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Methylophaga and *Hyphomicrobium* can be used as target genera in monitoring saline water methanol-utilizing denitrification

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Abstract Which bacterial taxonomic groups can be used in monitoring saline water methanol-utilizing denitrification and whether nitrate is transformed into N_2 in the process are unclear. Therefore, methylotrophic bacterial communities of two efficiently functioning (nitrate/nitrite reduction was 63–96 %) tropical and cool seawater reactors at a public aquarium were investigated with clone library analysis and 454 pyrosequencing of the 16S rRNA genes. Transformation of nitrate into N_2 was confirmed using ^{15}N labeling in incubation of carrier material from the tropical reactor. Combining the data with previous study results, *Methylophaga* and *Hyphomicrobium* were determined to be suitable target genera for monitoring the function of saline water methanol-fed denitrification systems. However, monitoring was not possible at the single species level. Interestingly, potential nitrate-reducing methylotrophs within *Filomicrobium* and closely related Fil I and Fil II clusters were detected in the reactors suggesting that they also contributed to methylotrophic denitrification in the saline environment.

Keywords Methylotrophy · Denitrification · Saline water · Reactor · 16S rRNA

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32

33 ## Introduction

34

35 Denitrification, step-wise reduction of water-soluble nitrate (NO_3^-) via nitrite (NO_2^-) to
36 gaseous nitric oxide (NO), nitrous oxide (N_2O) and di-nitrogen (N_2) by facultative
37 anaerobic heterotrophic bacteria, provides an important biotechnological water treatment
38 process for nitrogen (N) removal. Denitrification is mostly a community process, as many
39 denitrifiers perform only partial denitrification reducing NO_3^- to NO_2^- or to N_2O, and only
40 some bacterial species are capable of the whole denitrification chain from NO_3^- to N_2 gas
41 [10]. Due to the low C:N ratio of the influent water in many N removal systems, an
42 external carbon source, usually methanol, is added to the process. Methanol-utilizing
43 denitrification systems are widely applied in municipal wastewater treatment plants [18].
44 In addition, these systems are especially important for reducing toxic inorganic N
45 compounds in closed marine facilities, for example, in public aquaria [21] and aquaculture
46 farms [25].

47 The physicochemical and technical aspects of methanol-utilizing denitrification
48 processes have been comprehensively characterized [18, 24, 25, 26]. However,
49 denitrification is usually measured by NO_x reduction, that is, the disappearance of NO_3^-
50 /NO_2^- [23]. This indirectly measured denitrification rate denotes the conversion of water-
51 soluble NO_x^- into gaseous forms, but the proportions of NO, N_2O and N_2 in the end-
52 product are not specified. Thus far, only a few studies have been conducted on direct
53 measurements of gaseous end-products [17, 27]. These studies mostly focused on N_2O
54 production [17, 27], and the conversion of NO_3^- to N_2 has only very rarely been measured
55 or even confirmed in water treatment facilities [6].

56 Optimal control and operation of wastewater treatment processes would also
57 greatly benefit from microbiological data [27, 43], such as monitoring the presence and
58 abundance of taxonomic groups crucial for system function [27]. The search for potential
59 target taxonomic groups for monitoring saline water methanol-utilizing denitrification
60 processes should be carried out in efficiently functioning systems and should focus on
61 methylotrophic (C1-compound utilizing) organisms as they play a key role in the current
62 processes, by utilizing methanol as an electron donor in denitrification and by
63 transforming methanol into various extracellular organic compounds, which can then be
64 utilized by co-occurring non-methylotrophic denitrifiers [27]. Methylotrophic bacteria of
65 the genera *Methylophaga* and *Hyphomicrobium* have been shown to dominate the two
66 previously studied saline water methanol-fed denitrification systems: a moving bed
67 biofilm reactor at a seawater aquarium [3, 21, 22] and a laboratory-scale continuously
68 stirred tank reactor (CSTR) that treat synthetic saline wastewater [32]. In addition to these
69 two genera, *Azoarcus* and *Paracoccus* were important methylotrophs in CSTR [32].
70 Further analyses in the aquarium showed the genetic potential of *Methylophaga* to reduce
71 NO_3^- to NO_2^- and *Hyphomicrobium* to complete the denitrification by converting NO_2^-
72 into N_2 [2, 3, 42]. However, physicochemical and biological variations among bioreactors
73 could lead to differences in the community composition. Thus, other methylotrophs could
74 be important for the function of these systems given that methylotrophy is a quite widely
75 dispersed trait among bacteria [19], and many can also conduct partial or complete
76 denitrification [4, 5, 8, 12, 20, 45]. Therefore, more studies are needed to determine
77 suitable target taxonomic groups for monitoring the function of saline water methanol-fed
78 denitrification processes.
79 This study investigated NO_x^- reduction and the bacterial communities of two
80 methanol-utilizing denitrifying bioreactors in a marine fish aquarium operating at two

81 water circulation temperatures. We aimed to confirm the microbiological transformation
82 of NO_3^- to N_2 using batch incubations and the ^{15}N tracer technique. Furthermore, we
83 aimed to find suitable target taxonomic groups for monitoring the function of saline water
84 methanol-utilizing denitrification processes. This was achieved via a clone library and 454
85 pyrosequencing analysis of the 16S rRNA genes and comparison of the results for the two
86 study reactors to those for previously studied systems [3, 21, 32]. We specifically focused
87 on the analysis of taxonomic groups that harbor known methylotrophs.

88
Materials and methods

89
90
Sampling site and sampling for molecular microbiology

91
92 The public fish aquarium SEA LIFE Helsinki Finland
93 (https://www.visitsealife.com/helsinki/) is divided into two water recirculation systems
94 (warm tropical, AQUAR_T, and cold North Atlantic, AQUAR_C) and has a total water
95 volume of 420 m^3 with 27–34 ppt salinity. Both circulation systems have their own
96 fluidized-bed type denitrification reactors that receive NO_3^--rich water from the
97 nitrification stage. The volume of each reactor is ~330 L of which ~90 L is the fluidized
98 carrier material bed (oolitic sand, 100 kg, density = 1.5 kg/L). The temperature inside the
99 denitrification reactors of AQUAR_T and AQUAR_C was 23–24 °C and 18–19 °C,
100 respectively. Two samples of oolitic sand for molecular microbiological analyses were
101 collected twice, on 10 November 2008 and 8 September 2010, from the AQUAR_T and
102 AQUAR_C reactors, in sterile 50 mL plastic containers and stored at –20 °C before
103 processing within 1 to 2 months. The reactors utilized methanol as their carbon source
104 except AQUAR_C in 2008 when a mixture of methanol and saccharose was used until the
105 sampling time point after which only methanol was used.

