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

Antti J. Rissanen[1, 2,] *, Anne Ojala[3,4], Tommi Fred[5], Jyrki Toivonen[6] & Marja Tiirola[2]

[1]Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101, Tampere, Finland
[2]Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FI-40014, University of Jyväskylä, Finland
[3]Department of Environmental Sciences, University of Helsinki, P.O. Box 65, FI-00014, University of Helsinki, Finland
[4]Department of Forest Sciences, University of Helsinki, P.O. Box 27, FI-00014, University of Helsinki, Finland
[5]Helsinki Region Environmental Services Authority HSY, P.O. Box 100, FI-00066 HSY, Finland
[6]Salon Vesi, P.O. Box 77, FI-24101 Salo, Finland

*Corresponding author.
email address: antti.rissanen@tut.fi
tel. : +358 40 1981145
fax.: +358 3 3641392

Acknowledgements

Abstract

Molecular monitoring of bacterial communities can explain and predict the stability of

bioprocesses in varying physicochemical conditions. To study methanol-fed

denitrification biofilters of municipal wastewater treatment plants, bacterial

communities of two full-scale biofilters were compared through fingerprinting and

sequencing of the 16S rRNA genes. Additionally, 16S rRNA gene fingerprinting was

used for 10-week temporal monitoring of the bacterial community in one of the

biofilters. Combining the data with previous study results, the family *Methylophilaceae*

and genus *Hyphomicrobium* were determined as suitable target groups for monitoring.

An increase in the relative abundance of *Hyphomicrobium*-related biomarkers occurred

simultaneously with increases in water flow, NO_x^- load, and methanol addition, as well

as a higher denitrification rate, although the dominating biomarkers linked to

Methylophilaceae showed an opposite pattern. The results indicate that during increased

loading, stability of the bioprocess is maintained by selection of more efficient

denitrifier populations, and this progress can be analyzed using simple molecular

fingerprinting.

Introduction

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

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

within family *Methylophilaceae* (*Betaproteobacteria*) [10, 29, 33, 36] as well as within

genera *Methyloversatilis* (*Betaproteobacteria*) [2] and *Paracoccus*

(*Alphaproteobacteria*) [6, 21, 27] can also play a significant role in the process.

However, most studies have been done at laboratory scale. Other than the studies of

Neef et al. [27] and Lemmer et al. [21], which found *Paracoccus* and *Hyphomicrobium*

to be important methylotrophs in a methanol-fed denitrifying sand filter of a WWTP,

very little is known about the overall bacterial dynamics or about the identity and

community dynamics of methylotrophic denitrifiers in full-scale biofilters. There are

ecological differences between methylotrophs and non-methylotrophs [21]. In addition,

the ecology of *Hyphomicrobium* differs from that of *Methyloversatilis* [2], *Paracoccus*

[21], and *Methylophilaceae* [10]. This indicates that methylotrophs and non-

methylotrophs as well as different taxonomic groups of methylotrophs respond

differently to the temporal and inter-system variations in the physicochemical

conditions confronted by the full-scale biofilters.

This study investigated the bacterial communities of two full-scale methanol-fed

denitrifying WWTP biofilters by length heterogeneity PCR (LH-PCR) [37] and clone

library and 454-pyrosequencing analysis of the 16S rRNA gene sequences. We

specifically focused on the taxonomic groups of the methylotrophic bacteria that

inhabited both of the biofilters as well as previously studied systems. In addition to

comparing the bacterial communities of the two biofilters, we analyzed the temporal

variation in the structure of the bacterial communities and linked it with the

physicochemical and functional data during a 10-week follow-up period in one of the

biofilters. We aimed to determine the following: 1) which methylotrophic taxonomic

groups are typical for methanol-fed denitrification systems and could thus be used as

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.

