

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

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Acknowledgements

We thank H. Devlin, B. Thamdrup and S. Hallin for comments on an earlier version of this manuscript. This study was funded by Maa- ja Vesitekniikan Tuki ry (A.J.R.) and the Academy of Finland (projects 286642 to A.J.R. and 120089 to M.T.), as well as the European Research Council (ERC) Consolidator project 615146 (M.T.).

1 **Abstract** Which bacterial taxonomic groups can be used in monitoring saline water
2 methanol-utilizing denitrification and whether nitrate is transformed into N₂ 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 N₂ was confirmed using ¹⁵N 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 (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**

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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 m³ 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.

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107 **Denitrification measurements**

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109 The 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 (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 (${}_L\text{NO}_{x^- \text{inflow}}$ and ${}_L\text{NO}_{x^- \text{out}}$, 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 (%):

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

121 and as the actual 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 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 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 $\text{K}^{15}\text{NO}_3^-$ (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 ($\sim 688 \mu\text{M}$) 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 Tirola 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 $\text{N}/\text{L}_{\text{car}}/\text{h}$). 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

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

147 where $[\text{N}_{2\text{sample}}]$ is the 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}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**

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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**

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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 N₂ was produced and NO₃⁻ was transformed
216 into N₂ by microbes in AQUAR_T (Fig. 2). The estimated N₂ 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. nitratreducenticrescens*, 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 $\mu\text{mol N/L}_{\text{car}}/\text{h}$)
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 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 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 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 *NO₂⁻ 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. zavarzini* 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, O₂ 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 ([O₂] < 0.1 mg/L, below the detection limit of the O₂ probe) that
344 prevail inside the reactors [9]. Thus, variation in the O₂ 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 O₂.
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 O₂ and by aerobically converting methanol to organic substrates utilizable by
350 non-methylotrophic denitrifiers. However, in addition to varying NO₃⁻ and O₂,
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**

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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.

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

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References

1. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389-3402
2. Auclair J, Lépine F, Parent S, Villemur R (2010) Dissimilatory reduction of nitrate in seawater by a *Methylophaga* strain containing two highly divergent *narG* sequences. *ISME J* 4:1302-1313
3. Auclair J, Parent S, Villemur R (2012) Functional diversity in the denitrifying biofilm of the methanol-fed marine denitrification system at the Montreal Biodome. *Microb Ecol* 63:726-735
4. 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
5. 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
6. Bernet N, Delgenes N, Akunna JC, Delgenes JP, Moletta R (2000) Combined anaerobic-aerobic SBR for the treatment of piggery wastewater. *Water Res* 34:611-619
7. Boden R, Ferriera S, Johnson J, Kelly DP, Murrell JC, Schäfer H (2011) Draft genome sequence of the chemolithoheterotrophic, halophilic methylotroph *Methylophaga thiooxydans* DMS010. *J Bacteriol* 193:3154-3155
8. Chistoserdova L, Kalyuzhnaya MG, Lidstrom ME (2009) The expanding world of methylotrophic organisms. *Annu Rev Microbiol* 63:477-499
9. Dernjatin M (2008) Leijuva-alustaisen denitrifikaatioprosessin optimointi suljetussa kierto-vesijärjestelmässä. MSc thesis, University of Helsinki, Helsinki, Finland (in Finnish)
10. 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
11. Gittel A, Kofoed MVW, Sørensen KB, Ingvorsen K, Schramm A (2012) Succession of *Deferribacteres* and *Epsilonproteobacteria* through a nitrate-treated high-temperature oil production facility. *Syst Appl Microbiol* 35:165-174
12. Hagman M, Nielsen JL, Nielsen PH, Jansen J la C (2008) Mixed carbon sources for nitrate reduction in activated sludge-identification of bacteria and process activity studies. *Water Res* 42:1539-1546
13. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontol Electron* 4
14. Henriques AC, De Marco P (2015) Complete genome sequences of two strains of “*Candidatus* Filomicrobium marinum,” a methanesulfonate-degrading species. *Genome Announc* 3:e00160-15
15. Hung WL, Wade WG, Boden R, Kelly DP, Wood AP (2011) Facultative methylotrophs from the human oral cavity and methylotrophy in strains of *Gordonia*, *Leifsonia*, and *Microbacterium*. *Arch Microbiol* 193:407-417
16. Jensen S, Neufeld JD, Birkeland NK, Hovland M, Murrell JC (2008) Methane assimilation and trophic interactions with marine *Methylomicrobium* in deep-water coral reef sediment off the coast of Norway. *FEMS Microbiol Ecol* 66:320-330