106

107
Denitrification measurements

108

109 The NO_x^- concentrations inside the reactors, near the reactor outlet ($NO_x^-{}_{out}$) and in the

110 inflow water feeding the reactors ($NO_x^-{}_{inflow}$) were measured using Spectroquant® nitrate

111 and nitrite test kits (Merck Millipore, Germany) with a Spectroquant® Nova 60

112 photometer (Merck Millipore, Germany) from both reactors 1 to 2 times per month for 2.3

113 years (time period 24 September 2008 to 28 December 2010). The methanol addition

114 (Met_f, mmol/h) and water flow (W_f, L/h) rates were adjusted by the operators and for this

115 study reported for 1.5 to 2 month periods before bacterial sampling in 2008 and 2010 (24

116 September to 10 November 2008 and 13 July to 8 September 2010). Hourly NO_x^- loads

117 (${}_LNO_x^-{}_{inflow}$ and ${}_LNO_x^-{}_{out}$, mmol N/h) were calculated using the W_f and NO_x^- concentration

118 values for these time periods. Denitrification was then estimated indirectly as the relative

119 NO_x^- reduction (%):

120 $$NO_x^- \ reduction = \frac{(NO_x^-{}_{inflow} - NO_x^-{}_{out})}{NO_x^-{}_{inflow}} \times 100$$

121 and as the actual NO_x^- reduction:

122 $$actual \ NO_x^- \ reduction = {}_LNO_x^-{}_{inflow} - {}_LNO_x^-{}_{out,}$$

123 which was converted into the NO_x^- reduction rate of the carrier material (µmol N/L_{car}/h).

124 Direct denitrification measurement was performed with bottle incubation in 2010.

125 Batches of oolitic sand (~36 mL) collected from the middle of the filter bed in AQUAR_T

126 were put in 120 mL glass bottles (11 bottles altogether). The bottles were filled with

127 anoxic reactor water by submerging them in the reactor, and while submerged, they were

128 closed with caps that had butyl rubber stoppers. Each bottle was injected with ~219 µmol

129 (~7 mg) of methanol and ~50 µmol (~0.7 mg) of NO_3^--N, which had a 2% ^{15}N isotope

130 label, and then shaken briefly but vigorously. The NO_3^- stock solution (0.1 M NO_3^--N)

131 was prepared from $NaNO_3$ and $K^{15}NO_3^-$ (Cambridge Isotope Laboratories, Inc., MA,

132 USA). The total NO_3^--N concentration after the addition was ~664 µM, which is

133 approximately the same as the average $NO_x^-{}_{inflow}$ concentration (~688 μM) during the ~2
134 month period before sampling in 2010. The bottles were incubated non-shaken at 21–22
135 °C and sacrificed in batches after 2 h (4 bottles), 6 h (4 bottles) and 21 h (3 bottles) of
136 incubation. One non-incubated water sample taken from the reactor before the incubation
137 periods served as the 0 time point control. The water subsamples were stored in 12 mL
138 borosilicate glass Exetainer® tubes with screw-capped butyl rubber septa (Labco Ltd.,
139 High Wycombe, UK), and the microbial activity in the vials was terminated by adding 0.1
140 mL of $ZnCl_2$ (1 g/mL). The concentration and ^{15}N content of the N_2 gas in the water was
141 measured as in Tiirola et al. [41]. Denitrification was calculated as the rate of the total N_2
142 gas accumulation and converted to the N_2 production rate of the carrier material (µmol
143 N/L_{car}/h). In addition, reduction of NO_3^- to N_2 gas was verified by the accumulation of
144 excess ^{15}N-containing N_2 gas. The concentration of the excess ^{15}N-containing N_2 gas,
145 [excess ^{15}N], was calculated for each sample as

146 $$[excess\ ^{15}N] = \frac{(at\%^{15}N_{\mathrm{sample}} \times [N_{2\mathrm{sample}}] - at\%^{15}N_{\mathrm{zero}} \times [N_{2\mathrm{sample}}])}{100}$$

147 where [$N_{2sample}$] is the N_2 gas concentration in the incubated sample and the at% $^{15}N_{sample}$
148 and the at% $^{15}N_{zero}$ are the ^{15}N content (in %) of the N_2 gas in the incubated and non-
149 incubated (0 time point) samples, respectively.

150

151 **Molecular microbiological analyses**

152

153 DNA was extracted from 0.5–0.6 g of frozen oolitic sand from each sample using glass
154 bead beating and phenol–chlorophorm extraction, which was followed by isopropanol-
155 NaCl precipitation (pH 8) and dissolution of the DNA pellet in TE buffer.
156 PCR for the clone library analyses of the 16S rRNA genes was performed with
157 primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) / 907R (5´-
158 CCGTCAATTCMTTTGAGTTT-3´) as previously described [36] but using the following

159 program: initial denaturation at 95 °C for 5 min and 30 cycles of amplification (94 °C for
160 30 s, 53 °C for 1 min, 72 °C for 3 min) and final elongation at 72 °C for 15 min. The PCR
161 products of the replicate samples were pooled for subsequent cloning and sequencing,
162 which was carried out as previously described [36].
163 To get deeper insight into the bacteria inhabiting the methanol-fed denitrification
164 reactors, the bacterial community in AQUAR_T, which used methanol as the sole carbon
165 source, was studied with 454 pyrosequencing. Equal amounts of nucleic acid extracts from
166 replicate samples of AQUAR_T in 2008 were pooled before the PCR reactions. PCR
167 amplification of the 16S rRNA genes using primers 341F (5´-
168 CCTACGGGNGGCWGCAG-3´) / 805R (5´-GACTACHVGGGTATCTAATCC-3´),
169 subsequent purification steps and sequencing were performed as previously described
170 [33].
171
172 **Sequence analysis**
173
174 In the analysis of the clone library sequences, the Mothur program package [38] was used
175 for sequence alignment, chimera-checking (chimera.uchime executable), classification of
176 sequences into operational taxonomic units (OTUs; 97 % identity threshold) and
177 taxonomic classification of the OTUs (using the Ribosomal Database Project database).
178 Variations in the community structure among the samples were analyzed with hierarchical
179 clustering (UPGMA linkage, Bray-Curtis distances) using PAST version 3.09 [13].
180 Representative sequences of the OTUs assigned to taxonomic groups of known
181 methylotrophs derived from the previous literature [8, 19, 21, 22, 32, 45], in this case
182 *Hyphomicrobiaceae* and *Methylophaga*, were subjected to phylogenetic tree analyses
183 [neighbor-joining (NJ) method, Kimura-2 distances, pairwise exclusion of gaps] using
184 Mega 5.05 [40].