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 ℃

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 O_2

114   (O_{2in}), suspended solids (SS_{in}), PO_4^{3-} ($PO_{4\ in}^{3-}$), total phosphorous (TP_{in}), and outflow

115   concentrations of SS (SS_{out}), PO_4^{3-} ($PO_{4\ out}^{3-}$), 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, $NO_{x\ in}^-$, $PO_{4\ in}^{3-}$, $SS_{in,}$

119   and O_{2in} and $NO_{x\ out}^-$, $PO_{4\ out}^{3-}$, and SS_{out} were calculated for the whole denitrification

120   system. The NO_x^- load ($\mu mol\ s^{-1}$) in the inflow ($_L NO_{x\ in}^-$) and outflow ($_L NO_{x\ out}^-$) 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 ($\mu mol\ s^{-1}$) NO_x^- reduction as follows:

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

$$124 \qquad Actual\ NO_x^-\ reduction = {}_L NO_{x\ in}^- - {}_L NO_{x\ out}^-$$

125   Denitrification in this study refers to the conversion of water soluble NO_x^- into gaseous

126   forms, but the proportions of NO, N_2O, and N_2 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 95ºC for 5 min,

139 30 cycles of amplification (94ºC for 30 s, 53ºC for 1 min, 72ºC for 3 min), and final

140 elongation at 72ºC 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

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

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

8

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 µmol/L; O_{2in}: 1 to 215 µmol/L) than in WWTPA ($NO_x^-{}_{in}$: 500 – 1000 µmol/L;

198 O_{2in}: 40 - 110 µmol/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 $NO_x^-{}_{in}$ 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-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 (µmol/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

9

209   temporally more stable, and $Met_{f:L}NO_x^-_{inflow}$ was lower in WWTPA ($Met_{f:L}NO_x^-_{inflow}$

210   ratio: 0.90–1.13; relative NO_x^- reduction: 82–93 %; $NO_x^-_{out}$: 66–99 µmol/L) than in

211   WWTPB ($Met_{f:L}NO_x^-_{inflow}$ ratio: 0.98–1.18; relative NO_x^- reduction: 64–90 %; $NO_x^-_{out}$:

212   128–870 µmol/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 µmol/m^3/s; O_2: 50 µmol/m^3/s; methanol: 590 µmol/m^3/s; actual

216   NO_x^- reduction: 510 µmol/m^3/s) than in WWTPB (NO_x^-: 890 µmol/m^3/s; O_2: 60

217   µmol/m^3/s; methanol: 930 µmol/m^3/s; actual NO_x^- reduction: 730 µmol/m^3/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 $Met_{f:L}NO_x^-_{inflow}$

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 *Hyphomicrobiacea* and

244   *Methylophilacea*, 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, *Methylotenera* I,

256   and *Methylotenera* II (clustering according to this study), included 8 OTUs covering the

257   majority of the observed *Methylophilaceae* sequences (Table 2, Fig. 3). *Methylotenera* I

258   and *Methylotenera* II were closely related to the cultured members of the genus

259   *Methylotenera* (Fig. 3), while the Met I cluster probably represented a novel species of

260 *Methylotenera* 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 *Methylophilacea* in the carrier material of WWTPB, whereas it was absent in WWTPA

266 (Table 2, Fig. 3). In contrast, clusters *Methylotenera* 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). *Methylotenera* I was much more abundant than *Methylotenera* II in WWTPA,

269 but it was only slightly less abundant than *Methylotenera* 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-pyrosequncing library, was identical to *Methylotenera* OTU 6

273 (within cluster *Methylotenera* I) in the clone library analyses (Fig. 3). Furthermore, 454-

274 pyrosequencing of 16S rRNA gene amplicons revealed a marginal abundance ($\leq$ 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 ($\geq$ 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), $_LNO_x^-{}_{in}$ (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 *Methylotenera* 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 $Met_f{:}_LNO_x^-$

372    $_{inflow}$ and higher Met_f estimated per carrier volume). As a higher O_2 load caused the

373   higher $Met_f{:}_LNO_x^-{}_{inflow}$ 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 O_2 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 *Methylotenera* 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 O_2 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 O_2, NO_x^-, and/or methanol. Therefore, the lower O_2, NO_x^-, and

399 methanol load (as expressed per carrier volume) could both explain the lower

400 abundance of *Methylophilacea* 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 *Methylophilacea* 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 *Methylophilacea*. In addition, as there are variations in the

405 response of different *Hyphomicrobium* species to varying NO_3^- [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 NO_3^-

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 [μmol 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, *Methylotenera* 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 *Methylotenera* [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-

19

460   methylotrophs 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-methylotrophs suggests that non-methylotrophs

469   can efficiently support the N removal of methanol-fed denitrification systems,

470   especially during periods of high N load. In those conditions, methylotrophs might have

471   increasingly allocated more of the methanol C into extracellular substances than into

472   biomass and thus supported the activity of non-methylotrophs.

