Post-print of: Adrados B, Sánchez O, Arias CA, Becares E, Garrido L, Mas J, Brix H and Morato J. Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters. Water Research 55: 304-312

Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters.

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Abstract

The prokaryotic microbial communities (Bacteria and Archaea) of three different systems operating in Denmark for the treatment of domestic wastewater (horizontal flow constructed wetlands (HFCW), vertical flow constructed wetlands (VFCW) and biofilters (BF)) was analysed using endpoint PCR followed by Denaturing Gradient Gel Electrophoresis (DGGE). Further sequencing of the most representative bacterial bands revealed that diverse and distinct bacterial communities were found in each system unit, being γ-Proteobacteria and Bacteroidetes present mainly in all of them, while Firmicutes was observed in HFCW and BF. Members of the Actinobacteria group, although found in HFCW and VFCW, seemed to be more abundant in BF units. Finally, some representatives of α, ß and δ-Proteobacteria, Acidobacteria and Chloroflexi were also retrieved from some samples. On the other hand, a lower archaeal diversity was found in comparison with the bacterial population. Cluster analysis of the DGGE bacterial band patterns showed that community structure was related to the design of the treatment system and the organic matter load, while no clear relation was established between the microbial assemblage and the wastewater influent.

Keywords: microbial community, PCR-DGGE, domestic wastewater, constructed wetlands, biofilters.

1. Introduction

Natural wastewater treatment systems such as constructed wetlands, biological sand filters and other decentralised solutions are becoming an increasingly relevant alternative to conventional systems when treating wastewater from small communities and dwellings due to its efficiency, low establishment costs and low operation and management requirements. In order to treat wastewater effectively, several factors have to be taken into account, e.g. the system's capacity, the plant species used, colonization characteristics of certain microbial groups, and the interactions of biogenic compounds and particular contaminants (wastewater components) with the filter bed material (Stottmeister, 2003). Although filtration is considered an important process in these removal mechanisms, additional interactions occur among media, plants and water. Many processes and relations between them take place: microbial-mediated processes, chemical networks, volatilization, sedimentation, sorption, photodegradation, plant uptake, transpiration flux and accretion (Kadlec and Wallace, 2009). The importance of microbial processes has been further studied as many reactions are microbiologically mediated (Stottmeister 2003, Kadlec and Wallace, 2009).

The most stable microbiota in these systems is found in the biofilm associated to the plant's roots and/or attached to the surface of the filter bed material. This complex microbial community created by interactions with wastewater, is mainly responsible for the degradation performance of the system (Sleytr et al., 2009). Furthermore, the diversity of microorganisms in this environment may be critical for its proper functioning and maintenance (Ibekwe, 2003). To improve the design of these systems, a detailed knowledge of the structure of these communities should be acquired in order to understand the biological processes that are taking place within them (Truu et al., 2009, Dong and Reddy, 2010). Recently, several studies have characterized microbial populations in laboratory scale units, sand filters and full scale constructed wetlands under specific conditions (Ragusa et al., 2004, Vacca et al., 2005, Baptista et al., 2008, Calheiros et al., 2009, Krasnits et al., 2009, Sleytr et al., 2009, Zhang et al., 2010, Dong and Reddy, 2010). However, there is a general lack of information on the diversity and changes of the microbial communities in long-term operation systems treating domestic wastewater at real time scale (Krastnits et al., 2009).

Increased removal efficiency of nitrogen from wastewater is one of the key issues for further development of constructed wetlands and other decentralised technologies. The diversity of microorganisms involved in the N-cycle is expected to be high in these systems. In fact, previous studies have suggested that archaeal nitrifiers, denitrifying fungi, aerobic

denitrifying bacteria and heterotrophic nitrifying microorganisms may play an important role in nitrogen transformations in constructed wetlands (Truu et al., 2005). Most importantly, the effects of biofilms on nitrogen transformation and removal have not been adequately studied and modelled. As microorganisms affect processes like nitrification, denitrification, uptake, and sedimentation, they have to be taken into consideration when modelling the transformation and removal of nitrogen from wastewater (Mayo and Bigambo, 2004). Thus, a first step for establishing the role of biological communities in N-removal in constructed wetlands is to evaluate the diversity of microorganisms under different conditions and systems. With this purpose recent studies have introduced the characterization of bacterial communities by means of molecular methods based on 16S rRNA gene analysis (Sleytr, 2009).

The aim of this study was to compare the composition of microbial communities of three different types of domestic wastewater treatment systems used in Denmark: Horizontal Flow Constructed Wetlands (HFCW), Vertical Flow Constructed Wetlands (VFCW) and Biofilters (BF, with combined configurations of vertical or horizontal flow) using the PCR-DGGE based method. The systems were composed of different bed filling media, namely soil, sand and LWA (lightweight aggregate). In this work, we enlarged the microbial analysis by analyzing both the bacterial and archaeal populations, focusing in the possible influence of the water influent composition, the design and the bed filling of the treatment systems in the structure of these microbial communities.

2. Material and methods

2.1 Site description

The wastewater treatment systems (WWTS) investigated were rural facilities used in Denmark for the treatment of domestic wastewaters. All the systems were built following Danish guidelines and comply with Danish wastewater discharge standards (for details see Brix and Arias, 2005). The layout of all the studied systems included a primary treatment step, using a sedimentation tank with a hydraulic residence time proportional to the number of people served and a minimum of 2 m^3. The second treatment step differs depending on the system chosen by the users among an array of technical possibilities approved by the Danish EPA.

Three types of systems were selected for the study: two horizontal flow constructed wetlands (HFCW) with soil beds, two vertical flow constructed wetlands (VFCW) with sand bed and

two LWA Biofilters (BF) fitted with a Filtralite-P® bed for the removal of phosphorous (Jenssen et al., 2010). The systems differed in flow configuration, operational and bed media characteristics.

The HFCWs studied have been operational for over 20 years. The systems were built following national guidelines (Miljøministeriet Miljøstyrelsen, 1990) and were composed of two soil filled beds operating in parallel with the necessary structures for distribution and collection of domestic water. After the treatment water was discharged to nearby watercourses (for details see Brix et al., 2009).

