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Title: Methylophilaceae and Hyphomicrobium as target taxonomic groups in monitoring the function of methanol-fed denitrification biofilters in municipal wastewater treatment plants

Year: 2017

Version:

Please cite the original version:

Rissanen, A., Ojala, A., Fred, T., Toivonen, J., & Tirola, M. (2017). Methylophilaceae and Hyphomicrobium as target taxonomic groups in monitoring the function of methanol-fed denitrification biofilters in municipal wastewater treatment plants. *Journal of Industrial Microbiology and Biotechnology*, 44(1), 35-47.
<https://doi.org/10.1007/s10295-016-1860-5>

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***Methylophilaceae* and *Hyphomicrobium* as target taxonomic groups in monitoring the function of methanol-fed denitrification biofilters in municipal wastewater treatment plants**

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Acknowledgements

We thank P. Lindholm, P. Lindell, L. Sundell, K. Murtonen, and M. Heinonen for technical assistance. We thank R. Kettunen for valuable comments on this manuscript. We also thank H. Devlin, B. Thamdrup, and S. Hallin for comments on the earlier version of this manuscript. This study was funded by Maa- ja Vesitekniikan Tuki ry for A.J.R and Academy of Finland (projects 286642 and 140964 to A.J.R and 260797 to M.T.) as well as European Research Council (ERC) Consolidator project 615146 to M.T.

1 **Abstract**

2
3 Molecular monitoring of bacterial communities can explain and predict the stability of
4 bioprocesses in varying physicochemical conditions. To study methanol-fed
5 denitrification biofilters of municipal wastewater treatment plants, bacterial
6 communities of two full-scale biofilters were compared through fingerprinting and
7 sequencing of the 16S rRNA genes. Additionally, 16S rRNA gene fingerprinting was
8 used for 10-week temporal monitoring of the bacterial community in one of the
9 biofilters. Combining the data with previous study results, the family *Methylophilaceae*
10 and genus *Hyphomicrobium* were determined as suitable target groups for monitoring.
11 An increase in the relative abundance of *Hyphomicrobium*-related biomarkers occurred
12 simultaneously with increases in water flow, NO_x⁻ load, and methanol addition, as well
13 as a higher denitrification rate, although the dominating biomarkers linked to
14 *Methylophilaceae* showed an opposite pattern. The results indicate that during increased
15 loading, stability of the bioprocess is maintained by selection of more efficient
16 denitrifier populations, and this progress can be analyzed using simple molecular
17 fingerprinting.

18

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20 **Keywords** Methanol · Denitrification · Biofilter · *Hyphomicrobium* · *Methylophilaceae*

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28 **Introduction**

29

30 Denitrification is an essential biotechnological process in municipal wastewater
31 treatment plants (WWTPs) for reducing the nitrogen (N) load to recipient waters. This
32 step-wise reduction of water-soluble nitrate (NO_3^-) via nitrite (NO_2^-) to gaseous nitric
33 oxide (NO), nitrous oxide (N_2O), and di-nitrogen (N_2) is catalyzed by facultative
34 anaerobic heterotrophic bacteria. Denitrification is a community process, as many
35 denitrifiers perform only a portion of the reduction steps, reducing NO_3^- to NO_2^- or to
36 N_2O , and only some bacterial species are capable of the whole denitrification chain
37 from NO_3^- to N_2 gas [8]. Due to the unfavorably low carbon-to-nitrogen (C:N) ratio of
38 the water in many N removal systems, an additional organic C and energy source,
39 usually methanol, is used in the process. In WWTPs, methanol-fed denitrification is
40 often accomplished by filtration of the wastewater through a support material in
41 biofilters [17].

42 The physicochemical and technical aspects of the methanol-utilizing
43 denitrification processes have been comprehensively characterized [17, 20]. However,
44 the optimal control and operation of the processes would also benefit greatly from
45 microbiological data [22, 39], such as the identity and potential controlling factors of
46 the taxonomic groups crucial for the system function, which could be used in process
47 monitoring [22]. Methylotrophs play a key role in methanol-fed denitrification systems,
48 both by directly utilizing methanol as an electron donor in denitrification as well as by
49 transforming methanol into various organic extracellular compounds, which are utilized
50 by co-occurring non-methylotrophic denitrifiers [22]. Of the known methylotrophic
51 denitrifiers, the genus *Hyphomicrobium* (*Alphaproteobacteria*) is frequently detected in
52 methanol-fed denitrification systems [2, 6, 21, 27-29, 35, 38] and is thus considered a
53 suitable target for monitoring methanol-fed denitrification [22]. In addition, bacteria

54 within family *Methylophilaceae* (*Betaproteobacteria*) [10, 29, 33, 36] as well as within
55 genera *Methyloversatilis* (*Betaproteobacteria*) [2] and *Paracoccus*
56 (*Alphaproteobacteria*) [6, 21, 27] can also play a significant role in the process.
57 However, most studies have been done at laboratory scale. Other than the studies of
58 Neef et al. [27] and Lemmer et al. [21], which found *Paracoccus* and *Hyphomicrobium*
59 to be important methylotrophs in a methanol-fed denitrifying sand filter of a WWTP,
60 very little is known about the overall bacterial dynamics or about the identity and
61 community dynamics of methylotrophic denitrifiers in full-scale biofilters. There are
62 ecological differences between methylotrophs and non-methylotrophs [21]. In addition,
63 the ecology of *Hyphomicrobium* differs from that of *Methyloversatilis* [2], *Paracoccus*
64 [21], and *Methylophilaceae* [10]. This indicates that methylotrophs and non-
65 methylotrophs as well as different taxonomic groups of methylotrophs respond
66 differently to the temporal and inter-system variations in the physicochemical
67 conditions confronted by the full-scale biofilters.

68 This study investigated the bacterial communities of two full-scale methanol-fed
69 denitrifying WWTP biofilters by length heterogeneity PCR (LH-PCR) [37] and clone
70 library and 454-pyrosequencing analysis of the 16S rRNA gene sequences. We
71 specifically focused on the taxonomic groups of the methylotrophic bacteria that
72 inhabited both of the biofilters as well as previously studied systems. In addition to
73 comparing the bacterial communities of the two biofilters, we analyzed the temporal
74 variation in the structure of the bacterial communities and linked it with the
75 physicochemical and functional data during a 10-week follow-up period in one of the
76 biofilters. We aimed to determine the following: 1) which methylotrophic taxonomic
77 groups are typical for methanol-fed denitrification systems and could thus be used as
78 target taxonomic groups for monitoring the process function in full-scale WWTP

79 biofilters; 2) whether variations in physicochemical conditions affect the bacterial
80 community structure; and 3) whether methylotrophs and non-methylotrophs as well as
81 4) different taxonomic groups of methylotrophs respond differently to these variations.

82 **Materials and methods**

83

84 **Microbiological sampling**

85

86 Samples were collected from the methanol-fed denitrification filters of two municipal
87 wastewater treatment plants: the Viikinmäki wastewater treatment plant in Helsinki,
88 Finland (WWTPA), and the Salo wastewater treatment plant in Salo, Finland
89 (WWTPB) (Table 1). WWTPA is a large plant with one of the largest denitrification
90 filter systems in the world, whereas WWTPB is a small-sized plant (Table 1).
91 Methanol-fed denitrification filters have been functioning since 2004 and 2007 in
92 WWTPA and WWTPB, respectively. In both sites, the denitrification is preceded by an
93 aerobic stage (activated sludge) where nitrification occurs. The samples from the
94 denitrification filter of WWTPA were collected from the same denitrification cell at 5 to
95 9 day intervals during a 10-week follow-up period (27 August 2008 – 28 October 2008).
96 The samples from the denitrification filter of WWTPB were collected once (2 October
97 2008). In addition, samples from the inflow of the denitrification systems were collected
98 once (from WWTPA 10 November 2008 and from WWTPB 2 October 2008).

99 The biofilter samples were taken from the backwash water channel.

100 Backwashing consists of air-sparging and washing, which detaches biomass from the
101 carrier material. Samples of the backwash water (1 sample per sampling date in
102 WWTPA, 2 replicate samples in WWTPB) and polystyrene carrier material beads
103 escaping from the WWTPB biofilter were collected into sterile 50 ml plastic containers.
104 Bacteria in the inflow of the systems were collected by filtering 100–200 ml water using

105 Sarstedt Filtropur S 0.2 polyethersulfone filters. The samples were stored at -20 °C
106 before further processing within 1 to 2 months.