185 Tags and primer sequences, as well as low-quality sequences (containing
186 ambiguous nucleotides and homopolymers longer than eight nucleotides) were removed
187 from the 454 pyrosequencing library. Sequences (~200 bp) were thereafter processed as
188 described above for the clone library analysis except phylogenetic trees were not
189 constructed.

190 The 16S rRNA gene sequences of the clone libraries were deposited in the NCBI
191 GenBank (accession numbers KP098736-KP098970, KP098976-KP098984 and
192 KP098989-KP099005). The 454 pyrosequencing data were deposited in the NCBI SRA
193 database (SRX646347).

194

195 Results

196

197 Functioning of denitrifying reactors

198

199 The operator-adjusted W_f and Met_f were lower in 2010 than in 2008 in both reactors
200 during the 1.5 to 2 month period before bacterial sampling (Table 1). The average hourly
201 NO_x^- load in the inflow decreased in AQUAR_T and increased in AQUAR_C from 2008
202 to 2010 (Table 1). The average molar ratio of the added methanol to the N load
203 methanol:NO_x^--N_{inflow} (mol:mol) decreased from 2008 to 2010 in both reactors (Table 1).
204 The ratio was higher in AQUAR_C than in AQUAR_T in 2008 and vice versa in 2010
205 (Table 1).

206 The relative NO_x^- reduction, on average, was slightly higher in AQUAR_C (91 %,
207 range: 81–95 %) than in AQUAR_T (86 %, range: 63–96 %) during the 2.3 year
208 monitoring period (24 September 2008 to 28 December 2010, excluding the very low
209 values caused by the pumping and carbon dosage problems on 4 November 2008 in both
210 reactors and on 4 June 2009 in AQUAR_C; Fig. 1). When averaged over the 1.5 to 2
211 month period before the bacterial sampling, the average actual NO_x^- reduction rate

212 expressed per carrier (oolitic sand) volume was lower in 2008 than in 2010 in both
213 reactors (Table 1). The rate was higher in AQUAR_T than in AQUAR_C in 2008 and vice
214 versa in 2010 (Table 1).

215 Bottle incubation tests confirmed that N_2 was produced and NO_3^- was transformed
216 into N_2 by microbes in AQUAR_T (Fig. 2). The estimated N_2 production rate of the
217 carrier material was 56.4 µmol N/L_{car}/h.

218

219 **Variation in microbial community structure**

220

221 The clone library analysis of the 16S rRNA genes indicated that the variation in the
222 bacterial community structure was higher between the reactors than between the years
223 (Table 2; Online Resource 1). Variation between the years in the community structure was
224 considerably higher in the cooler reactor AQUAR_C, which was first fed with a mixture
225 of methanol and saccharose, than in the warmer reactor AQUAR_T (Table 2; Online
226 Resource 1).

227 Different taxonomic groups were assigned to a putative methylotrophic function
228 based on the previous literature. The methylotrophic groups detected in the reactors were
229 the genera *Methylophaga*, *Hyphomicrobium, Filomicrobium*, as well as clusters Fil I and
230 Fil II, which were more closely related to *Filomicrobium* than to *Hyphomicrobium* (Table
231 2; Figs. 3 and 4). Betaproteobacterial methylotrophs were not detected. In the
232 phylogenetic tree, the OTUs assigned to the genus *Hyphomicrobium* were positioned
233 between the previously assigned *Hyphomicrobium* clusters I and II [35] (Fig. 3). Most of
234 the OTUs and sequences assigned to *Methylophaga* clustered close to *M.*
235 *nitratireducenticrescens*, whereas one of the OTUs had its closest relative in *M.*
236 *thiooxydans* (Fig. 4).

237 The relative abundance of the putative methylotrophs was considerably higher in
238 the warmer reactor AQUAR_T than in the cooler reactor AQUAR_C in both years (Table
239 2). In addition, methylotrophs were more abundant in 2010 than in 2008 in AQUAR_C
240 whereas the opposite took place in AQUAR_T (Table 2). The relative abundance of
241 *Methylophaga* was considerably higher in the warmer reactor AQUAR_T than in the
242 cooler reactor AQUAR_C (Table 2). In AQUAR_C, *Methylophaga* were absent in 2008
243 and present at very low numbers in 2010. Correspondingly, there was a higher abundance
244 of *Methylophaga* in 2010 than in 2008 in AQUAR_T (Table 2). *Methylophaga* in
245 AQUAR_T had their closest relative in *M. nitratireducenticrescens*, whereas those in
246 AQUAR_C were most closely related to *M. thiooxydans* (Fig. 4). Of the family
247 *Hyphomicrobiaceae*, *Hyphomicrobium* were much more abundant in the warmer reactor
248 AQUAR_T than in the cooler reactor AQUAR_C. There was a higher abundance of
249 *Hyphomicrobium* in 2010 than in 2008 in AQUAR_C and vice versa in AQUAR_T (Table
250 2). In contrast, the bacteria of the Fil I cluster were much more abundant in AQUAR_C
251 than in AQUAR_T (Table 2). In AQUAR_T, Fil I bacteria were absent in 2008 and
252 present at very low abundance in 2010. Correspondingly, there was a higher abundance of
253 Fil I bacteria in 2010 than in 2008 in AQUAR_C. Fil II cluster bacteria were present in
254 low abundance in both reactors and were absent in AQUAR_T in 2010 (Table 2).
255 *Filomicrobium* cluster bacteria were present in low abundance and only in AQUAR_T
256 (Table 2; Fig. 3).
257 The relative abundance of other bacteria also varied between reactors and years and
258 was generally higher in AQUAR_C than in AQUAR_T (Table 2). Within
259 *Gammaproteobacteria*, there was a considerably large group of unclassified 16S rRNA
260 gene sequences that probably represented clades without cultured representatives (Table
261 2). Of *Alphaproteobacteria* (other than methylotrophs), *Rhodobacteraceae* and