VFCWs were also built following the Danish design and construction guidelines (Miljøministeriet Miljøstyrelsen 2005). The domestic wastewater was pre-treated in a sedimentation tank; after that, water was loaded sequentially on the system surface at a rate of approx. 20 pulses/d to an unsaturated bed filled with sand, where it was homogeneously distributed in the surface trickling vertically. Once the water percolated through a one meter deep bed, it was collected at the bottom and evacuated. In order to improve the water quality, and enhance denitrification capacity, treated water was recycled back to the pumping well in one of the two systems studied, where conditions should favour the process (for details see Brix and Arias, 2005).

BFs are media filled systems that combine unsaturated conditions and a water saturated bed. The first section of the system operates unsaturated; it is housed in a fibreglass dome filled with a lightweight aggregate (LWA) from which wastewater is pumped at a rate of around 25 pulses/day. The second step of the treatment system involves the flow of water through a saturated bed filled with Filtralite -P® media, which is a LWA product chemically enriched, specifically engineered for phosphorus removal (see details in Jenssen et al., 2010). Different wastewater treatment systems studied are shown in Figure 1 and their operational and design characteristics are shown in Table 1.

The flow conditions within the systems control the oxygen availability and therefore, anoxic conditions predominated in saturated HFCWs while oxic conditions prevail in VFCWs (Vymazal et al., 2006; Brix and Arias, 2005).

On the other hand, because of the combination of two different modules, oxic conditions are found in the first section of BF systems, while anoxic conditions develop in the P removal bed.

2.2 Soil and water sampling

Soil samples were taken in May 2010 from each system (Figure 1 and Figure 2), the two HFCW (HFCW 1 and HFCW 2), the two BF (BF 1 and BF2) and the two VFCW (1 and 2). When sampling HFCW, because of the horizontal flow, two separated zones were differentiated and samples were taken at the influent (I) and effluent (E) zone, and considered separately. In the case of BF, samples were also taken in two different parts of the system: in the first module (also represented as I) and in the main bed (E). Sampling points are shown with arrows in Figure 1.

Three subsamples were collected in each sampling point at random by means of a core (1 m length, 2.54 cm diameter) and then mixed to yield one composite sample per point. Samples were stored at 4ºC, and processed within 24h.

Grab water samples from influent and effluent were taken in three sampling campaigns, once a month between March and May 2010. Each campaign consisted of three consecutive sampling days. Samples were frozen at -20ºC until they were processed.

2.3 Water analysis

The water quality parameters measured included *in situ* measurements of water temperature, oxygen saturation and electric conductivity as standard water control by means of calibrated electrodes. Additional water quality analysis included BOD5 determination using APHA5210B method, and nitrogen species such as total nitrogen (Kjeldhal Method), ammonia (APHA 4500 NH3 D method), nitrite (APHA 4500 NO_2 B method) and nitrate (APHA 4500-NO_3^- F method).

2.4 Soil DNA extraction

A total of 100 g for each composite sample were collected in 100 ml of sterile saline solution (9 % NaCl) and sonicated for 5 minutes in an ultrasonic water bath (Selecta, Barcelona, Spain). Samples were also vortexed 1 min to release the biofilm attached to the solution into the liquid phase. Subsequently, 10 ml were recovered and concentrated by centrifugation (5 min, 8,000 g), and then samples were stored at -20ºC until further processing. DNA extractions were performed using the EZNA® Soil DNA kit (Omega Bio-Tek, Doraville, USA) following the manufacturer's recommendations.

197

198 *2.5 PCR amplification, DGGE and sequencing of 16S rRNA genes*

199

200 Amplification of 16S rRNA gene fragments for DGGE analysis was performed by using the
201 bacterial specific primer set 358F with a 40bp GC clamp, and the universal primer 907RM
202 (Sánchez et al., 2007). Polymerase chain reaction (PCR) was carried out with a Biometra
203 thermocycler using the following program: initial denaturation at 94 ºC for 5 min, 10
204 touchdown cycles of denaturation (at 94 ºC for 1 min), annealing (at 63.5 ºC to 53.5 ºC for 1
205 min, decreasing 1ºC each cycle), and extension at 72 ºC for 3 min. This procedure was
206 followed by 20 additional cycles at an annealing temperature of 53.5 ºC. During the last cycle
207 of the program, the length of the extension step was 15 min at 72 ºC.

208

209 Primers 344F-GC and 915R were used for archaeal 16S rRNA gene fragment amplification
210 (Casamayor et al., 2002). The PCR protocol included an initial denaturation step at 94 ºC for
211 5 min, followed by 20 touchdown cycles of denaturation (at 94 ºC for 1 min), annealing (at 71
212 ºC to 61 ºC for 1 min, decreasing 1 ºC each cycle), and extension (at 72 ºC for 3 min); 20
213 standard cycles (annealing at 55 ºC, 1 min) and a final extension at 72 ºC for 5 min.

214

215 PCR mixtures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a
216 concentration of 200µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U Taq
217 DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. Bovine Serum
218 Albumin (BSA) at a final concentration of 600 µg ml^{-1} was added to minimize the inhibitory
219 effect of humic substances (Kreader, 1996). The volume of reactions was 50 µl. PCR products
220 were verified and quantified by agarose gel electrophoresis, with a low DNA mass ladder
221 standard (Invitrogen).

222

223 The DGGE was run in a DCode system (Bio-Rad) as described by Muyzer et al. (1998). A
224 6% polyacrylamide gel with a gradient of 40-80% DNA denaturant agent was cast by mixing
225 solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40%
226 deionized formamide). Seven hundred ng of PCR product were loaded for each sample and
227 the gels were run at 100 V for 18 h at 60°C in 1 x TAE buffer (40 mM Tris [pH 7.4], 20 mM
228 sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45
229 min, rinsed with 1 x TAE buffer, removed from the glass plate to a UV-transparent gel scoop,
230 and visualized with UV in a Chemi Doc EQ (Bio-Rad). Prominent bands were excised from
231 the gels, resuspended in milli-q water overnight and reamplified for their sequencing.