107

108 **Background data and NO_x⁻ reduction**

109

110 Online monitoring data of the WWTPs were used as background data in this study. For
111 WWTPA, water flow (W_f), methanol addition rate (Met_f), inflow and outflow
112 concentrations of $NO_3^- + NO_2^-$ (henceforth NO_{x^-in} and NO_{x^-out} , respectively) in the studied
113 denitrification cell, as well as inflow temperature (T) and inflow concentrations of O₂
114 (O_{2in}), suspended solids (SS_{in}), PO_4^{3-} (PO_4^{3-in}), total phosphorous (TP_{in}), and outflow
115 concentrations of SS (SS_{out}), PO_4^{3-} (PO_4^{3-out}), and TP (TP_{out}) in the whole denitrification
116 system were measured hourly. Daily averages (for the time period 20 August 2008 – 31
117 October 2008) were then calculated. For WWTPB, daily averages (for the time period 1
118 September 2008 – 31 October 2008) for W_f and Met_f along with T, NO_{x^-in} , PO_4^{3-in} , SS_{in} ,
119 and O_{2in} and NO_{x^-out} , PO_4^{3-out} , and SS_{out} were calculated for the whole denitrification
120 system. The NO_{x^-} load ($\mu\text{mol s}^{-1}$) in the inflow (LNO_{x^-in}) and outflow (LNO_{x^-out}) water
121 was calculated from W_f and NO_{x^-in} or NO_{x^-out} . Denitrification in the filters was
122 calculated either as relative (%) or actual ($\mu\text{mol s}^{-1}$) NO_{x^-} reduction as follows:

$$123 \quad NO_{x^-} \text{ reduction} = \frac{(NO_{x^-in} - NO_{x^-out})}{NO_{x^-in}} \times 100$$

$$124 \quad \text{Actual } NO_{x^-} \text{ reduction} = LNO_{x^-in} - LNO_{x^-out}$$

125 Denitrification in this study refers to the conversion of water soluble NO_{x^-} into gaseous
126 forms, but the proportions of NO, N₂O, and N₂ in the end product are not separated.

127

128 **Molecular microbiological analyses**

129

130 DNA extraction of each sample – from 10 mg of freeze-dried backwash sample material
131 from WWTPA and WWTPB, from 5 frozen carrier beads from WWTPB (sample
132 WWTPB_Car), and from the Filtropur filters containing the inflow water samples – was
133 carried out as previously described [32].

134 For the LH-PCR analysis, PCR was performed using the universal bacterial
135 primers F8 (5'-AGA GTT TGA TCM TGG CTC AG-3') (1:4 ird700-labelled) [41] and
136 PRUN518r (5'-ATT ACC GCG GCT GCT GG-3') [26], with a GeneAmp PCR system
137 9600 (Perkin Elmer), in previously described reaction mixtures [31]. For the PCR
138 reaction, the following program was used: an initial denaturation step at 95°C for 5 min,
139 30 cycles of amplification (94°C for 30 s, 53°C for 1 min, 72°C for 3 min), and final
140 elongation at 72°C for 15 min. The LH-PCR analysis was done as previously described
141 [31]. The relative area (%), that is, the relative abundance of each LH-PCR peak was
142 defined as a ratio of the total peak area (sum of the areas of all peaks) of the sample.

143 PCR for the clone library analyses of 16S rRNA was performed using the
144 universal bacterial primers 27F (5'- AGAGTTTGATCMTGGCTCAG - 3') [19] and
145 907R (5'- CCGTCAATTCMTTTGAGTTT - 3') [13], and cloning and sequencing
146 (Sanger sequencing) of the PCR amplicons was done as in Rissanen et al. [32]. For the
147 clone libraries, PCR products of the samples from WWTPA on all sampling dates
148 (WWTPA - library), PCR products of the replicate samples of backwash water
149 (WWTPB - library), and the carrier materials of WWTPB (WWTPB_Car - library) were
150 pooled separately.

151 The bacterial communities of WWTPA were also studied via 454-
152 pyrosequencing. Equal amounts of nucleic acid extracts from each sampling date were
153 pooled before PCR reactions, and the PCR and sequencing was performed as previously
154 described [32].

155

156 **Sequence analysis**

157

158 The analysis of the clone library and 454-pyrosequencing library sequences was done as
159 previously described [32]. Putative methylotrophic operational taxonomic units (OTUs)
160 (97 % identity threshold) were determined based on the previous literature [1, 2, 5, 10,
161 18, 27, 34-35]. Clone library OTUs assigned to the methylotrophic families found from
162 both biofilters (*Methylophilaceae* and *Hyphomicrobiaceae*) were subjected to
163 phylogenetic tree analyses, as described previously [32]. In addition, phylogenetic
164 classification was linked to the LH-PCR peaks *in silico* using the length and
165 taxonomical data obtained in the clone library analysis.

166 16S rRNA gene sequences of the clone libraries were deposited into the EMBL
167 database (accession numbers KP098594 – KP098735, KP098971 – KP098975, and
168 KP098985 – KP098988). The 454-pyrosequencing data were deposited into the NCBI
169 SRA database (SRX646346).

170

171 **Statistical analyses**

172

173 Bray–Curtis dissimilarities among the samples were calculated from the relative
174 abundances of the LH-PCR peaks. Temporal variations in the structure of the bacterial
175 communities of WWTPA were then analyzed by non-metric multidimensional scaling
176 (NMS) of the LH-PCR peak data. Changes in the WWTPA community structures were
177 correlated with variations in the background parameters using Mantel’s test. In addition,
178 temporal variations in the relative abundances of the LH-PCR peaks affiliated with
179 methylotrophs and non-methylotrophs were correlated with variations in the
180 background parameters using either Pearson correlation analysis (for normally
181 distributed variables, normality tested using the Shapiro–Wilk test) or Spearman’s

182 correlation analysis (for non-normally distributed variables). For background
183 parameters, the average daily values for the time period between the two samplings was
184 used in the correlation analyses. Temporal and inter-system variations in the community
185 structures were also analyzed by hierarchical clustering (UPGMA linkage) using the
186 LH-PCR data. The NMS analysis and Mantel's test were performed in PC-ORD 6.0
187 [24], and cluster analysis was done using PAST version 3.09 [11]. The correlation
188 analyses were performed in PASW 18.0 (PASW Statistics 18, Release Version 18.0.0,
189 SPSS, Inc., 2009, Chicago).

190 **Results**

191

192 **Performance of the denitrification biofilters**

193

194 As is typical for WWTPs in Northern countries in autumn, W_f increased and T
195 decreased during the study period in both filter systems (Fig. 1, Online Resource 1).
196 NO_x^- in and O_{2in} were generally higher and more variable in WWTPB (NO_x^- in: 700 –
197 2900 $\mu\text{mol/L}$; O_{2in} : 1 to 215 $\mu\text{mol/L}$) than in WWTPA (NO_x^- in: 500 – 1000 $\mu\text{mol/L}$;
198 O_{2in} : 40 - 110 $\mu\text{mol/L}$). In addition, NO_x^- in decreased in WWTPB and O_{2in} in WWTPA
199 during the study period (Fig. 1, Online Resource 1). The higher NO_x^- in WWTPB
200 compared to WWTPA could be due to possible differences in the total N concentrations
201 feeding the WWTPs, the nitrification efficiency between WWTPA and WWTPB, or the
202 lack of a pre-denitrification system in WWTPB (Table 1). In the filters, Met_f is
203 controlled by a feedback loop that controls the $NO_3\text{-N}$ concentration inside the filter
204 cells [7]. As a result, Met_f followed ${}_LNO_x^-$ in tightly, and they both controlled the actual
205 NO_x^- reduction rate ($\mu\text{mol/s}$) in the systems (Fig. 1, Online Resource 1). This kept the
206 C:N ratio in the inflow ($Met_f:{}_LNO_x^-$ inflow ratio), as well as the relative NO_x^- reduction
207 and the NO_x^- out concentration, relatively stable in both systems. However, the relative
208 NO_x^- reduction and NO_x^- out concentration were higher and lower, respectively, and

209 temporally more stable, and Metf:LNO_x^- inflow was lower in WWTPA (Metf:LNO_x^- inflow
210 ratio: 0.90–1.13; relative NO_x^- reduction: 82–93 %; NO_x^- out: 66–99 $\mu\text{mol/L}$) than in
211 WWTPB (Metf:LNO_x^- inflow ratio: 0.98–1.18; relative NO_x^- reduction: 64–90 %; NO_x^- out:
212 128–870 $\mu\text{mol/L}$, when the exceptional values of 25 October were excluded) (Fig. 1,
213 Online Resource 1). When estimated per carrier volume, the load of NO_x^- , O_2 and
214 methanol feeding as well as the actual NO_x^- reduction rate were on average lower in
215 WWTPA (NO_x^- : 570 $\mu\text{mol/m}^3/\text{s}$; O_2 : 50 $\mu\text{mol/m}^3/\text{s}$; methanol: 590 $\mu\text{mol/m}^3/\text{s}$; actual
216 NO_x^- reduction: 510 $\mu\text{mol/m}^3/\text{s}$) than in WWTPB (NO_x^- : 890 $\mu\text{mol/m}^3/\text{s}$; O_2 : 60
217 $\mu\text{mol/m}^3/\text{s}$; methanol: 930 $\mu\text{mol/m}^3/\text{s}$; actual NO_x^- reduction: 730 $\mu\text{mol/m}^3/\text{s}$). The
218 higher O_2 load increases the requirement for electron donors for O_2 reduction (to allow
219 anaerobic conditions for denitrification), which explains the higher Metf:LNO_x^- inflow
220 ratio in WWTPB than in WWTPA. Furthermore, the average surface load was higher
221 and the average hydraulic retention time (HRT) lower in the biofilter of WWTPA
222 (Table 1).

223

224 **Differences in the bacterial community structures between the biofilters**

225

226 Based on the UPGMA clustering of the LH-PCR data, conditions within the biofilters
227 shaped the original bacterial communities (communities of the inflow water) in both
228 WWTPA and WWTPB (Online Resource 2 & 3). The bacterial communities of the
229 WWTPA and WWTPB samples clustered separately (Table 2, Online Resource 2 & 3),
230 except for the carrier material of WWTPB, which more resembled the backwash water
231 of WWTPA than that of WWTPB (Table 2, Online Resource 2).

