Title: A novel culture-independent method to search unknown dominant bacterial groups and its application to microbial analysis of membrane bioreactors.

Running title: A novel culture-independent enumeration method.

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ABSTRACT

As we could not get numerical information for unknown unculturable microorganisms through conventional culture-independent analysis methods such as next-generation sequencing, or real time PCR, we developed an original culture-independent method, and searched the numerically dominant bacteria in three industrial membrane bioreactors for livestock farms.

Although *Actinobacteria* was the numerically dominant phylum ($9.3x10^5$MPN/mL) on 6/August/2014 in the MBR of A farm, when a bacteria with the same genotype to *Arthrobacter* sp. (AF197047; $4.3x10^5$MPN/mL), and those similar to *Burkholderi*a sp. (AB299593; $4.3x10^5$MPN/mL) were the numerically dominant, after about 13 months (24/October/2015) a number of the *Arthrobacter* genotype increased to $930x10^5$ MPN (230 times) and become dominant, and those similar to the *Microbacterium* sp. (AM403628) increased to $92x10^5$MPN, while that of the *Burkholderi*a genotype disappeared. In the other MBR of B farm, bacteria having a similar genotype to *Enshifer* sp. (AB195268, CP000738), or *Shinorhizobium* sp. (AF227755, AB195268), or *Mesorhizobium* sp. (BA000012, Mso.tians29), or *Agrobacterium vitis* (D12795) was dominant on 18/August/2015 ($24x10^5$ MPN) and 30/August/2015 ($15.5x10^5$ MPN). In the other MBR of C farm (9/October/2015), bacteria having a similar genotype to uncultured *Betaproteobacteria* (AY921864) was dominant ($430x10^5$MPN), followed by uncultured bacterium ($74x10^5$MPN ; AM268745), and *Mycobacteriaceae* (AB298730), or *Propionibacteriaceae* (AB298731) ($7.4 x10^5$MPN). There was no common bacterial groups among tested three MBRs. Present results indicated that different kinds of

32 homogeneous bacteria were numerically dominant in the three tested membrane bioreactors, where
33 their numbers and ratios were varied with the duration of the driving periods.

34 **IMPORTANCE**

35 Although the conventional molecular-based culture independent methods have been used in
36 place of traditional culture-based methods for microbiological research and expanded information of
37 unculturable low-abundance bacterial groups, not all of them were always highly active in the
38 environment and it was difficult to search for microorganisms among them which were highly active
39 and play an important role in the environment. As numerical data of each bacteria might become an
40 important index to know their activity in environment, we had created a novel culture-independent
41 enumeration method for numerically dominant unidentified bacteria. Through the method, we found
42 that different kinds of homogeneous bacteria were numerically dominant in the three tested
43 membrane bioreactors, whose numbers were high enough to affect the performance of the reactor as
44 a single strain. The method was found useful to specify and trace unknown numerically dominant
45 bacterial groups in a culture independent manner.

46

47

INTRODUCTION

Until now, the unculturability of microorganisms was reported to be caused from the following different factors: artificial ones caused by unknown suitable growth condition such as cases in filamentous bacteria in activated sludge (1, 2), or *Dehalococcoides* sp. in soil (3), intrinsic ones caused from low-abundance, and slow growing environmental microorganisms, such as rare biosphere (4, 5), and acquired ones caused from a dormancy state of culturable bacteria (6-10).

As most environmental bacteria were unculturable in growth media, over the past two decades, molecular-based culture independent methods have been used in place of traditional culture-based methods (11, 12), and has expanded information of unculturable bacterial groups in environments (13). In particular, next-generation sequencing (NSG) was expected to become a powerful tool not only to provide genetic information of all the low-abundance microorganisms but also to relate them to their various functions (14).

Among environmental samples, biological wastewater treatment reactors have been widely been studied by using them, based on the social demand that the reactor was an essential facility to purify water by removing excess organics, nitrogen, phosphorus and pathogenic microorganisms in wastewater (15), and that their microbial complex community primarily affected the performance of reactors (16, 17).

As for conventional activated sludge (CAS) equipped with final settling tanks for solid-liquid

66 separation, the culture independent methods such as clone library sequencing (18), denaturing
67 gradient gel electrophoresis (DGGE) (19, 20), terminal restriction fragment polymorphisms analysis
68 (21-23), fluorescence in situ hybridization (FISH) (24, 25), microarray-based genomics (26), and
69 NGS (27-32) have been used to clarify bacterial relation to their performance. These methods have
70 also been used for membrane bioreactor (MBR), by which solids and liquids could be completely
71 separated through a membrane, was an increasingly implemented technology because of advantages
72 such as reduced excess sludge, higher effluent quality and operation under higher biomass
73 concentrations over CAS (23, 24, 30).
74 Most reports have focused mainly on the comparative study of overall microbial composition (23,
75 26-28, 32), and their changes in the reactor (29, 30). With respect to the well-known specific gene, or
76 bacteria, their numbers were estimated by using real-time PCR (33-35), or by FISH (24, 25). There
77 have been few reports to show whether numerically dominant specific microorganisms, which might
78 affect the performance of the reactor, existed in the reactor or not until now (1, 2). Higher biomass in
79 effluent, and higher performance of the reactors (16, 20, 22-24, 27, 28, 30, 31) implied that the
80 unculturability of microorganisms in the reactor might mainly be cased from unknown growth
81 condition, and not by low-abundance and slow growing microorganisms, nor by dormant cell.
82 As microbial numbers might become a simple and useful index to show bacterial activity in the
83 environment, the author has developed an original method to specify and trace unknown dominant

84 microorganisms, in which taxonomies of each bacterial groups were elucidated from the multiple
85 enzyme restriction fragment length polymorphism (MERFLP) of 16S rDNA (36, 37), and each
86 number of the bacterial groups were estimated by most probable number (MPN) (38). Although the
87 method had mostly been applied as a culture-based method (39-43), we presented the results of our
88 application as culture-independent analyses of three industrial membrane bioreactors (MBR) situated
89 in the Hokkaido area. The results were compared with those by the culture-based same method, and
90 those by culture-independent PCR-DGGE.
91

92 **MATERIALS AND METHODS**

93 **Samples.** The three industrial MBR had been constructed by BICOM Co.Ltd. (Osaka, Japan) to
94 purify and clean up wastewater from daily farms. All the samples were collected from equalizing
95 tanks before septic tanks having an immersion-type membrane filtration apparatus. Sludge in MBR
96 of A farm (Yubetsu Town in Monbetsu-Gun Hokkaido) was sampled on 6/August/2014 and
97 24/October/2015. That of B farm (Tsurui Village in Akan-Gun Hokkaido) was sampled on 18/
98 August /2014 and 30/August/2014. That of C farm (Onbetsu Town in Kushiro Branch Office,
99 Hokkaido) was sampled on 9/October/2015. Except for the MBR of A farm on 6/August/2014, when
100 a large amount of waste milk flowed into the MBR because of epidemic bovine mastitis and the
101 performance of the MBR became lower, the total performance of purifying and cleaning up

wastewater was kept in good condition.

Culture-independent MPN and culture based MPN. For culture-independent MPN, DNA was extracted from samples (10mL fresh wt.) as described previously (36) after mixing in a 15 mL Falcon tube (215rpm, 20min). After purification by conventional methods, the DNA solution was further purified using a GenElute Agarose Spin Column (SIGMA). Serial 10-fold dilutions (10^{-2} to 10^{-9}) were prepared from the DNA solution.

For culture-based MPN, serial 10-fold dilutions (10^{-2} to 10^{-9}) prepared from samples (1mL fresh wt.) were inoculated to test vials (three replicates), including lactose broth (Difco, Sparks MD). After three days incubation at 30°C, bacterial DNA in each vial was extracted as described previously and purified by conventional methods (36, 37).

