Bacterial communities in the rhizosphere of Phragmites australis from an oil-polluted wetland

Peer-reviewed author version

Abed, Raeid M. M.; Al-Kharusi, Samiha; GKOREZIS, Panos; Prigent, Stephane & Headley, Tom (2018) Bacterial communities in the rhizosphere of Phragmites australis from an oil-polluted wetland. In: Archives of Agronomy and Soil Science, 64(3), p. 360-370.

DOI: 10.1080/03650340.2017.1352087
Handle: http://hdl.handle.net/1942/24029

Bacterial communities in the rhizosphere of *Phragmites australis* from an oil-polluted wetland

Raeid Abed [a], Samiha Al-Kharusi [a], Panagiotis Gkorezis [b], Stephane Prigent [c], Tom Headley[d].

[a] Biology Department, College of Science , Sultan Qaboos University, Al Khoud, Sultanate of Oman; [b] Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium; [c] BAUER Resources, Constructed Wetland Competence Centre, Muscat, Sultanate of Oman; [d] Ecological Engineering Department, The Water and Carbon Group, Brisbane, Australia

Running title: Rhizosphere soil bacteria

Abstract

Although *Phragmites australis* is commonly planted in constructed wetlands, very little is known about its roots-associated bacterial communities, especially in wetlands used for the remediation of oil produced waters. Here, we describe the bacterial diversity, using molecular (illumina MiSeq sequencing) and cultivation techniques, in the rhizosphere soils of *P. australis* from an oil-polluted wetland in Oman. The obtained isolates were tested for their plant-growth promoting properties. Most sequences belonged to *Proteobacteria*, *Bacteriodetes* and *Firmicutes*. Sequences of potential hydrocarbon-degrading bacteria (e.g. *Ochrobactrum*, and *Pseudomonas*) were frequently encountered. All soils contained sequences of known sulfur-oxidizing (e.g. *Thiobacillus*, *Thiofaba*,

Rhodobacter and *Sulfurovum*) and sulfate-reducing bacteria, although the latter group made up only 0.1% to 3% of total sequences. The obtained isolates from the rhizosphere soils were phylogenetically affiliated to *Serratia*, *Acinetobacter*, *Xenorhabdus*, *Escherichia* and *Salmonella*. All strains were able to solubilize phosphate and about half were capable of producing organic acids and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Around 42% of the strains had the ability to produce indole acetic acid and siderophores. We conclude that the rhizosphere soils of *P. australis* in oil polluted wetlands harbor diverse bacterial communities that could enhance the wetland performance through hydrocarbon degradation, nutrient cycling and supporting plant growth.

Keywords: Reed bed; Hydrocarbons; MiSeq sequencing; Constructed wetland; *P. australis*

Introduction

The utilization of constructed wetlands for the treatment of contaminants has been increasing steadily over the past decades (Kadlec et al. 2008; Li et al. 2013; Zou et al. 2013), mainly because it is cost-effective and environmentally friendly (Bouali et al. 2014a). Several plants, with the large perennial wetland plant species *P. australis*, known also as the common reed, have been used in wetlands throughout temperate and tropical regions of the world (Chandra et al. 2012). The selection of the plant species is based on their ability to grow in local environment, their ability to withstand and degrade contaminants, their high biomass and root depth and based on the water management plan

47 (water reuse or zero-discharge) (Ravit et al. 2003). However, the treatment performance
48 of constructed wetlands relies largely on the microbial communities in the rhizosphere of
49 the selected plant species, which not only play a direct role in pollutant removal but also
50 in biogeochemical cycles of major nutrients (Tian et al. 2014). Some of these
51 microorganisms possess plant-growth promoting (PGP) traits that accelerate the growth
52 of plants (Jiang et al. 2013). The diversity of microorganisms associated with the roots of
53 several wetland plants has been studied using culture-dependent and molecular
54 techniques (Jiang et al. 2013; Li et al. 2013). Although it is believed that root exudates
55 enhance the growth and activity of rhizosphere soil bacterial communities (Jiang et al.
56 2013; Zou et al. 2013), few studies have compared these communities in different roots
57 of the same plant species (Likar et al. 2009).

58 Few wetlands have been constructed to treat oil-contaminated produced water
59 (Abed et al. 2014a). Although previous studies have demonstrated drastic effects of oil
60 spills on natural wetlands (Zou et al. 2013; Tian et al. 2014), engineered constructed
61 wetlands are very effective in the breakdown and removal of hydrocarbons from
62 produced water. In the Arabian Gulf region, one of the largest surface flow constructed
63 wetland systems was constructed in Oman for the treatment of oil-produced water and
64 this wetland is predominantly planted with the wetland plant species *P. australis* (Abed et
65 al. 2014a). So far, there are no studies performed to describe and compare the diversity of
66 microorganisms in the rhizosphere soils surrounding *P. australis* in this wetland in
67 particular and in oil-polluted wetlands in general. The study of these bacterial
68 communities will contribute towards a better understanding of their diversity under
69 elevated levels of oil and very harsh desert conditions as well as their role in the

performance of the whole ecosystem. Hence, we investigated, using culture-dependent and independent approaches, the composition of bacterial communities in the rhizosphere soils of *P. autralis* from an oil-polluted wetland located in the desert of Oman. Furthermore, we tested the obtained bacterial isolates for their PGP characteristics.

Materials and methods

Study site and roots collection

The studied wetland occupies an area of 350 hectare, vegetated predominantly with *P. australis.* The wetland is mainly used for the treatment of oil-produced water (115,000 m^3 day^{-1}). It is a surface flow design consisting of 0.2 m depth of soil overlain by an average 15 cm depth of surface water. Detailed layout of the wetland has been described earlier (Abed et al. 2014a). The roots of *P. australis* were sampled from two different sites (termed hereafter as A and B); each was ten-hectare in size. These two sites were chosen because of the clear differences in oil concentration, temperature, pH, chemical oxygen demand (COD) and sulfate concentration in the overlying water (Table 1). The roots of three plants from two locations within each of the two studies sites (total 6 plants from each site) were gently pulled out of the ground. The fine-textured soils around the roots consisted of fine particles, silts and clays that when saturated are low in permeability. Around 5 g each of these soils were collected in a petri dish using a sterile spatula and then stored in sterile plastic boxes. All samples from site A and B were transferred to the laboratory in a cool box, and were immediately stored at -20ºC, for MiSeq sequencing.