232 Samples of the sheared biomass in the backwash water were used in comparing
233 the methylotrophic communities between WWTPA and WWTPB. The relative
234 abundance of putative methylotrophs was much higher in WWTPB than in WWTPA

235 (Table 2). *Methylophilaceae* and *Hyphomicrobiaceae* were the dominant
236 methylotrophic families that were found in both biofilters, whereas *Paracoccus*
237 (*Rhodobacteraceae*) and *Methyloversatilis* (*Rhodocyclaceae*) were found only in
238 WWTPB (Table 2, Figs. 2-3). According to the clone library analyses,
239 *Hyphomicrobiaceae* had a much higher relative abundance in WWTPA than in
240 WWTPB, whereas the opposite was observed for *Methylophilaceae* (Table 2). In
241 contrast to the backwash sample, the carrier material of WWTPB did not harbor
242 *Paracoccus* or *Methyloversatilis* but rather *Bradyrhizobium*. The carrier material of
243 WWTPB also had a higher and lower relative abundance of *Hyphomicrobiaceae* and
244 *Methylophilaceae*, respectively, than the backwash material of WWTPB (Table 2).
245 *Hyphomicrobiaceae* was represented by only 2 OTUs in the clone libraries.
246 These OTUs belonged to *Hyphomicrobium* cluster II [30] (Table 2, Fig. 2). OTU 16 was
247 shared between WWTPA and WWTPB. The other OTU, OTU 22, likely representing a
248 different *Hyphomicrobium* species, was only found in the carrier material of WWTPB
249 (Fig. 2), where it was more abundant than OTU 16. 454-pyrosequencing had a lower
250 resolution for detecting *Hyphomicrobiaceae* than the clone library analysis (Table 2),
251 but it showed 7 *Hyphomicrobiaceae* OTUs in WWTPA, of which the dominant one,
252 harboring almost all (91 %) of the *Hyphomicrobiaceae* sequences in the 454-
253 pyrosequencing library, was identical to OTU 16 in the clone library (Fig. 2).
254 Bacteria within *Methylophilaceae*, consisting of 10 OTUs, were divided into four
255 groups (Table 2, Fig. 3). Three of the groups, that is, clusters Met I, *Methylostenella* I,
256 and *Methylostenella* II (clustering according to this study), included 8 OTUs covering the
257 majority of the observed *Methylophilaceae* sequences (Table 2, Fig. 3). *Methylostenella* I
258 and *Methylostenella* II were closely related to the cultured members of the genus
259 *Methylostenella* (Fig. 3), while the Met I cluster probably represented a novel species of

260 *Methylothera* with no cultured representatives so far. The fourth group included two
261 rare OTUs that were not closely affiliated to known *Methylophilaceae* genera (Table 2,
262 Fig. 3). Strikingly, despite the high relative abundance of *Methylophilaceae*, the
263 backwash material of WWTPB had only one *Methylophilaceae* OTU, and it belonged to
264 cluster Met I (Fig. 3). Cluster Met I was also the most abundant group of
265 *Methylophilaceae* in the carrier material of WWTPB, whereas it was absent in WWTPA
266 (Table 2, Fig. 3). In contrast, clusters *Methylothera* I and II were found in the
267 backwash material of WWTPA and also in the carrier material of WWTPB (Table 2,
268 Fig. 3). *Methylothera* I was much more abundant than *Methylothera* II in WWTPA,
269 but it was only slightly less abundant than *Methylothera* II in the carrier material of
270 WWTPB (Table 2). 454-pyrosequencing found 6 *Methylophilaceae* OTUs in WWTPA,
271 of which the dominant OTU, harboring almost all (99 %) of the *Methylophilaceae*
272 sequences in the 454-pyrosequencing library, was identical to *Methylothera* OTU 6
273 (within cluster *Methylothera* I) in the clone library analyses (Fig. 3). Furthermore, 454-
274 pyrosequencing of 16S rRNA gene amplicons revealed a marginal abundance (≤ 1 % of
275 16S rRNA sequences) of the following putative methylotrophs: *Methylocystaceae*,
276 *Methylococcaceae*, *Acinetobacter*, and *Flavobacterium* in WWTPA (Table 2). 454-
277 pyrosequencing also resulted in a higher proportion of unclassified bacterial sequences
278 than the clone library analysis (Table 2).

279 The abundant non-methylotrophic bacterial groups (≥ 5 % of 16S rRNA
280 sequences in any of the libraries) included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*
281 (other than *Flavobacterium*), *Chloroflexi*, *Comamonadaceae*, *Deltaproteobacteria*,
282 *Planctomycetes*, and *Rhodocyclaceae* (other than *Methyloversatilis*) (Table 2).

283
284 **Temporal variation in the bacterial community in the WWTPA biofilter**
285

286 The bacterial community structure changed over time (non-metric multidimensional
287 scaling analysis, Fig. 1), along with a temporal change in several operational parameters
288 (Fig. 1). The fluctuations in the community structure were correlated with variations in
289 W_f (Mantel's test, $r = 0.36$, $p < 0.05$, $n = 10$), ${}_{L}NO_x^-$ in ($r = 0.61$, $p < 0.05$, $n = 10$), Met_f (r
290 $= 0.55$, $p < 0.05$, $n = 10$), and T ($r = 0.59$, $p < 0.05$, $n = 10$). In addition, the community
291 structure correlated with the actual NO_x^- reduction rate ($r = 0.62$, $p < 0.05$).

292 To study the variation of the methylotrophic taxa in WWTPA, the phylogenetic
293 classification was linked to the LH-PCR peaks *in silico* using the length and taxonomic
294 data obtained from the clone library analyses (Online Resource 3). All the clone library
295 sequences with a size of 466 bp in the area amplifiable by LH-PCR primers belonged to
296 OTU 16 within the *Hyphomicrobium* II cluster, and all the sequences of genus
297 *Hyphomicrobium* had the size of this peak (see Fig. 2). The sequences assigned to
298 *Methylophilaceae* were found only within peaks 521 bp and 524 bp, and they dominated
299 only within peak 521 bp (73 %), which was also the largest peak in the LH-PCR
300 profiles of WWTPA (Online Resource 3). Peak 521 bp consisted mostly of OTU 6
301 within the *Methylothera* I cluster (67 %) and for the smaller part of the unclassified
302 *Methylophilaceae* OTU 137 (6 %) (see Fig. 3), *Burkholderiales* (13 %), *Rhodocyclales*
303 (7 %, not *Methyloversatilis*), and *Bacteroidetes* (7%, not *Flavobacterium*). Thus, LH-
304 PCR peaks 466 bp and 521 bp were chosen as biomarkers of *Hyphomicrobium* and
305 *Methylophilaceae*, respectively. Furthermore, the sum of LH-PCR peaks 466 bp and
306 521 bp were used as a general biomarker for methylotrophs, whereas the sum of all
307 peaks excluding methylotrophic peaks 466 bp, 521 bp, and 524 bp (see above) were
308 used as a biomarker for non-methylotrophs.

309 During the study period, there was a negative correlation between the relative
310 abundances of *Hyphomicrobium* and *Methylophilaceae* ($r = -0.91$, $p < 0.001$) (Fig. 4).

311 The relative abundance of *Hyphomicrobium* increased as Met_f , W_f , and LNO_x^- in
312 increased (Met_f : $r = 0.74$, $p < 0.05$; W_f , $\rho = 0.67$, $p < 0.05$; LNO_x^- in, $r = 0.80$, $p < 0.05$, $n =$
313 $= 10$) (Figs. 1 & 4), while the opposite took place with *Methylophilaceae* (Met_f : $r = -$
314 0.74 , $p < 0.05$; W_f , $\rho = -0.66$, $p < 0.05$; LNO_x^- in, $r = -0.77$, $p < 0.05$, $n = 10$). The relative
315 abundance of *Methylophilaceae* also increased as T increased ($r = 0.67$, $p < 0.05$, $n =$
316 10), while there was no correlation between T and *Hyphomicrobium* ($r = -0.62$, $p =$
317 0.06 , $n = 10$) (Fig. 4). The relative abundance of total methylotrophs decreased as Met_f
318 and LNO_x^- in increased (Met_f : $r = -0.73$, $p < 0.05$; LNO_x^- in, $r = -0.77$, $p < 0.05$, $n = 10$) and
319 T decreased ($r = 0.67$, $p < 0.05$), while the opposite took place with non-methylotrophs
320 (Met_f : $r = 0.79$, $p < 0.05$; LNO_x^- in: $r = 0.80$, $p < 0.05$; T: $r = -0.72$, $p < 0.05$, $n = 10$) (Fig.
321 4). An increase in the relative abundance of *Hyphomicrobium* ($r = 0.77$, $p < 0.05$, $n =$
322 10) and non-methylotrophs ($r = 0.80$, $p < 0.05$, $n = 10$) and a decrease in
323 *Methylophilaceae* ($r = -0.77$, $p < 0.05$, $n = 10$) and total methylotrophs ($r = -0.76$, $p <$
324 0.05 , $n = 10$) also occurred with the increase in the actual NO_x^- reduction rate (Figs. 1 &
325 4).

326

327 **Discussion**

328 Bacteria belonging to genus *Hyphomicrobium* inhabited both WWTP biofilters. This
329 agrees with the results from many previous studies [e.g. 2, 27, 29] indicating that
330 bacteria in *Hyphomicrobium* are crucial for the function of methanol-utilizing
331 denitrification processes. Moreover, this further confirms that *Hyphomicrobium* is a
332 suitable target genus for monitoring denitrification in full-scale methanol-fed WWTP
333 biofilters [23].