MERFLP of the amplified 16S rDNA. Using the V2 forward primer (41f), and the V6 reverse primer (1066r) (44), 16S rDNA was amplified, as described previously (36, 37). Their restriction fragment lengths were measured by microchip electrophoresis systems (MCE-202 MultiNA; Shimadzu Co., Ltd. Kyoto, Japan) after digestion of the PCR product (10μl) using each of the following 4 restriction enzymes: *Hae*III or *Hha* I or *Rsa* I or *Alu* I (10 units, Takara Bio Co. Ltd. Shiga, Japan) in buffer solution (10xLow salt buffer, Takara Bio Co. Ltd.) and 5 folds dilution by de-ionized water, as described previously (36-43).

Reference database used for the phylogenetic estimation. The reference database used for

120 this research included 30,844 post-amplification sequence files for the 41f/1066r primers, which
121 were mainly re-edited from small subunit rRNA files in RDP II release 9_61 (45) under 5 - bases
122 mismatches in the both in primer annealing sites (36, 37), and consisted of 1,379 bacterial genera,
123 including uncultured and unidentified bacteria (40-43) .

124 **Data processing for multi-template DNA and phylogenetic estimation.** As each MPN vial
125 included multi-template DNAs originating from heterogeneous bacteria, the measured MERFL
126 digested from the homogeneous 16S rDNA was selected among the mixed MERFLs digested from
127 the heterogeneous 16S rDNA, as described previously (36, 37). All the theoretical MERFLs
128 originated from the homogeneous 16S rDNA sequence data. The major restriction fragments (RFs)
129 (represented as H in Table 1-3) were those with the highest relative mole concentration (ratio of
130 fluorescent intensity to fragment size). After subtraction of the major RFs from the mixed
131 heterogeneous RFs, the 2nd major RFs were similarly selected (represented as M in Table 1-3). After
132 subtraction of the second major RFs from the remaining heterogeneous RFs, the 3rd major RFs were
133 similarly selected (represented as L in Table 1-3). The similarity between the measured RFLP (A)
134 and the reference RFLP (B) was calculated as described previously (36-43), based on the pairwise
135 distance (D_{AB}) according to Nei and Li (46). The pairwise distance of the MERFLPs (D_{ABME}) was an
136 average of all the D_{ABs} for used restriction enzymes. Similarity (%) was (1-D_{ABME}) x 100 (Table 1-3).
137 In the phylogenetic estimation, combinations of the three restriction enzymes were used when the

138 identical theoretical MERFL (100% similarity) was not found using the four restriction enzymes.
139 When the identical theoretical MERFL was not found using three restriction enzymes, combinations
140 of the two restriction enzymes was used. If the identical theoretical MERFL (100% similarity) was
141 not found using the two restriction enzymes, the theoretical MERFL having the highest similarity
142 (over 90%) to the measured MERFL was indicated in most cases (Table 1-3) (38-43).

143 **Enumeration of bacterial groups by MPN.** Through a three-tube, three-decimal-dilution
144 experiment, MPNs of each bacterial groups were estimated (Table 4-6). Using FDA's Bacterial
145 Analytical Manual (47), confidence limits were obtained and shown in the Tables. Confidence limits
146 shown in Table 4-6 were obtained using Woodward's method (48), except for the
147 culture-independent MPN in B farm on 30/August/2014, when we could not obtain the data of a 10^{-5}
148 dilution sample, and a 10^{-6} dilution sample.

149 **PCR- DGGE.** F984GC corresponding to positions 968–984 in *E.coli* 16S rRNA (49), and
150 R1378 corresponding to positions 1379-1401 were used as PCR primers (50). The PCR profile
151 consisted of a 2 min denaturation at 94°C, and 30 cycles of 30 sec denaturation at 94°C, a 1 min
152 annealing at 55°C, and a 1 min extension at 72°C, followed by a 3 min extension at 72°C.
153 Amplicons, which were checked by agarose gel electrophoresis, were analyzed by DGGE using a
154 Bio-Rad DCodeTMsystem (Bio-Rad Laboratories, Hercules, CA, USA) according to the
155 manufacture's manual, as described in the report (51).

156

157 **RESULTS**

158 **Culture independent analysis**. There was not so large a difference among three replicate

159 electropherograms, which indicated higher reproducibility of the method (Fig. 1). The band strength

160 of the most RFs had gradually decreased in correlation with dilutions, which suggested each of the

161 bacterial numbers could be estimated by MPN (Fig. 1).

162 The total detected bacterial number amplified by the 41f/1066r primers was $24x10^5$MPN/mL for

163 MBR in A farm on 6/August/2014. A numerically dominant phylum was *Acitinobacteria*

164 ($9.3x10^5$MPN) (Table 1, Table 4), where a homogeneous bacteria with the same MERFLP to

165 *Arthrobacter* sp. (AF197047) was one that was numerically dominant ($4.3x10^5$MPN), and that which

166 was similar in MERFLP to *Burkholderia* sp. (AB299593) was the other ($4.3x10^5$MPN) (Table 1, 6).

167 After about 13 months (24/October/2015), the total detected bacterial numbers increased to 100

168 times ($2400x10^5$MPN/mL) (Table1, Table 4). All of them were *Actinobacteria*, where those with the

169 *Arthrobacter* sp. genotype were also the numerically dominant ($930 x10^5$MPN), followed by those

170 with a similar MERFLP to *Microbacterium* sp.(AM403628) ($92x10^5$MPN), and those with a

171 *Burkholderia* genotype disappeared (Table 1, Table 4). These results suggested that an increase of

172 the total detected bacteria number during 13 months was caused by a proliferation of *Actinobacteria*,

173 such as the *Arthrobacter* genotype (230 times) and the *Microbacterium* genotype (Fig. 2).

174 The total detected bacterial number was 46x10^{5}MPN in the other MBR of B farm on
175 18/August/2015 (Table 5). A numerically dominant phylum was *Proteobacteria*, in which a
176 homogeneous bacteria with a similar MERFLP to *Alphaproteobacteria*, such as *Ensifer*
177 sp.(CP000738,D14509,D14516), or *Shinorhizobium* sp.(Srh.fredi5, Srh.melil7), or *Mesorhizobium*
178 sp.(BA000012, Mso.tians2), or *Agrobacterium vitis* (D12795) was numerically dominant
179 (24x10^{5}MPN (Table 2, Table 5)). After 12 days (30/August/2015), the total detected bacterial
180 numbers decreased to 24x10^{5}MPN/mL (Fig. 2). Although those with the *Alphaproteobacteria*
181 genotype were also numerically dominant, its number decreased to 15.5x10^{5}MPN, and a number of
182 bacteria having a similar MERFLP to *Haloanaerobacter chitinovoran* (U32596) increased
183 (4.1x10^{5}MPN) (Table 2, Table 5). The result implied that a decrease of the total number of detected
184 bacteria might be caused by a decrease of the numerically dominant *Alphaproteobacteria* genotype
185 (Fig. 2).
186 The total number of detected bacterial amplified by the 41f/1066r primers was 930x10^{5}MPN in the
187 other MBR of C farm on 9/October/2015 (Table 3, 6). A numerically dominant phylum was
188 *Proteobacteria*, in which a homogeneous bacteria having a similar MERFLP to uncultured
189 *Betaproteobacteria* (AY921864) was numerically dominant (430 x10^5 MPN), followed by
190 uncultured bacterium (74 x10^5 MPN; AM268745), *Mycobacteriaceae* (AB298730), or
191 *Propionibacteriaceae* bacterium (7.4 x10^{5}MPNA; B298731), *Streotiverticillium baldaccii* (6.2

192 x10^5MPNA;X53164, X53169), and *Lactobacillus fermentum* (1.5 x10^5MPN; DQ208931,

193 L.fermentm) (Table 3, 6). There was no bacteria with the same MERFLP among the three tested

194 MBRs, which implied that the three MBRs had a different bacterial consortium (Fig. 2).

195 **Comparison to culture-independent PCR-DGGE**. There was not such a large difference among

196 three replicate DGGE profiles, which indicated a higher reproducibility of the method (Fig.3, Fig.4).

