

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

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1 **Abstract** Which bacterial taxonomic groups can be used in monitoring saline water  
2 methanol-utilizing denitrification and whether nitrate is transformed into N<sub>2</sub> in the process  
3 are unclear. Therefore, methylotrophic bacterial communities of two efficiently  
4 functioning (nitrate/nitrite reduction was 63–96 %) tropical and cool seawater reactors at a  
5 public aquarium were investigated with clone library analysis and 454 pyrosequencing of  
6 the 16S rRNA genes. Transformation of nitrate into N<sub>2</sub> was confirmed using <sup>15</sup>N labeling  
7 in incubation of carrier material from the tropical reactor. Combining the data with  
8 previous study results, *Methylophaga* and *Hyphomicrobium* were determined to be  
9 suitable target genera for monitoring the function of saline water methanol-fed  
10 denitrification systems. However, monitoring was not possible at the single species level.  
11 Interestingly, potential nitrate-reducing methylotrophs within *Filomicrobium* and closely  
12 related Fil I and Fil II clusters were detected in the reactors suggesting that they also  
13 contributed to methylotrophic denitrification in the saline environment.

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

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### 33 **Introduction**

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35 Denitrification, step-wise reduction of water-soluble nitrate ( $\text{NO}_3^-$ ) via nitrite ( $\text{NO}_2^-$ ) to

36 gaseous nitric oxide (NO), nitrous oxide ( $\text{N}_2\text{O}$ ) and di-nitrogen ( $\text{N}_2$ ) 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  $\text{NO}_3^-$  to  $\text{NO}_2^-$  or to  $\text{N}_2\text{O}$ , and only

40 some bacterial species are capable of the whole denitrification chain from  $\text{NO}_3^-$  to  $\text{N}_2$  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  $\text{NO}_x$  reduction, that is, the disappearance of  $\text{NO}_3^-$

50 / $\text{NO}_2^-$  [23]. This indirectly measured denitrification rate denotes the conversion of water-

51 soluble  $\text{NO}_x^-$  into gaseous forms, but the proportions of NO,  $\text{N}_2\text{O}$  and  $\text{N}_2$  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  $\text{N}_2\text{O}$

54 production [17, 27], and the conversion of  $\text{NO}_3^-$  to  $\text{N}_2$  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  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and *Hyphomicrobium* to complete the denitrification by converting  $\text{NO}_2^-$   
72 into  $\text{N}_2$  [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  $\text{NO}_x^-$  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  $\text{NO}_3^-$  to  $\text{N}_2$  using batch incubations and the  $^{15}\text{N}$  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**

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### 90 **Sampling site and sampling for molecular microbiology**

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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<sup>3</sup> with 27–34 ppt salinity. Both circulation systems have their own

96 fluidized-bed type denitrification reactors that receive  $\text{NO}_3^-$ -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.

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### 107 **Denitrification measurements**

108

109 The  $\text{NO}_x^-$  concentrations inside the reactors, near the reactor outlet ( $\text{NO}_{x^- \text{out}}$ ) and in the  
110 inflow water feeding the reactors ( $\text{NO}_{x^- \text{inflow}}$ ) 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 ( $\text{Met}_f$ , 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  $\text{NO}_x^-$  loads  
117 ( ${}_L\text{NO}_{x^- \text{inflow}}$  and  ${}_L\text{NO}_{x^- \text{out}}$ , mmol N/h) were calculated using the  $W_f$  and  $\text{NO}_x^-$  concentration  
118 values for these time periods. Denitrification was then estimated indirectly as the relative  
119  $\text{NO}_x^-$  reduction (%):

$$120 \quad \text{NO}_x^- \text{ reduction} = \frac{(\text{NO}_{x^- \text{inflow}} - \text{NO}_{x^- \text{out}})}{\text{NO}_{x^- \text{inflow}}} \times 100$$

121 and as the actual  $\text{NO}_x^-$  reduction:

$$122 \quad \text{actual NO}_x^- \text{ reduction} = {}_L\text{NO}_{x^- \text{inflow}} - {}_L\text{NO}_{x^- \text{out}},$$

123 which was converted into the  $\text{NO}_x^-$  reduction rate of the carrier material ( $\mu\text{mol N}/\text{L}_{\text{car}}/\text{h}$ ).