334 *Methylophilaceae* were also important components of the bacterial communities
335 in both biofilters, which is in accordance with results from laboratory-scale methanol-

336 fed denitrification systems [10, 29, 36]. In addition, *Methylophilaceae* were abundant in
337 pilot-scale activated sludge reactors during a period of high nitrate and methanol
338 concentration [12] and in a full-scale, methanol-fed, activated sludge plant [33]. Since
339 the first indication of the methylotrophic denitrification capability of *Methylophilaceae*
340 was shown in 2004 [10], *Methylophilaceae* were not even targeted (*Methylophilaceae*-
341 specific fluorescence *in situ* hybridized [FISH] probes were not used) in a previous
342 study of a full-scale WWTP biofilter (a sand filter) [21, 27]. However, the addition of
343 methanol led to enrichment of *Betaproteobacteria* in the biofilter [27], and it can be
344 suggested that this was at least partially due to the growth of *Methylophilaceae*.
345 Together, these results suggest that, besides *Hyphomicrobium*, bacteria belonging to
346 *Methylophilaceae* are crucial for the function of methanol-utilizing denitrification
347 processes. Furthermore, the results from the WWTPA and WWTPB biofilters and
348 methanol-affected activated sludge systems [12, 33] indicate that, of the family
349 *Methylophilaceae*, the bacteria belonging to genus *Methylotenera*, which includes
350 species that couple methylotrophs to denitrification [16], can be important components
351 of methanol-fed denitrification systems. In addition, many yet uncultivated species of
352 *Methylotenera* probably also exist, as exemplified by the abundant Cluster Met I
353 detected in WWTPB. However, *Methylobacillus* [29, 36] and *Methylophilus* [29] as
354 well as another, thus far uncultivated *Methylophilaceae* genus [10] (Fig. 3) were
355 determined to be the primary methanol-consuming *Methylophilaceae* in previous
356 laboratory-scale studies of methanol-utilizing denitrification. Thus, *Methylophilaceae*
357 can be used as a target family for monitoring denitrification in full-scale methanol-fed
358 WWTP biofilters, although there can be variation in the genera and species mediating
359 the process between different systems.

360 The considerable differences between the bacterial communities within the
361 biofilters and in the water feeding the biofilters indicate that prevailing physicochemical
362 conditions are very strong determinants of the bacterial community structure inside the
363 biofilters. A change in the primary C source from multicarbon sources (present in the
364 feed water) to methanol can exert an especially strong structuring force on the bacterial
365 communities [36]. We suggest that differences in the biofilter communities between
366 WWTPA and WWTPB are mostly due to variations in physicochemical conditions, but
367 the effect of variations in the original inocula (bacteria from preceding activated sludge
368 stage) cannot be completely ruled out.

369 Many possible physicochemical factors might have affected the differences
370 between the filters. The higher abundance of methylotrophs in WWTPB than in
371 WWTPA could be explained by the higher availability of methanol (higher $\text{Met}_f:\text{LNO}_x^-$
372 inflow and higher Met_f estimated per carrier volume). As a higher O_2 load caused the
373 higher $\text{Met}_f:\text{LNO}_x^- \text{inflow}$ in WWTPB, the higher abundance of methylotrophs could be
374 due to a higher contribution of aerobic methylotrophs and methylotrophs performing
375 aerobic denitrification in WWTPB. Analogous to aerobic methane oxidation coupled
376 with denitrification (AME-D) [43], these methylotrophs could have contributed to the
377 overall denitrification performance by consuming O_2 and by converting methanol to
378 substrates utilizable by non-methylotrophic denitrifiers. However, higher HRT and
379 lower surface load, which act through decreasing the input of bacteria (mostly non-
380 methylotrophic) from the preceding activated sludge stage and through lowering the
381 physical force exerted on the carrier material, might have also favored the growth and
382 development of methylotrophs over non-methylotrophs in WWTPB.

383 Capable of aerobic denitrification, *Paracoccus* tolerates O_2 better than
384 *Hyphomicrobium*, which thrive in anoxic conditions, and thus *Paracoccus* were favored

385 in the surface zones of the biofilm in a previously studied full-scale biofilter (a sand
386 filter) [21]. This is in accordance with our results on the higher and lower relative
387 abundance of *Paracoccus* and *Hyphomicrobium*, respectively, in the sheared biomass of
388 the backwash water (representing more aerobic surface biofilm) than in the carrier
389 material (representing deeper anoxic biofilm) in WWTPB. Similarly, the lower O₂ load
390 (as expressed per carrier volume) could explain the higher abundance of
391 *Hyphomicrobium* and the absence of *Paracoccus* in WWTPA. Since some
392 *Methylothera* strains are aerobic [3, 14] or perform aerobic denitrification [25], the
393 higher abundance of *Methylophilaceae* in the sheared biomass than in the carrier
394 material could also be due to differences in O₂ availability. However, it could also be
395 due to differences in NO_x⁻ and methanol availability, which is expected to be higher in
396 the biofilm surface. The results indicate that Cluster Met I, which was the sole
397 *Methylophilaceae* group in the sheared biomass of WWTPB, was especially favored by
398 the higher availability of O₂, NO_x⁻, and/or methanol. Therefore, the lower O₂, NO_x⁻, and
399 methanol load (as expressed per carrier volume) could both explain the lower
400 abundance of *Methylophilaceae* and the absence of Cluster Met I in WWTPA. However,
401 as discussed below for the temporal variation in the bacterial community in WWTPA,
402 the lower abundance of *Methylophilaceae* and higher abundance of *Hyphomicrobium* in
403 WWTPA could also be due to a lower HRT and higher surface load, which could favor
404 *Hyphomicrobium* over *Methylophilaceae*. In addition, as there are variations in the
405 response of different *Hyphomicrobium* species to varying NO₃⁻ [23], the differential
406 distribution of the two *Hyphomicrobium* species (OTUs) between the sheared biomass
407 and carrier material in WWTPB was probably due to the decreased availability of NO₃⁻
408 deeper in the biofilm. Finally, *Methyloversatilis* and *Paracoccus* gain an ecological
409 advantage by shifting between using C1-carbon and multicarbon substrates [2, 4, 34].

410 Their presence in WWTPB but not in WWTPA might also reflect higher temporal
411 variation in the availability of methanol or higher and temporally more variable
412 availability of other C sources (present in feed water or produced from methanol) in
413 WWTPB.

414 In accordance with the results from the comparison of the biofilters, many
415 possible physicochemical factors might have affected the temporal variation in the
416 bacterial community structure within the WWTPA biofilter. The overall bacterial
417 community structure changed due to variations in the availability of electron acceptors
418 (NO_x^-) and donors (methanol) as well as in temperature, which has also previously been
419 shown to affect denitrifying communities [9, 40]. In addition, changes in the water flow,
420 which act through changing the HRT and surface load, possibly affected the community
421 structure. However, due to the covariation among these factors (Fig. 1) and the
422 relatively small sample size, it is impossible to specify the effects of each variable. In
423 contrast to explaining differences between the biofilters, the availability of O_2 (the O_2
424 concentration and the O_2 flow [$\mu\text{mol s}^{-1}$] [data not shown]) did not affect the temporal
425 variation in the community structure in WWTPA.

426 Assigning taxonomies to the LH-PCR peaks allowed for analysis of the
427 relationship between the physicochemical factors and bacterial communities at the level
428 of major functional and methylotrophic groups. Methylotrophs and non-methylotrophs
429 as well as the key methylotrophic groups, *Methylophilaceae* and *Hyphomicrobium*,
430 responded differently to variations in the physicochemical factors. Since the bulk of
431 methylotrophs consisted of *Methylophilacea* in every sampling occasion, the variation
432 in the relative abundance of methylotrophs tightly followed that of *Methylophilaceae*.

433 The decrease in *Methylophilaceae* (and total methylotrophs) and increase in
434 *Hyphomicrobium* and non-methylotrophs with increasing NO_x^- and methanol loads

435 contrasts with the above comparison between WWTPA and WWTPB. This discrepancy
436 could be due to the dominant *Methylophilaceae* group in WWTPA, *Methylothenera* I,
437 having a slower growth rate and a lesser response to increases in NO_x^- and methanol
438 than the dominant group in WWTPB, Cluster Met I. However, differences in the water
439 flow acting through changes in the HRT and surface load provide a more unifying
440 explanation for the community variations both between the biofilters and within
441 WWTPA. With an increased water flow (lowered HRT and increased surface load), the
442 input of non-methylotrophic bacteria from the preceding activated sludge stage was
443 increased, which could have lowered the relative abundance of *Methylophilaceae* (and
444 total methylotrophs). Furthermore, increased physical disturbance due to increased
445 water flow could have caused the selective removal of *Methylophilaceae*, which would
446 further contribute to the decrease in methylotrophs as well as to the increase in
447 *Hyphomicrobium. Prosthecae* and buds of *Hyphomicrobium* [42] might have provided
448 firmer attachment to the carrier material than the flagellum and ‘prostheca-like’
449 structures of *Methylothenera* [15]. In addition, decreased temperature could have
450 decreased the growth rate of *Methylophilaceae* (and total methylotrophs), which could
451 have also contributed to the observed community variations.