The two sampling sites were characterized for the water quality by measuring (in triplicate samples) water temperature, dissolved oxygen, pH, oxidation reduction

potential (ORP) and conductivity using a standard calibrated multiline meter (WTW Multiline P4 Universal Meter and Hach HQ30d Flexi Meter). Oil concentration in the overlying water of each sampling site was measured on grab samples using a spectrophotometer (DR 3900 spectrophotometer, Hach Lange, Germany). Ammonia, phosphate, sulfate, boron and chemical oxygen demand (COD) were measured in the water samples using a spectrophotometer (DR 3900 spectrophotomer, Hach Lange, Germany) according to Hach standard methods (Hach 1989).

MiSeq sequencing and sequence analyses

DNA was extracted from the rhizosphere soil samples from site A and B using the PowerBiofilm DNA isolation kit (MOBIO laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions. The DNA extracts of the triplicate samples from each location were pooled together. The four pooled and purified DNA extracts (total 4, 2 from each site) were then submitted to Molecular Research MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA) for illumina MiSeq sequencing of the bacterial 16S rRNA genes using the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') with barcode on the forward primer. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations and then purified using calibrated AMPure XP beads. The pooled and purified PCR products were used to prepare a DNA library by following illumina TruSeq DNA library preparation protocol. Sequence analysis was carried out using the Mothur MiSeq SOP pipeline (https://www.mothur.org/wiki/MiSeq_SOP). Briefly, barcodes were removed and sequences with less than 200 base pairs (bp) and sequences with ambiguous base calls were eliminated. Sequences were denoised,

operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database.

Isolation and identification of rhizosphere soil bacteria

The soils attached to the rhizosphere of *P. australis* from the two sampling sites were mixed together prior to isolation to increase the chance of isolating different species. The aim of the isolation work was not to study and compare the bacterial diversity between the two sites but rather to obtain pure strains and study their PGP properties. Soil samples were then added into Erlenmeyer flasks containing 100 ml of Tryptic soy broth (TSB). TSB contains 17 g l^{-1} Bacto™ tryptone, 3 g l^{-1} Bacto soytone (peptic digest of soybean meal), 2.5 g l^{-1} glucose, 5 g l^{-1} sodium chloride and 2.5 g l^{-1} dipotassium hydrogen phosphate. The flasks were incubated at 30°C and shaken at 60 rpm for 24h. A loopful of each flask was plated on tryptic soy agar (TSA) to obtain single colonies. Twenty six bacterial colonies with different growth characteristics were picked and further purified on freshly prepared TSA plates. The 16S rRNA-based identification of the isolates and the construction of the maximum likelihood phylogenetic tree were performed as described in (Abed et al. 2014b).

Screening for PGP traits

The ACC deaminase production in all strains was detected in cell-free extracts using an established protocol (Belimov et al. 2005). The production of indole acetic acid (IAA) by the isolates was qualitatively tested using a previously described classical method (Gordon & Weber 1951). Bacterial isolates with the ability to produce various organic acids were identified by a color change of the alizarine red pH indicator from red (pH >

6) to yellow (pH ~ 5 or below) using the method described in (Cunningham & Kuiack 1992). A qualitative evaluation of siderophore production was based in the overlay-CAS assay (Pérez-Miranda et al. 2007). The Phosphate-solubilizing abilities of the isolated strains were assayed on plates as previously described (Schmid et al. 2009).

Statistical analysis

From MiSeq data, rarefaction curves and diversity indices (OTU richness, Chao 1 and ACE) were calculated using the Mothur software. Chao-1 is based on rare OTUs in a given sample and ACE is abundance-based coverage. The percentage of sequences that appeared only once (i.e. singletons, SSO) or twice (i.e. doubletons, DSO) were also calculated using a custom R script. Principal component analysis (PCA) implemented in PAST program (Paleontological Statistics, ver. 1.47, http:\\folk.uio.no\ohammer\past) was performed to evaluate the similarity among the bacterial communities of the four samples based on taxonomic composition.

Results

Physical-chemical characteristics of the sampling sites

Oil concentration in the overlying water was five folds higher in site A than in site B with a total amount of 1.63 and 0.33 mg l^{-1}, respectively (Table 1). The water temperature was slightly warmer in site A compared to site B. Ammonia and dissolved oxygen of both sites were comparable. Site B had higher contents of phosphate, sulfate and boron but lower total phosphate (TP) and total nitrogen (TN) (Table 1). pH, conductivity and COD were also higher in Site B.

MiSeq sequencing and bacterial diversity

A total of 455,201 16S rRNA sequences were generated by MiSeq sequencing (Table 2). The OTU richness and diversity indices (e.g. Chao and ACE) indicate higher diversity in Site A than Site B rhizophere soils samples (Table 2). Rarefaction curves showed that still more sequences are needed to cover the whole bacterial diversity in the samples (Figure 1A). Cluster analysis based on the distribution of bacterial groups and genera (Figures 1B and 1C) placed the Site A samples closer to each other than the Site B samples (Figure 1C).

The majority of sequences belonged to the phylum *Proteobacteria*, with a relative abundance of 24.5-70.4% of total sequences (Figure 1B). The occurrence of proteobacterial sequences was higher in Site A than in Site B soils. *Alphaproteobacteria* exhibited a relative abundance between 3.1 and 27.4 % of total sequences in all samples, with the dominance of sequences belonging to *Ochrobactrum* (Figure 1C). Sequences affiliated to *Agrobacterium* and *Brevundimonas* were detected in all samples, except B1, whereas sequences of *Rhodobacter* and *Labrenzia* exhibited a higher relative abundance in Site A than Site B samples (Figure 1C). *Betaproteobacteria* constituted 2.3-16.5% of total sequences in all soils, however with higher relative abundance in Site A (Figure 1B). The betaproteobacterial sequences belonged to the genera *Delftia*, *Acidovorax*, *Thiobacillus* and *Thauera*. *Gammaproteobacteria* was the most dominant (19-29.1% of the total sequences) bacterial class in all samples (Figure 1B), with sequences belonging to *Enhydrobacter*, *Pseudoxanthomonas*, *Halothiobacillus*, *Stenotrophomonas*, *Pseudomonas* and *Leclercia* detected in the soils of both sites (Figure 1C). Sequences belonging to the genera *Thiofaba* were only found in Site A samples. *Deltaproteobacteria* made up between 0.1% and 3% of total sequences in all samples. All sequences from this

group were related to known sulfate reducing bacteria from the genera *Desulfofustis, Desulforhopalus, Desulfobulbus, Desulfovibrio, Desulfustis* and *Desulfonema* (Figure 1C). *Epsilonproteobacteria* constituted ≤5.6% of total sequences in all soils, with *Sulfurovum* and *Sulfurimonas* mainly encountered in Site A soils (Figure 1B and 1C).