- 459 17. Kampschreur MJ, Temmink H, Kleerebezem R, Jetten MSM, van Loosdrecht,
460 MCM (2009) Nitrous oxide emission during wastewater treatment. *Water Res*
461 43:4093-4103
- 462 18. Koch G, Siegrist H (1997) Denitrification with methanol in tertiary filtration. *Water*
463 *Res* 31:3029-3038
- 464 19. Kolb S (2009) Aerobic methanol- oxidizing *Bacteria* in soil. *FEMS Microbiol Lett*
465 300:1-10
- 466 20. Kumaresan D, Wischer D, Hillebrand-Voiculescu AM, Murrell JC (2015) Draft
467 genome sequences of facultative methylotrophs, *Gemmobacter* sp. strain LW1 and
468 *Mesorhizobium* sp. strain 1M-11, isolated from Movile Cave, Romania. *Genome*
469 *Announc* 3:e01266-15
- 470 21. Labbé N, Juteau P, Parent S, Villemur R (2003) Bacterial diversity in a marine
471 methanol-fed denitrification reactor at the Montreal Biodome, Canada. *Microb Ecol*
472 46:12-21
- 473 22. Labbé N, Laurin V, Juteau P, Parent S, Villemur R (2007) Microbiological
474 community structure of the biofilm of a methanol-fed, marine denitrification system,
475 and identification of the methanol-utilizing micro-organisms. *Microb Ecol* 53:621-
476 630
- 477 23. Labbé N, Parent S, Villemur R (2003) Addition of trace metals increases
478 denitrification rate in closed marine systems. *Water Res* 37:914-920
- 479 24. Labelle M-A, Juteau P, Jolicoeur M, Villemur R, Parent S, Comeau Y (2005)
480 Seawater denitrification in a closed mesocosm by a submerged moving bed biofilm
481 reactor. *Water Res* 39:3409-3417
- 482 25. Lee PG, Lea RN, Dohmann E, Prebilsky W, Turk PE, Ying H, Whitson JL (2000)
483 Denitrification in aquaculture systems: an example of a fuzzy logic control problem.
484 *Aquacult Eng* 23:37-59
- 485 26. Lemmer H, Zaglauer A, Metzner G (1997) Denitrification in a methanol-fed fixed-
486 bed reactor. Part 1: Physico-chemical and biological characterization. *Water Res*
487 31:1897-1902
- 488 27. Lu H, Chandran K, Stensel D (2014) Microbial ecology of denitrification in
489 biological wastewater treatment. *Water Res* 64:237-254
- 490 28. Madhaiyan M, Poonguzhali S, Lee J-S, Lee KC, Sundaram S (2010) *Flavobacterium*
491 *glycines* sp. nov., a facultative methylotroph isolated from the rhizosphere of
492 soybean. *Int J Syst Evol Microbiol* 60:2187-2192
- 493 29. Martineau C, Villeneuve C, Mauffrey F, Villemur R (2013) *Hyphomicrobium*
494 *nitratorans* sp. nov., isolated from the biofilm of a methanol-fed denitrification
495 system treating seawater at the Montreal Biodome. *Int J Syst Evol Microbiol*
496 63:3777-3781
- 497 30. Martineau C, Mauffrey F, Villemur R (2015) Comparative analysis of denitrifying
498 activities of *Hyphomicrobium nitratorans*, *Hyphomicrobium denitrificans* and
499 *Hyphomicrobium zavarzinii*. *Appl Environ Microbiol* 81:5003-5014
- 500 31. Neufeld JD, Schäfer H, Cox MJ, Boden R, McDonald IR, Murrell JC (2007) Stable-
501 isotope probing implicates *Methylophaga* spp and novel *Gammaproteobacteria* in
502 marine methanol and methylamine metabolism. *ISME J* 1:480-491
- 503 32. Osaka T, Shirotani K, Yoshie S, Tsuneda S (2008) Effects of carbon source on
504 denitrification efficiency and microbial community structure in a saline wastewater
505 treatment process. *Water Res* 42:3709-3718
- 506 33. Peura S, Eiler A, Bertilsson S, Nykänen H, Tirola M, Jones RI (2012) Distinct and
507 diverse anaerobic bacterial communities in boreal lakes dominated by candidate
508 division OD1. *ISME J* 6:1640-1652