452 Physicochemical factors can control microbial process rates both directly by
453 affecting the short-term cell function and indirectly by affecting the microbial
454 community structure in the longer term [40]. The correlation between the community
455 structure and function (actual NO_x^- reduction rate) in the WWTPA biofilter suggests
456 that physicochemical factors controlled the denitrification rate of the biofilter indirectly
457 by modifying the community composition. However, this study cannot rule out the
458 importance of direct control of physicochemical factors on cell function. The decrease
459 in *Methylophilaceae* and total methylotrophs and increase in *Hyphomicrobium* and non-

460 methylophils with an increasing actual NO_x^- reduction rate is surprising and contrasts
461 with the results from a laboratory reactor in which the relative abundance of
462 *Methylophilaceae* increased and that of *Hyphomicrobium* did not change with
463 increasing denitrification rate [10]. However, this discrepancy is probably due to
464 differing expressions of the process rate, expressed as per biofilter or per volume of
465 carrier material in our study and as per mass of biomass (mixed liquor volatile
466 suspended solids [MLVSS]) in Ginige et al. [10]. Unfortunately, MLVSS was not
467 analyzed in this study. However, the higher actual NO_x^- reduction rate with an
468 increasing relative abundance of non-methylophils suggests that non-methylophils
469 can efficiently support the N removal of methanol-fed denitrification systems,
470 especially during periods of high N load. In those conditions, methylophils might have
471 increasingly allocated more of the methanol C into extracellular substances than into
472 biomass and thus supported the activity of non-methylophils.

473

474 **Conclusions**

475 Combining the results of the two WWTP biofilters with those of previous studies
476 confirms that bacteria in genus *Hyphomicrobium* and family *Methylophilaceae* are
477 crucial components of methanol-utilizing denitrification. Thus, *Hyphomicrobium* and
478 *Methylophilaceae* can be used as target taxonomic groups to monitor the function of
479 full-scale methanol-fed denitrification biofilters of WWTPs. Although *Methylophils*
480 was the major *Methylophilaceae* genus in the studied WWTP biofilters, other genera
481 (*Methylophilus* and *Methylobacillus*) may be more important in other systems. There
482 were differences in the bacterial communities between the biofilters. In addition, 10-
483 week monitoring of one of the biofilters showed temporal variation in the bacterial
484 community. Variation in the loads of NO_x^- and O_2 as well as in the methanol addition

485 rate, water flow rate (acting through changing HRT and surface load), and temperature
486 were all potential candidates affecting the structure of the bacterial communities.
487 Methylotrophs and non-methylotrophs as well as *Hyphomicrobium* and
488 *Methylophilaceae* responded differently to these variations. Furthermore, the correlation
489 of the bacterial community structure with the process function (actual NO_x^- reduction
490 rate) in the temporally monitored biofilter indicates that fluctuating physicochemical
491 conditions affected the denitrification rate indirectly by affecting the community
492 composition. Further temporal monitoring and/or experimental studies combined with
493 modern sophisticated culture-independent (stable isotope probing of DNA/RNA,
494 metatranscriptomics, metagenomics) as well as culture-dependent (high-throughput
495 culturing) techniques are needed to resolve the exact mechanisms underlying the
496 observed relationship among the physicochemical factors, bacterial communities
497 (methylotrophs, non-methylotrophs, *Hyphomicrobium*, and *Methylophilaceae*), and
498 process function.

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504 **Conflict of interest** The authors declare that they have no conflict of interest.

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References

1. Bamforth CW, Quayle JR (1978) Aerobic and anaerobic growth of *Paracoccus denitrificans* on methanol. Arch Microbiol 119:91-97
2. Baytshtok V, Lu H, Park H, Kim S, Yu R, Khandran K (2009) Impact of varying electron donors on the molecular microbial ecology and biokinetics of methylotrophic denitrifying bacteria. Biotechnol Bioeng 102:1527-1536
3. Beck DAC, McTaggart TL, Setboonsarng U, Vorobev A, Kalyuzhnaya MG, Ivanova N, Goodwin L, Woyke T, Lidstrom ME, Chistoserdova L (2014) The expanded diversity of *Methylophilaceae* from Lake Washington through cultivation and genomic sequencing of novel ecotypes. PLoS ONE 9:e102458
4. Blaszczyk M (1993) Effect of medium composition on the denitrification of nitrate by *Paracoccus denitrificans*. Appl Environ Microbiol 59:3951-3953
5. Chistoserdova L, Kalyuzhnaya MG, Lidstrom ME (2009) The expanding world of methylotrophic organisms. Annu Rev Microbiol 63:477-499
6. Claus G, Kutzner HJ (1985) Denitrification of nitrate and nitric acid with methanol as carbon source. Appl Microbiol Biotechnol 22:378-381
7. Corona F, Mulas M, Haimi H, Sundell L, Heinonen M, Vahala R (2013) Monitoring nitrate concentrations in the denitrifying post-filtration unit of a municipal wastewater treatment plant. J Process Contr 23:158-170
8. Gentile ME, Nyman JL, Criddle CS (2007) Correlation of patterns of denitrification instability in replicated bioreactor communities with shifts in the relative abundance and the denitrification patterns of specific populations. ISME J. 1:714-728
9. Gentile M, Yan T, Tiquia SM, Fields MW, Nyman J, Zhou J, Criddle CS (2006) Stability in a denitrifying fluidized bed reactor. Microb Ecol 52:311-321
10. Ginige MP, Hugenholtz P, Daims H, Wagner M, Keller J, Blackall LL (2004) Use of stable isotope-probing, full-cycle rRNA analysis, and fluorescence in situ hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. Appl Environ Microbiol 70:588-596
11. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron 4(1):9pp
12. Isazadeh S, Ozcer PO, Frigon D (2014) Microbial community structure of wastewater treatment subjected to high mortality rate due to ozonation of return activated sludge. J Appl Microbiol 117:587-596
13. Johnson JL (1994) Similarity analysis of DNAs. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds.) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC, pp 655–682
14. Kalyuzhnaya MG, Beck DA, Vorobev A, Smalley N, Kunkel DD, Lidstrom ME, Chistoserdova L (2012) Novel methylotrophic isolates from lake sediment, description of *Methylotenera versatilis* sp. nov. and emended description of the genus *Methylotenera*. Int J Syst Evol Micr 62:106-111
15. Kalyuzhnaya MG, Boverman S, Lara JC, Lidstrom ME, Chistoserdova L (2006) *Methylotenera mobilis* gen. nov., sp. nov., an obligately methylamine-utilizing bacterium within the family *Methylophilaceae*. Int J Syst Evol Micr 56:2819-2823
16. Kalyuzhnaya MG, Martens-Habbena W, Wang T, Hackett M, Stolyar SM, Stahl DA, Lidstrom ME, Chistoserdova L (2009) *Methylophilaceae* link methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis. Environ Microbiol Reports 1:385-392

- 554 17. Koch G, Siegrist H (1997) Denitrification with methanol in tertiary filtration.
555 Water Res 31:3029-3038
- 556 18. Kolb S (2009) Aerobic methanol-oxidizing *Bacteria* in soil. FEMS Microbiol Lett
557 300:1-10
- 558 19. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M
559 (eds.) Nucleic acid techniques in bacterial systematics. Wiley, New York, pp 115–
560 175
- 561 20. Lemmer H, Zaglauer A, Metzner G (1997) Denitrification in a methanol-fed
562 fixed-bed reactor. Part 1: Physico-chemical and biological characterization. Water
563 Res 31:1897-1902
- 564 21. Lemmer H, Zaglauer A, Neef A, Meier H, Amann R (1997) Denitrification in a
565 methanol-fed fixed-bed reactor. Part 2: Composition and ecology of the bacterial
566 community in the biofilms. Water Res 31:1903-1908
- 567 22. Lu H, Chandran K, Stensel D (2014) Microbial ecology of denitrification in
568 biological wastewater treatment. Water Res 64:237-254
- 569 23. Martineau C, Mauffrey F, Villemur R (2015) Comparative analysis of denitrifying
570 activities of *Hyphomicrobium nitratorans*, *Hyphomicrobium denitrificans* and
571 *Hyphomicrobium zavarzinii*. Appl Environ Microbiol 81:5003-5014
- 572 24. McCune B, Mefford MJ (2011) PC-ORD. Multivariate Analysis of Ecological
573 Data. Version 6. MjM Software, Gleneden Beach, Oregon, USA
- 574 25. Mustakhimov I, Kalyuzhnaya MG, Lidstrom ME, Chistoserdova L (2013)
575 Insights into denitrification in *Methylothermobacter mobilis* from denitrification
576 pathway and methanol metabolism mutants. J Bacteriol 195:2207-2211
- 577 26. Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial
578 populations by denaturing gel electrophoresis of polymerase chain reaction
579 amplified genes coding for 16S ribosomal RNA. Appl Environ Microbiol 59:695–
580 700
- 581 27. Neef A, Zaglauer A, Meier H, Amann R, Lemmer H, Schleifer K-H (1996)
582 Population analysis in a denitrifying sand filter: Conventional and in situ
583 identification of *Paracoccus* spp. in methanol-fed biofilms. Appl Environ
584 Microbiol 62:4329-4339
- 585 28. Nurse GR (1980) Denitrification with methanol: microbiology and biochemistry.
586 Water Res 14:531-537
- 587 29. Osaka T, Yoshie S, Tsuneda S, Hirata A, Iwami N, Inamori Y (2006)
588 Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing
589 conditions by stable-isotope probing. Microb Ecol 52:253-266
- 590 30. Rainey FA, Ward-Rainey N, Gliesche CG, Stackebrandt E (1998) Phylogenetic
591 analysis and intrageneric structure of the genus and the related genus
592 *Filomicrobium*. Int J Syst Bacteriol 48:635-639
- 593 31. Rissanen AJ, Kurhela E, Aho T, Oittinen T, Tiirola M (2010) Storage of
594 environmental samples for guaranteeing nucleic acid yields for molecular
595 microbiological studies. Appl Microbiol Biotechnol 88:977-984
- 596 32. Rissanen AJ, Ojala A, Dernjatin M, Jaakkola J, Tiirola M (2016) *Methylophaga*
597 and *Hyphomicrobium* can be used as target genera in monitoring saline water
598 methanol-utilizing denitrification. J Ind Microbiol Biot. doi:10.1007/s10295-016-
599 1839-2
- 600 33. Saunders AM, Albertsen M, Vollertsen J, Nielsen PH (2016) The activated sludge
601 ecosystem contains a core community of abundant organisms. ISME J 10:11-20
- 602 34. Smalley NE, Taipale S, De Marco P, Doronina NV, Kyrpides N, Shapiro N,
603 Woyke T, Kalyuzhnaya MG (2015) Functional and genomic diversity of