232

Purification of PCR products from DGGE bands and sequencing reactions were performed by Macrogen (South Korea) with primer 907RM for Bacteria and primer 915R for Archaea. PCR products of the reamplified bands were used as DNA template in a sequencing reaction with the Big Dye Terminator version 3.1 sequencing kit in an automatic ABI 3730XL Analyzer-96 capillary type. Sequences were subjected to a BLAST search (Altschul et al., 1997) to obtain an indication of the phylogenetic affiliation.

Fifty-six 16S bacterial rRNA gene sequences were submitted to the EMBL database (http://www.ebi.ac.uk/embl) and received the following accession numbers: from HE716787 to HE716842.

2.6 Analysis of DGGE patterns and statistical analyses

Digitalized DGGE images were analysed with the Quantity One software (Bio-Rad, Hercules, USA). Bands occupying the same position in the different lanes of the gels were identified. A matrix was constructed for all lanes, taking into account the presence or absence of the individual bands. Raup-Crick index was used for absence-presence data as this index utilizes a randomization procedure (Monte Carlo) comparing the observed number of species occurring in both samples in 200 pairs of random replicates of the pooled sample. The PAST program (Hammer et al 2004) was used for theses analyses.

DGGE banding data were used to calculate the Shannon–Weaver index as a measure of the diversity of microbial communities. It was calculated using the following function:

$$H' = -\sum_{i=1}^{i=n} p_i \ln p_i$$

Where n is the number of bands in the sample and p_i the relative intensity of the band.

3. Results and discussion

The aim of this study was to investigate the factors affecting the structure of prokaryotic communities established in three different types of natural wastewater treatment systems, each with different substrate and configuration. Analysis of bacterial and archaeal community composition from the substrate samples collected was performed by means of PCR-DGGE. The banding patterns for the 16S rRNA gene DGGE-PCR amplicons are presented in Fig. 2 for Bacteria and Archaea. Clear differences could be observed in both gels concerning band

267 position, intensity and band number for the different samples, demonstrating that different
268 bacterial and archaeal communities developed in the different systems.
269
270 In the bacterial DGGE, a high number of bands could be observed in all lanes (Fig. 2A). Band
271 richness fluctuated from 31 in HFCW1I to 17 in the BF1E system (Table 2). Significant
272 differences were found in total band richness among the influents and effluents ($p < 0.05$),
273 influents harbouring higher richness than effluents (27 and 21 mean band richness for
274 influents and effluents respectively). Similar results were found for Shannon diversity indexes
275 (2.65 and 2.25 for influents and effluents respectively). On the other hand, although archaeal
276 amplification was also found, the DGGE banding profile clearly revealed a lower diversity in
277 comparison with the bacterial community (Fig. 2B).
278
279 Excision of prominent bacterial DGGE bands and subsequent sequencing allowed the
280 characterization of the predominant microorganisms in the different systems studied.
281 Informative sequences were obtained from 56 bacterial bands. The number of bases used to
282 calculate each similarity value is also shown in Table 3, as an indication of the quality of the
283 sequence. Unfortunately, bands recovered from the archaeal DGGE gel yielded sequences
284 with a very poor quality that have not been included in this study. The most represented
285 taxonomic groups in all samples belonged to the γ-Proteobacteria (26% of recovered bands)
286 and Bacteroidetes (26%). Firmicutes (15%) were present in all systems with the exception of
287 samples from VFCW. Members of the Actinobacteria group, although found in HFCW and
288 VFCW, seemed to be more abundant in BF systems. Finally, some representatives of α, ß and
289 δ-Proteobacteria, Acidobacteria and Chloroflexi were also retrieved in some of the samples.
290
291 Most of the sequences corresponded to uncultured microorganisms (71% of the retrieved
292 sequences), while others matched with a high percentage of similarity to cultured bacteria
293 (29%). In general, typical bacteria from soil and wastewater environments were found in all
294 the systems analyzed. For example, we could retrieve in HFCW typical soil bacteria such as
295 sequences related to *Acinetobacter* sp. *(*γ-Proteobacteria), *Arthrobacter* sp (from the
296 Actinobacteria group, also found in samples from VFCW and BF), and *Bacillus* sp.
297 (Firmicutes), all of them potential denitrifying bacteria. Besides, other non-culturable matches
298 corresponding to different groups were present. *Acinetobacter* sp. is commonly present in
299 activated sludge (Snaird el al. 1997) especially in those where enhanced biological phosphate
300 removal is observed (Ivanov et al., 2005). On the other hand, *Arthrobacter* sp has been related
301 to the nitrogen cycle, particularly to nitrogen fixation (Cacciari et al. 1971). The fact that

some aerobic microorganisms have been found suggests that although HFCW systems are mostly all the time saturated, enough oxygen is present to allow proliferation of these microbial groups, with the subsequent possibility of nitrification in the system. Oxygen is present probably due to plant aeration and also because the upper part of the bed normally remains unsaturated.

Concerning the Bacteroidetes phylum, a group of chemoheterotrophic bacteria known by its ability to degrade complex organic matter, sequences with a high similarity at the species level were found. Thus, some of the retrieved sequences related to *Flavobacterium* sp., another potential denitrifying bacteria, and have been detected in VFCW and BF; it is a typical genus that can be found in activated sludge (Park et al. 2007). Another sequence similar to the denitrifying *Thauera terpenica* (cultured closest match 99.6% similarity) was also observed in VFCWs. Other species were also found in BF systems, such as sequences related to the γ-Proteobacteria *Xanthomonas* sp., *Dokdonella* sp., and some denitrifying bacteria such as *Rhodanobacter* sp. and *Stenotrophomonas* sp.

The application of molecular techniques (PCR-DGGE profiling) on different wastewater treatment systems has allowed the identification of some players and their potential role in the nitrogen removal processes. The diversity of N-cycling bacteria found in the analyzed systems is an indicator of the multiple possibilities of biological nitrogen transformations inside them. In addition, this profiling method is a useful tool to classify microbial community under different substrates by clustering and diversity analyses.