262 *Phyllobacteriaceae* as well as non-methylotrophic *Hyphomicrobiaceae* within *Maritalea*
263 and Mar I clusters were detected (Table 2; Fig. 3). According to BLAST searches [1] of
264 the NCBI nr-database, the detected *Rhodobacteraceae* were most closely related to the
265 non-methylotrophic genus *Roseovarius*. Of the *Proteobacteria*, also *Deltaproteobacteria*
266 were present. Other detected phyla were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*,
267 *Chloroflexi*, *Deferribacteres*, *Planctomycetes* and *Spirochaetes* (Table 2).
268 There were differences in the results of the clone library analysis and 454
269 pyrosequencing (Table 2). Most importantly, 454 pyrosequencing had lower resolution to
270 detect *Alphaproteobacteria* or resolve different genera within *Hyphomicrobiaceae* and
271 resulted in a higher proportion of unclassified bacterial sequences than the clone library
272 analysis (Table 2). Since high taxonomic resolution was necessary to identify clusters
273 especially within *Hyphomicrobiaceae*, the focus in this study was on the clone library
274 results.
275

276 **Discussion**
277

278 Both study reactors removed N very efficiently. The relative NO_x^- reduction was even
279 slightly higher than measured at other methanol-fed sea water denitrification bioreactors
280 that reduced 65–90 % [22] and up to 88 % [24] of NO_x^-. In contrast, the average actual
281 NO_x^- reduction rates were within the lower end of the range (300–9000 µmol N/L_{car}/h)
282 measured at other systems [21, 23, 24]. The differences in the actual NO_x^- reduction rates
283 are mostly due to 3–10 times higher N loads (as expressed per carrier volume) in the
284 previously studied reactors [23, 24]. However, the differences can be also partially
285 attributed to the use of deaeration systems to remove O_2 in the inflow and the amendment
286 of the trace metal solution in the previously studied reactors, which enhanced

287 denitrification [21, 23]. To our knowledge, this is the first study of saline water methanol-
288 utilizing denitrification systems that confirmed NO_3^- is reduced to N_2. However, the N_2
289 production measured in the batch tests was only 6–7 % of the actual NO_x^- reduction rates
290 in the AQUAR_T reactor. This difference is very likely due to the lack of shaking during
291 incubation, which reduced the contact between NO_3^- and the bacteria on the carrier
292 material. However, the possible formation of other gases, N_2O [17] and NO, during the
293 incubation periods can also explain a small part of this discrepancy.

294 Combining the results from the two study reactors and the two previously studied
295 systems [3, 21, 32] shows that putative methylotrophic bacteria belonging to
296 *Methylophaga* and *Hyphomicrobium* are very common in efficiently functioning saline
297 water methanol-fed denitrification systems. This result indicates that these bacteria are
298 crucial for the function of the process. Thus, these two genera can serve as targets when
299 monitoring the function of saline-water methanol-utilizing denitrification systems.
300 However, there were considerable species-level differences between the systems.
301 *Hyphomicrobium nitrativorans*, which dominated in a previously studied system [3, 21],
302 were not found at all in the AQUAR reactors, and *Methylophaga nitratireducenticrescens*,
303 which inhabited a previously studied reactor [3] and AQUAR_T, were not found in
304 AQUAR_C. Thus, no single *Methylophaga* or *Hyphomicrobium* species can be
305 determined for use as a general target species for monitoring the function of saline water
306 methanol-fed denitrification systems.

307 Interestingly, this study is also the first to show that members of *Filomicrobium*
308 sp. and *Filomicrobium*-related Fil I and Fil II clusters can be abundant in efficiently
309 functioning saline water methanol-fed denitrification systems. In addition to results from
310 cultivation-based [45] and genomic [14] studies of *Filomicrobium* strains, further
311 confirmation of the bacteria's methylotrophic metabolism was provided by the concurrent

312 increase in the relative abundance of *Hyphomicrobium*, *Methylophaga* and Fil I bacteria in
313 AQUAR_C from 2008 to 2010 when the C source changed from saccharose+methanol to
314 solely methanol. In 2008, the methylotrophs were probably over-competed by more
315 efficiently growing non-methylotrophs, for example, in *Bacteroidetes*, *Rhodobacteraceae*
316 and *Maritalea*. Furthermore, the concurrent decrease in *Hyphomicrobium* and Fil II
317 bacteria in AQUAR_T from 2008 to 2010 could due to the decrease in the availability of
318 methanol (a decrease in Met_f and methanol:NO_x-N_{inflow} before sampling).

319 Previous studies also suggest that *Filomicrobium* participate in denitrification by
320 dissimilatory reduction of NO_3^- to NO_2^- [14, 45]. Thus, in addition to *Methylophaga* [2, 3],
321 *Filomicrobium*, Fil I and Fil II bacterial clusters could couple methylotrophy with the
322 reduction of NO_3^- to NO_2^- and supply it for denitrifiers capable of further denitrification
323 steps [3]. However, previously studied strains of *Methylophaga thiooxydans* [7] and *M.*
324 *nitratireducenticrescens* [42] also had the *NO_2^- reductase gene*, although it was truncated
325 in *M. nitratireducenticrescens* [42]. In addition, *M. nitratireducenticrescens* had genes for
326 NO and N_2O^- reduction [42]. This suggests that *Methylophaga* species coupling
327 methylotrophy to NO_2^-, NO and N_2O reduction could exist in nature and in saline water
328 methanol-fed denitrification systems. Since only a few *Filomicrobium* strains have been
329 characterized for their metabolic potential [14, 45], it is possible that some species within
330 *Filomicrobium*, Fil I and Fil II clusters are also capable of this.