The spore-forming classes *Clostridia* and *Bacilli* were predominantly detected in B1 sample, making up 1.5% and 19.6% of total sequences, respectively (Figure 1B). The two classes made up <4.2% of total sequences in all other samples. While *Clostridia*-related sequences of the genera *Clostridium* and *Ruminiclostridium* were only encountered in the B1 sample, *Bacilli*-related sequences belonging to *Enterococcus, Bacillus* and *Lysinibacillus* were detected in B1 and A2 samples (Figure 1C). *Actinobacteria* and *Flavobacteriia* exhibited their highest abundance in the B1 sample (Figure 1B). The Actinobacterial sequences belonged to the genus *Corynebacterium* whereas the *Flavobacteriia*-related sequences belonged to the genera *Cloacibacterium, Weeksella, Bergyella* and *Flavobacterium* (Figure 1C).

Principal component analysis (PCA) indicated that A1 and A2 samples were more related to each other than B1 and B2 samples (Figure 2). While *Flavobacteriia* and *Bacilli* were more associated with B1 site, *Sphagnopsida* and *Gammaproteobacteria* were associated with B2 site and *Betproteobacteria* with A1 and A2 sites. Bacteria belonging to *Sulfurimona*s were most dominant in A1 and A2 sites whereas *Halothiobacillus* and *Ochrobactrum* exhibited their highest abundance in B2 sample.

Strain identification and PGP characteristics

All isolated strains belonged to the class *Gammaproteobacteria* (Figure 3). Five strains clustered together and shared 100% sequence similarity with *Serratia marcescens*

(JX868557), which was isolated from an oil-polluted site. Two strains shared >88% sequence similarity to other strains of *Serratia marcescens* isolated from cotton soil rhizosphere (HQ123473 and HQ130340). Seven strains were phylogenetically related to *Acinetobacter junii*, which was isolated from wetlands (EF429000). Only one was related to the genus *Xenorhabdus*, two to *E. coli* and two to *Salmonella enterica*.

Out of the 26 bacterial strains, 16 strains were capable of producing organic acids and 11 strains had the ability to produce indole acetic acid (Table 3). All strains had the ability to solubilize phosphate. The bacterial isolates that had a strong affinity to solubilize phosphate could always produce siderophores, except in the case of *E. coli* YS1 (Table 3). On the other hand, the strains that showed low phosphate solubility had a relatively strong ACC deaminase activity (11 strains), except in case of YS21and YS22. Approximately 70% of the isolates showed positive ACC deaminase activity (Table 3). Only one strain (i.e. YS20) was positive in all assays.

Discussion

Miseq sequencing provided a detailed insight into the bacterial diversity of the rhizopshere soils of *P. australis* from a poorly studied oil-polluted constructed wetland. The presence of diverse bacterial communities in the rhizosphere soils could be attributed to the organic-rich plant root exudates or oil, which could stimulate microbial growth and activity (Ukaegbu-Obi & Mbakwem-Aniebo 2014). Previous studies have shown that the whole diversity in the rhizosphere soils could not be covered using MiSeq, even after obtaining a large number of reads (Jiang et al. 2013) and rhizospheres contain 100-1,000 times higher microbial diversity than bulk soils (Mwajita et al. 2013). OTU richness,

231 Chao1 and ACE revealed a higher diversity in Site A compared with Site B soils. The
232 microbial diversity exhibited variations even between the duplicate soils from each site.
233 Sample heterogeneity depends on the physicochemical characteristics of soils, plant
234 exudates, pollution level and environmental conditions (Yue et al. 2012; Huang et al.
235 2014; Erguven et al. 2016).

236 **Proteobacteria *in rhizosphere soils***

237 All soils were predominated by sequences of the class *Proteobacteria*. This finding is
238 consistent with previous reports on the rhizosphere of *P. australis* (Li et al. 2013; Bouali
239 et al. 2014b). *Proteobacteria* constituted 24-70% of the total sequences in our samples as
240 well as in the rhizosphere of *P. australis* from other oil-free and oil-polluted wetlands
241 (Bouali et al. 2014b; Tian et al. 2014). While *Gamma-* and *Alphaproteobacteria*
242 dominated all our samples, *Alpha-* and *Deltaproteobacteria* dominated the rhizosphere of
243 *P. australis* from a wastewater wetland in Tunisia (Bouali et al. 2014b), and *Gamma- and*
244 *Deltaproteobacter*ia dominated the rhizosphere of mangroves from a natural wetland in
245 Hong Kong (Jiang et al. 2013). Both *Alpha-* and *Gammaproteobacteria* in our soils
246 included potential oil-degrading species. For instance, species belonging to
247 *Ochrobactrum, Agrobacterium, Pseudomonas, Stenotrophomonas, Pseudoxanthomonas*
248 and *Leclercia* were shown to degrade low and even high molecular weight hydrocarbons
249 (Cébron et al. 2011; Chandra et al. 2012). Indeed, the negative correlation between the
250 measured COD values and oil concentration in the overlying water of the two studied
251 sites indicates the occurrence of biodegradation (Erguven 2017).

252 ***Sulfur and nitrogen cycles in the rhizospehere soils***

In the studied soils, several genera of known aerobic sulfur oxidizing bacteria such as *Delftia*, *Thiobacillus*, *Thiofaba*, *Pseudomonas* and *Halothiobacillus* (Behera et al. 2014), anaerobic sulfur oxidizing bacteria like *Rhodobacter*, *Sulfurovum* and *Sulfurimonas* (Pavitra et al. 2015) as well as sulfate-reducing bacteria (SRBs) have been detected. This points out to the presence of a sulfur cycle in the rhizosphere soils. Oil pollution and the high input of organic matter from root exudates are known to stimulate sulfur cycle (Kleikemper et al. 2002). Many of the detected sulfur-cycle related bacteria have been previously detected in the rhizosphere soils of different plants (Vladár et al. 2008; Li et al. 2013). Interestingly, the sequences of SRBs made up only 0.1% to 3% of the total sequences in all investigated soils. This finding is congruent with earlier reports, where SRBs made up a minor fraction of the total bacterial community in other wetlands (Bottos et al. 2008), despite the high rates of sulfate reduction (Balasooriya et al. 2008).