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 *Methylotenera*

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.

499

500

501

502
503

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

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 nitrativorans*, *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 *Methylotenera 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 denaturating 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 filer: 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

methylotrophic *Rhodocyclaceae*: description of *Methyloversatilis discipulorum* sp. nov. Int J Syst Evol Microbiol 65:2227-2233

35. Sperl GT, Hoare DS (1971) Denitrification with methanol: a selective enrichment for *Hyphomicrobium* species. J Bacteriol 108:733-736

36. Srinandan CS, D´souza G, Srivastava N, Nayak BB, Nerurkar AS (2012) Carbon sources influence the nitrate removal activity, community structure and biofilm architecture. Bioresour Technol 117:292-299

37. Suzuki M, Rappe MS, Giovannoni SJ (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Appl Environ Microbiol 64:4522-4529

38. Timmermans P, Van Heute A (1983) Denitrification with methanol: Fundamental study of the growth and denitrification capacity of *Hyphomicrobium* sp. Water Res 17:1249-1255

39. Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, Daims H (2002) Microbial community composition and function in wastewater treatment plants. Antonie van Leeuwenhoek 81:665–680

40. Wallenstein MD, Myrold DD, Firestone M, Voytek M (2006) Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecol Appl 16:2143-2152

41. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697-703

42. Wu X-L, Yu S-L, Gu J, Zhao G-F, Chi C-Q (2009) *Filomicrobium insigne* sp. nov., isolated from an oil-polluted saline soil. Int J Syst Evol Micr 59:300-305

43. Zhu J, Wang Q, Yuan M, Tan GYA, Sun F, Wang C, Wu W, Lee PH (2016) Microbiology and potential applications of aerobic methane oxidation coupled to denitrification (AME-D) process: a review. Water Res 90:203-215

Figure captions:

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 the16S 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 the16S 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)

666

667

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

676

677 Online Resource figure captions

678

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

685

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

690

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)
703

704

705

706

707

708

709

710

711

712

713

714

715

716

717

718

719

Table 2 Bacterial community composition (% of the 16S rRNA gene sequences) in the
denitrifying biofilters of municipal wastewater treatment plants (WWTPA and WWTPB) based on
clone library and 454-pyrosequencing analyses of the sheared biomass in backwash water and the
biomass on carrier material (only in WWTPB). Putative methylotrophic taxa are marked with
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**
Alphaproteobacteria		7	3	5	15
Hyphomicrobiaceae		7	2	2	10
Hyphomicrobium II[c]	Meth	7	2	2	10
Methylocystaceae	Meth	-	<0.1	-	-
Rhodobacteraceae		-	<0.2	3	-
Paracoccus	Meth	-	-	3	-
Bradyrhizobiaceae		-	<0.1	-	2
Bradyrhizobium	Meth	-	-	-	2
Betaproteobacteria		47	41	74	33
Methylophilaceae	Meth	26	25	66	26
Cluster Met I[d]	Meth	-	-	66	10
Methylotenera I[d]	Meth	22	25	-	6
Methylotenera II[d]	Meth	2	-	-	8
unclassified[d]	Meth	2	-	-	2
Rhodocyclaceae		9	4	3	2
Methyloversatilis	Meth	-	-	3	-
Comamonadaceae		9	4	2	2
Deltaproteobacteria		9	10	3	-
Epsilonproteobacteria		-	<0.5	-	-
Gammaproteobacteria		-	3	2	2
Moraxellaceae		-	1	-	-
Acinetobacter	Meth	-	<0.1	-	-
Methylococcaceae	Meth	-	<0.2	-	-
Acidobacteria		-	1	-	15
Actinobacteria		-	1	2	8
Bacteroidetes		9	8	7	6
Flavobacteriaceae		-	1	-	-
Flavobacterium	Meth	-	1	-	-
Chloroflexi		11	2	2	-
Deinococcus-Thermus		2	<0.1	-	4
Nitrospirae		-	-	-	4
Planctomycetes		-	<0.5	-	8
unclassified bacteria+others		15	30	5	5