604 methylotrophic *Rhodocyclaceae*: description of *Methyloversatilis discipulorum*
605 sp. nov. Int J Syst Evol Microbiol 65:2227-2233
606 35. Sperl GT, Hoare DS (1971) Denitrification with methanol: a selective enrichment
607 for *Hyphomicrobium* species. J Bacteriol 108:733-736
608 36. Srinandan CS, D'souza G, Srivastava N, Nayak BB, Nerurkar AS (2012) Carbon
609 sources influence the nitrate removal activity, community structure and biofilm
610 architecture. Bioresour Technol 117:292-299
611 37. Suzuki M, Rappe MS, Giovannoni SJ (1998) Kinetic bias in estimates of coastal
612 picoplankton community structure obtained by measurements of small-subunit
613 rRNA gene PCR amplicon length heterogeneity. Appl Environ Microbiol
614 64:4522-4529
615 38. Timmermans P, Van Heute A (1983) Denitrification with methanol: Fundamental
616 study of the growth and denitrification capacity of *Hyphomicrobium* sp. Water
617 Res 17:1249-1255
618 39. Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, Daims H (2002) Microbial
619 community composition and function in wastewater treatment plants. Antonie van
620 Leeuwenhoek 81:665-680
621 40. Wallenstein MD, Myrold DD, Firestone M, Voytek M (2006) Environmental
622 controls on denitrifying communities and denitrification rates: insights from
623 molecular methods. Ecol Appl 16:2143-2152
624 41. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA
625 amplification for phylogenetic study. J Bacteriol 173:697-703
626 42. Wu X-L, Yu S-L, Gu J, Zhao G-F, Chi C-Q (2009) *Filomicrobium insigne* sp.
627 nov., isolated from an oil-polluted saline soil. Int J Syst Evol Micr 59:300-305
628 43. Zhu J, Wang Q, Yuan M, Tan GYA, Sun F, Wang C, Wu W, Lee PH (2016)
629 Microbiology and potential applications of aerobic methane oxidation coupled to
630 denitrification (AME-D) process: a review. Water Res 90:203-215
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641 **Figure captions:**

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644 **Fig. 1** NO_x^- reduction, operating conditions, and microbial community dynamics in the
645 denitrification filter of WWTPA (the 10-week follow-up period of microbial communities [27
646 August 2008 – 28 October 2008] is framed). (a) Temperature and the concentration of NO_x^- and O_2
647 in the inflow, concentration of NO_x^- in the outflow, and the relative NO_x^- reduction. (b) NO_x^- load
648 in the inflow and outflow, actual NO_x^- reduction rate, water flow, methanol addition rate, and
649 methanol: NO_x^- ratio in the inflow. (c) Results of non-metric multidimensional scaling analysis of
650 LH-PCR peak abundance data (1. axis shown, explaining 90 % of the variability in community
651 structure) and relative abundance of methylotrophs, *Hyphomicrobium* (peak 466 bp) and
652 *Methylophilaceae* (peak 521 bp), as well as their sum as a biomarker of methylotrophs and the
653 relative abundance of non-methylotrophs (sum of all peaks except 466 bp, 521 bp and 524 bp)
654 based on the LH-PCR peak data

655

656 **Fig. 2** Phylogenetic tree (neighbor joining method) of the 16S rRNA gene clone libraries of the
657 *Hyphomicrobiaceae* assigned operational taxonomic units (OTUs) (at 97 % sequence similarity) in
658 the studied denitrification filters. *Hyphomicrobium* clusters were previously defined by Rainey et
659 al. [30]. The numbers in brackets after the OTU number indicate the number of sequences within
660 that OTU. The numbers at the nodes indicate the percentages of occurrence in 1000 bootstrapped
661 trees (bootstrap values > 50% are shown)

662

663 **Fig. 3** Phylogenetic tree (neighbor joining method) of the 16S rRNA gene clone libraries of the
664 *Methylophilaceae* assigned OTUs. *Methylophilaceae* clusters were defined in this study (see tree
665 details in the legend of Fig. 2)

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668 **Fig. 4** Correlation between the relative abundance of the peaks assigned to (a) *Hyphomicrobium*
669 (peak 466 bp) and *Methylophilaceae* (peak 521 bp) and (b) methylotrophs (sum of 466 bp and 521
670 bp) and non-methylotrophs (sum of all peaks except 466bp, 521bp, and 524 bp) in the length
671 heterogeneity-PCR (LH-PCR) analysis of WWTPA samples during the 10-week monitoring
672 period. Physicochemical and process variables correlating ($p < 0.05$) with the relative abundance
673 of both groups in either (a) or (b); the sign of the correlations are shown with black-colored text
674 and dashed-line arrow, whereas those correlating only with one of the groups are shown as gray-
675 colored text and dashed-line arrow

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677 Online Resource figure captions

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679 **Online Resource 1** Operating conditions and functional performance in the denitrification
680 biofilter of WWTPB. (a) Temperature and concentration of NO_x^- and O_2 in the inflow,
681 concentration of NO_x^- in the outflow, and the relative NO_x^- reduction. (b) NO_x^- load in the inflow
682 and outflow, actual NO_x^- reduction rate, water flow, methanol addition rate, and the methanol: NO_x^-
683 ratio in the inflow. The date of sampling for microbial studies (2 October 2008) is indicated with
684 an arrow

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686 **Online Resource 2** Hierarchical clustering analysis (UPGMA) of the relative abundance of peaks
687 in the length heterogeneity-PCR (LH-PCR) analysis of the 16S rRNA genes of the inflow water
688 and backwash water of the denitrification biofilters of WWTPA and WWTPB and the carrier
689 material from WWTPB

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691 **Online Resource 3** Electropherograms of the length heterogeneity PCR (LH-PCR) analysis of the
692 16S rRNA genes in samples of the denitrification biofilters of WWTPA and WWTPB (backwash
693 water from both systems and carrier material from WWTPB) and the inflow water (feed water).
694 The peaks assigned to *Hyphomicrobium* and *Methylophilaceae* at WWTPA are marked by arrows

695 **Table 1** Characteristics of the municipal wastewater treatment plants (WWTPA and WWTPB) and
 696 the studied methanol-fed denitrification biofilters
 697

	WWTPA	WWTPB
Type/N removal	Biol.chem./pre- & postdenitr.	Biol.chem/postdenitr.
Population equivalent	740000	31000
Aver. flow rate (m ³ /d)	280000	14000
Annual aver. N-reduction (%) ^a	90	75
Annual T range (°C)	9 - 18	2 - 20
Number of denitr. filter cells	10	6
Bed volume (m ³ /filter cell)	432	56
Carrier material in filter cells	Polystyrene beads	Polystyrene beads
Aver. NO _x ⁻ red. (mol/m ³ /d) ^b	44	63
Aver. NO _x ⁻ red. (%) ^b	89	81
Aver. surface load (m/h) ^b	8.1	3.4
Aver. hydraulic retent. time (h) ^b	0.4	0.6

698 ^a Annual average relative N-reduction for the whole treatment process in WWTPs
 699 ^b Average NO_x⁻ reduction expressed per carrier material volume, average relative NO_x⁻
 700 reduction, average surface load, and average hydraulic retention time in the studied filter
 701 cell in WWTPA (study period 20 August 2008 – 31 October 2008) and in the whole
 702 biofilter system in WWTPB (study period 1 September 2008 – 31 October 2008)
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720 **Table 2** Bacterial community composition (% of the 16S rRNA gene sequences) in the
 721 denitrifying biofilters of municipal wastewater treatment plants (WWTPA and WWTPB) based on
 722 clone library and 454-pyrosequencing analyses of the sheared biomass in backwash water and the
 723 biomass on carrier material (only in WWTPB). Putative methylotrophic taxa are marked with
 724 Meth