Sequences of potential N_2 fixing, nitrifying and denitrifying bacteria have also been detected in our soils. For instance, the genera *Ochrobactrum*, *Agrobacterium* and *Brevundimonas* are known to include nitrogen-fixing species. Sequences related to nitrifiers such as *Labrenzia*, *Bacillus* and *Lysinibacillus* (Mishra et al. 2015) and denitrifiers such as *Thiobacillus* and *Pseudomonas* (Ruiz-Rueda et al. 2009) have also been encountered. Earlier studies have reported the presence of these bacterial taxa (Haaijer et al. 2006) and the occurrence of nitrogen cycle processes such as N_2 fixation, nitrification and denitrification (Whitmire & Hamilton 2005) in other wetlands.

Flavobacteriia *in rhizosphere soils*

An interesting feature of the obtained sequencing data was the dominance (38.4% of total sequences) of *Flavobacteriia* in B1 sample. Oil pollution was shown to favor the growth

of *Flavobacteriia* (Lv et al. 2014) and this group contains species that are able to degrade hydrocarbons (Hemalatha et al. 2011). *Flavobacteriia* also known to possess PGP features, such as their ability to hydrolyse organic phosphate (Fitriatin et al. 2011) and their ability to solubilize sulfate ester (Fitzgerald 1976). Thus, presence of these bacteria suggests a role in hydrocarbon degradation as well as in nutrient cycling and enhancement of plant growth in this nutrient-poor wetland (Dipak & Sinha 2013).

PGP characteristics of rhizosphere isolates

Most isolated strains from the rhizosphere soils belonged to *Serratia* (60% of total strains) and *Acinetobacter* (30%). *Serratia* and *Acinetobacter* spp. have been detected in the rhizosphere of *P. australis* in other natural and constructed wetlands (Chandra, et al. 2012; Zhang et al. 2013) as well as in the rhizosphere of different plants (Gyaneshwar et al., 2001; Rokhbakhsh-Zamin et al. 2011). Previous reports demonstrated a key role of *Serratia marcescens* in phytoremediation (Almansoory et al. 2014; Ukaegbu-Obi & Mbakwem-Aniebo 2014) and the degradation of aromatics by a *Serratia*-containing consortium (Ortega-González et al. 2013). The ability of our strains to exhibit ACC deaminase activity, siderophores production and phosphate solubilization has been previously demonstrated for species of the same genera (Nadeem et al. 2010; George et al. 2013). The production of IAA by *Acinetobacter* and *Serratia* spp renders them as potential biofertilizers (Gulati et al. 2009). IAA production not only improves plant growth (Mohite 2013), but also supports plant survival and adaptation under the desert's harsh environmental conditions (George et al. 2013).

Conclusions

In conclusion, the bacterial communities of the rhizosphere soils of *P. australis* are diverse and vary with location and in different plants of the same species. These bacteria are likely to contribute to the removal of hydrocarbons from the produced water. Moreover, they also play a role in nutrient cycling in the wetland and in supporting the growth of *P. australis* under the harsh desert conditions. Future research should focus on the interaction between *P. australis* and their associated bacteria under these harsh conditions and on studying the diversity of endophytic bacteria. The potential of these extremophilic bacteria for biotechnology should be exploited.

References

Abed RMM, Al Kharusi S, Prigent S, Headley T. 2014a. Diversity, distribution and hydrocarbon biodegradation capabilities of microbial communities in oil-contaminated cyanobacterial mats from a constructed wetland. PLOS One 9:e114570.

Abed RMM, Al-Sabahi J, Al-Maqrashi F, Al-Habsi A, Al-Hinai M. 2014b. Characterization of hydrocarbon-degrading bacteria isolated from oil-contaminated sediments in the Sultanate of Oman and evaluation of bioaugmentation and biostimulation approaches in microcosm experiments. Int Biodeterior Biodegradation. 89:58-666.

Almansoory AF, Idris M, Abdullah SRS, Anuar N. 2014. Plant-microbe interaction of *Serratia marcescens* and *Scirpus mucronatus* on phytoremediation of gasoline contaminated soil. Int J Chemtech Res. 6:556-564.

321 Balasooriya WK, Denef K, Peters J, Verhoest NEC, Boeckx P. 2008. Vegetation
322 composition and soil microbial community structural changes along a wetland
323 hydrological gradient. Hydrol Earth Syst Sc. 12:2272-2291.
324 Behera BC, Mishra RR, Dutta SK, Thatoi HN. 2014. Sulfur oxidizing bacteria in
325 Mangrove system: A review. Afr J Biotechnol. 13:2897-2907.
326 Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick
327 BR. 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the
328 roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem. 37:241–
329 250.
330 Bottos EM, Vincent WF, Greer CW, Whyte LG. 2008. Prokaryotic diversity of arctic ice
331 shelf microbial mats. Environ Microbiol. 10:950-966.
332 Bouali M, Feki M, Bakhrouf A. 2014a. Comparative study of two constructed wetlands
333 for wastewater treatment. J Chem Biol Phys Sci. 4:1680-1691.
334 Bouali M, Zrafi I, Bakhrouf A, Chaussonnerie S, Sghir A. 2014b. Bacterial structure and
335 spatiotemporal distribution in a horizontal subsurface flow constructed wetland.
336 App Microbiol Biotechnol. 98:3191-1203.
337 Cébron A, Louvel B, Faure P, France-Lanord C, Chen Y, Murrell YC, Leyval C. 2011.
338 Root exudates modify bacterial diversity of phenanthrene degraders in PAH-
339 polluted soil but not phenanthrene degradation rates. Environ Microbiol. 13:722-
340 736.
341 Chandra R, Bharagava RN, Kapley A, Purohit HJ. 2012. Characterization of *Phragmites*
342 cummunis rhizosphere bacterial communities and metabolic products during the

two stage sequential treatment of post methanated distillery effluent by bacteria and wetland plants. Bioresour Technol. 103:78–86.

Cunningham JE, Kuiack C. 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl Environ Microbiol. 58:1451-1458.

Dipak P, Sinha SN. 2013. Bacteria showing phosphate solubilizing efficiency in river sediment. J Biosci. 1:1-5.

Erguven GO. 2017. Comparison the performance of some soil fungi on ethalfluralin biodegradation with chemical oxygen demand and turbidity. EC Microbiol. 5(6):203-208.

Erguven GO, Bayhan H, Demir G, Ikizoglu B, Kanat G. 2016. Monitoring aclonifen remediation in soil with a laboratory-scale research. J Chem. Article ID 5059049.