[a] Library generated using 454 – pyrosequencing

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

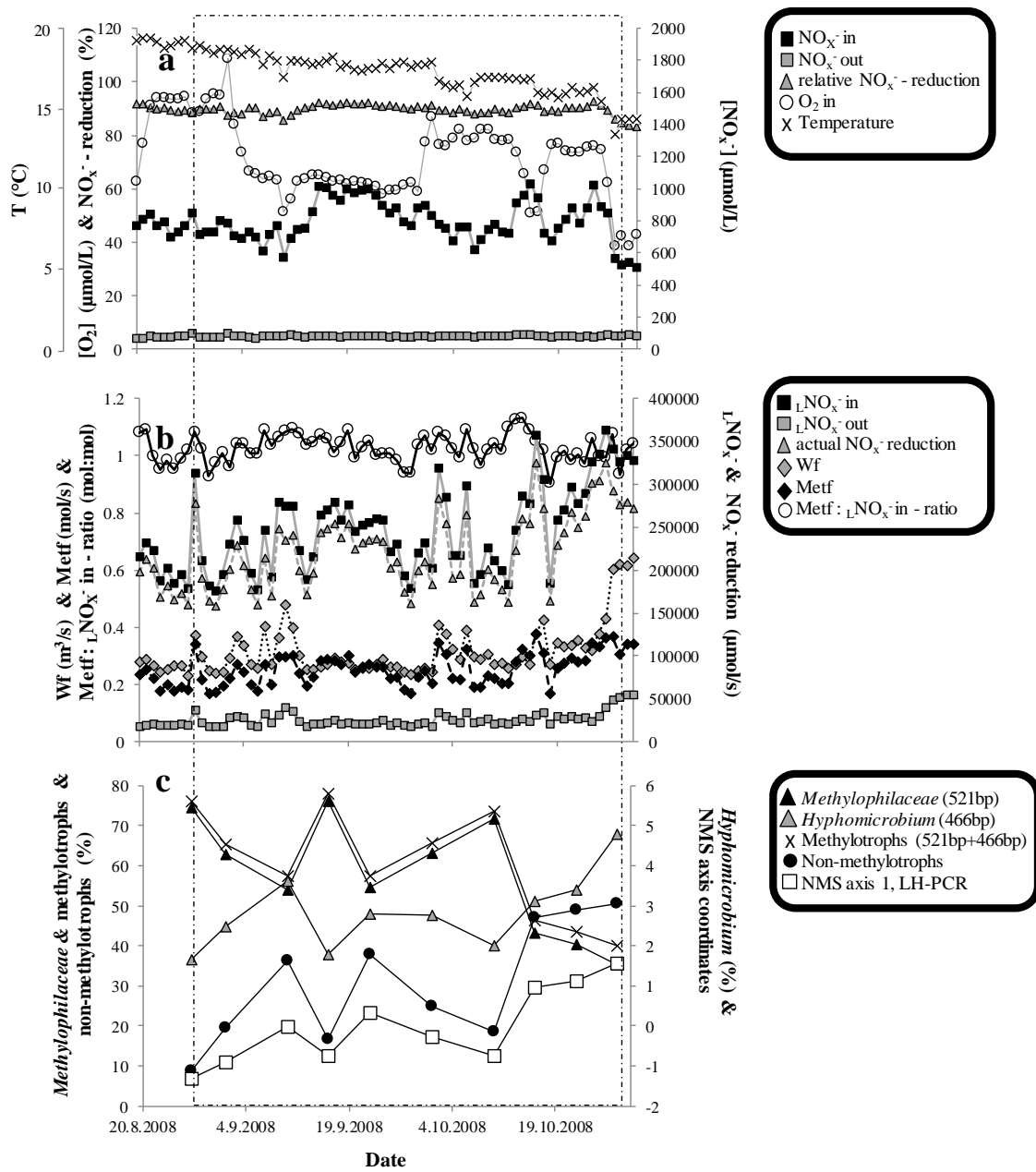[c] Clustering (clusters I and II) of *Hyphomicrobium* according to Rainey et al. [30]. See
also Fig. 2.

[d] Clustering based on Fig. 3.

