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

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29

30 **Abstract**

31

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

46

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

49

50 **1. Introduction**

51

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

66

67 The most stable microbiota in these systems is found in the biofilm associated to the plant's
68 roots and/or attached to the surface of the filter bed material. This complex microbial
69 community created by interactions with wastewater, is mainly responsible for the degradation
70 performance of the system (Sleytr et al., 2009). Furthermore, the diversity of microorganisms
71 in this environment may be critical for its proper functioning and maintenance (Ibekwe,
72 2003). To improve the design of these systems, a detailed knowledge of the structure of these
73 communities should be acquired in order to understand the biological processes that are
74 taking place within them (Truu et al., 2009, Dong and Reddy, 2010). Recently, several studies
75 have characterized microbial populations in laboratory scale units, sand filters and full scale
76 constructed wetlands under specific conditions (Ragusa et al., 2004, Vacca et al., 2005,
77 Baptista et al., 2008, Calheiros et al., 2009, Krasnits et al., 2009, Sleytr et al., 2009, Zhang et
78 al., 2010, Dong and Reddy, 2010). However, there is a general lack of information on the
79 diversity and changes of the microbial communities in long-term operation systems treating
80 domestic wastewater at real time scale (Krastnits et al., 2009).

81

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

86 denitrifying bacteria and heterotrophic nitrifying microorganisms may play an important role
87 in nitrogen transformations in constructed wetlands (Truu et al., 2005). Most importantly, the
88 effects of biofilms on nitrogen transformation and removal have not been adequately studied
89 and modelled. As microorganisms affect processes like nitrification, denitrification, uptake,
90 and sedimentation, they have to be taken into consideration when modelling the
91 transformation and removal of nitrogen from wastewater (Mayo and Bigambo, 2004). Thus, a
92 first step for establishing the role of biological communities in N-removal in constructed
93 wetlands is to evaluate the diversity of microorganisms under different conditions and
94 systems. With this purpose recent studies have introduced the characterization of bacterial
95 communities by means of molecular methods based on 16S rRNA gene analysis (Sleytr,
96 2009).
97
98 The aim of this study was to compare the composition of microbial communities of three
99 different types of domestic wastewater treatment systems used in Denmark: Horizontal Flow
100 Constructed Wetlands (HFCW), Vertical Flow Constructed Wetlands (VFCW) and Biofilters
101 (BF, with combined configurations of vertical or horizontal flow) using the PCR-DGGE
102 based method. The systems were composed of different bed filling media, namely soil, sand
103 and LWA (lightweight aggregate). In this work, we enlarged the microbial analysis by
104 analyzing both the bacterial and archaeal populations, focusing in the possible influence of the
105 water influent composition, the design and the bed filling of the treatment systems in the
106 structure of these microbial communities.
107
108 **2. Material and methods**
109
110 *2.1 Site description*
111
112 The wastewater treatment systems (WWTS) investigated were rural facilities used in
113 Denmark for the treatment of domestic wastewaters. All the systems were built following
114 Danish guidelines and comply with Danish wastewater discharge standards (for details see
115 Brix and Arias, 2005). The layout of all the studied systems included a primary treatment
116 step, using a sedimentation tank with a hydraulic residence time proportional to the number of
117 people served and a minimum of 2 m^3. The second treatment step differs depending on the
118 system chosen by the users among an array of technical possibilities approved by the Danish
119 EPA.
120 Three types of systems were selected for the study: two horizontal flow constructed wetlands
121 (HFCW) with soil beds, two vertical flow constructed wetlands (VFCW) with sand bed and