Fitriatin BN, Arief DH, Simarmata T, Santosa DW, Joy B. 2011. Phosphatase-producing bacteria isolated from Sanggabuana forest and their capability to hydrolyze organic phosphate. J Soil Sci Environ Manag. 2:299-303.

Fitzgerald JW. 1976. Sulfate Ester Formation and Hydrolysis: a Potentially Important Yet Often Ignored Aspect of the Sulfur Cycle of Aerobic Soils. Bacteriol Rev. 40:698-721.

George P, Gupta A, Gopal M, Thomas L, Thomas GV. 2013. Multifarious beneficial traits and plant growth promoting potential of *Serratia marcescens* KiSII and *Enterobacter* sp. RNF 267 isolated from the rhizosphere of coconut palms (*Cocos nucifera* L.). World J Microbiol Biotechnol. 29:109-117.

366 Gyaneshwar P, James E, Mathan N, Reddy P, Reinhold-Hurek B, Ladha JK. 2001.
367 Endophytic Colonization of Rice by a Diazotrophic Strain of *Serratia marcescens*.
368 J Bacteriol. 183:2634–2645.
369 Gordon SA, Weber RP. 1951. Colorimetric estimation of indole- acetic acid. Plant
370 Physiol. 26:192-195.
371 Gulati A, Vyas P, Rahi P, Kasana RC. 2009. Plant Growth-Promoting and Rhizosphere-
372 Competent *Acinetobacter rhizosphaerae* Strain BIHB 723 from the Cold Deserts
373 of the Himalayas. Curr Microbiol. 58:371–377.
374 Haaijer SC, Van der Welle ME, Schmid MC, Lamers LP, Jetten MS, Op den Camp HJ.
375 2006. Evidence for the involvement of betaproteobacterial *Thiobacilli* in the
376 nitrate-dependent oxidation of iron sulfide minerals. FEMS Microbiol Ecol.
377 58:439-448.
378 The Water Analysis Handbook. 1989. Loveland (CO): Hach Company.
379 Hemalatha S, Veeramanikandan P. 2011. Characterization of Aromatic Hydrocarbon
380 Degrading Bacteria from Petroleum Contaminated Sites. J Environ Prot. 2:243-
381 254.
382 Huang XF, Chaparro JM, Reardon KF, Zhang R, Shen Q, Vivanco JM. 2014.
383 Rhizosphere interactions: root exudates, microbes, and microbial communities.
384 Botany. 92:267–275.
385 Jiang XT, Peng X, Deng GH, Sheng HF, Wang Y, Zhou HW, Tam N FY. 2013. Illumina
386 sequencing of 16S rRNA Tag revealed spatial variations of bacterial communities
387 in a Mangrove wetland. Environ Microbiol. 66:96–104.
388 Kadlec RH, Wallace S. 2008. Treatment Wetlands. Boca Raton (FL): CRC Press.

389 Kleikemper J, Schroth MH, Sigler WV, Schmucki M, Bernasconi SM, Zeyer J . 2002.
390 Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-
391 contaminated aquifer. Appl Environ Microbiol. 68:1516-1523.
392 Li YH, Zhu JN, Liu QF, Liu Y, Liu M, Liu L, Zhang Q. 2013. Comparison of the
393 diversity of root-associated bacteria in *Phragmites australis* and *Typha*
394 *angustifolia* in artificial wetlands. World J Microbiol Biotechnol. 29:1499–1508.
395 Likar M, Regvar M, Mandic-Mulec I, Stres B, Bothe H. 2009. Diversity and seasonal
396 variations of mycorrhiza and rhizosphere bacteria in three common plant species
397 at the Slovenian Ljubljana Marsh. Biol Fertil Soils. 45:573–583.
398 Lv X, Yu J, Fu Y, Ma B, Qu F, Ning K, Wu H. 2014. A Meta-Analysis of the Bacterial
399 and Archaeal Diversity Observed in Wetland Soils. Sci World J. 2014. Article ID
400 437684.
401 Mishra SS, Markande AR, Keluskar RP, Karunasagar I, Nayak BB. 2015. Simultaneous
402 nitrification and denitrification by novel heterotrophs in remediation of fish
403 processing effluent. J Basic Microbiol. 54:1–8.
404 Mohite B. 2013. Isolation and characterization of indole acetic acid (IAA) producing
405 bacteria from rhizospheric soil and its effect on plant growth. J Soil Sci Plant
406 Nutr. 13:638-649.
407 Mwajita MR, Murage H, Tani A, Kahangi EM. 2013. Evaluation of rhizosphere,
408 rhizoplane and phyllosphere bacteria and fungi isolated from rice in Kenya for
409 plant growth promoters. SpringerPlus. 2:606.

Nadeem SM, Zahir ZA, Naveed M, Asghar HN, Arshad M. 2010. Rhizobacteria Capable of Producing ACC-deaminase May Mitigate Salt Stress in Wheat. Soil Sci Soc Am J. 74:533-542.

Ortega-González DK, Zaragoza D, Aguirre-Garrido J, Ramírez-Saad H, Hernández-Rodríguez C, Jan-Roblero J. 2013. Degradation of benzene, toluene, and xylene isomers by a bacterial consortium obtained from rhizosphere soil of *Cyperus* sp. grown in a petroleum-contaminated area. Folia Microbiol. 58:569-577.

Pavitra BV, Sreenivasa MN, Savalgi VP, Shirnalli G. 2015. Isolation of potential phototrophic purple non-sulphur bacteria in paddy and their effects on paddy seedlings in hydroponic culture. Afr J Microbiol Res. 9:814-820.

Pérez-Miranda S, Cabirol N, George-Téllez R, Zamudio-Rivera LS, Fernández FJ. 2007. O-CAS, a fast and universal method for siderophore detection. J Microbiol Methods. 70:127–131.

Ravit B, Ehrenfeld JG, Haggblom MM. 2003. A Comparison of Sediment Microbial Communities Associated with *Phragmites australis* and *Spartina altemiflora* in Two Brackish Wetlands of New Jersey. Estuaries. 26: 465-474.

Rokhbakhsh-Zamin F, Sachdev D, Kazemi-Pour N, Engineer A, Pardesi KR, Zinjarde S, Dhakephalkar PK, Chopade BA. 2011. Characterization of plant-growth-promoting traits of *Acinetobacter* species isolated from rhizosphere of *Pennisetum glaucum*. J Microbiol Biotechnol. 21:556-566.