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  $\mu\text{mol}$   
129 (~7 mg) of methanol and ~50  $\mu\text{mol}$  (~0.7 mg) of  $\text{NO}_3^-$ -N, which had a 2%  $^{15}\text{N}$  isotope  
130 label, and then shaken briefly but vigorously. The  $\text{NO}_3^-$  stock solution (0.1 M  $\text{NO}_3^-$ -N)  
131 was prepared from  $\text{NaNO}_3$  and  $\text{K}^{15}\text{NO}_3^-$  (Cambridge Isotope Laboratories, Inc., MA,  
132 USA). The total  $\text{NO}_3^-$ -N concentration after the addition was ~664  $\mu\text{M}$ , which is

133 approximately the same as the average  $\text{NO}_x^-$  inflow concentration ( $\sim 688 \mu\text{M}$ ) during the  $\sim 2$   
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  $\text{ZnCl}_2$  (1 g/mL). The concentration and  $^{15}\text{N}$  content of the  $\text{N}_2$  gas in the water was  
141 measured as in Tirola et al. [41]. Denitrification was calculated as the rate of the total  $\text{N}_2$   
142 gas accumulation and converted to the  $\text{N}_2$  production rate of the carrier material ( $\mu\text{mol}$   
143  $\text{N}/\text{L}_{\text{car}}/\text{h}$ ). In addition, reduction of  $\text{NO}_3^-$  to  $\text{N}_2$  gas was verified by the accumulation of  
144 excess  $^{15}\text{N}$ -containing  $\text{N}_2$  gas. The concentration of the excess  $^{15}\text{N}$ -containing  $\text{N}_2$  gas,  
145 [excess  $^{15}\text{N}$ ], was calculated for each sample as

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

147 where  $[\text{N}_{2\text{sample}}]$  is the  $\text{N}_2$  gas concentration in the incubated sample and the  $\text{at}\%^{15}\text{N}_{\text{sample}}$   
148 and the  $\text{at}\%^{15}\text{N}_{\text{zero}}$  are the  $^{15}\text{N}$  content (in %) of the  $\text{N}_2$  gas in the incubated and non-  
149 incubated (0 time point) samples, respectively.

150

### 151 **Molecular microbiological analyses**

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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 CCGTCAATTCMTTGGAGTTT-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**

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

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<sub>2</sub> was produced and NO<sub>3</sub><sup>-</sup> was transformed  
216 into N<sub>2</sub> by microbes in AQUAR\_T (Fig. 2). The estimated N<sub>2</sub> production rate of the  
217 carrier material was 56.4 μmol N/L<sub>car</sub>/h.

218  
219 **Variation in microbial community structure**  
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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  $\text{NO}_x^-$  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  $\text{NO}_x^-$ . In contrast, the average actual  
281  $\text{NO}_x^-$  reduction rates were within the lower end of the range (300–9000  $\mu\text{mol N/L}_{\text{car}}/\text{h}$ )  
282 measured at other systems [21, 23, 24]. The differences in the actual  $\text{NO}_x^-$  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  $\text{O}_2$  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  $\text{NO}_3^-$  is reduced to  $\text{N}_2$ . However, the  $\text{N}_2$   
289 production measured in the batch tests was only 6–7 % of the actual  $\text{NO}_x^-$  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  $\text{NO}_3^-$  and the bacteria on the carrier  
292 material. However, the possible formation of other gases,  $\text{N}_2\text{O}$  [17] and  $\text{NO}$ , 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 nitratorans*, which dominated in a previously studied system [3, 21],  
302 were not found at all in the AQUAR reactors, and *Methylophaga nitratreducenticrescens*,  
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  $\text{Met}_f$  and methanol: $\text{NO}_x\text{-N}_{\text{inflow}}$  before sampling).