197 The strength of each bands had gradually decreased in correlation with dilutions, which suggested

198 the numbers of each bands could be estimated by MPN (Fig.3, Fig.4). DGGE profile of MBR in A

199 farm on 6/August/2014 indicated the existence of the two numerically dominant bacteria (B1, and

200 C1), and two subdominant bacterial groups (A1, and D1) (Fig.3). Numbers of B1 and C1 were

201 estimated to be over 11x10^5MPN, and those of A1 and D1 were 2.3x10^5MPN (Fig.3). The DGGE

202 profile of MBR in A farm on 24/October/2015 indicated the existence of one numerically dominant

203 bacteria (E2), a subdominant bacteria (D2), and the three bacterial groups (A2, B2, and C2) (Fig.4).

204 The number of E2 was estimated to be over 11,000 x 10^5MPN, with that of D2 being 2,300x10^5MPN.

205 Those of A2, B2, and C2 were 230x10^5, 230x10^5, and 23x10^5, respectively (Fig.4).

206 **Comparison to culture-based MPN**. As there was a large difference among three replicate

207 electropherograms, this indicated poor reproducibility of the method. Most of the restriction

208 fragments had not always disappeared in correlation with dilutions, which suggested each bacterial

209 number could not precisely be estimated by MPN.

A total detected bacterial number amplified by the 41f/1066r primers was over $110x10^8$MPN/mL for MBR in farm A on 6/August/2014 (Table 4), and that of farm B on 24/October/2015 was $46x10^8$MPN/mL (Table 5). However, the numbers of total bacteria, and the numerical dominant for MBR in farm C on 9/October/2015 were underestimated because the bacterial numbers scaled out of the detection range of the MPN. A total detected bacterial number was over $1.1x10^8$MPN/mL (Table 6). There were no common MERFLPs between those of the culture independent method and the culture-based method (Fig. 2).

DISCUSSION

All the results of culture-independent MPN indicated that each sample included a homogeneous MERFP (Table 1-Table 3) originated from a single strain (Table 4-Table 6), whose numbers were high enough to affect performance of the reactor (33). However the total number of the detected bacteria was lower than those by the culture-based MPN (Table 4-Table 6), and those by the other reports (16, 33).

The lower bacterial number by the culture-independent MPN was attributed to the elimination of low-abundance bacteria with huge diversity, as the following. There were smeared bands in DGGE profiles lower than 10^{-4} dilution in Figure 3, and lower than 10^{-6} dilution in Figure 4, which were un-enumerable diverse kinds of 16S rDNA from low-abundance bacteria, such as rare biosphere (4, 5,

228 19). They were eliminated from the total bacterial count by our culture independent MPN.

229 In culture-based MPN, some of such low-abundance bacteria occasionally proliferated in the LB

230 medium, which resulted in the higher total bacterial count (Table 4-6) comparable to those of the

231 reported bacterial numbers (16, 33). However, such an occasional proliferation resulted in incubation

232 bias and poor reproducibility for composition analysis (11, 12), and resulted in a non-detection of the

233 numerically dominant bacteria. LB medium was not suitable for the numerically dominant bacteria

234 in the MBRs. By using growth media suitable for target microorganisms (40, 42), or those which

235 required higher selection bias, such as multi-drug resistant bacteria (38, 43), the culture-based MPN

236 afforded reproducible results.

237 The results of culture-independent DGGE were well consistent with those of culture-independent

238 MPN. The two numerically dominant bacteria (B1, and C1) in the DGGE profile (Fig.3) were

239 supposed to be the *Arthrobacter* genotype and the *Burkholderia* genotype. The one numerically

240 dominant bacteria (E2) in the DGGE profile (Fig.4) was supposed to be the *Arthrobacter* genotype,

241 and the subdominant bacteria (D2) was supposed to be the *Microbacterium* genotype (Fig.4).

242 Estimated numbers of each identified bacterial groups by our method were lower than those

243 estimated from band patterns in DGGE profiles. Because short RF that originated from higher

244 dilution samples only had lower intensity, it became difficult to select the RFs that originated from

245 homogeneous 16S rDNA precisely, which lowered similarity in the similarity search by MERFLP.

246 Although the highest number of the *Arthrobacter* genotype was estimated to be 930 x10^5MPN/mL
247 by MERFLP/MPN in the MBR for A farm on 24/October/2015 (Table 4), its number might be
248 underestimated and estimated to be over 11,000x10^5MPN mL by DGGE/MPN (Fig. 4), which is
249 comparable to the reported total bacteria number in the CAS (from 10,100 x10^5 to
250 80,000x10^5cell/mL) (16, 33). The numerically dominant bacteria might primarily influence the
251 performance of the MBR as a single strain.
252 Although we could not get any information about low-abundance bacteria by our method, the
253 method was found to be effective in specifying and tracing the numerically dominant bacterial
254 groups in the tested three MBRs. This is because precise and reproducible phylogenetic affiliation
255 was possible with respect to the major bacterial groups in higher dilution DNA, where MERFLPs
256 originated from almost a single isolated strain.
257 As far as CAS reactors, Xia et al. suggested that all the reactors contained a core of bacterial
258 phylum with almost identical compositions, where *Proteobacteria* was the largest phylum and
259 *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* were the subdominant phylum in five CAS reactors
260 (26). In contrast, Takada et al. reported that there was no core of bacterial phylum or similar
261 phylogenetic structure among 12 different MBRs (23). Our present results, that there was no
262 common numerically dominant bacterial groups in the tested three MBR reactors, was consistent
263 with the latter results (Fig. 2). Our other results, that the composition of overall classes was not

264 changed between sampling periods, was also consistent with those by the other conventional
265 techniques (29, 30) (Fig. 2).

266 As a new finding through our method, which has not been reported by the other conventional
267 techniques (23, 26-30, 32), the present results indicated that different homogeneous bacteria were
268 numerically dominant in all three MBRs individually, whose numbers were high enough to affect the
269 performance of the reactor as a single strain. The numerically dominant bacteria in A farm were the
270 homogeneous *Arthrobacter* genotype and the homogeneous *Burkholderi*a genotype (AB299593)
271 (Table 1a, 1b), while those in B had the homogeneous *Alphaproteobacteria* genotype (Table 2a, 2b),
272 and those in C had the homogeneous *Betaproteobacteria* genotype (Table 3), which occupied a
273 higher ratio among the detected bacteria (Table 6). This finding could be obtained by the
274 differentiation and elimination of low-abundance bacterial groups having huge diversity shown as
275 smeared bands in the DGGE profiles (Fig. 3, Fig.4).

276 The present results also indicated that the method was effective to demonstrate the population
277 dynamics of unknown bacterial groups without cultivation, as the following. In the MBR of A farm,
278 the number of the numerically dominant *Arthrobacter* genotype increased 230 times, and a number
279 of the *Microbacterium* genotpe increased to become the subdominant strain, while the other
280 numerically dominant *Burkholderia* genotype disappeared during 13 months (Table 4), when the
281 performance of the MBR recovered to normal condition from serious damage by a large effluent of

282 waste milk. In the MBR of B farm, the number of the numerically dominant *Alphaproteobacteria*
283 genotype ($24x10^5$ MPN) slightly decreased to $15.5x10^5$MPN, the number of those of the
284 *Haloanaerobacter chitinovoran* genotype increased after 12 days (Table 5). Our present results
285 clearly demonstrated a dynamic transition of microbial composition in an MBR that particular
286 bacteria proliferated and became extinct.