A cluster analysis of bacterial DGGE banding patterns based in band richness is shown in Fig. 3. Samples separated in two clusters; samples coming from VFCW and BFI, corresponding to unsaturated samples with a high organic load (Table 1), clustered together in one of the two main clusters, while all the other samples, corresponding to saturated systems with low organic load, clustered in another group. As there is almost no relation between the influent and effluent bacterial communities inside the same wetland, these results suggest that factors other than the influent wastewater, such as the organic load and the design of the treatment system, contribute to shape the microbial community.

Previous studies have shown that shifts in the structure of bacterial communities can be associated with changes in a number of soil properties, including soil texture and soil nitrogen availability (Dong et al, 2010). The substrate is an important component since it supports plant growth (in case of planted wetland systems), as well as the establishment of a microbial

biofilm, and it influences the hydraulic processes (Stottmeister et al., 2003). A porous matrix substrate such as LWA will probably favour the development of biofilms. Additionally, recent studies concluded that the type of substrate is one of the main factors influencing bacterial communities (Vacca et al, 2005, Calheiros et al, 2009). However, none of these studies took place in real constructed wetlands; both of them consisted in different pilot systems, with the same influent water. In our study, no relation between the microbial assemblage and the substrate was found, as different communities were retrieved within systems with the same substrate. On the contrary, from the cluster analysis we did observe two separated groups that appeared to be influenced by factors such as the organic load, as well as for the absence/presence of oxygen, since one of the groups is composed only by samples from unsaturated samples, which receive a higher load of organic matter (VFCW and influent of BF), and the other group by saturated conditions with a lower load of organic matter (HFCW influent and effluent zone, and BF effluent zone). Since influent water is different for each system, the results suggest a community configuration more related with the design of the treatment system and its operational conditions. These results are in consonance with the work carried out by Baptista et al. (2008), who suggested that stochastic processes could play an important role in the microbial community assembly in engineered and natural systems.

Different authors, such as Ibekwe et al (2003) and Calheiros et al. (2009) indicated that the diversity of the bacterial community in the constructed wetlands systems might influence the final effluent quality, and so the engineering should be directed to develop a higher diversity in order to enhance processes such as nitrification and denitrification (Ibekwe et al. 2003). The Shannon index obtained for our samples showed a very similar diversity for all the samples. Significant differences (p-value<0.05) were only found between HFCW and BF.

On the other hand, despite we could not retrieve sequences directly affiliated to known nitrifiers, nitrogen removal occurred in all the systems evaluated, although the removal rates were different among systems (Table 1). Saturated systems did not reach high nitrification rates but they were able to denitrify almost all the nitrified ammonia. Unsaturated systems were capable of high nitrification rates but total nitrogen removal was lower than unsaturated CW.

The removal of nitrogen in constructed wetlands is usually limited by the nitrification process, and in order to reach high total nitrification rates is important that biological nitrification takes place. Additionally, in order to increase denitrification rates in the unsaturated systems, the establishment of recycling or an additional step is a must. In this sense, the application of

molecular techniques in this study has revealed the presence of several groups of denitrifiers. Finally, the diversity for bacterial groups has proven to be higher than for archaeal representatives. Further studies are needed to assess the activity of these groups under different conditions, and to go deeper into the functional groups present in each system.

4. Conclusions

-The application of molecular techniques (PCR-DGGE profiling) on different wastewater treatment systems showed that there is no relation between the influent and effluent bacterial communities inside the same treatment system.

-Different systems with the same configuration and substrate, showed different microbial community.

-High diversity of bacteria was found in all systems studied. A lower archaeal diversity was found in comparison with the bacterial population

-Microbial community structure was related to the design of the treatment systems and the organic matter load.

-Microbial community structure was affected by oxygen conditions in the substrate (saturated or unsaturated).

Acknowledgements

This research was supported by predoctoral scholarships, FI and FPI, from the Comissionat per a Universitats i Recerca del Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya i del Fons Social Europeu, and Ministry of Education and Science of Spain, respectively. Financial support was provided by grants CTM2008-06676-C05-02/TECNO from the Ministry of Science and Innovation of Spain to Jordi Morató and by Consolider TRAGUA (CSD2006-00044), CTQ2009-14390-C02-02.

REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhan, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402

Baptista, J.C., Davenport, R.J., Donnelly, T., Curtis, T.P., 2008. The microbial diversity of laboratory-scale wetlands appears to be randomly assembled. Water Researchh 42:3182-3190

Brix, H., Arias, C.A., 2005. Danish guidelines for small-scale constructed wetland systems for onsite treatment of domestic seawage. Water Science Technology 51:1-9

414 Brix, H., Koottatep, T., Fryd, O., Laugesen, C.H., 2010. The flower and the butterfly
415 constructed wetland system at Koh Phi Phi – System design and lessons learned during
416 implementation and operation. Ecological Engineering 37:729-735

417 Cacciari, I., Giovannozzi-Sermanni, G., Grapelli, A., Lippi, D., 1971. Nitrogen fixation by
418 *Arthrobacter* sp. Part I. Taxonomic study and evidence of nitrogenase activity of two new
419 strains. Annali di microbiologia ed enzimologia 21:97-105

420 Calheiros, C.S.C., Duque, A.F., Moura, A., Henriques, I.S., Correia, A., Rangel, A.O., Castro,
421 P.M., 2009. Substrate effect on bacterial communities from constructed wetlands planted with
422 *Typha latifolia* treating industrial wastewater. Ecological engineering 35:744-753

423 Casamayor, E.O., Massana, R., Benlloch, S., Ovreas, L., Díez, B., Goddard, V-J., Gasol, J.M.,
424 Joint, I., Rodriguez-Valera, F., Pedrós-Alió, C., 2002. Changes in archaeal, bacterial and
425 eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting
426 methods in a multipond solar saltern. Environmental Microbiology 4:338-348

427 Dong, X., Reddy, G.B., 2010. Soil bacterial communities in constructed wetlands treated with
428 swine wastewater using PCR-DGGE technique. Bioresource Technology 101:1175-1182

429 Hammer, O., Harper, D.A.T., Ryan, P.D., 2008. PAST-Paleontological Statistics. V 1.81

430 Ibekwe, A.M., Grieve, C.M., Lyon, S.R., 2003. Characterization of microbial communities
431 and composition in constructed dairy wetland wastewater effluent. Applied and
432 Environmental Microbioly 69:5060-5069