331 Thorough comparative analyses of the factors that affect the growth and activity of
332 *Filomicrobium* and *Hyphomicrobium* species do not exist. Therefore, many possible
333 factors might have affected the genera- and species-level variation of *Hyphomicrobiaceae*
334 between the AQUAR reactors and previously studied systems [3, 21]. In a comparison of
335 denitrifying *Hyphomicrobium* species, NO_3^- significantly affected the growth and activity
336 of *Hyphomicrobium* species with *H. nitrativorans* growing and denitrifying at higher NO_3^-

337 concentrations than the other studied species, *H. zavarzinii* and *H. denitrificans* [30].
338 Thus, the considerably lower N loads could explain the presence and dominance of
339 *Hyphomicrobium* species other than *H. nitrativorans* in the AQUAR reactors. Similarly,
340 *Filomicrobium*, Fil I and Fil II bacteria could have been favored in the lower N load
341 conditions that prevail in the AQUAR system. Furthermore, O_2 constantly enters AQUAR
342 reactors due to the lack of a preceding deaeration step but is rapidly consumed there based
343 on the anoxic conditions ($[O_2] < 0.1$ mg/L, below the detection limit of the O_2 probe) that
344 prevail inside the reactors [9]. Thus, variation in the O_2 availability between the systems
345 can also explain the differences in the bacterial communities. Part of the *Hyphomicrobium*
346 and *Filomicrobium* OTUs in the AQUAR reactors may have been favored by O_2.
347 Analogously, as in aerobic methane oxidation coupled with denitrification (AME-D) [46],
348 these bacteria could have contributed to the overall denitrification performance by
349 consuming O_2 and by aerobically converting methanol to organic substrates utilizable by
350 non-methylotrophic denitrifiers. However, in addition to variating NO_3^- and O_2,
351 differences in the inocula (the original bacterial community that colonized the reactors)
352 and in the carrier materials between the reactors as well as the amendment of the trace
353 metal solution and the higher addition of methanol in the previously studied systems [3,
354 21, 22] could have also affected the differences in the bacterial communities.
355 The lower relative abundance of methylotrophs in the cooler reactor AQUAR_C
356 than in AQUAR_T can be first explained by the saccharose addition in 2008 leading to
357 over-competition of non-methylotrophic organisms in AQUAR_C. In 2010, the difference
358 can be attributed to the lower availability of methanol (lower Met_f and methanol:NO_x^-
359 $_{inflow}$) in AQUAR_C. Variation in the availability of methanol can also explain the lower
360 abundance of *Hyphomicrobium* and *Methylophaga* and the higher abundance of Fil I
361 bacteria in AQUAR_C than in AQUAR_T. However, differences in temperature could

362 also play a role here since the lowest limits of the growth temperature ranges of
363 *Methylophaga* (*M. nitratireducenticrescens*, 15–37 °C) [42] and *Hyphomicrobium* (*H.*
364 *nitrativorans*, 15–35 °C) [29] are higher than that of *Filomicrobium* (e.g., *F. insigne*, 4–45
365 °C) [45], which indicates that *Filomicrobium*-related Fil I bacteria grew better in the
366 cooler AQUAR_C reactor. The growth temperature range of *M. thiooxydans*, the closest
367 cultured relative of *Methylophaga* in AQUAR_C, is not known. However, the absence of
368 *M. thiooxydans* in the previously studied sea water reactor with a slightly lower
369 temperature, 16–18 °C [3], than in AQUAR_C, 18–19 °C, suggests that temperature
370 variations do not explain the differences in the *Methylophaga* species between AQUAR_C
371 and AQUAR_T. *M. thiooxydans* can grow on a wider range of carbon substrates
372 (methanol, fructose, monomethylamine) than *M. nitratireducenticrescens* (methanol) [42].
373 Thus, differences in the *Methylophaga* species between the reactors could be explained by
374 *M. thiooxydans* surviving better with lower methanol availability.
375 Many other detected phyla, that is, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*,
376 *Chloroflexi*, *Deferribacteres*, *Planctomycetes* and *Proteobacteria* (other than the detected
377 methylotrophs), contain species capable of either partial or complete denitrification [11,
378 27, 37, 39, 44] suggesting that they contribute to the overall denitrification performance of
379 the AQUAR reactors and utilize the organic compounds produced by methylotrophs.
380 However, recent cultivation studies have shown that methylotrophy is present within
381 *Bacteroidetes* [28] and *Actinobacteria* [15]. In addition, DNA stable isotopic probing
382 (SIP) analyses, although not able to distinguish between direct C1 utilization and cross-
383 feeding, raise speculation about the existence of unclassified methylotrophic
384 *Gammaproteobacteria* [31], as well as methylotrophic members within *Bacteroidetes*,
385 *Deferribacteres* [16] and *Acidobacteria* [34]. Further studies are needed to show whether
386 members of these groups couple methylotrophy with denitrification in saline water

387 systems and could also be considered potential target taxonomic groups for monitoring

388 purposes.

389

390 **Conclusions**

391

392 This study confirmed that NO_3 was transformed into N_2 in a functioning saline water

393 methanol-fed denitrification system. Combining the results of this study with those of

394 previous studies show that *Methylophaga* and *Hyphomicrobium* can serve as target genera

395 in monitoring the function of saline water methanol-utilizing denitrification systems.

396 However, species-level differences among the systems, stemming from differences in the

397 physicochemical and biological conditions, prevent the determination of individual

398 *Methylophaga* or *Hyphomicrobium* species to be used as general target species in process

399 monitoring. Interestingly, other bacteria with potential methylotrophic and NO_3^--reducing

400 metabolism, within *Filomicrobium* and Fil I and Fil II clusters, were found, suggesting

401 they also contributed to saline water methylotrophic denitrification. Whether bacteria

402 within *Methylophaga*, *Filomicrobium*, Fil I and Fil II clusters can only reduce NO_3^- to

403 NO_2^- or reduce NO_2^-, NO and N_2O, and whether other detected bacteria are able to couple

404 methylotrophy with denitrification in the saline water denitrification bioreactors, is still to

405 be resolved. These studies should employ high-throughput culturing methods to isolate

406 target organisms, as well as metagenomics and metatranscriptomics.

407

408

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

410 **References**

411

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564 Figure captions:
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567 **Fig. 1** Concentration of NO_x^- in the inflow and the outflow and the relative NO_x^- reduction in the
568 denitrification reactors, AQUAR_T and AQUAR_C of the sea water aquarium from 24 September
569 2008 to 28 December 2010. The sampling dates for the microbial studies (10 November 2008 and
570 8 September 2010) are indicated with arrows
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572 **Fig. 2** Concentrations (average +/- standard deviation) and estimated production rates of N_2 gas
573 and excess ^{15}N (in N_2 gas) in incubation bottles after 2 h (n = 4), 6 h (n = 4) and 21 h (n = 3) of
574 incubation (at room temperature) of the carrier material and water from AQUAR_T with methanol
575 and ^{15}N-labeled NO_3^-. One non-incubated water sample taken from the reactor before the
576 incubation served as the 0 time point control (n = 1). The standard deviations of the N_2 and ^{15}N
577 concentrations at time point 2 h are very low and masked behind the symbol
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579 **Fig. 3** Phylogenetic tree (neighbor-joining method) of *Hyphomicrobiaceae* based on a comparative
580 analysis of 16S rRNA gene sequences in clone libraries showing the phylogenetic position of the
581 operational taxonomic units (OTUs; at 97 % sequence similarity) in AQUAR_C and AQUAR_T
582 in 2008 and 2010 (symbols). *Hyphomicrobium* clusters I and II were previously defined by Rainey
583 et al. [35]. The numbers in the parentheses after the OTU number indicate the number of
584 sequences within that OTU. The numbers at the nodes indicate the percentage of occurrence in
585 1000 bootstrapped trees (bootstrap values > 50 % are shown)
586