287 Until now, most of the microbial research of CAS (18-22, 24-29, 31, 32), and MBR (23, 24, 30)
288 has been focused on the exploration of microbial diversity using the conventional
289 culture-independent molecular techniques with aim to cover almost all the low-abundance
290 microorganisms. These approaches seemed to have targeted such bacterial groups as those that
291 appeared as smeared bands in lower dilutions of DGGE profiles (Fig. 3, Fig.4), which seemed to be
292 not suitable to specify and trace numerically dominant bacterial groups. Our method was simple but
293 effective to clarify the dynamic transition of the numerically dominant unknown microbial group in
294 bioreactors.

295

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449 ## Figure legends

450 Figure 1. Electropherograms of RFLP (*Hh* I) of serial 10-fold dilutions (10^{-3}-10^{-9}) in the MBR for C

451 farm on 9/October/2015.

452

453 Figure 2. Ratios of bacterial groups estimated by MPN for the tested three MBRs.

454 Capital letters indicate the results of culture-independent MPN as the following: A1, A2

455 were in the MBR for A farm on 6/August/2014, and 24/October/2015, B1and B2 were the MBR

456 for A farm on 18/ August /2014 and 30/August/2014, and C was the MBR for C farm on

457 9/October/2015. Small letters indicate the results of culture-based MPN as the following,

458 a1 was in the MBR for A farm on 6/August/2014, b1 was the MBR for B farm on 18/ August

459 /2014, and c was in that for C farm on 9/October/2015.Ratio of Actinobacteria

460 ( ), Firmicutes( ), α-Proteobacteria ( ), β-Proteobacteria ( ), γ-Proteobacteria ( ),

461 δ-Proteobacteria ( ), ε -Proteobacteria ( ), Other bacteria ( ), unidentified bacteria ( ).

462

463 Figure 3. DGGE profiles of serial 10-fold dilutions (10^{-2}-10^{-5}) in the MBR for A farm on

464 6/August/2014.

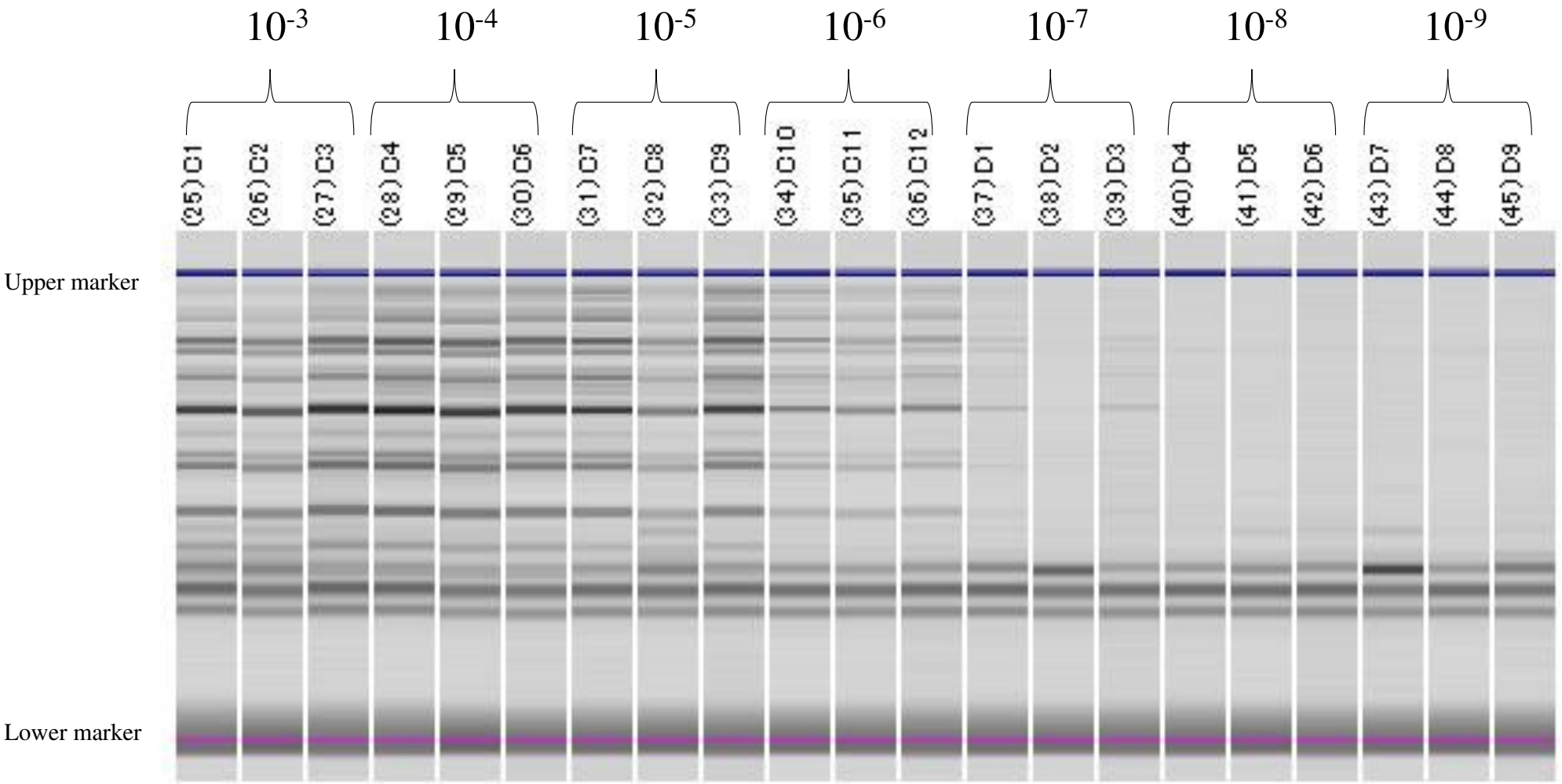
465

466 Figure 4. DGGE profiles of serial 10-fold dilutions (10^{-4}-10^{-8}) in the MBR for A farm on

467 24/October/2015.