319 Previous studies also suggest that *Filomicrobium* participate in denitrification by  
320 dissimilatory reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  [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  $\text{NO}_3^-$  to  $\text{NO}_2^-$  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  $\text{NO}_2^-$  reductase gene, although it was truncated  
325 in *M. nitratireducenticrescens* [42]. In addition, *M. nitratireducenticrescens* had genes for  
326 NO and  $\text{N}_2\text{O}$  reduction [42]. This suggests that *Methylophaga* species coupling  
327 methylotrophy to  $\text{NO}_2^-$ , NO and  $\text{N}_2\text{O}$  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,  $\text{NO}_3^-$  significantly affected the growth and activity  
336 of *Hyphomicrobium* species with *H. nitrativorans* growing and denitrifying at higher  $\text{NO}_3^-$

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<sub>2</sub> 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<sub>2</sub>] < 0.1 mg/L, below the detection limit of the O<sub>2</sub> probe) that  
344 prevail inside the reactors [9]. Thus, variation in the O<sub>2</sub> 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<sub>2</sub>.  
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<sub>2</sub> and by aerobically converting methanol to organic substrates utilizable by  
350 non-methylotrophic denitrifiers. However, in addition to varying NO<sub>3</sub><sup>-</sup> and O<sub>2</sub>,  
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<sub>f</sub> and methanol:NO<sub>x</sub><sup>-</sup>  
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**

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392 This study confirmed that  $\text{NO}_3^-$  was transformed into  $\text{N}_2$  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  $\text{NO}_3^-$ -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  $\text{NO}_3^-$  to  
403  $\text{NO}_2^-$  or reduce  $\text{NO}_2^-$ , NO and  $\text{N}_2\text{O}$ , 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.

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

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564 Figure captions:  
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567 **Fig. 1** Concentration of  $\text{NO}_x^-$  in the inflow and the outflow and the relative  $\text{NO}_x^-$  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  $\text{N}_2$  gas  
573 and excess  $^{15}\text{N}$  (in  $\text{N}_2$  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  $^{15}\text{N}$ -labeled  $\text{NO}_3^-$ . 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  $\text{N}_2$  and  $^{15}\text{N}$   
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<sub>x</sub><sup>-</sup> reduction rates, NO<sub>x</sub><sup>-</sup> load rate ( $L_{NO_x^-}$  inflow) and inflow methanol:NO<sub>x</sub><sup>-</sup>-N ratio as well as  
 620 operator-controlled rates of methanol addition (Met<sub>f</sub>), saccharose addition and water flow (W<sub>f</sub>) 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	
	2008	2010	2008	2010
Year	2008	2010	2008	2010
Actual NO <sub>x</sub> <sup>-</sup> reduction (μmol N/L <sub>car</sub> /h)	580 <sup>a</sup> (60–1020)	890 (740–1090)	540 <sup>b</sup> (290–700)	910 (880–950)
Met <sub>f</sub> (mmol C/h)	158	98	185	85
Saccharose addition (mmol C/h)			67	
W <sub>f</sub> (L/h)	120	70	110	93
$L_{NO_x^-}$ inflow (mmol N/h)	76 (70–80)	66 (54–76)	51 (47–54)	66 (63–68)
Methanol:NO <sub>x</sub> <sup>-</sup> -N <sub>inflow</sub> (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 <sup>a</sup> Excluding values on 4 November 2008 (pumping and carbon dosage problems), the average actual NO<sub>x</sub><sup>-</sup> reduction rate is 840 μmol N/L<sub>car</sub>/h (range 660–  
 625 1020)