433 Ivanov, V., Stabnikov, V., Zhuan, W.Q., Tay, J.H., Tay, S.T., 2005. Phosphate removal from
434 the returned liquor of municipal wastewater plant using iron-reducing bacteria. Journal of
435 Applied Microbiology 98:1152-1161

436 Jenssen, P.D., Krogstad, T., Paruch, A.M., Maehlun, T., Adam, K., Aricas, C.A., Heistad, A.,
437 Jonsson, L., Hellström, D., Brix, H., Yli-Halla, M., Vrale, L., Valve, M., 2010. Filter bed
438 systems treating domestic wastewater in the Nordic countries – Performance and reuse of
439 filter media. Ecological engineering 36:1651-1659

440 Kadlec, R.H., Wallace, S., 2009. Treatment wetlands. CRC press, Boca Raton, FL, USA

441 Krasnits, E., Friedler, E., Sabbah, I., Beliavski, M., Tarre, S., Green, M., 2009. Spatial
442 distribution of major microbial groups in a well established constructed wetland treating
443 municipal wastewater. Ecological engineering 35:1085-1089

444 Kreader, C.A., 1996. Relief of amplification inhibition in PCR with bovine serum albumin or
445 T4 gene 32 protein. Applied and Environmental Microbiology 62:1102-1106

446 Mayo, A.W., Bigambo, T., 2005. Nitrogen transformation in horizontal subsurface flow
447 constructed wetlands I: Model development. Physics and Chemistry of the Earth 30:658–667

448 Miljøministeriet Miljøstyrelsen, 1990. Spildevandsforskning fra Miljøstyrelsen Nr 8
449 Spildevandsrensning i rodzoneanlæg. Copenhagen (In Danish).

450 Miljøministeriet Miljøstyrelsen. 2004 Retningslinier for etablering af beplantede filteranlæg
451 op til 30 PE, Økologisk byfornyelse og spildevandsrensning Nr. 52. Copenhaguen (In Danish)

452 Park, M., Ryu, S.H., Vu, T.H., Ro, H.S., Yun, P.Y., Jeon, C.O., 2007. *Flavobacterium*
453 *defluvii* sp nov, isolated from activated sludge. International Journal of Systematic and
454 Evolutionaru Microbiology 57:233-237

455 Ragusa, S.R., McNevin, D., Qasem, S., Mitchell, C., 2004. Indicators of biofilm development
456 and activity in constructed wetlands microcosms. Water Research 38:2865-2873

457 Sánchez, O., Gasol, J.M., Massana, R., Mas, J., Pedrós-Alió, C., 2007. Comparison of
458 different denaturing gradient gel electrophoresis primer sets for the study of marine
459 bacterioplankton communities. Applied andl Environmental Microbiology 73:5962-5967

460 Sleytr, K., Tietz, A., Langergraber, G., Haberl, R., Sessitsch, A., 2009. Diversity of abundant
461 bacteria in subsurface vertical flow constructed wetlands. Ecological engineering 35:1021-
462 1025

463 Snaidr, J., Amann, R., Huber, I., Ludwig, W., Schleiffer, K.H., 1997. Phylogenetic analysis
464 and in situ identification of bacteria in activated sludge. Applied and Environmental
465 Microbiology 63:2884-2896

466 Stottmeister, U., Wiessner, A., Kuschk, P., Kappelmeyer, M.K., Bederski, R.A., Müller, H.,
467 Moormann, H., 2003. Effects of plants and microorganisms in constructed wetlands for
468 wastewater treatment. Biotechnology Advances 22:93-117

469 Truu, M., Juhanson, J., Truu, J., 2009. Microbial biomass, activity and community
470 composition in constructed wetlands. Science of the Total Environment 407:3958-3971

471 Truu, M,, Nurk, K., Juhanson, J., Mander, U., 2005. Variation of microbiological parameters
472 within planted soil filter for domestic wastewater treatment. Journal of Environmental Scienc
473 and Health. Part A Toxic/ Hazardous Substances and Environmental Engineering 40:1191-
474 1200

475 Vacca, G., Wand, H., Nikolausz, M., Kuschk, P., Kästner, M., 2005. Effect of plants and filter
476 materials on bacteria removal in pilot-scale constructed wetlands. Water Research 39:1361-
477 1373

478 Vymazal, J., Brix, H., Cooper, P.F., Green, M.B., Haberl, R., 2006. Constructed wetlands for
479 wastewater treatment. In: JTA Verhoeven, B Beltman, R Bobbink, DF Wigham (eds),
480 Wetlands as a natural resource, Vol 1. Wetlands and natural resource management, Springer
481 Verlag, pp 69-96

482 Zhang, C-B., Wang, J., Liu, W-L., Zhu, S-X, Ge, H.L., Chang, S, Chang, J., Ge, Y., 2010.
483 Effects of plant diversity on microbial biomass and community metabolic profiles in a full-
484 scale constructed wetland. Ecological engineering 36:62-68

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Figure legends

Figure 1 Schemes of the three types of systems studied; a) HFCW, b) VFCW, c) BF. 1) inlet, 2) sedimentation tank, 3) pumping well, 4) bed, 5) outlet well, 6) recycling, 7) P removal system, 8) LWA dome biofilters. Arrows indicate the sampling sites of each system.

Figure 2 Negative images of DGGE gels with PCR products amplified with bacterial (A) and archaeal (B) primer sets from samples of the different systems: HFCW (Horizontal Flow Constructed Wetlands), BF (Biofilters) and VFCW (Vertical Flow Constructed Wetlands); 1 and 2 are replicates from each system; when applied, I: Influent zone, E: Effluent zone.

Figure 3 Cluster analysis of bacterial DGGE profiles, determined by the Raup-Crick method.