587 **Fig. 4** Phylogenetic tree (neighbor-joining method) of *Methylophaga* based on a comparative
588 analysis of 16S rRNA gene sequences in clone libraries showing the phylogenetic position of the
589 operational taxonomic units (OTUs; at 97 % sequence similarity) in AQUAR_C and AQUAR_T
590 at 2008 and 2010 (symbols). The numbers in the parentheses after the OTU number indicate the

591 number of sequences within that OTU. The numbers at the nodes indicate the percentage of
592 occurrence in 1000 bootstrapped trees (bootstrap values > 50 % are shown)
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595 Online Resource figure captions
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597 **Online Resource 1** Hierarchical clustering analysis (UPGMA) of relative abundance of OTUs
598 (using Bray-Curtis distances) in 16S rRNA gene clone library analyses of AQUAR_T and
599 AQUAR_C in 2008 and 2010
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619 **Table 1** Average (range) (n = 3 in both years) in the actual NO_x^- reduction rates, NO_x^- load rate ($_LNO_x^-{}_{inflow}$) and inflow methanol:NO_x^--N ratio as well as
620 operator-controlled rates of methanol addition (Met_f), saccharose addition and water flow (W_f) in the denitrification reactors of AQUAR_T and AQUAR_C in
621 2008 and 2010 for the 1.5 to 2 month periods before sampling for bacterial DNA (24 September to 10 November 2008 and 13 July to 8 September 2010)
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	AQUAR_T		AQUAR_C	
Year	2008	2010	2008	2010
Actual NO_x^- reduction (µmol N/L_{car}/h)	580[a] (60–1020)	890 (740–1090)	540[b] (290–700)	910 (880–950)
Met_f (mmol C/h)	158	98	185	85
Saccharose addition (mmol C/h)			67	
W_f (L/h)	120	70	110	93
$_LNO_x^-{}_{inflow}$ (mmol N/h)	76 (70–80)	66 (54–76)	51 (47–54)	66 (63–68)
Methanol:NO_x^--N_{inflow} (mol:mol)	2.1 (2.0–2.2)	1.5 (1.3–1.8)	3.6 (3.4–3.9)	1.3 (1.3–1.4)

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624 [a] Excluding values on 4 November 2008 (pumping and carbon dosage problems), the average actual NO_x^- reduction rate is 840 µmol N/L_{car}/h (range 660–
625 1020)
626 [b] Excluding values on 4 November 2008 (pumping and carbon dosage problems), the average actual NO_x^- reduction rate is 660 µmol N/L_{car}/h (range 620–700)
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635 **Table 2** Bacterial community composition (% of sequences) in denitrifying reactors of sea water aquarium (AQUAR_T and AQUAR_C) based on 16S rRNA
636 gene clone libraries and 454 pyrosequencing. Methanol was used as the sole external carbon source except for AQUAR_C in 2008, when a mixture of
637 methanol and saccharose was used during the period before microbial sampling

	AQUAR_T	AQUAR_T (454)[a]	AQUAR_T	AQUAR_C	AQUAR_C
Year (number of sequences)	2008 (72)	2008 (1849)	2010 (67)	2008 (59)	2010 (63)
Frequency (%)[b]:					
Putative methylotrophic	74	38	56	15	30
Alphaproteobacteria	64	35	46	69	30
Hyphomicrobiaceae	60	22	31	20	27
Hyphomicrobium	53	–	25	5	8
Filomicrobium cluster	3	–	3	–	–
Fil I cluster	–	–	3	7	17
Fil II cluster	4	–	–	3	2
Maritalea cluster	–	–	–	2	–
Mar I cluster	–	–	–	3	–
Rhodobacteraceae	1	2	–	32	–
Phyllobacteriaceae	–	4	9	2	3
Gammaproteobacteria	22	29	28	7	8
Piscirickettsiaceae	14	16	25	–	3
Methylophaga	14	16	25	–	3
incertae sedis	–	<1	–	–	3
unclassified	8	13	3	7	–
Deltaproteobacteria	3	2	7	–	5
Acidobacteria	–	–	–	–	3
Actinobacteria	1	<1	1	3	2
Bacteroidetes	3	7	6	19	14
Chloroflexi	3	3	1	–	19
Deferribacteres	–	–	–	–	2
Planctomycetes	–	4	–	–	10
Spirochaetes	–	<1	–	–	2
Unclassified + others	4	20	11	2	5