- 509 34. Radajewski S, Webster G, Reay DS, Morris SA, Ineson P, Nedwell DB, Prosser JI,
510 Murrell JC (2002) Identification of active methylotroph populations in an acidic
511 forest soil by stable-isotope probing. *Microbiology* 148:2331-2342
- 512 35. Rainey FA, Ward-Rainey N, Gliesche CG, Stackebrandt, E (1998) Phylogenetic
513 analysis and intrageneric structure of the genus and the related genus *Filomicrobium*.
514 *Int J Syst Bacteriol* 48:635-639
- 515 36. Rissanen AJ, Kurhela E, Aho T, Oittinen T, Tirola M (2010) Storage of
516 environmental samples for guaranteeing nucleic acid yields for molecular
517 microbiological studies. *Appl Microbiol Biotechnol* 88:977-984
- 518 37. Scheuner C, Tindall BJ, Lu M, Nolan M, Lapidus A, Cheng J-F, Goodwin L, Pitluck
519 S, Huntemann M, Liolios K, Pagani I, Mavromatis K, Ivanova N, Pati A, Chen A,
520 Palaniappan K, Jeffries CD, Hauser L, Land M, Mwrchia R, Rohde M, Abt B,
521 Detter JC, Woyke T, Eisen JA, Markowitz V, Hugenholtz P, Göker M, Kyrpides
522 NC, Klenk H-P (2014) Complete genome sequence of *Planctomyces brasiliensis*
523 type strain (DSM 5305T), phylogenomic analysis and reclassification of
524 *Planctomyces* including the descriptions of *Gimesia* gen. nov., *Planctopirus* gen.
525 nov. and *Rubinisphaera* gen. nov. and emended descriptions of the order
526 *Planctomycetales* and the family *Planctomycetaceae*. *Stand Genomic Sci* 9:10
- 527 38. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski
528 RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van
529 Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent,
530 community-supported software for describing and comparing microbial
531 communities. *Appl Environ Microbiol* 75:7537-7541
- 532 39. Shoun H, Kano M, Baba I, Takaya N, Matsuo M (1998) Denitrification by
533 Actinomycetes and purification of dissimilatory nitrite reductase and azurin from
534 *Streptomyces thioluteus*. *J Bacteriol* 180:4413-4415
- 535 40. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5:
536 Molecular evolutionary genetics analysis using maximum likelihood, evolutionary
537 distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731-2739
- 538 41. Tirola MA, Rissanen AJ, Sarpakunnas M, Arvola L, Nykänen H (2011) Stable
539 isotope profiles of nitrogen gas indicate denitrification in oxygen-stratified humic
540 lakes. *Rapid Commun Mass Spectrom* 25:1497-1502
- 541 42. Villeneuve C, Martineau C, Mauffrey F, Villemur R (2013) *Methylophaga*
542 *nitratireducenticrescens* sp. nov. and *Methylophaga frappieri* sp. nov., isolated from
543 the biofilm of the methanol-fed denitrification system treating the seawater at the
544 Montreal Biodome. *Int J Syst Evol Microbiol* 63:2216-2222
- 545 43. Wagner M, Loy A, Nogueira R, Purkhold U, Lee N, Daims H (2002) Microbial
546 community composition and function in wastewater treatment plants. *Antonie van*
547 *Leeuwenhoek* 81:665-680
- 548 44. Ward NL, Challacombe JF, Janssen PH, Henrissat B, Countinho PM, Wu M, Xie G,
549 Haft DH, Sait M, Badger J, Barabote RD, Bradley B, Brettin TS, Brinkac LM, Bruce
550 D, Creasy T, Daugherty SC, Davidsen TM, DeBoy RT, Detter JC, Dodson RJ,
551 Durkin AS, Ganapathy A, Gwinn-Giglio M, Han CS, Khouri H, Kiss H, Kothari SP,
552 Madupu R, Nelson KE, Nelson WC, Paulsen I, Penn K, Ren Q, Rosovitz MJ,
553 Selengut JD, Shrivastava S, Sullivan SA, Tapia R, Thompson LS, Watkins KL,
554 Yang Q, Yu C, Zafar N, Zhou L, Kuske CR (2009) Three genomes from the phylum
555 *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils.
556 *Appl Environ Microbiol* 75:2046-2056
- 557 45. Wu X-L, Yu S-L, Gu J, Zhao G-F, Chi C-Q (2009) *Filomicrobium insigne* sp. nov.,
558 isolated from an oil-polluted saline soil. *Int J Syst Evol Microbiol* 59:300-305