498 **Fig. 1**
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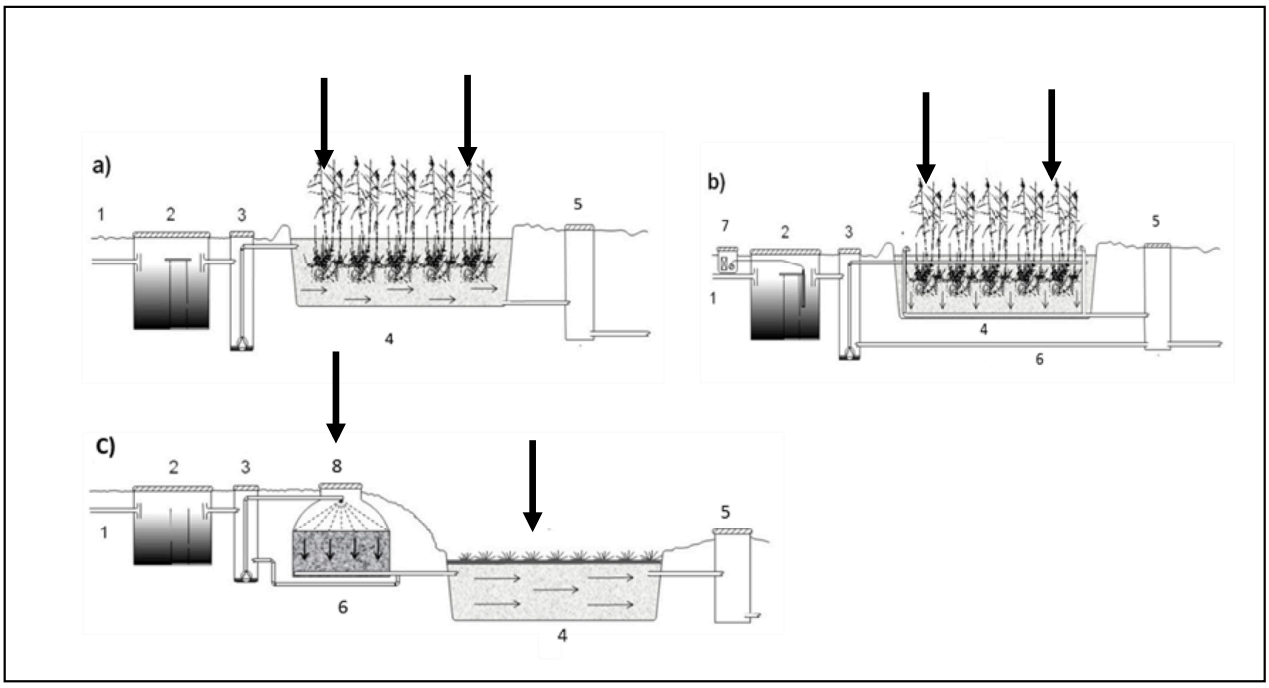

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502 **Fig. 2**
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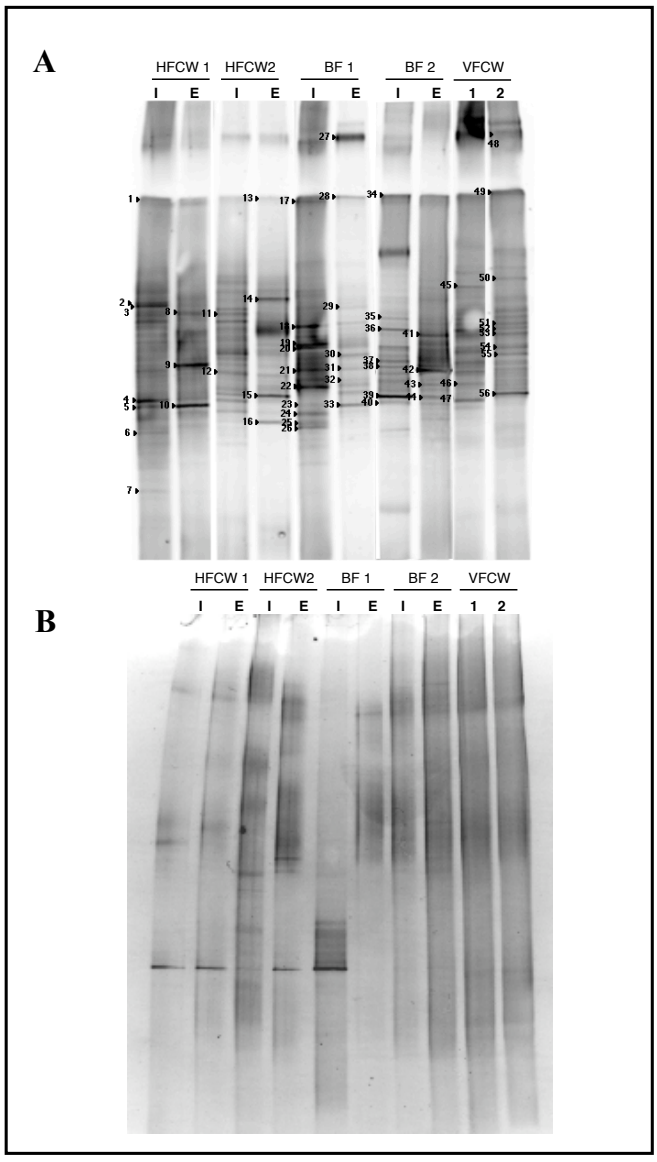