Ruiz-Rueda O, Hallin S, Baneras L. 2009. Structure and function of denitrifying and nitrifying bacterial communities in relation to the plant species in a constructed wetland. FEMS Microbiol Lett. 67:308–319.

433 Schmid M, Iversen C, Gontia I, Stephan R, Hofmann A, Hartmann A, Jha B, Eberl L,
434 Riedel K, Lehner A. 2009. Evidence for a plant-associated natural habitat for
435 *Cronobacter* spp. Res Microbio. 160:608-614.
436 Tian W, Zhao Y, Sun H, Bai J, Wang Y, Wu C. 2014. The effect of irrigation with oil-
437 polluted water on microbial communities in estuarine reed rhizosphere soils. Ecol
438 Eng. 70:275–281.
439 Ukaegbu-Obi KM, Mbakwem-Aniebo CC. 2014. Bioremediation Potentials of Bacteria
440 Isolated from Rhizosphere of Some Plants of Oil Contaminated Soil of Niger
441 Delta. Appl Environ Microbiol. 2:194-197.
442 Vladár P, Rusznyák A, Márialigeti K, Borsodi AK. 2008. Diversity of sulfate-reducing
443 bacteria inhabiting the rhizosphere of *Phragmites australis* in lake velencei
444 (Hungary) revealed by a combined cultivation-based and molecular approach**.**
445 Microb Ecol. 56:64-75.
446 Whitmire SL, Hamilton SK. 2005. Rapid removal of nitrate and sulfate in freshwater
447 wetland sediments. J Environ Qual. 34: 2062-2071.
448 Yue Y, Wang H, Liu J, Wang Q, Shen T, Guo W, Wang R. 2012. Shifts in microbial
449 community function and structure along the successional gradient of coastal
450 wetlands in Yellow River Estuary. Eur J Soil Biol. 49:12-21.
451 Zhang W, Wu X, Liu G, Chen T, Zhang G, Dong Z, Yang X, Hu P. 2013.
452 Pyrosequencing Reveals Bacterial Diversity in the Rhizosphere of Three
453 *Phragmites australis* Ecotypes. Geomicrobiol J. 30:593–599.

Zou J, Liu X, He C, Zhang X, Zhong C, Wang C, Wei J. 2013. Effect of *Scripus triqueter* of its rhizosphere and root exudates on microbial community structure of simulated diesel-spiked wetland. Int Biodeterior Biodegradation. 82:110–116.

Table and figure legends

Table 1. Physical-chemical properties of the water from the two sampling sites.

Table 2. MiSeq sequencing and bacterial diversity estimators for the four *P. australis* rhizosphere soils.

Table 3. Plant growth characteristics of the bacterial strains isolated from the rhizosphere soils of *P. australis*

Figure 1. A) Calculated rarefaction curves of observed OTUs (sequences that have 97% similarity are defined as one OTU) richness in the soils B) the most dominant bacterial classes/phyla in different rhizosphere soil samples, all classes that made up ≤ 3% each were groups into "others" C) Heatmaps representing a comparison of the relative abundance (% of total sequences) of bacterial genera between different rhizosphere soil samples.

Figure 2. Principal component analysis (PCA) based on the relative abundance of all detected bacterial classes/phyla (A) and genera (B) of the four studied rhizosphere soil samples

Figure 3. Unrooted phylogenetic tree showing the affiliation based on the 16S rRNA genes of the 26 strains obtained from the rhizosphere soils of *P. australis*

482

483 **Table 1**

484

Table 1. Physical-chemical water characteristics of the two sampling sites

Sampling site	A	B
Oil concentration (mg l^{-1})	1.6±1.1	0.3±0.4
Water temperature (˚C)	29.3±7.2	26.6±3.2
COD (mg l^{-1})	131.7±5.2	336.7±11.5
Ammonia (mg l^{-1})	0.3±0.1	0.3±0.1
TN (mg l^{-1})	1.2±0.1	0.5±0.1
Phosphate (mg l^{-1})	0.1±0.1	0.3±0.1
TP (mg l^{-1})	0.1±0.1	0.1±0.1
Dissolved oxygen (mg l^{-1})	8.0±3.4	7.5±1.1
pH	8.3±0.2	8.7±0.1
Conductivity (mS cm^{-1})	11.8±0.4	14.4±1.1
ORP (mV)	102.9±84	76.4±66
Sulfate (mg l^{-1})	370.2±29	448.7±42
Boron (mg l^{-1})	5.4±1.6	7.6±3.0

ORP: Oxidation reduction potential

COD: Chemical oxygen demand

485
486
487
488
489
490

Table 2

Table 2. Pyrosequencing and bacterial diversity estimators for the four *Phramites australis* rhizosphere soils using MiSeq.

Sample	Number of sequences	Number of OTUs 0.03[a]	Chao1	ACE	SSO	DSO
A1	112113	796	1084	1069	10	10.1
A2	104845	888	1177	1160	7.1	8.9
B1	151664	568	874	868	12.7	10.2
B2	86579	629	998	958	11.7	9.0

[a] Operational taxonomic unit at 3% sequence dissimilarity based on equal subsets of sequences

SSO Singletons sequences that were observed once

DSO Doubletons are sequences that were observed twice

491

Table 3

Table 3. Plant-growth characteristics of the bacterial strains isolated from the rhizosphere soils of *Phragmites australis*

Strain	OA	ACC deaminase	P	SID	IAA
YS1	+	-	+++	-	++
YS2	+	++	+	-	-
YS3	+	++	+	-	-
YS4	+	++	+	-	-
YS5	+	++	+	-	-
YS6	-	-	+++	+	-
YS7	+	++	+	-	-
YS8	-	-	+++	+	-
YS9	+	++	+	-	++
YS10	-	-	+++	+	++
YS11	+	++	+	-	++
YS12	-	-	+++	+	++
YS13	+	++	+	-	-
YS14	+	+	+++	+	-
YS15	-	+	+++	+	++
YS16	+	++	+	-	-
YS17	+	++	+	-	-
YS18	-	-	+++	+	++
YS19	-	-	+++	+	++
YS20	+	+	+++	+	++
YS21	+	+	+	-	-
YS22	+	+	+	-	-
YS23	-	-	+++	+	++
YS24	+	++	+	-	-
YS25	-	+	+++	+	-
YS26	-	+	+++	+	++

OA: organic acid production, ACC: 1-aminocyclopropane-1-carboxylate deaminase; IAA: indole acetic acid; SID: siderophore production; P: inorganic phosphate solubilization.