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

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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 ($L_{NO_x^-}$ 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	
	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
$L_{NO_x^-}$ 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
<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

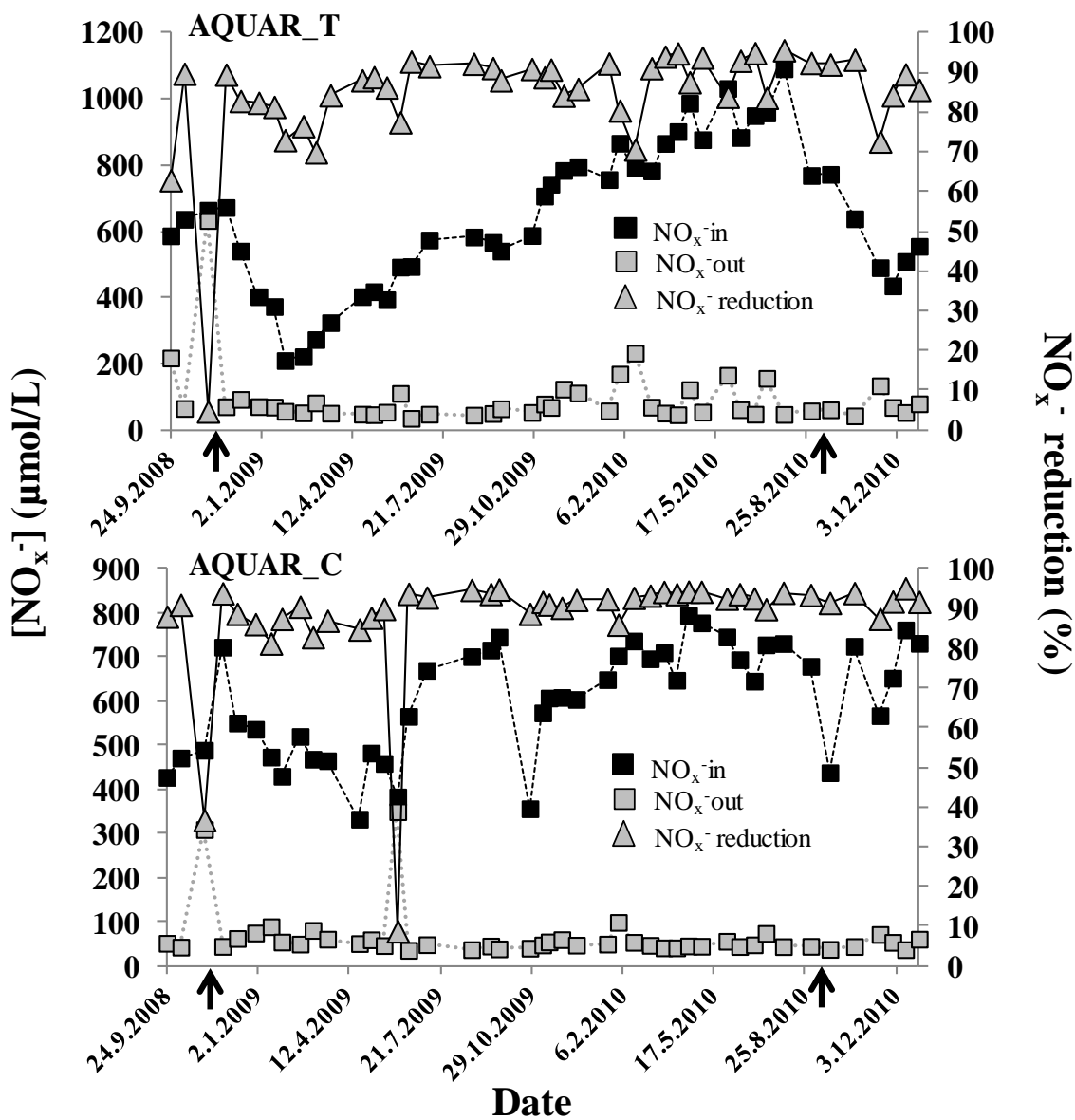
^a Libraries generated using 454 pyrosequencing