122 two LWA Biofilters (BF) fitted with a Filtralite-P® bed for the removal of phosphorous
123 (Jenssen et al., 2010). The systems differed in flow configuration, operational and bed media
124 characteristics.
125
126 The HFCWs studied have been operational for over 20 years. The systems were built
127 following national guidelines (Miljøministeriet Miljøstyrelsen, 1990) and were composed of
128 two soil filled beds operating in parallel with the necessary structures for distribution and
129 collection of domestic water. After the treatment water was discharged to nearby
130 watercourses (for details see Brix et al., 2009).
131
132 VFCWs were also built following the Danish design and construction guidelines
133 (Miljøministeriet Miljøstyrelsen 2005). The domestic wastewater was pre-treated in a
134 sedimentation tank; after that, water was loaded sequentially on the system surface at a rate of
135 approx. 20 pulses/d to an unsaturated bed filled with sand, where it was homogeneously
136 distributed in the surface trickling vertically. Once the water percolated through a one meter
137 deep bed, it was collected at the bottom and evacuated. In order to improve the water quality,
138 and enhance denitrification capacity, treated water was recycled back to the pumping well in
139 one of the two systems studied, where conditions should favour the process (for details see
140 Brix and Arias, 2005).
141
142 BFs are media filled systems that combine unsaturated conditions and a water saturated bed.
143 The first section of the system operates unsaturated; it is housed in a fibreglass dome filled
144 with a lightweight aggregate (LWA) from which wastewater is pumped at a rate of around 25
145 pulses/day. The second step of the treatment system involves the flow of water through a
146 saturated bed filled with Filtralite -P® media, which is a LWA product chemically enriched,
147 specifically engineered for phosphorus removal (see details in Jenssen et al., 2010). Different
148 wastewater treatment systems studied are shown in Figure 1 and their operational and design
149 characteristics are shown in Table 1.
150
151 The flow conditions within the systems control the oxygen availability and therefore, anoxic
152 conditions predominated in saturated HFCWs while oxic conditions prevail in VFCWs
153 (Vymazal et al., 2006; Brix and Arias, 2005).
154
155 On the other hand, because of the combination of two different modules, oxic conditions are
156 found in the first section of BF systems, while anoxic conditions develop in the P removal
157 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.

194 *2.5 PCR amplification, DGGE and sequencing of 16S rRNA genes*

195

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

204

205 Primers 344F-GC and 915R were used for archaeal 16S rRNA gene fragment amplification
206 (Casamayor et al., 2002). The PCR protocol included an initial denaturation step at 94 ºC for
207 5 min, followed by 20 touchdown cycles of denaturation (at 94 ºC for 1 min), annealing (at 71
208 ºC to 61 ºC for 1 min, decreasing 1 ºC each cycle), and extension (at 72 ºC for 3 min); 20
209 standard cycles (annealing at 55 ºC, 1 min) and a final extension at 72 ºC for 5 min.

210

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

218

219 The DGGE was run in a DCode system (Bio-Rad) as described by Muyzer et al. (1998). A
220 6% polyacrylamide gel with a gradient of 40-80% DNA denaturant agent was cast by mixing
221 solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40%
222 deionized formamide). Seven hundred ng of PCR product were loaded for each sample and
223 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
224 sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45
225 min, rinsed with 1 x TAE buffer, removed from the glass plate to a UV-transparent gel scoop,
226 and visualized with UV in a Chemi Doc EQ (Bio-Rad). Prominent bands were excised from
227 the gels, resuspended in milli-q water overnight and reamplified for their sequencing.

228

229 Purification of PCR products from DGGE bands and sequencing reactions were performed by
230 Macrogen (South Korea) with primer 907RM for Bacteria and primer 915R for Archaea. PCR
231 products of the reamplified bands were used as DNA template in a sequencing reaction with
232 the Big Dye Terminator version 3.1 sequencing kit in an automatic ABI 3730XL Analyzer-96
233 capillary type. Sequences were subjected to a BLAST search (Altschul et al., 1997) to obtain
234 an indication of the phylogenetic affiliation.
235
236 Fifty-six 16S bacterial rRNA gene sequences were submitted to the EMBL database
237 (http://www.ebi.ac.uk/embl) and received the following accession numbers: from HE716787
238 to HE716842.
239
240 *2.6 Analysis of DGGE patterns and statistical analyses*
241
242 Digitalized DGGE images were analysed with the Quantity One software (Bio-Rad, Hercules,
243 USA). Bands occupying the same position in the different lanes of the gels were identified. A
244 matrix was constructed for all lanes, taking into account the presence or absence of the
245 individual bands. Raup-Crick index was used for absence-presence data as this index utilizes
246 a randomization procedure (Monte Carlo) comparing the observed number of species
247 occurring in both samples in 200 pairs of random replicates of the pooled sample. The PAST
248 program (Hammer et al 2004) was used for theses analyses.
249
250 DGGE banding data were used to calculate the Shannon–Weaver index as a measure of the
251 diversity of microbial communities. It was calculated using the following function:

252 $$H' = -\sum_{i=1}^{i=n} p_i \ln p_i$$

253 Where n is the number of bands in the sample and p_i the relative intensity of the band.
254
255 **3. Results and discussion**
256
257 The aim of this study was to investigate the factors affecting the structure of prokaryotic
258 communities established in three different types of natural wastewater treatment systems,
259 each with different substrate and configuration. Analysis of bacterial and archaeal community
260 composition from the substrate samples collected was performed by means of PCR-DGGE.
261 The banding patterns for the 16S rRNA gene DGGE-PCR amplicons are presented in Fig. 2
262 for Bacteria and Archaea. Clear differences could be observed in both gels concerning band