- No activity, + low activity, ++ high activity, +++ very high activity

Figure 1

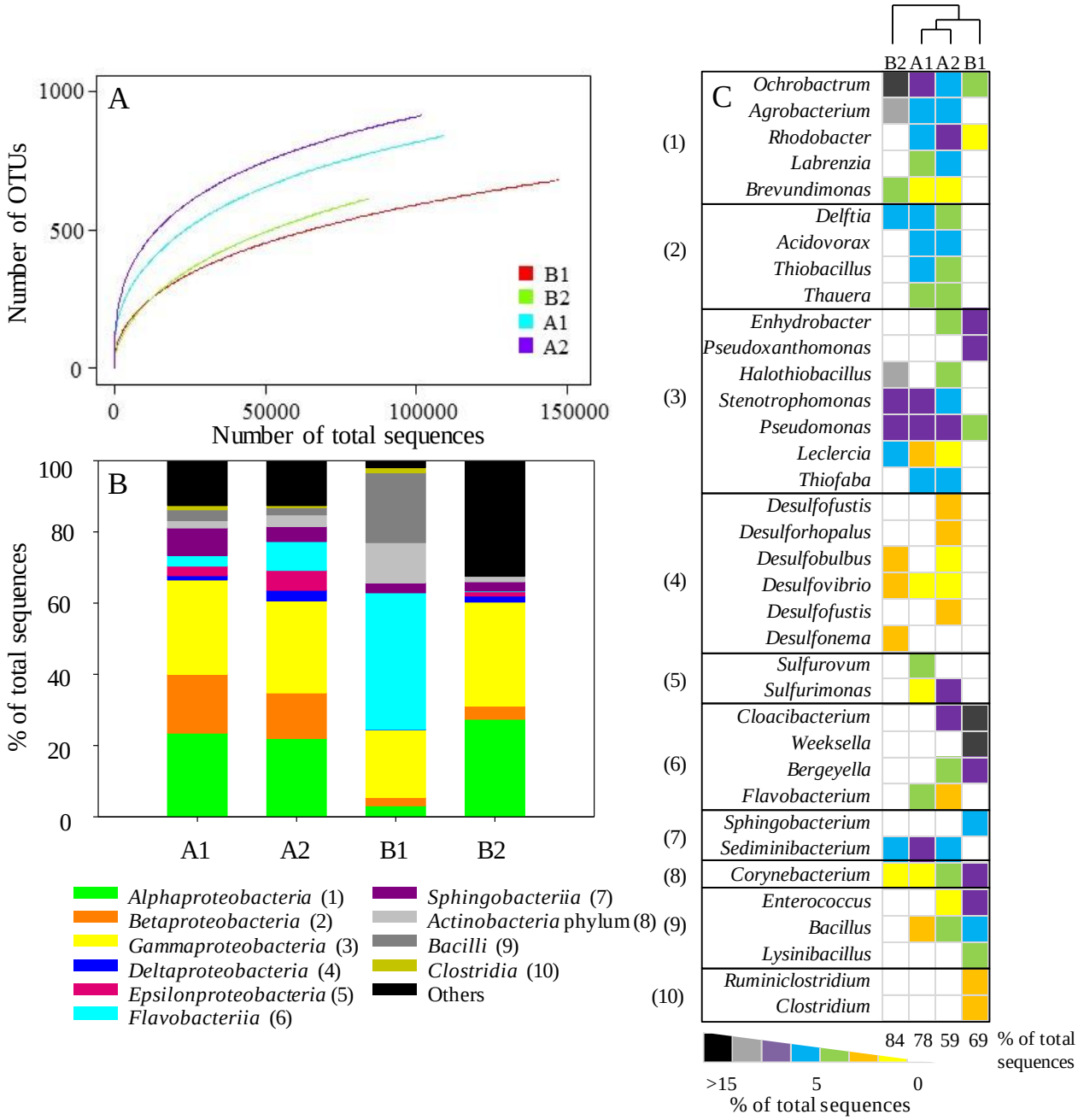
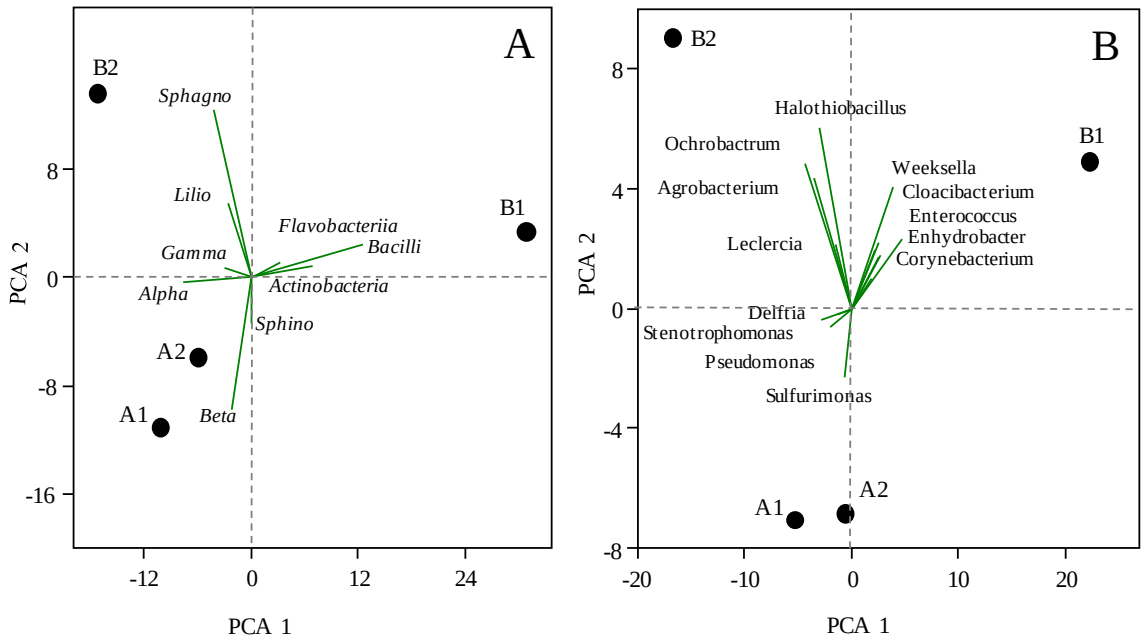
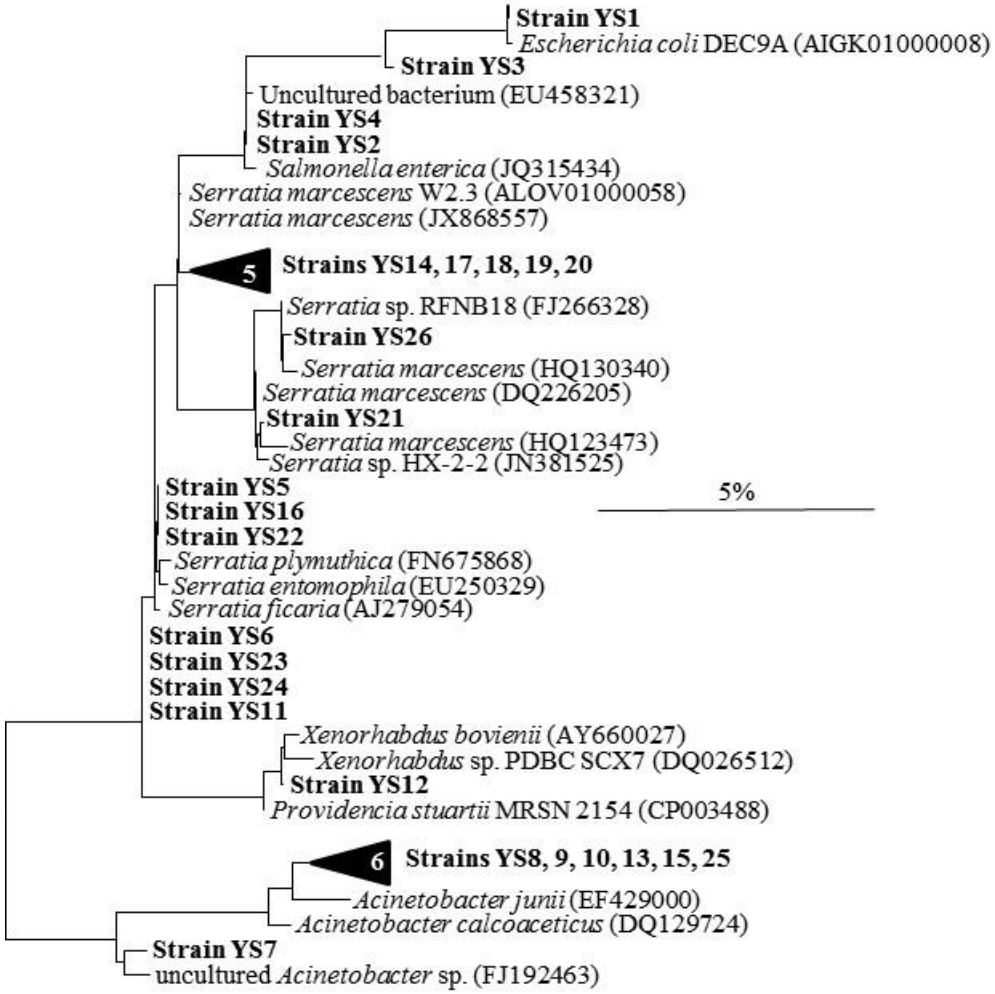