468

469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(28)C4
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(30)C6
(31)C7
(32)C8
(33)C9
(34)C10
(35)C11
(36)C12
(37)D1
(38)D2
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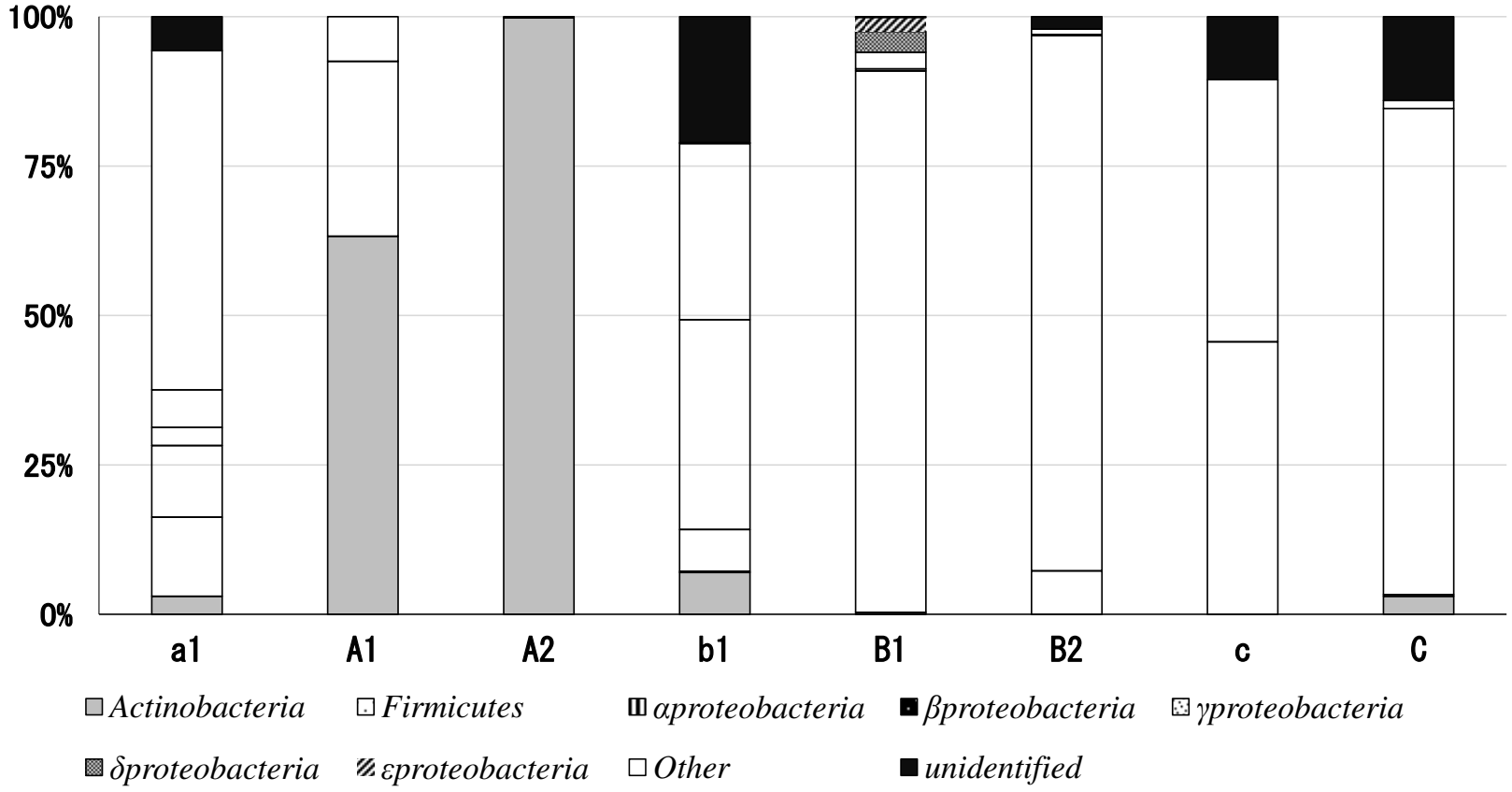
(44)D8
(45)D9
Upper marker
Lower marker

Figure 1.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Other
unidentified

Figure 2.

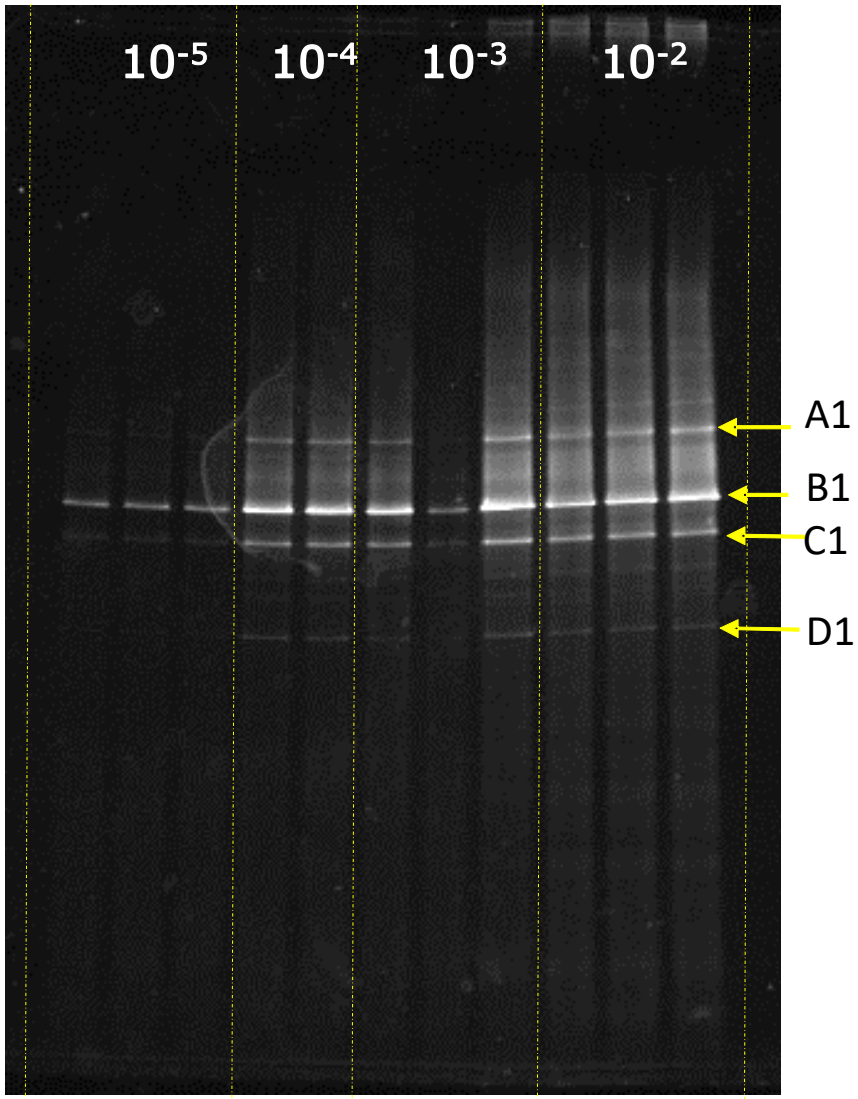

Figure 3.