263 position, intensity and band number for the different samples, demonstrating that different
264 bacterial and archaeal communities developed in the different systems.
265
266 In the bacterial DGGE, a high number of bands could be observed in all lanes (Fig. 2A). Band
267 richness fluctuated from 31 in HFCW1I to 17 in the BF1E system (Table 2). Significant
268 differences were found in total band richness among the influents and effluents ($p < 0.05$),
269 influents harbouring higher richness than effluents (27 and 21 mean band richness for
270 influents and effluents respectively). Similar results were found for Shannon diversity indexes
271 (2.65 and 2.25 for influents and effluents respectively). On the other hand, although archaeal
272 amplification was also found, the DGGE banding profile clearly revealed a lower diversity in
273 comparison with the bacterial community (Fig. 2B).
274
275 Excision of prominent bacterial DGGE bands and subsequent sequencing allowed the
276 characterization of the predominant microorganisms in the different systems studied.
277 Informative sequences were obtained from 56 bacterial bands. The number of bases used to
278 calculate each similarity value is also shown in Table 3, as an indication of the quality of the
279 sequence. Unfortunately, bands recovered from the archaeal DGGE gel yielded sequences
280 with a very poor quality that have not been included in this study. The most represented
281 taxonomic groups in all samples belonged to the γ-Proteobacteria (26% of recovered bands)
282 and Bacteroidetes (26%). Firmicutes (15%) were present in all systems with the exception of
283 samples from VFCW. Members of the Actinobacteria group, although found in HFCW and
284 VFCW, seemed to be more abundant in BF systems. Finally, some representatives of α, ß and
285 δ-Proteobacteria, Acidobacteria and Chloroflexi were also retrieved in some of the samples.
286
287 Most of the sequences corresponded to uncultured microorganisms (71% of the retrieved
288 sequences), while others matched with a high percentage of similarity to cultured bacteria
289 (29%). In general, typical bacteria from soil and wastewater environments were found in all
290 the systems analyzed. For example, we could retrieve in HFCW typical soil bacteria such as
291 sequences related to *Acinetobacter* sp. (γ-Proteobacteria), *Arthrobacter* sp (from the
292 Actinobacteria group, also found in samples from VFCW and BF), and *Bacillus* sp.
293 (Firmicutes), all of them potential denitrifying bacteria. Besides, other non-culturable matches
294 corresponding to different groups were present. *Acinetobacter* sp. is commonly present in
295 activated sludge (Snaird el al. 1997) especially in those where enhanced biological phosphate
296 removal is observed (Ivanov et al., 2005). On the other hand, *Arthrobacter* sp has been related
297 to the nitrogen cycle, particularly to nitrogen fixation (Cacciari et al. 1971). The fact that

298 some aerobic microorganisms have been found suggests that although HFCW systems are
299 mostly all the time saturated, enough oxygen is present to allow proliferation of these
300 microbial groups, with the subsequent possibility of nitrification in the system. Oxygen is
301 present probably due to plant aeration and also because the upper part of the bed normally
302 remains unsaturated.
303
304 Concerning the Bacteroidetes phylum, a group of chemoheterotrophic bacteria known by its
305 ability to degrade complex organic matter, sequences with a high similarity at the species
306 level were found. Thus, some of the retrieved sequences related to *Flavobacterium* sp.,
307 another potential denitrifying bacteria, and have been detected in VFCW and BF; it is a
308 typical genus that can be found in activated sludge (Park et al. 2007). Another sequence
309 similar to the denitrifying *Thauera terpenica* (cultured closest match 99.6% similarity) was
310 also observed in VFCWs. Other species were also found in BF systems, such as sequences
311 related to the γ-Proteobacteria *Xanthomonas* sp., *Dokdonella* sp., and some denitrifying
312 bacteria such as *Rhodanobacter* sp. and *Stenotrophomonas* sp.
313
314 The application of molecular techniques (PCR-DGGE profiling) on different wastewater
315 treatment systems has allowed the identification of some players and their potential role in the
316 nitrogen removal processes. The diversity of N-cycling bacteria found in the analyzed
317 systems is an indicator of the multiple possibilities of biological nitrogen transformations
318 inside them. In addition, this profiling method is a useful tool to classify microbial
319 community under different substrates by clustering and diversity analyses.
320
321 A cluster analysis of bacterial DGGE banding patterns based in band richness is shown in Fig.
322 3. Samples separated in two clusters; samples coming from VFCW and BFI, corresponding to
323 unsaturated samples with a high organic load (Table 1), clustered together in one of the two
324 main clusters, while all the other samples, corresponding to saturated systems with low
325 organic load, clustered in another group. As there is almost no relation between the influent
326 and effluent bacterial communities inside the same wetland, these results suggest that factors
327 other than the influent wastewater, such as the organic load and the design of the treatment
328 system, contribute to shape the microbial community.
329
330 Previous studies have shown that shifts in the structure of bacterial communities can be
331 associated with changes in a number of soil properties, including soil texture and soil nitrogen
332 availability (Dong et al, 2010). The substrate is an important component since it supports
333 plant growth (in case of planted wetland systems), as well as the establishment of a microbial