735   Figure 1
736

737
738
739
740
741
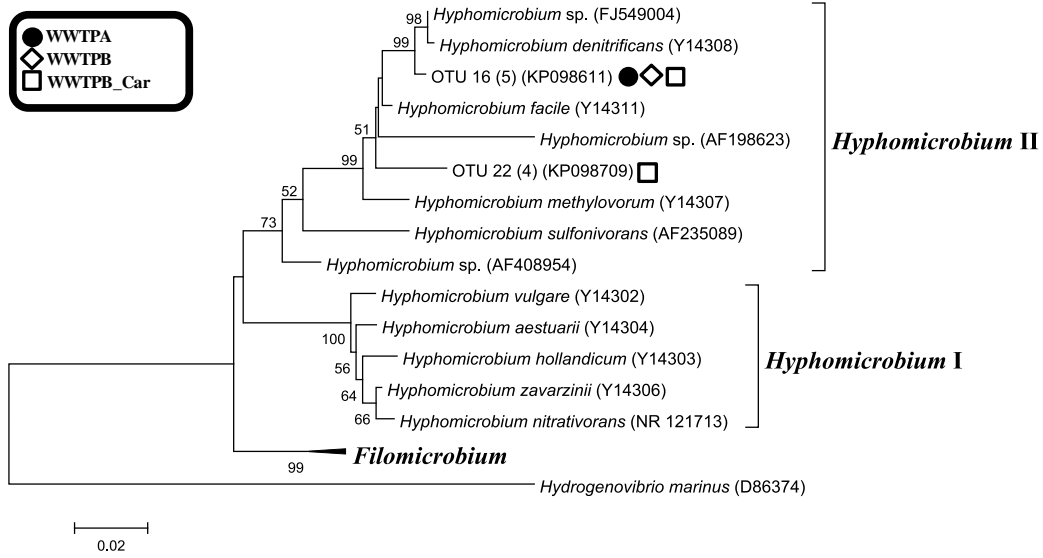742
743

744 Figure 2
745

746 0.02

747
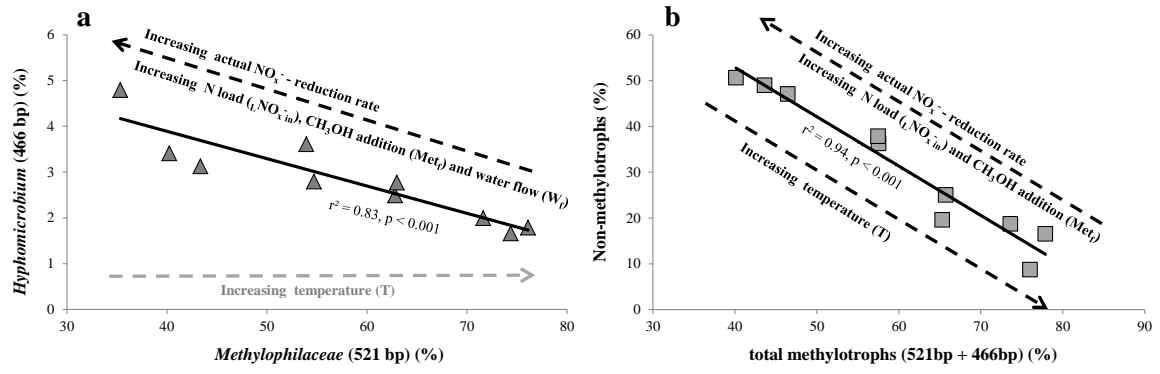
748

749

750

751

752

753

754

755

756

757

758

759

760

761

762

763

764   Figure 3
765

Figure 3

766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782

a

Hyphomicrobium (466 bp) (%)

Increasing actual NO$_x^-$- reduction rate

Increasing N load ($_tNO_{x~in}$), CH$_3$OH addition (Met$_t$) and water flow (W$_t$)

$r^2 = 0.83, p < 0.001$

Increasing temperature (T)

Methylophilaceae (521 bp) (%)

b

Non-methylotrophs (%)

Increasing actual NO$_x^-$- reduction rate

Increasing N load ($_tNO_{x~in}$) and CH$_3$OH addition (Met$_t$)

$r^2 = 0.94, p < 0.001$

Increasing temperature (T)

total methylotrophs (521bp + 466bp) (%)