A
Number of OTUs
1000
500
0
0
50000
100000
150000
Number of total sequences
B1
B2
A1
A2
B
% of total sequences
100
80
60
40
20
0
A1
A2
B1
B2
Alphaproteobacteria (1)
Betaproteobacteria (2)
Gammaproteobacteria (3)
Deltaproteobacteria (4)
Epsilonproteobacteria (5)
Flavobacteriia (6)
Sphingobacteriia (7)
Actinobacteria phylum (8)
Bacilli (9)
Clostridia (10)
Others
B2 A1 A2 B1
C
Ochrobactrum
Agrobacterium
Rhodobacter
Labrenzia
Brevundimonas
(1)
Delftia
Acidovorax
Thiobacillus
Thauera
(2)
Enhydrobacter
Pseudoxanthomonas
Halothiobacillus
Stenotrophomonas
Pseudomonas
Leclercia
Thiofaba
(3)
Desulfofustis
Desulforhopalus
Desulfobulbus
Desulfovibrio
Desulfofustis
Desulfonema
(4)
Sulfurovum
Sulfurimonas
(5)
Cloacibacterium
Weeksella
Bergeyella
Flavobacterium
(6)
Sphingobacterium
Sediminibacterium
(7)
Corynebacterium
(8)
Enterococcus
Bacillus
Lysinibacillus
(9)
Ruminiclostridium
Clostridium
(10)
84 78 59 69 % of total sequences
>15 5 0
% of total sequences

Figure 2


A
B2
Sphagno
Lilio
Gamma
Alpha
Flavobacteriia
Bacilli
Actinobacteria
Sphino
B1
A2
A1
Beta
PCA 2
PCA 1
8
0
-8
-16
-12
0
12
24
B
B2
Halothiobacillus
Ochrobactrum
Agrobacterium
Leclercia
Weeksella
Cloacibacterium
Enterococcus
Enhydrobacter
Corynebacterium
B1
Delftia
Stenotrophomonas
Pseudomonas
Sulfurimonas
A1
A2
PCA 2
PCA 1
8
4
0
-4
-8
-20
-10
0
10
20

495

Figure 3


Strain YS1
Escherichia coli DEC9A (AIGK01000008)
Strain YS3
Uncultured bacterium (EU458321)
Strain YS4
Strain YS2
Salmonella enterica (JQ315434)
Serratia marcescens W2.3 (ALOV01000058)
Serratia marcescens (JX868557)
5
Strains YS14, 17, 18, 19, 20
Serratia sp. RFNB18 (FJ266328)
Strain YS26
Serratia marcescens (HQ130340)
Serratia marcescens (DQ226205)
Strain YS21
Serratia marcescens (HQ123473)
Serratia sp. HX-2-2 (JN381525)
5%
Strain YS5
Strain YS16
Strain YS22
Serratia plymuthica (FN675868)
Serratia entomophila (EU250329)
Serratia ficaria (AJ279054)
Strain YS6
Strain YS23
Strain YS24
Strain YS11
Xenorhabdus bovienii (AY660027)
Xenorhabdus sp. PDBC SCX7 (DQ026512)
Strain YS12
Providencia stuartii MRSN 2154 (CP003488)
6
Strains YS8, 9, 10, 13, 15, 25
Acinetobacter junii (EF429000)
Acinetobacter calcoaceticus (DQ129724)
Strain YS7
uncultured Acinetobacter sp. (FJ192463)