334 biofilm, and it influences the hydraulic processes (Stottmeister et al., 2003). A porous matrix
335 substrate such as LWA will probably favour the development of biofilms. Additionally, recent
336 studies concluded that the type of substrate is one of the main factors influencing bacterial
337 communities (Vacca et al, 2005, Calheiros et al, 2009). However, none of these studies took
338 place in real constructed wetlands; both of them consisted in different pilot systems, with the
339 same influent water. In our study, no relation between the microbial assemblage and the
340 substrate was found, as different communities were retrieved within systems with the same
341 substrate. On the contrary, from the cluster analysis we did observe two separated groups that
342 appeared to be influenced by factors such as the organic load, as well as for the
343 absence/presence of oxygen, since one of the groups is composed only by samples from
344 unsaturated samples, which receive a higher load of organic matter (VFCW and influent of
345 BF), and the other group by saturated conditions with a lower load of organic matter (HFCW
346 influent and effluent zone, and BF effluent zone). Since influent water is different for each
347 system, the results suggest a community configuration more related with the design of the
348 treatment system and its operational conditions. These results are in consonance with the
349 work carried out by Baptista et al. (2008), who suggested that stochastic processes could play
350 an important role in the microbial community assembly in engineered and natural systems.
351
352 Different authors, such as Ibekwe et al (2003) and Calheiros et al. (2009) indicated that the
353 diversity of the bacterial community in the constructed wetlands systems might influence the
354 final effluent quality, and so the engineering should be directed to develop a higher diversity
355 in order to enhance processes such as nitrification and denitrification (Ibekwe et al. 2003).
356 The Shannon index obtained for our samples showed a very similar diversity for all the
357 samples. Significant differences ($p\text{-value}<0.05$) were only found between HFCW and BF.
358
359 On the other hand, despite we could not retrieve sequences directly affiliated to known
360 nitrifiers, nitrogen removal occurred in all the systems evaluated, although the removal rates
361 were different among systems (Table 1). Saturated systems did not reach high nitrification
362 rates but they were able to denitrify almost all the nitrified ammonia. Unsaturated systems
363 were capable of high nitrification rates but total nitrogen removal was lower than unsaturated
364 CW.
365
366 The removal of nitrogen in constructed wetlands is usually limited by the nitrification process,
367 and in order to reach high total nitrification rates is important that biological nitrification takes
368 place. Additionally, in order to increase denitrification rates in the unsaturated systems, the
369 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.

-Microbial community structure was related to the oxygen conditions (saturated or unsaturated) and organic matter load.

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

Formatat: anglès (EUA)

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.

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478 **Figure legends**
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480 **Figure 1** Schemes of the three types of systems studied; a) HFCW, b) VFCW, c) BF. 1) inlet, 2)
481 sedimentation tank, 3) pumping well, 4) bed, 5) outlet well, 6) recycling, 7) P removal system, 8) LWA
482 dome biofilters. Arrows indicate the sampling sites of each system.
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484 **Figure 2** Negative images of DGGE gels with PCR products amplified with bacterial (A) and archaeal (B)
485 primer sets from samples of the different systems: HFCW (Horizontal Flow Constructed Wetlands), BF
486 (Biofilters) and VFCW (Vertical Flow Constructed Wetlands); 1 and 2 are replicates from each system; when
487 applied, I: Influent zone, E: Effluent zone.
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489 **Figure 3** Cluster analysis of bacterial DGGE profiles, determined by the Raup-Crick method.

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491 **Table 1** Description of the systems evaluated. The averages of nitrification and total nitrogen removal 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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Table 2 Shannon diversity index (H) and band richness calculated for each sample from 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 symbion*t (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)