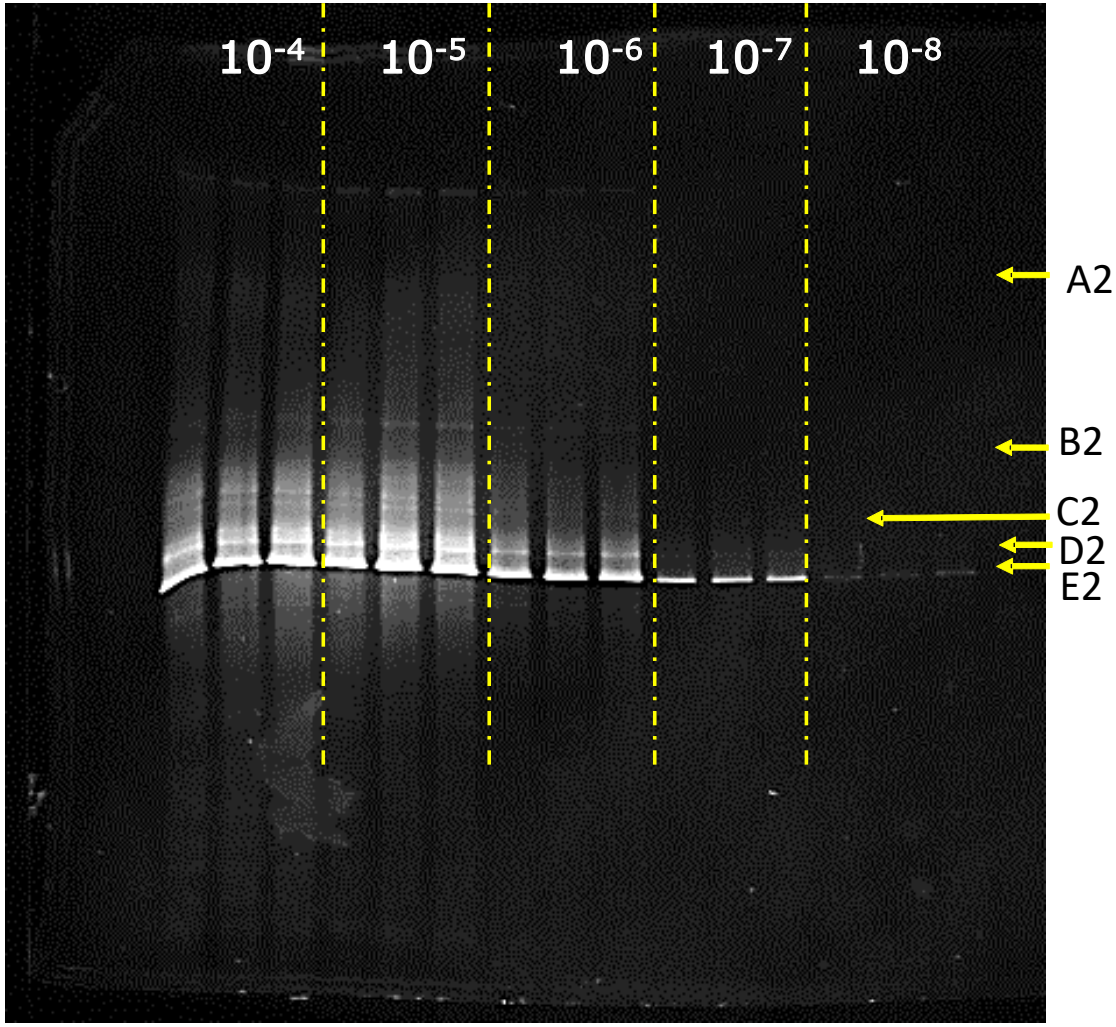

Figure 4.

Table 1. Affiliation[a] of bacteria in serially diluted DNA extract of MBR in A farm on 6/August/2014 (A1) and on 24/October/2015 (A2) by MERFLP (culture-independent MPN).

A1	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Acession number)[d]
A	A110^{-2}-2H	A,Ha,Hh	100	*Arthrobacter* sp.(AF197047)
	A110^{-2}-3H	A,Ha,Hh	100	
	A110^{-3}-1H	A,Ha,Hh	100	
	A110^{-3}-2H	A,Ha,Hh	100	
	A110^{-3}-3H	A,Ha,Hh	100	
	A110^{-4}-1H	A,Ha,Hh	100	
	A110^{-4}-2H	A,Ha,Hh	100	
	A110^{-4}-3H	A,Ha,Hh	100	
	A110^{-5}-3H	A,Ha,Hh	100	
	A110^{-2}-1H	A,R,Hh	91.7	*Streptmyces badius*(AY999783), *S.lavendulae*(D85110, D85111)
	A110^{-5}-2H	A,R	100	*Micromonospora carbonacea* (Mms.carbo2, Mmscarbon), *M.chalcea* (Mms.chalc2), *M.echinospora* (Mms echns2)
βP	A110^{-2}-1M	A,Ha,	93	*Burkholderia* sp.(AB299593)
	A110^{-2}-2M	A,Ha,	93	
	A110^{-2}-3M	A,Ha,	93	
	A110^{-3}-1M	A,Ha,	93	
	A110^{-3}-2M	A,Ha,	93	
	A110^{-3}-3M	A,Ha,	93	
	A110^{-4}-1M	A,Ha,	93	
	A110^{-4}-2M	A,Ha,	93	
	A110^{-4}-3M	A,Ha,	93	
γP	A110^{-5}-1H	A,R,Hh	93.3	*Cedecea neteri* (AB086230)

A2	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Acession number)[d]
A	A210^{-3}-1H	A,Ha,Hh	91.7	*Arthrobacter* sp.(AF197047,AF197044,AM11056,AM491459)
	A210^{-3}-2H	A,Ha,Hh	100	
	A210^{-3}-3H	A,Ha,Hh	91.7	
	A210^{-4}-1H	A,Ha,Hh	100	
	A210^{-4}-2H	A,Ha,Hh	100	
	A210^{-4}-3H	A,Ha,Hh	91.7	
	A210^{-5}-1H	A,Ha,Hh	91.7	
	A210^{-5}-2H	A,Ha,Hh	91.7	
	A210^{-5}-3H	A,Ha,Hh	91.7	
	A210^{-6}-1H	A,Ha,Hh	91.7	
	A210^{-6}-2H	A,Ha,Hh	91.7	
	A210^{-6}-3H	A,Ha,Hh	91.7	
	A210^{-7}-1H	A,Ha	100	
	A210^{-7}-3H	A,Ha,Hh	91.7	
	A210^{-3}-1M	Ha,Hh	100	*Microbacterium* sp.(AM403628)
	A210^{-3}-3M	Ha,Hh	100	
	A210^{-6}-3M	Ha,Hh	100	
	A210^{-6}-2M	Ha,Hh	100	*Microbacterium lavaniforman* (D21344)
	A210^{-4}-1M	Ha,Hh	100	*Cellulosimicrobium* sp.(AB188217), *Streptmyces shandongensis* (AY875718), *Promicromonospora enterophil* (X83807)
	A210^{-7}-2H	R,Hh	90	*Streptomyces cirratus* (AY944265), *S.lavendulae* (Stm.lave12)
F	A210^{-3}-2L	A,Hh	93	*Lactobacillus reuteri* (L23507, L.reuteri)
	A210^{-3}-3L	A,Hh	93	
	A210^{-5}-2L	A,Hh	93	*Eubacterium* sp.(Eub.W 1365), uncultured *Buleidia* sp.(AM420006)
αP	A210^{-3}-1L	A,R	100	*Leisingera methylohalidivorans*(AY005463)
γP	A210^{-3}-2M	A,Ha	94.4	uncultured *Gammaproteobacteria* (AY354844)
	A210^{-4}-3M	A,Ha	94.4	*Photobacterium profundum*(CR378673)
	A210^{-4}-2M	A,Ha	94.4	uncultured *Gammaproteobacteria*(AJ704665)
	A210^{-5}-1M	A,Ha	94.4	
	A210^{-5}-2M	A,Ha	94.4	
	A210^{-5}-3M	A,Ha	94.4	*Citrobacter* sp.(DQ190736)

[a] Grouping was based on affiliation by MERFL; *Actinobacteria* (A), *Firmicutes* (F), *Alpharoteobacteria* (α P), *Betaproteobacteria* (βP), *Gammaproteobacteria* (γP), *Deltaproteobacteria* (δP), *Epsilonproteobacteri*a (εP), the other bacteria (O), and unidentified or uncultured bacterial group (no).

[b] Exponential of vial number represents the decimal dilution of the vial. The 2nd number of vial number (1-3) represents number in 3 replicates for the each decimal dilution. “H” of last letter represents MERFL originating from the major 16S rDNA, “M” represents from the 2nd major 16S rDNA, and “L” represents from the 3rd major 16S rDNA.

[c] Restriction enzymes used for similarity search; "Ha", "R", and "Hh" stand for *Hae* III, *Rsa* I, and *Hha* I. For the measured MERFLP, which had no completely identical theoretical MERFLP, the theoretical MERFLP having the highest similarity using all the RFLPs, was presented with the similarity as described in the materials and method.

[d] Species name (accession number) of the theoretical MERFL having the highest similarity with the measured MERFL

Table 2. Affiliation[a] of bacteria in serially diluted DNA extract of MBR in B farm on 18/August/2014 (B2) and on 30/August/2014 (B2) by MERFLP (culture-independent MPN).