		WWTPA	WWTPA (454) ^a	WWTPB	WWTPB_Car (carrier mat.)
Number of sequences:		45	3643	58	48
Frequency (%): ^b					
Total methylotrophs		33	28	74	38
<i>Alphaproteobacteria</i>		7	3	5	15
<i>Hyphomicrobiaceae</i>		7	2	2	10
<i>Hyphomicrobium</i> II ^c	Meth	7	2	2	10
<i>Methylocystaceae</i>	Meth	-	<0.1	-	-
<i>Rhodobacteraceae</i>		-	<0.2	3	-
<i>Paracoccus</i>	Meth	-	-	3	-
<i>Bradyrhizobiaceae</i>		-	<0.1	-	2
<i>Bradyrhizobium</i>	Meth	-	-	-	2
<i>Betaproteobacteria</i>		47	41	74	33
<i>Methylophilaceae</i>	Meth	26	25	66	26
Cluster Met I ^d	Meth	-	-	66	10
<i>Methylotenera</i> I ^d	Meth	22	25	-	6
<i>Methylotenera</i> II ^d	Meth	2	-	-	8
unclassified ^d	Meth	2	-	-	2
<i>Rhodocyclaceae</i>		9	4	3	2
<i>Methyloversatilis</i>	Meth	-	-	3	-
<i>Comamonadaceae</i>		9	4	2	2
<i>Deltaproteobacteria</i>		9	10	3	-
<i>Epsilonproteobacteria</i>		-	<0.5	-	-
<i>Gammaproteobacteria</i>		-	3	2	2
<i>Moraxellaceae</i>		-	1	-	-
<i>Acinetobacter</i>	Meth	-	<0.1	-	-
<i>Methylococcaceae</i>	Meth	-	<0.2	-	-
<i>Acidobacteria</i>		-	1	-	15
<i>Actinobacteria</i>		-	1	2	8
<i>Bacteroidetes</i>		9	8	7	6
<i>Flavobacteriaceae</i>		-	1	-	-
<i>Flavobacterium</i>	Meth	-	1	-	-
<i>Chloroflexi</i>		11	2	2	-
<i>Deinococcus-Thermus</i>		2	<0.1	-	4
<i>Nitrospirae</i>		-	-	-	4
<i>Planctomycetes</i>		-	<0.5	-	8
unclassified bacteria+others		15	30	5	5

725 ^a Library generated using 454 – pyrosequencing

726 ^b Classification was made using RDP database in Mothur and by phylogenetic tree
 727 analysis (Figs. 2 & 3). Assignment to methylotrophic function was based on previous
 728 literature. Frequencies are given as percentages (%) of total number of sequences in a
 729 sample.

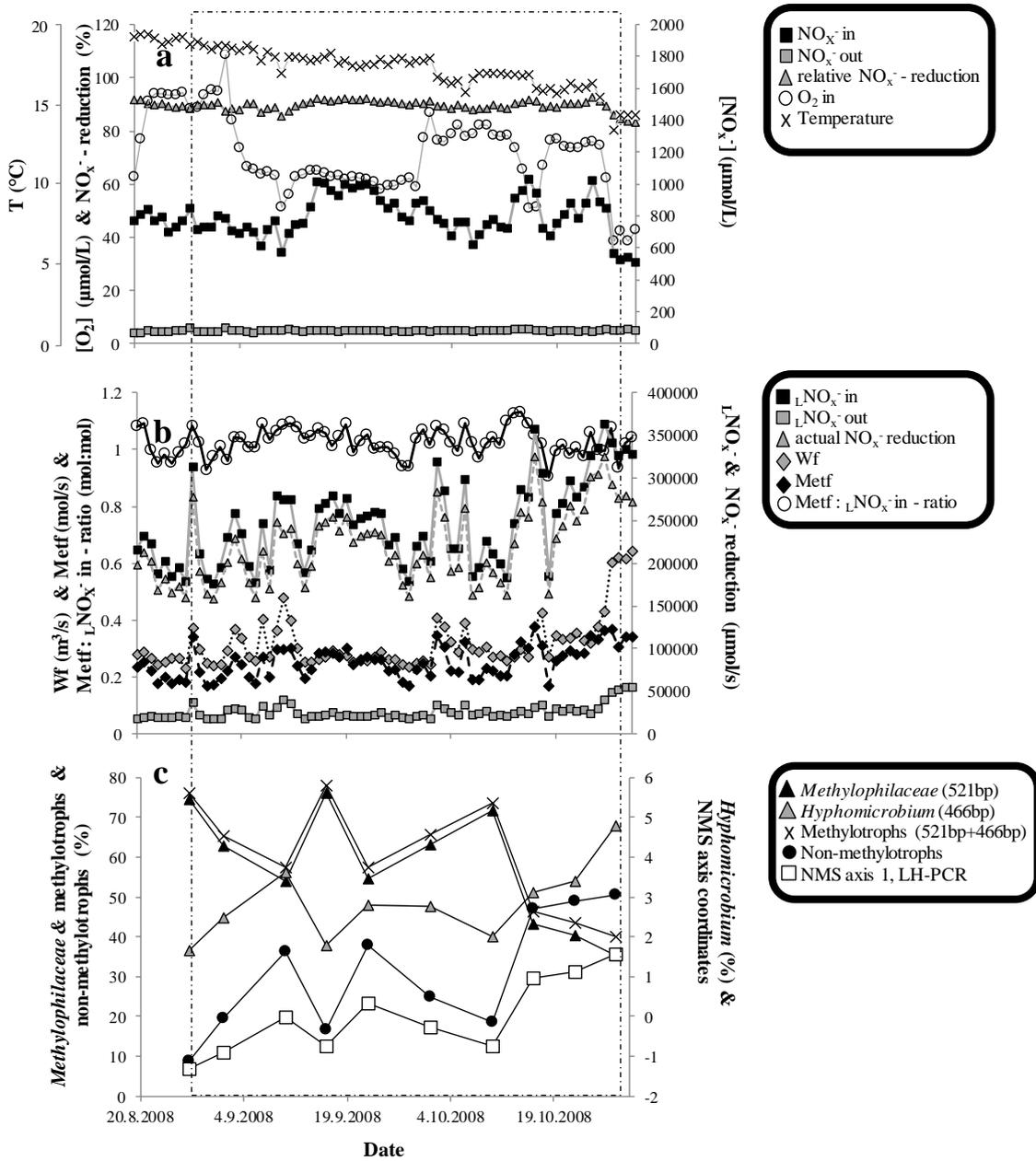
730 ^c Clustering (clusters I and II) of *Hyphomicrobium* according to Rainey et al. [30]. See
 731 also Fig. 2.

732 ^d Clustering based on Fig. 3.

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735 Figure 1
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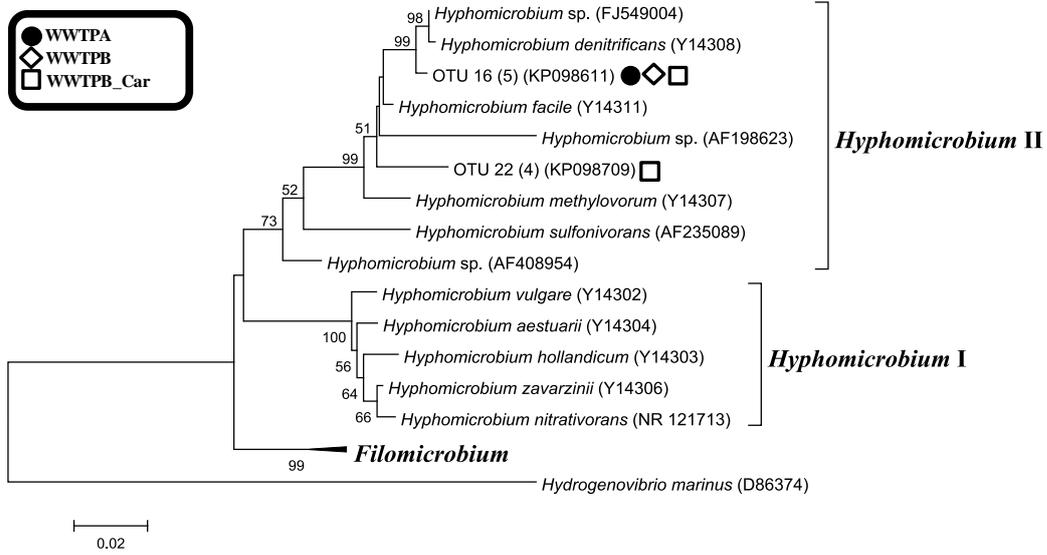
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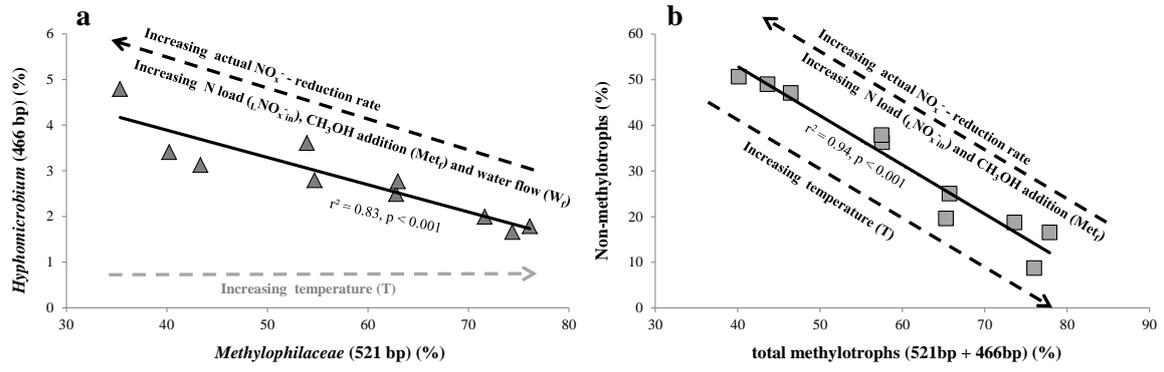
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744 Figure 2
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783 Figure 4
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806 Online Resources (1-3)