638 [a] Libraries generated using 454 pyrosequencing

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

673 Figure 1
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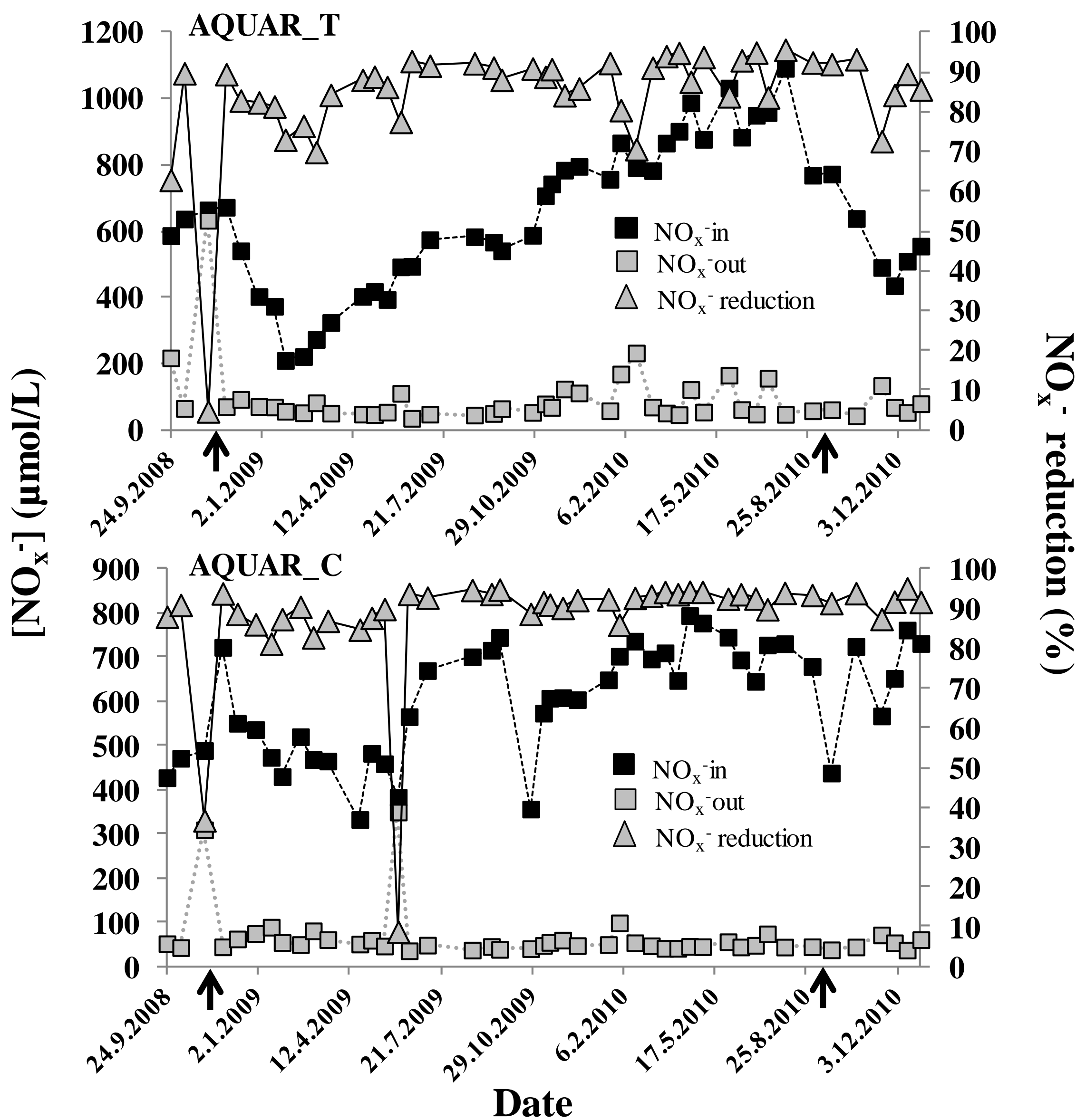

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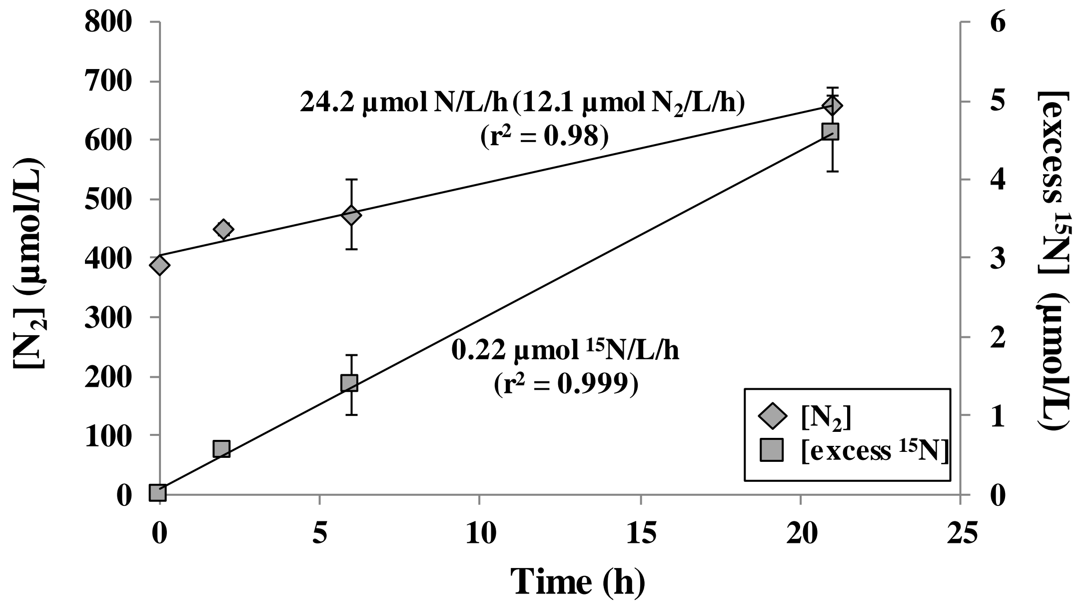