B1	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Acession number)[d]
A	$B110^{-3}$-2L	Ha,Hh	0.1	*Actinotelluria brasiliensis*(DQ029102)
	$B110^{-3}$-3L	Ha,Hh	93	uncultured actinobacterium(AJ575510,AJ575514)
αP	$B110^{-3}$-1H	R,Hh	100	*Ensifer fredii* (D14516), *E.meliloti* (D14509), *E. medicae* (CP000738), *Shinorhizobium fredi* (Srh.fredi5), *S. meliloti* (Srh.melil7), *Mesorhizobium tianshanense* (Mso.tians2), *M. loti* (BA000012), *Agrobacterium vitis* (D12795)
	$B110^{-3}$-3H	R,Hh	100	
	$B110^{-4}$-1H	R,Hh	100	
	$B110^{-4}$-2H	R,Hh	100	
	$B110^{-4}$-3H	R,Hh	100	
	$B110^{-5}$-1H	R,Hh	100	
	$B110^{-5}$-2H	R,Hh	100	
	$B110^{-5}$-3H	R,Hh	100	
βP	$B110^{-3}$-2H	Ha,Hh	100	*Aquaspirillum peregrinum* (AB074521)
	$B110^{-3}$-3M	A,Hh	90	*Neisseria elongata*(Nis,elong2)
γP	$B110^{-3}$-1M	A,Hh	90	*Vibrio shilonii* (AY911395), *V.harveyi*(AY911396), *V.alginolyticus*(V.alginol3), *Neisseria elongata* (Nis,elong2)
	$B110^{-3}$-2M	A,Hh	90	
	$B110^{-4}$-3M	A,Hh	90	
	$B110^{-5}$-2M	A,Hh	90	*Escherichia coli* (E.colirnB3,E.colirnC3,E.colirnD3), *Shigella dyenteriae*(X96966)
δP	$B110^{-4}$-1M	A,Hh	93	uncultured *Deltaproteobacterium* (AJ581352)
	$B110^{-4}$-2M	A,R	90	*Deltaproteobacterium* (AY162123)
εP	$B110^{-5}$-1M	Ha,Hh	90	uncultured *Epsilonproteobacterium* (AB013262, AB015535)
	$B110^{-5}$-3M	Ha,Hh	90	
O	$B110^{-3}$-2L	R,Hh	90	*Treponema* sp.(AF182834,AF182837)

[a] Grouping was the same as described in Table 1.
[b] Vial number was the same as described in Table 1.
[c] Restriction enzymes used for similarity search as described in Table 1.
[d] Species name (accession number) was the same as described in Table 1.

B2	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Acession number)[d]
F	$B210^{-3}$-2M	R,Hh	90	*Haloanaerobacter chitinovoran* (U32596)
	$B210^{-4}$-1M	R,Hh	90	
	$B210^{-5}$-3M	R,Hh	90	
αP	$B210^{-1}$-1H	A,R,Hh	96.3	*Ensifer fredii* (AB195268), *E.medicae*(CP000738), *Shinorhizobium* sp.(AF227755,AB195268)
	$B210^{-1}$-2H	A,R,Hh	96.3	
	$B210^{-1}$-3H	A,R,Hh	96.3	
	$B210^{-2}$-1H	A,R,Hh	96.3	
	$B210^{-2}$-2H	A,R,Hh	96.3	
	$B210^{-2}$-3H	A,R,Hh	96.3	
	$B210^{-3}$-1H	A,R,Hh	96.3	
	$B210^{-3}$-3H	A,R,Hh	96.3	
	$B210^{-4}$-1H	A,R,Hh	95.2	
	$B210^{-4}$-3H	A,R,Hh	96.3	
	$B210^{-5}$-2H	A,R,Hh	96.3	
	$B210^{-5}$-3H	A,R,Hh	96.3	
βP	$B210^{-3}$-2H	Ha,Hh	100	*Aquaspirillum peregrinum* (AB074521)
	$B210^{-1}$-1M	R,Hh	90	uncultured bacterium (AB186826, AB186827, AB186829)
	$B210^{-1}$-2M	R,Hh	90	
	$B210^{-1}$-3M	R,Hh	90	
	$B210^{-2}$-1M	R,Hh	90	
	$B210^{-2}$-2M	R,Hh	90	
	$B210^{-2}$-3M	R,Hh	90	
	$B210^{-3}$-1M	R,Hh	90	
	$B210^{-3}$-3M	R,Hh	90	
	$B210^{-4}$-3M	R,Hh	90	
	$B210^{-1}$-1L	R,Hh	90	uncultured bacterium(DQ154841)
	$B210^{-1}$-2L	R,Hh	90	
	$B210^{-2}$-3L	R,Hh	90	
	$B210^{-3}$-3L	R,Hh	90	
	$B210^{-4}$-3L	R,Hh	90	

Table 3. Affiliation[a] of bacteria in serially diluted DNA extract of MBR in C farm 9/October/2015 by MERFLP (culture-independent MPN).

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)	Name (Acession number)[d]
A	$C10^{-3}$-1L	R,Hh	100	*Mycobacteriaceae* bacterium (AB298730), *Propionibacteriaceae* bacterium (AB298731)
	$C10^{-4}$-1L	R,Hh	100	
	$C10^{-5}$-1L	R,Hh	100	
	$C10^{-6}$-2L	R,Hh	100	
	$C10^{-6}$-1M	Ha,Hh	90	*Streotiverticillium baldaccii* (X53164,X53169)
	$C10^{-6}$-2M	Ha,Hh	90	
F	$C10^{-3}$-2H	Ha,R	100	uncultured *Eubacterium* (AY356378,AY356789,AY356381,AY356382)
	$C10^{-3}$-3L	R,Hh	100	*Lactobacillus fermentum* (M58809, M58819, DQ208931, L.Fermentm), *L.bifermentum* (L.bifermen)
	$C10^{-4}$-2L	R,Hh	100	
	$C10^{-4}$-3L	R,Hh	100	
	$C10^{-5}$-3L	R,Hh	100	
βP	$C10^{-3}$-1H	Ha,R	100	uncultured *Betaproteobacteria* (AY921864)
	$C10^{-3}$-3H	Ha,R	100	
	$C10^{-4}$-1H	Ha,R	100	
	$C10^{-4}$-3H	Ha,R	100	
	$C10^{-5}$-1H	Ha,R	100	
	$C10^{-5}$-3H	Ha,R	100	
	$C10^{-6}$-1H	Ha,R	100	
	$C10^{-6}$-2H	Ha,R	100	
	$C10^{-6}$-3H	Ha,R	100	
	$C10^{-7}$-1H	Ha,R	100	
O	$C10^{-3}$-1M	Ha,R	90	*Aquiflexum balticum* (AJ744861)
	$C10^{-4}$-2H	Ha,R	100	uncultured *Chloroflexi* bacteria(AY921893)
	$C10^{-5}$-2H	Ha,R	100	
	$C10^{-6}$-1M	Ha,R	90	uncultured *Cytophaga* sp.(AB015265)
	$C10^{-3}$-2M	Ha,Hh	100	uncultured bacterium(AY942753,AY942754)
	$C10^{-5}$-1M	Ha,Hh	100	
	$C10^{-5}$-3M	Ha,Hh	100	
	$C10^{-4}$-2M	A,Ha	100	uncultured bacterium(AY375083)
	$C10^{-6}$-3L	R,Hh	100	uncultured prokaryote(AM268745)
	$C10^{-7}$-1L	R,Hh	100	

[a] Grouping was the same as described in Table 1.
[b] Vial number was the same as described in Table 1.
[c] Restriction enzymes used for similarity search as described in Table 1.
[d] Species name (accession number) were the same as described in Table 1.