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808 Journal: J Ind Microbiol Biotechnol

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810 Title: *Methylophilaceae* and *Hyphomicrobium* as target taxonomic groups in monitoring
811 the function of methanol-fed denitrification biofilters in municipal wastewater treatment

812 plants

813

814 Authors: Antti J. Rissanen^{1, 2, *}, Anne Ojala, Tommi Fred, Jyrki Toivonen & Marja Tiirola

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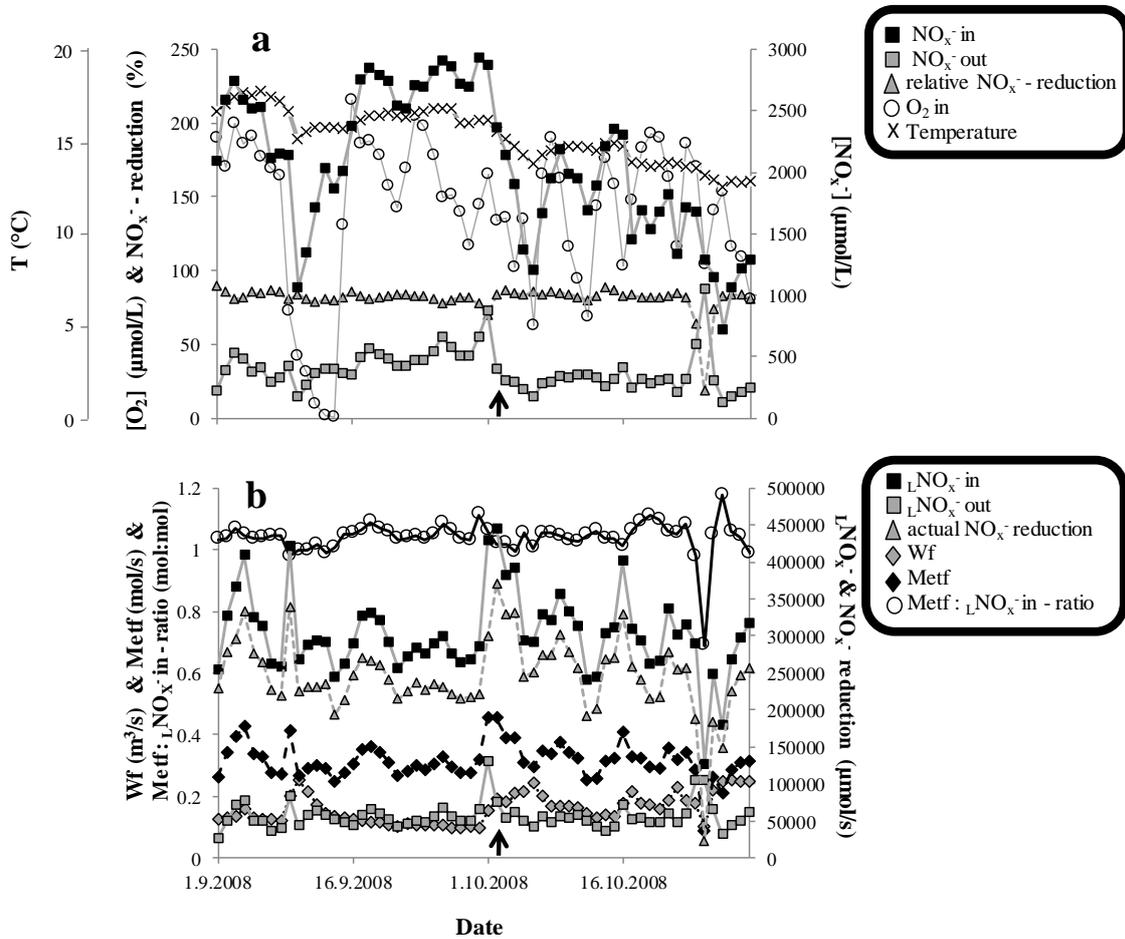
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845 **Online Resource 1** Operating conditions and functional performance in the denitrification
 846 biofilter of WWTPB. (a) Temperature and concentration of NO_x⁻ and O₂ in the inflow,
 847 concentration of NO_x⁻ in the outflow, and the relative NO_x⁻ reduction. (b) NO_x⁻ load in the inflow
 848 and outflow, actual NO_x⁻ reduction rate, water flow, methanol addition rate, and the methanol:NO_x⁻
 849 ratio in the inflow. The date of sampling for microbial studies (2 October 2008) is indicated with
 850 an arrow

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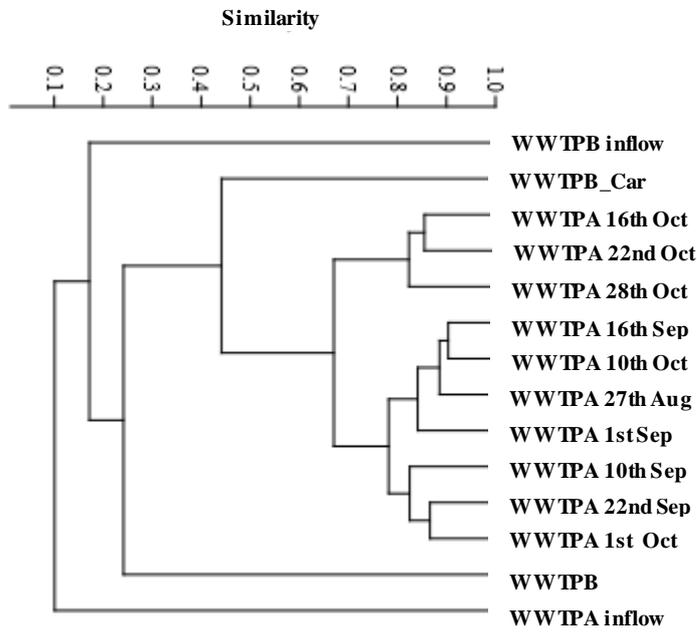
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856 Online Resource 2

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859 **Online Resource 2** Hierarchical clustering analysis (UPGMA) of the relative abundance of peaks
860 in the length heterogeneity-PCR (LH-PCR) analysis of the 16S rRNA genes of the inflow water
861 and backwash water of the denitrification biofilters of WWTPA and WWTPB and the carrier
862 material from WWTPB

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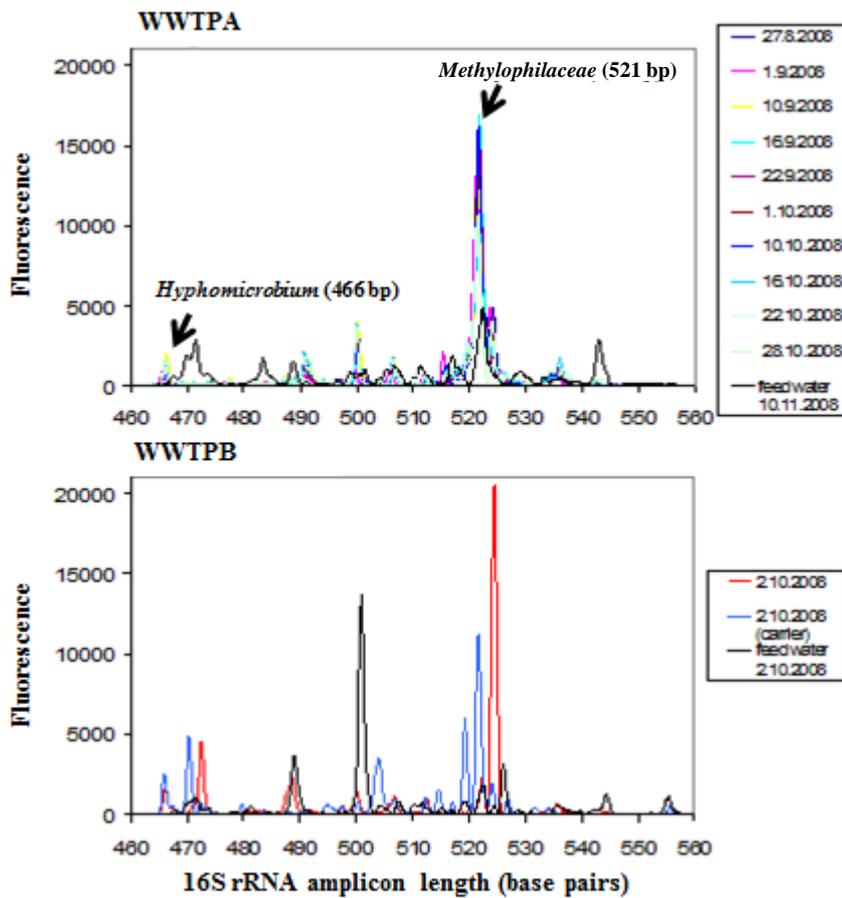
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872 Online Resource 3

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875 **Online Resource 3** Electropherograms of the length heterogeneity PCR (LH-PCR) analysis of the
876 16S rRNA genes in samples of the denitrification biofilters of WWTPA and WWTPB (backwash
877 water from both systems and carrier material from WWTPB) and the inflow water (feed water).
878 The peaks assigned to *Hyphomicrobium* and *Methylophilaceae* at WWTPA are marked by arrows