806   Online Resources (1-3)
807
808   Journal: J Ind Microbiol Biotechnol
809
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
815
816
817   [1]Department of Chemistry and Bioengineering, Tampere University of Technology, P.O.
818   Box 541, FI-33101 Tampere, Finland
819   [2]Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box
820   35, FI-40014 University of Jyväskylä, Finland
821
822   [*]Corresponding author
823   email-address: antti.rissanen@tut.fi
824

825

826

827

828

829
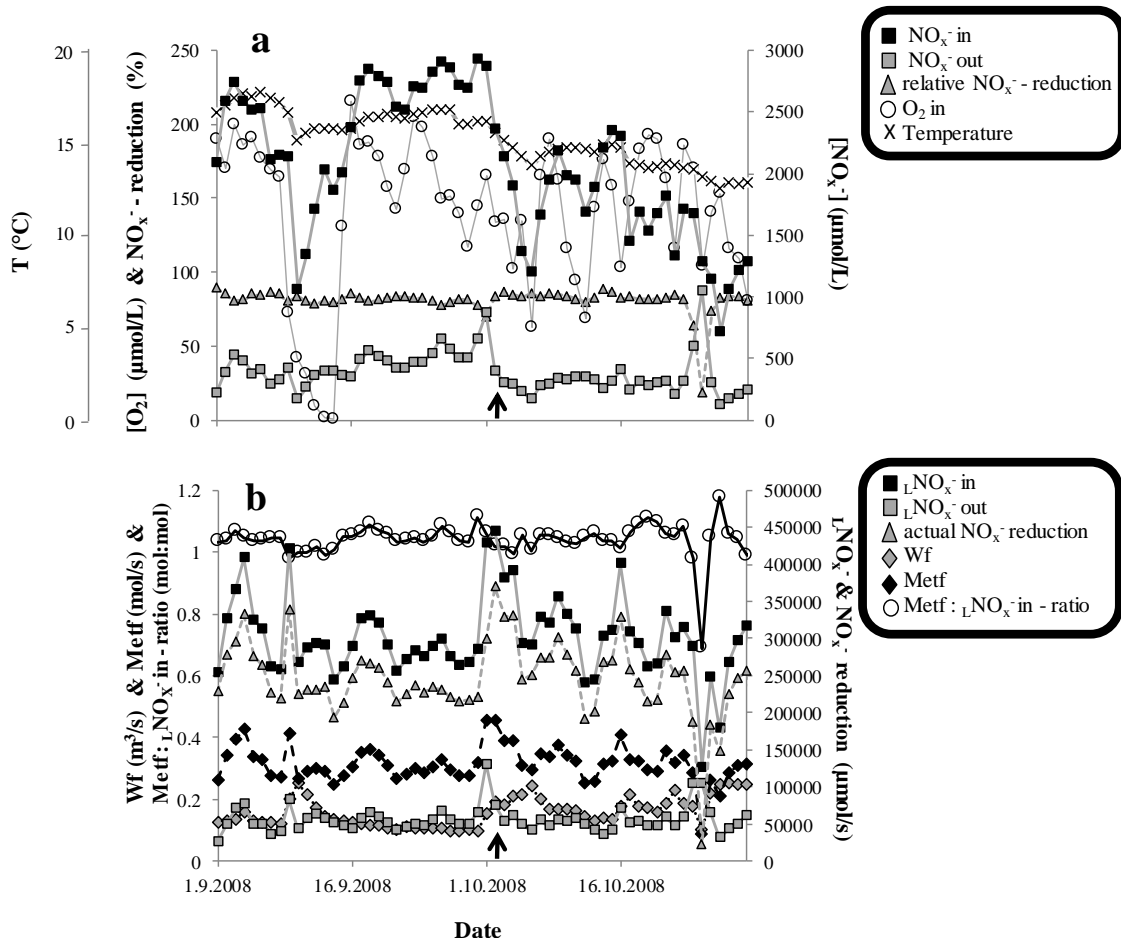
830

831

832

833

834

835

836

837

838

839

840

841

842   Online Resource 1
843

844

Date

845   **Online Resource 1** Operating conditions and functional performance in the denitrification

846   biofilter of WWTPB. (a) Temperature and concentration of NO_x^- and O_2 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