Table 4. Most probable numbers of the numbers of each group in the MBE in A farm on 6/August/2014 by culture-based MPN (a1), on 6/August /2014 (A1) and on 24/October /2015 (A2) by culture-independent MPN and 5% confidence limits obtained using the FDA's Bacterial Analytical Manual (47, 48).
*Under estimated MPN number due to an absence of MPN dilution vials higher than 10^{-9}.

	a1 (culture-based MPN)				**A1** (culture- independent MPN)				**A2** (culture- independent MPN)			
	Three dilutions	Score	x10^6MPN/mL	5%limits Low-High	Three dilutions	Score	x10^4MPN/mL	5% limits Low-High	Three dilutions	Score	x10^6MPN/mL	5% limits Low-High
Actinobacteria	$10^{-7}10^{-8}10^{-9}$	0-0-1	30	1.5-96	$10^{-4}10^{-5}10^{-6}$	3-2-0	93	18-420	$10^{-6}10^{-7}10^{-8}$	3-3-0	240	42-1000
Arthrobacter sp.					$10^{-4}10^{-5}10^{-6}$	3-1-0	43	9-180	$10^{-6}10^{-7}10^{-8}$		93	18-420
Microbacterium sp.									$10^{-6}10^{-7}10^{-8}$		9.2	1.4-38
Firmicutes	$10^{-7}10^{-8}10^{-9}$	2-0-1	140	36-420					$10^{-3}10^{-4}10^{-5}$	2-0-1	0.01	0.004-0.042
*α*proteobacteria	$10^{-5}10^{-6}10^{-7}$	2-0-0	0.92	0.14-3.8					$10^{-3}10^{-4}10^{-5}$	1-0-0	0.004	0.00002-0.018
*β*proteobacteria	$10^{-7}10^{-8}10^{-9}$	0-1-0	30	1.5-90	$10^{-4}10^{-5}10^{-6}$	3-1-0	43	9-180				
Burkholderia sp.					$10^{-4}10^{-5}10^{-6}$	3-1-0	43	9-180				
*γ*proteobacteria	$10^{-7}10^{-8}10^{-9}$	1-1-0	74	13-200	$10^{-4}10^{-5}10^{-6}$	0-1-0	11	0.15-11	$10^{-4}10^{-5}10^{-6}$	2-3-0	0.29	0.09-0.94
*δ*proteobacteria												
ε proteobacteria												
Other	$10^{-7}10^{-8}10^{-9}$	3-1-0	430	90-1800								
unidentified	$10^{-4}10^{-5}10^{-6}$	1-0-0	0.04	0.002-0.18								
Total number	$10^{-7}10^{-8}10^{-9}$	3-3-3	>11,000*	420-	$10^{-4}10^{-5}10^{-6}$	3-3-0	240	42-1000	$10^{-6}10^{-7}10^{-8}$	3-3-0	240	42-1000

Table 5. Most probable numbers of the numbers of each group in the MBE in B farm on 18/August/2014 by culture-based MPN (b1), on 18/August/2014 (B1) and on 30/August/2014 (B2) by culture-independent MPN and 5% confidence limits obtained using the FDA's Bacterial Analytical Manual (47, 48).

*We could not calculate 5% confidence limits because of the lack a 10^{-5} dilution sample, and a 10^{-6} dilution sample.

	b1 (culture-based MPN)				**B1** (culture- independent MPN)				**B2** (culture- independent MPN)			
	Three dilutions	Score	x10^6MPN/mL	5% limits Low-High	Three dilutions	Score	x10^4MPN/mL	5% limits Low-High	Three dilutions	Score	x10^6MPN/mL	5% limits Low-High
Actinobacteria	$10^{-7}10^{-8}10^{-9}$	0-0-1	30	1.5-96	$10^{-3}10^{-4}10^{-5}$	2-0-0	0.92	10.14-3.8				
Firmicutes	$10^{-5}10^{-6}10^{-7}$	1-0-1	0.72	0.13-1.8					$10^{-4}10^{-5}10^{-6}$	1-1-0	12.6	2.4-41
H.chitinovorans									$10^{-4}10^{-5}10^{-6}$	1-1-0	12.6	2.4-41
αproteobacteria	$10^{-7}10^{-8}10^{-9}$	0-1-0	30	1.5-300	$10^{-4}10^{-5}10^{-6}$	3-3-0	240	42-1000	$10^{-4}10^{-5}10^{-6}$	2-2-0	155	*
Ensifer/Shinorhizobium					$10^{-4}10^{-5}10^{-6}$	3-3-0	240	42-1000	$10^{-4}10^{-5}10^{-6}$	2-2-0	155	*
βproteobacteria	$10^{-7}10^{-8}10^{-9}$	2-1-0	150	37-420	$10^{-3}10^{-4}10^{-5}$	2-0-0	0.92	0.14-3.8	$10^{-3}10^{-4}10^{-5}$	1-0-0	0.37	*
γproteobacteria	$10^{-7}10^{-8}10^{-9}$	0-3-1	126	37-265	$10^{-4}10^{-5}10^{-6}$	1-1-0	7.4	1.3-20				
δproteobacteria					$10^{-4}10^{-5}10^{-6}$	2-0-0	9.2	1.4-30				
εproteobacteria					$10^{-4}10^{-5}10^{-6}$	0-2-0	6.2	1.2-18				
Other	$10^{-5}10^{-6}10^{-7}$	1-1-0	0.74	0.13-2	$10^{-3}10^{-4}10^{-5}$	0-1-0	0.36	0.017-1.8	$10^{-3}10^{-4}10^{-5}$	2-1-0	1.58	*
unidentified	$10^{-7}10^{-8}10^{-9}$	0-0-3	90	32-808					$10^{-3}10^{-4}10^{-5}$	3-0-0	3.56	*
Total number	$10^{-7}10^{-8}10^{-9}$	3-3-1	4,600	900-2000	$10^{-4}10^{-5}10^{-6}$	3-3-1	460	90-2000	$10^{-4}10^{-5}10^{-6}$	3-3-0	240	42-1000

Table 6. Most probable numbers of the numbers of each group in the MBE in farm C on 9/October/2015 by culture-based MPN (c), and by culture-independent MPN (C) and 5% confidence limits obtained using the FDA's Bacterial Analytical Manual (47, 48).
*Under estimated MPN number due to an absence of MPN dilution vials higher than 10^{-7}.

	c (culture-based MPN)				**C** (culture- independent MPN)			
	Three dilutions	Score	x10^6MPN/mL	5%limits Low-High	Three dilutions	Score	x10^6MPN/mL	5%limits Low-High
Actinobacteria					$10^{-5}10^{-6}10^{-7}$	1-3-0	1.6	0.45-4.2
Mycobacteriaceae					$10^{-5}10^{-6}10^{-7}$	1-1-0	0.74	0.13-2
S. baldaccii					$10^{-5}10^{-6}10^{-7}$	0-2-0	0.62	0.12-1.8
Firmicutes					$10^{-4}10^{-5}10^{-6}$	2-1-0	0.15	0.037-0.42
L. fermentum					$10^{-4}10^{-5}10^{-6}$	2-1-0	0.15	0.037-0.42
αproteobacteria								
βproteobacteria	$10^{-5}10^{-6}10^{-7}$	0-2-3	>1.56*	0.41-2.39	$10^{-6}10^{-7}10^{-8}$	3-1-0	43	9-180
AY921864					$10^{-6}10^{-7}10^{-8}$	3-1-0	43	9-180
γproteobacteria	$10^{-5}10^{-6}10^{-7}$	2-1-0	1.5	0.37-4.3				
δproteobacteria								
εproteobacteria								
Other unidentified	$10^{-5}10^{-6}10^{-7}$	1-0-0	0.36	0.017-1.8	$10^{-5}10^{-6}10^{-7}$	1-1-0	0.74	0.13-2
AM26874					$10^{-6}10^{-7}10^{-8}$	1-1-0	7.4	1.3-20
Total number	$10^{-5}10^{-6}10^{-7}$	3-3-3	>110*	42-	$10^{-6}10^{-7}10^{-8}$	1-1-0	93	18-420