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507 **Fig. 3**

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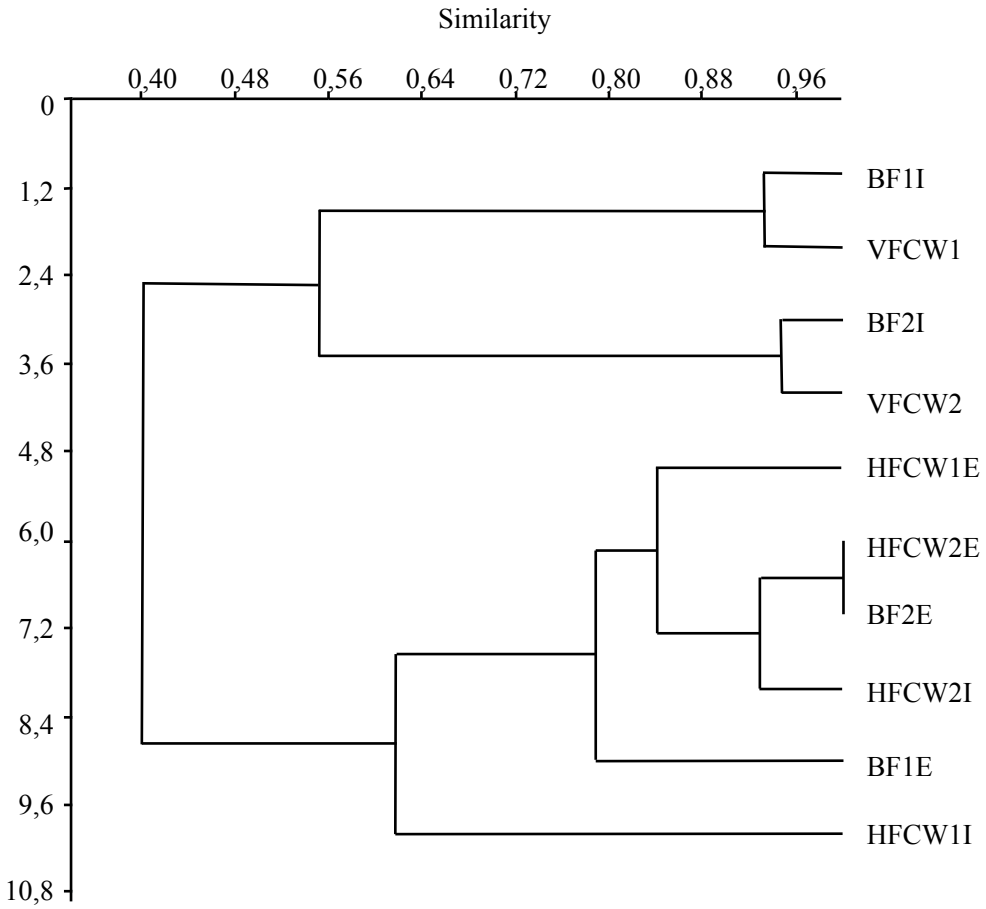

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512 **Table 1** Description of the systems evaluated. The averages of nitrification and total nitrogen removal
513 percentages are based on six month sampling (n= 9)

Location	System	Area (m^2)	PE served	Recycling	Years of operation	Hydraulic conditions	NH_4-N (%)	Total N (%)	BOD5 (mg/l)
Bjødstrup	HFCW1	470	80	No	>20	Saturated	60	64	103
Moesgaard	HFCW2	520	80	No	>20	Saturated	23	34	-
Friland 1	VFCW1	90	30	Yes	1	Unsaturated	99	84	169
Tisset	VFCW2	15	2	No	4	Unsaturated	99	21	240
Friland 2	BF1	50	4	No	6	Both	59	44	290
Janne	BF2	50	6	Yes	6	Both	91	85	280

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518 **Table 2** Shannon diversity index (H) and band
519 richness calculated for each sample from
520 bacterial data

System	H	Band richness
HFCW 1 I	2,84	31
HFCW 1 E	2,83	24
HFCW 2 I	2,96	26
HFCW 2 E	2,33	18
BF 1 I	2,27	26
BF 1 E	2,02	17
BF 2 I	2,51	26
BF 2 E	1,81	23
VFCW 1	2,32	25
VFCW 2	2,24	27

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Table 3 Phylogenetic affiliation of sequences obtained from DGGE bands with closest uncultured and cultured matches. Number of bases used to calculate the sequence similarity is shown in parentheses in the fourth column