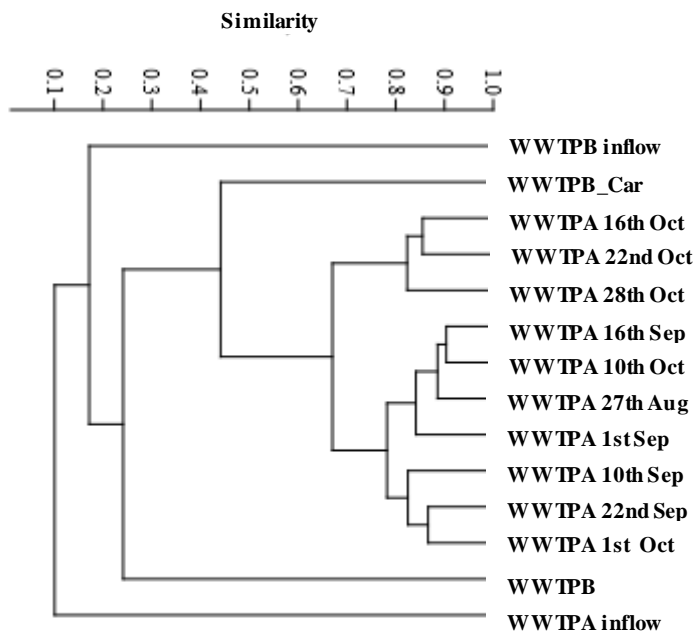850   an arrow

851

852

853

854

855

856   Online Resource 2

857

858

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

863

864

865
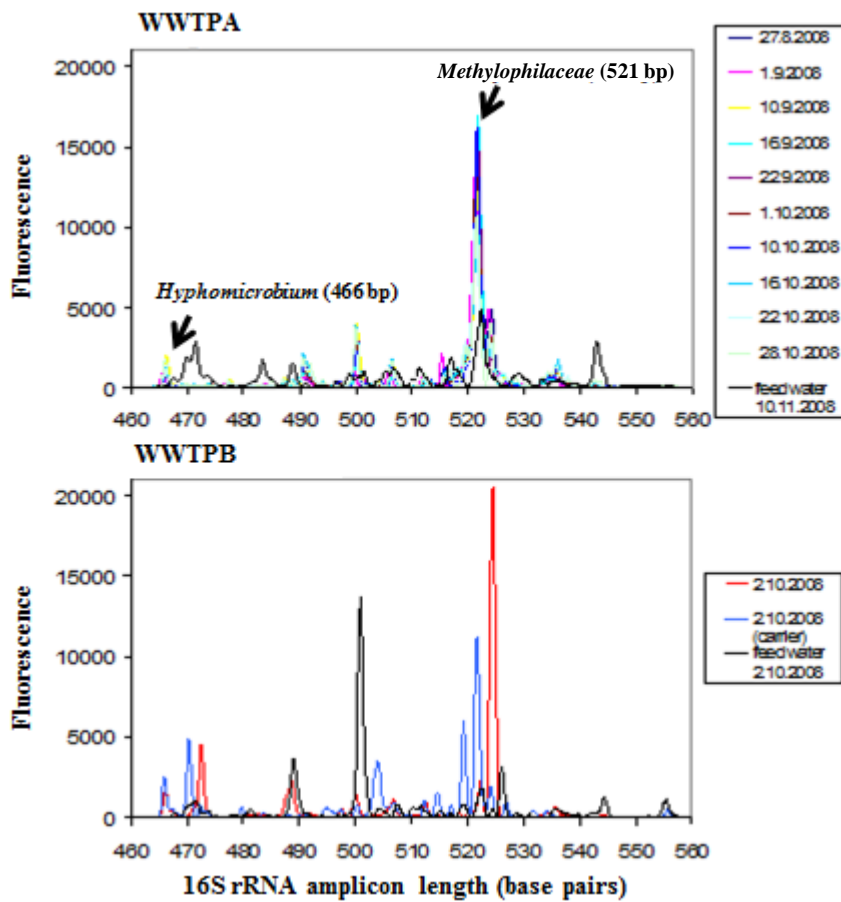
866

867

868

869

870

871

872   Online Resource 3

873

874

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