626 <sup>b</sup> Excluding values on 4 November 2008 (pumping and carbon dosage problems), the average actual NO<sub>x</sub><sup>-</sup> reduction rate is 660 μmol N/L<sub>car</sub>/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) <sup>a</sup>	AQUAR_T	AQUAR_C	AQUAR_C
Year (number of sequences)	2008 (72)	2008 (1849)	2010 (67)	2008 (59)	2010 (63)
Frequency (%) <sup>b</sup> :					
Putative methylotrophic	74	38	56	15	30
<i>Alphaproteobacteria</i>	64	35	46	69	30
<i>Hyphomicrobiaceae</i>	60	22	31	20	27
<i>Hyphomicrobium</i>	53	–	25	5	8
<i>Filomicrobium</i> cluster	3	–	3	–	–
Fil I cluster	–	–	3	7	17
Fil II cluster	4	–	–	3	2
<i>Maritalea</i> cluster	–	–	–	2	–
Mar I cluster	–	–	–	3	–
<i>Rhodobacteraceae</i>	1	2	–	32	–
<i>Phyllobacteriaceae</i>	–	4	9	2	3
<i>Gammaproteobacteria</i>	22	29	28	7	8
<i>Piscirickettsiaceae</i>	14	16	25	–	3
<i>Methylophaga</i>	14	16	25	–	3
incertae sedis	–	<1	–	–	3
unclassified	8	13	3	7	–
<i>Deltaproteobacteria</i>	3	2	7	–	5
<i>Acidobacteria</i>	–	–	–	–	3
<i>Actinobacteria</i>	1	<1	1	3	2
<i>Bacteroidetes</i>	3	7	6	19	14
<i>Chloroflexi</i>	3	3	1	–	19
<i>Deferribacteres</i>	–	–	–	–	2
<i>Planctomycetes</i>	–	4	–	–	10
<i>Spirochaetes</i>	–	<1	–	–	2
Unclassified + others	4	20	11	2	5

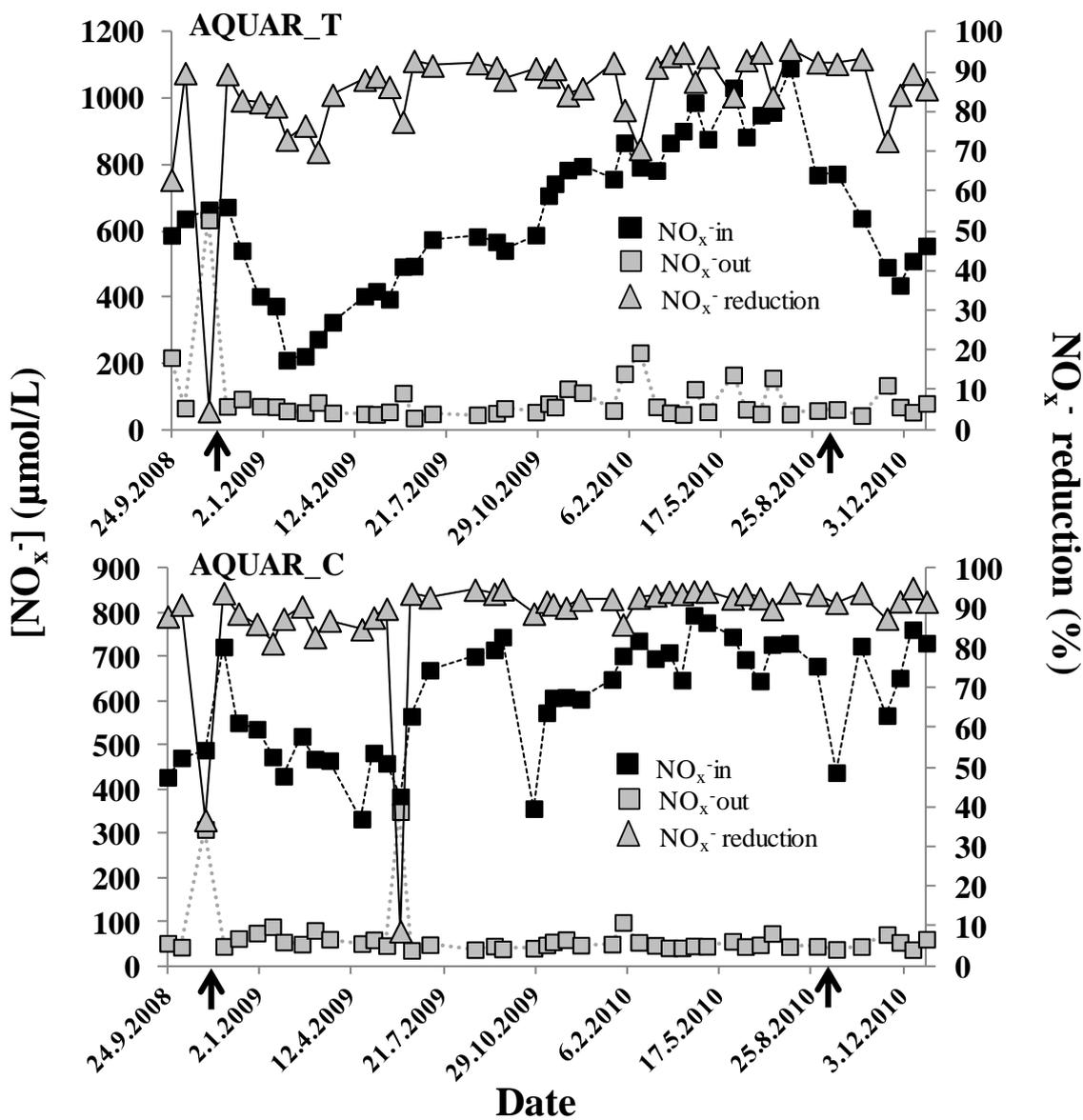
<sup>a</sup> Libraries generated using 454 pyrosequencing