System	Band	Closest match	%similarity (nº bases)	Taxonomic group	Acc nº (GenBank)	Cultured closest match (% similarity)
HFCW1 Influent	DKBF_1	Uncultured bacterium clone t15dG9Hb69	89.9 (473)	Bacteroidetes	FM956379	*Owenweeksia hongkongensis* (85.2)
	DKBF_2	*Acinetobacter* sp. Wuba16	98.7 (522)	γ-proteobacteria	AF336348	
	DKBF_3	*Acinetobacter* sp. OVT1-RT-4	98.9 (518)	γ-proteobacteria	EF523604	
	DKBF_4	Uncultured bacterium clone LaYa5b-79	97.4 (531)	Firmicutes	GV291613	*Bacillus* sp. PCWCS27 (97.4)
	DKBF_5	Uncultured *Geobacter* sp. clone MFC-A36	86.2 (355)	δ-proteobacteria	FJ262598	*Geobacter metallireducens* (86.2)
	DKBF_6	Uncultured Desulfuromonadales bacterium	93.5 (445)	δ-proteobacteria	AM934934	*Anaeromyxobacter dehalogenans* (88.0)
	DKBF_7	Uncultured Acidobacteria bacterium	88.9 (417)	Acidobacteria	FJ824900	*Holophaga* sp. (87.0)
HFCW1 Effluent	DKBF_8	Uncultured bacterium clone nbw447d07c1	83.2 (417)	γ-proteobacteria	GQ096652	*Acinetobacter* sp. (82.3)
	DKBF_9	Uncultured *Bacillus* sp. Clone GASP-MA351_F05	99.1 (523)	Firmicutes	EF663435	*Bacillus* sp. IDA4917 (99.1)
	DKBF_10	*Arthrobacter oxydans* strain Mm2H	99.6 (494)	Actinobacteria	GU391465	
HFCW2 Influent	DKBF_11	Uncultured bacterium clone Pav-112	92.4 (472)	Chloroflexi	DQ642421	*Chloroflexi bacterium* (84.3)
	DKBF_12	Uncultured bacterium clone LaYa5a-55	86.5 (455)	Firmicutes	GU291506	*Exiguobacterium* sp (86.3)
HFCW2 Effluent	DKBF_13	*Sphingobacterium faecium* strain c121	92.5 (467)	Bacteroidetes	FJ950587	
	DKBF_14	*Acinetobacter sp*. Wuba16	98.7 (531)	γ-proteobacteria	AF336348	
	DKBF_15	Uncultured bacterium clone LaYa5b-79	100 (541)	Firmicutes	GU291613	*Bacillus* sp. PCWCS27 (100)
	DKBF_16	Bacillales bacterium Gsoil 1105 gene	99.6 (523)	Firmicutes	AB245375	*Eubacterium* sp (97.6)
BF1 Influent	DKBF_17	Uncultured bacterium clone MBR-3	85.9 (396)	Bacteroidetes	FM200879	*Niastella sp* (83.5)
	DKBF_18	Uncultured bacterium clone AF-2	83.3 (405)	γ-proteobacteria	AF143844	*Acinetobacter* sp. A3-6 (83.1)
	DKBF_19	Uncultured *Ricketsiella* sp. clone B09-03G	93.6 (436)	γ-proteobacteria	FJ543061	*Ricketsiella melolonthae* (92.9)
	DKBF_20	Uncultured bacterium clone nbw133d11c1	83.2 (380)	β-proteobacteria	GQ024037	*Polaromonas* sp. (81.8)
	DKBF_21	Uncultured Xanthomonadaceae bacterium clone GASP-MA1S2_A03	95.5 (493)	γ-proteobacteria	EF662389	*Xanthomonas perforans* (94.8)
	DKBF_22	Uncultured Gamma proteobacteria clone AI-2M_F10	99.0 (494)	γ-proteobacteria	EF219801	*Dokdonella* sp. (95.3)
	DKBF_23	Uncultured bacterium gene	86.3 (345)	Firmicutes	AB525472	*Geobacillus stearothermophilus* (83.6)
	DKBF_24	Uncultured bacterium clone 1-20	95.3 (425)	Chloroflexi	AY548939	*Dehalococoides* sp. (86.3)
	DKBF_25	Uncultured Betaproteobacteria bacterium	97.2 (416)	β-proteobacteria	CU922449	*Burkholderia* sp. (88.5)
	DKBF_26	Uncultured Acidobacteria bacterium clone RUGL1-382	98.6 (490)	Acidobacteria	GQ421153	*Holophaga* sp. oral clone CA002 (89.0)
BF1 Effluent	DKBF_27	*Flavobacterium sp.*	100 (509)	Bacteroidetes	FJ889628	
	DKBF_28	Unidentified bacterium clone MEB004	99.2 (514)	Bacteroidetes	EF154088	*Epilithonimonas* sp. (99.0)
	DKBF_29	*Pedobacter* sp.	85.5 (437)	Bacteroidetes	AM988953	
	DKBF_30	Uncultured bacterium clone R3B6L	94.3 (498)	Firmicutes	GQ423904	*Trichococcus pasteurii* (94.3)
	DKBF_31	*Stenotrophomonas maltophilia*	91.6 (480)	γ-proteobacteria	FJ772057	
	DKBF_32	Uncultured bacterium clone AK 1DE1_09D	86.9 (442)	γ-proteobacteria	GQ396993	*Lysobacter* sp. (86.)
	DKBF_33	*Arthrobacter sp.*	90.3 (467)	Actinobacteria	FN392694	
BF 2 Influent	DKBF_34	*Chryseobacterium* sp	91.6 (478)	Bacteroidetes	FN550150	
	DKBF_35	Uncultured bacterium clone H2SRC13	93.4 (468)	Bacteroidetes	FM174354	
	DKBF_36	Uncultured bacterium clone KD4-4	98.3 (516)	Bacteroidetes	AY218633	*Owenweeksia hongkongensis* (85.2)
	DKBF_37	Uncultured bacterium clone Con3d08	99.6 (526)	γ-proteobacteria	GQ401680	*Rhodanobacter* sp (99.6)
	DKBF_38	Uncultured bacterium clone Con3d09	98.7 (538)	γ-proteobacteria	GQ401681	*Rhodanobacter* sp (98.7)
	DKBF_39	*Arthrobacter stackebrandtii*	99.6 (514)	Actinobacteria	AJ640198	
	DKBF_40	*Arthrobacter stackebrandtii*	99.4 (534)	Actinobacteria	AJ640198	
BF 2 Effluent	DKBF_41	Uncultured bacterium clone 96-12	99.8 (536)	Firmicutes	GU212517	*Planomicrobium* sp. (99.8)
	DKBF_42	Uncultured bacterium clone 96-12	100 (537)	Firmicutes	GU212518	*Planomicrobium* sp. (99.1)
	DKBF_43	*Bacillus* sp PU1	83.9 (447)	Firmicutes	FN555708	
	DKBF_44	*Arthrobacter* sp.	85.8 (440)	Actinobacteria	DQ158002	
VFCW 1	DKBF_45	Uncultured bacterium clone KD3-110	87.9 (458)	Bacteroidetes	AY218600	*Lishizhenia caseinilytica* (84.1)
	DKBF_46	Uncultured Gamma proteobacterium clone SM2E10	82.8 (415)	γ-proteobacteria	AF445726	*Arenimonas* sp. (82.7)
	DKBF_47	Uncultured bacterium clone AKAU 4119	94.0 (483)	Actinobacteria	DQ125870	*Arthrobacter* sp. (94.1)
VFCW 2	DKBF_48	*Flavobacterium gelidilacus*	96.6 (503)	Bacteroidetes	NR_025538	
	DKBF_49	Uncultured bacterium clone glb 266b	84.8 (417)	Bacteroidetes	EU978754	*Flavobacteria symbiont* (83.1)
	DKBF_50	Uncultured bacterium clone CYCU-0287	98.1 (516)	Bacteroidetes	DQ232441	*Chitinophaga* sp. (89.2)
	DKBF_51	*Brevundimonas* sp	88.6 (458)	α-proteobacteria	AY 576767	
	DKBF_52	Uncultured bacterium clone VC100	89.0 (405)	Bacteroidetes	EU593808	*Arenibacter* sp (86.8)
	DKBF_53	Antarctic bacterium R-8890 R-8890 strain	99.6 (520)	β-proteobacteria	AJ440995	*Rhodoferax ferrireducens* (97.1)
	DKBF_54	Uncultured pseudoxanthomonas	90.6 (490)	γ-proteobacteria	EU836188	*Stenotrophomonas* sp (90.6)
	DKBF_55	Uncultured Beta proteobacteria	84 (416)	γ-proteobacteria	FM992014	*Luteimonas* sp (82.0)
	DKBF_56	Uncultured bacterium gene	99.8 (523)	β-proteobacteria	AB196024	*Thauera terpenica* (99.6)

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