

Papadakis, J. 1961 *Climatic Tables for the World*. Papadakis, Buenos Aires.

Rincón, B., Raposo, F., Borja, R., González, J. M., Portillo, M. C. & Saiz-Jiménez, C. 2006 Performance and microbial communities of a continuous stirred tank anaerobic reactor treating two-phases olive mill solid wastes at low organic loading rates. *J. Biotechnol.* **121**, 534–543.

Sawayama, S., Tsukahara, K. & Yagishita, T. 2006 Phylogenetic description of immobilized methanogenic community using real-time PCR in a fixed-bed anaerobic digester. *Bioresour. Technol.* **97**, 69–76.

Shannon, C. E. & Weaver, W. 1949 *The Mathematical Theory of Communication*. University of Illinois Press, Urbana, IL.

Thomson, J. D., Higgins, D. G. & Gibson, T. J. 1994 Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choices. *Nucleic Acids Res.* **22**, 4673–4680.

Vacca, V., Wand, H., Nokolausz, M., Kuschk, P. & Kästner, M. 2005 Effect of plants and filter materials on bacteria removal in pilot-scale wetlands. *Water Res.* **39**, 1361–1373.

Vymazal, J. 2005 Horizontal sub-surface flow and hybrid constructed wetlands systems for wastewater treatment. *Ecol. Eng.* **25**, 478–490.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. 2007 Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **73**, 5261–5267.

Wan-Taek, I., Seong-Hye, K., Myung Kyum, K., Leonid, N. T. & Sung-Taik, L. 2006 *Pleomorphomonas koreensis* sp. nov., a nitrogen-fixing species in the order Rhizobiales. *Int. J. Syst. Evol. Microbiol.* **56**, 1663–1666.

Zhu, H., Qu, F. & Zhu, L. 1993 Isolation of genomic DNAs from plants, fungus and bacterias using benzyl chloride. *Nucleic Acids Res.* **21**, 5279–5280.

First received 10 November 2008; accepted in revised form 3 May 2009. Available online 9 November 2009