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704 Figure 3

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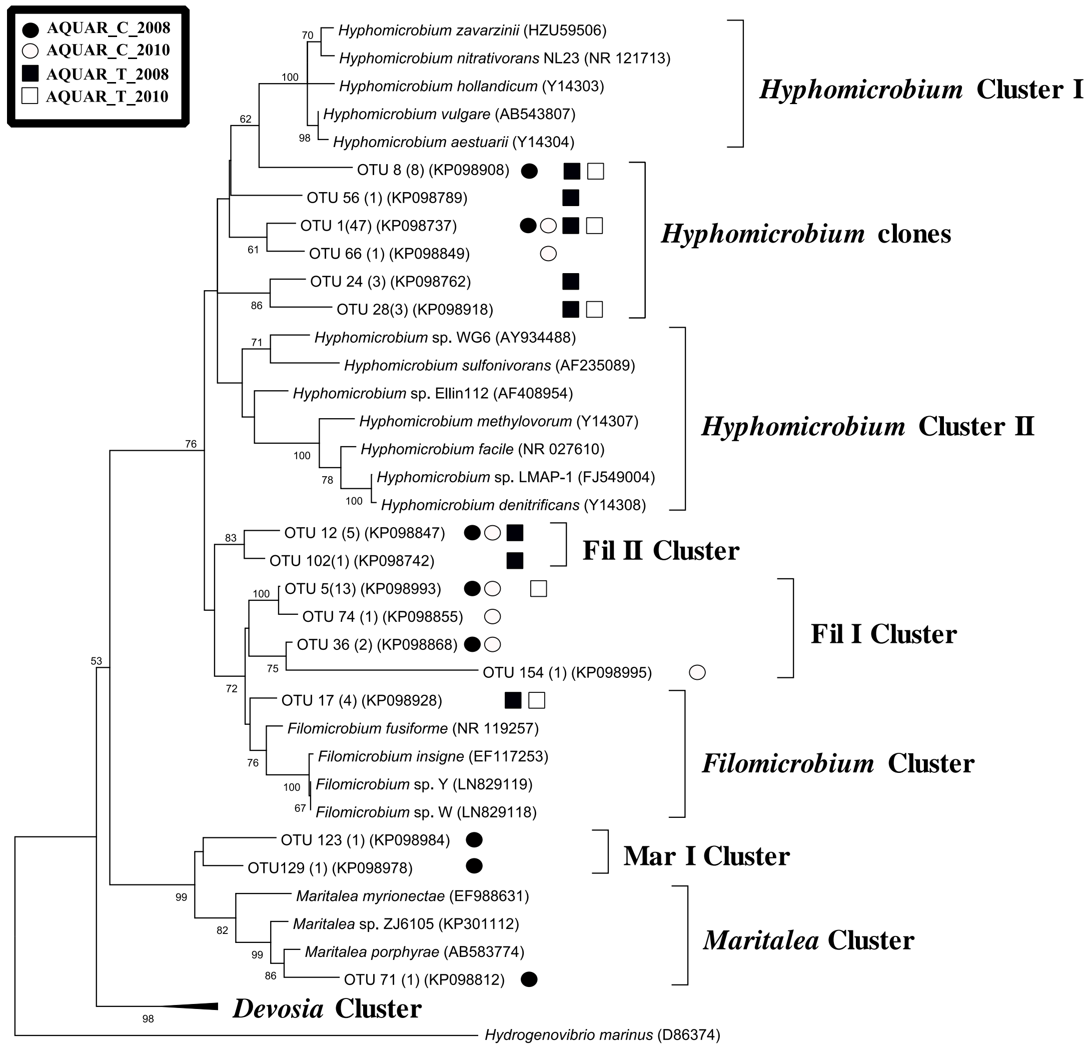

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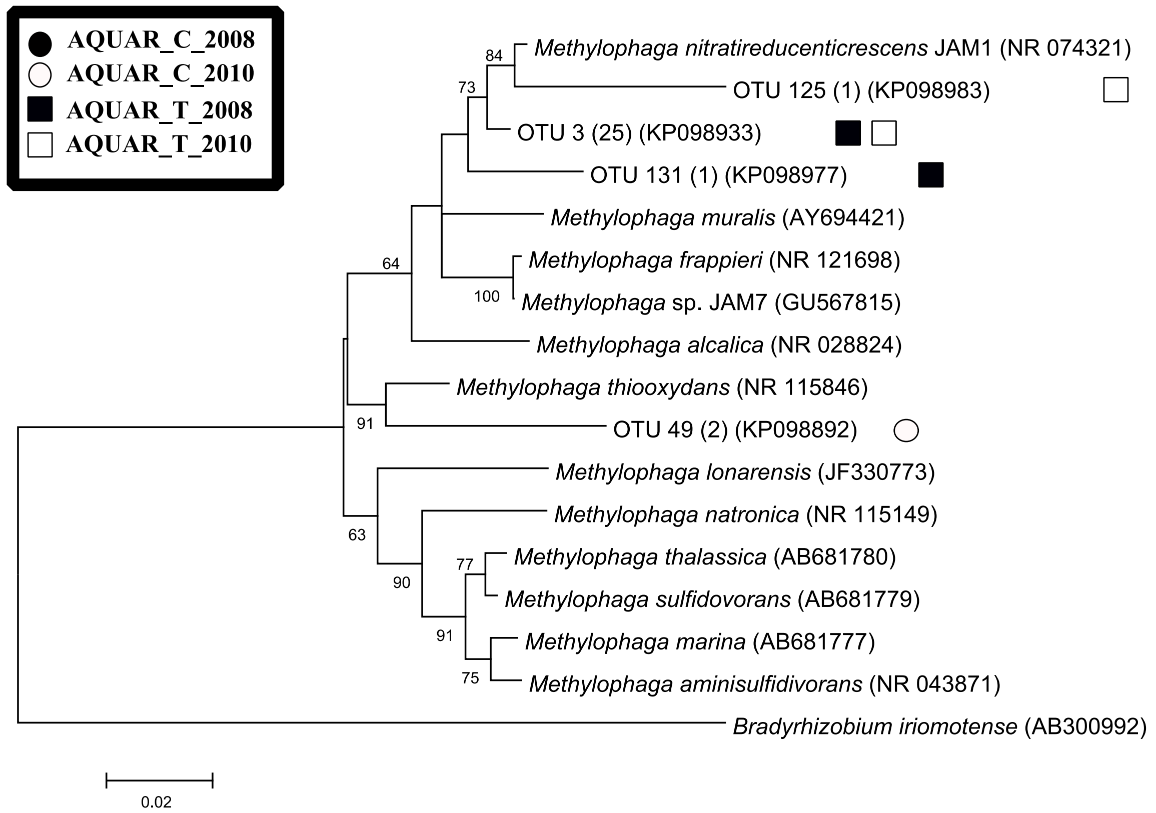

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735 Journal: J Ind Microbiol Biotechnol
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737 Title: *Methylophaga* and *Hyphomicrobium* can be used as target genera in monitoring
738 saline water methanol-utilizing denitrification
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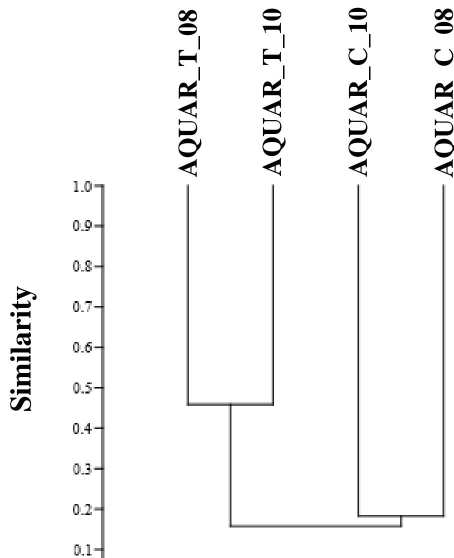

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755 **Online Resource 1** Hierarchical clustering analysis (UPGMA) of relative abundance of OTUs
756 (using Bray-Curtis distances) in 16S rRNA gene clone library analyses of AQUAR_T and
757 AQUAR_C in 2008 and 2010
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