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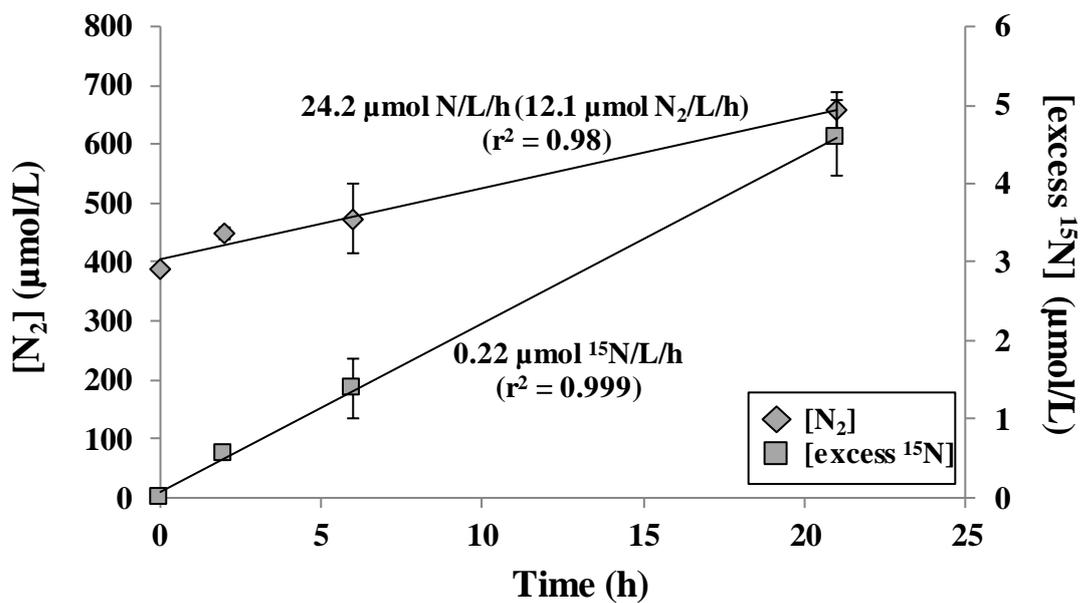
<sup>b</sup> Classification was performed using the RDP database in Mothur and with phylogenetic tree analysis (Fig. 3). Assignment to methylotrophic function was based on previous literature. Frequencies are given as percentages (%) of the total number of sequences in a sample

673 Figure 1  
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685 Figure 2  
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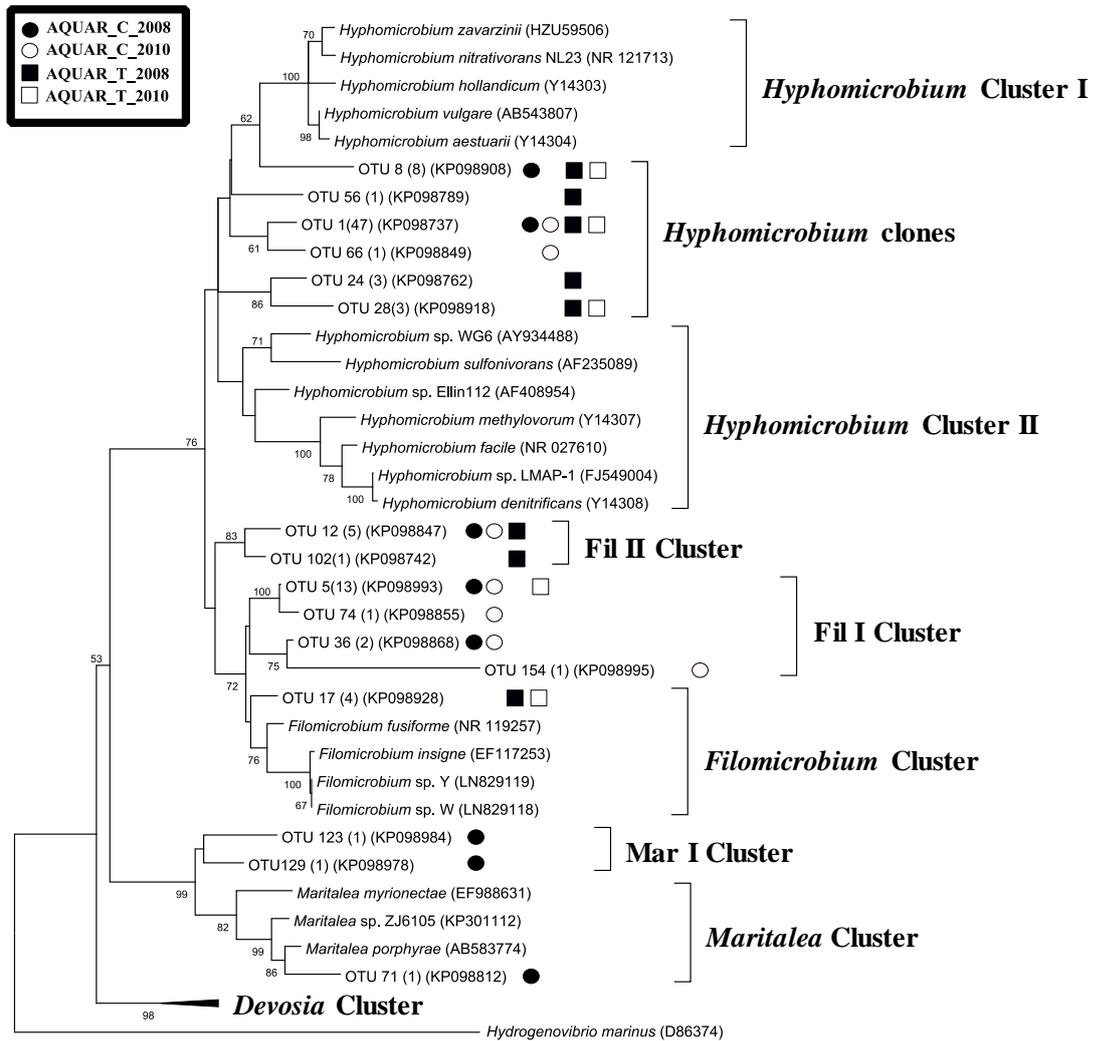
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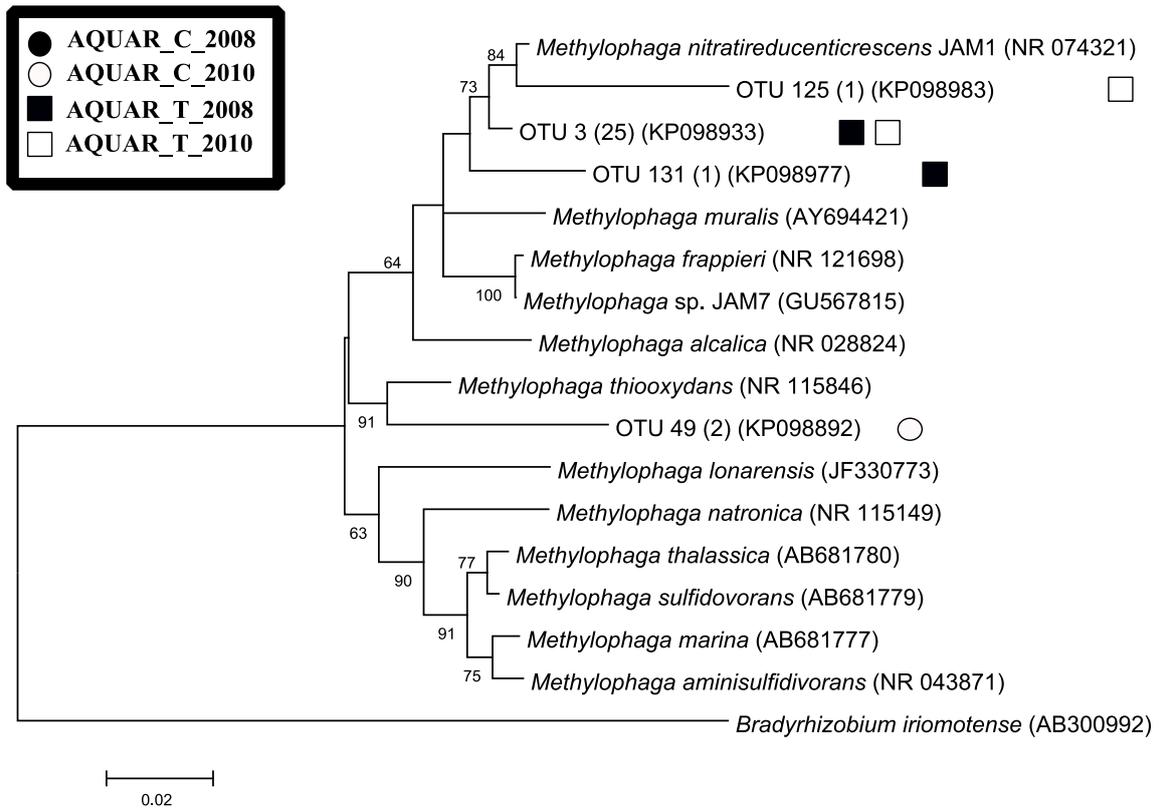
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733 Online Resources

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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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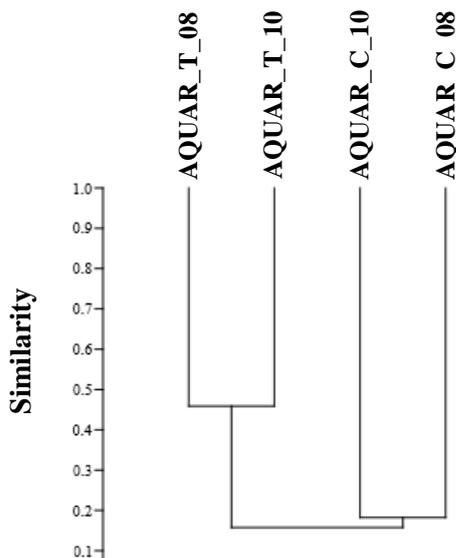
749 email-address: antti.rissanen@tut.fi

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752 Online Resource 1

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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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