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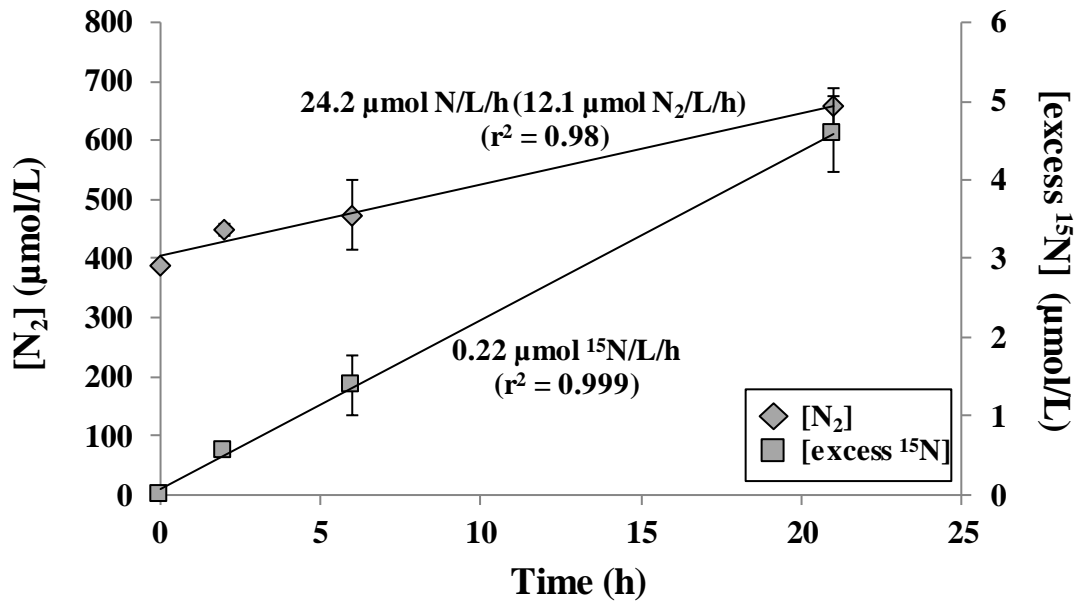
^b Classification was performed using the RDP database in Mothur and with phylogenetic tree analysis (Fig. 3). Assignment to methylotrophic function was based on previous literature. Frequencies are given as percentages (%) of the total number of sequences in a sample

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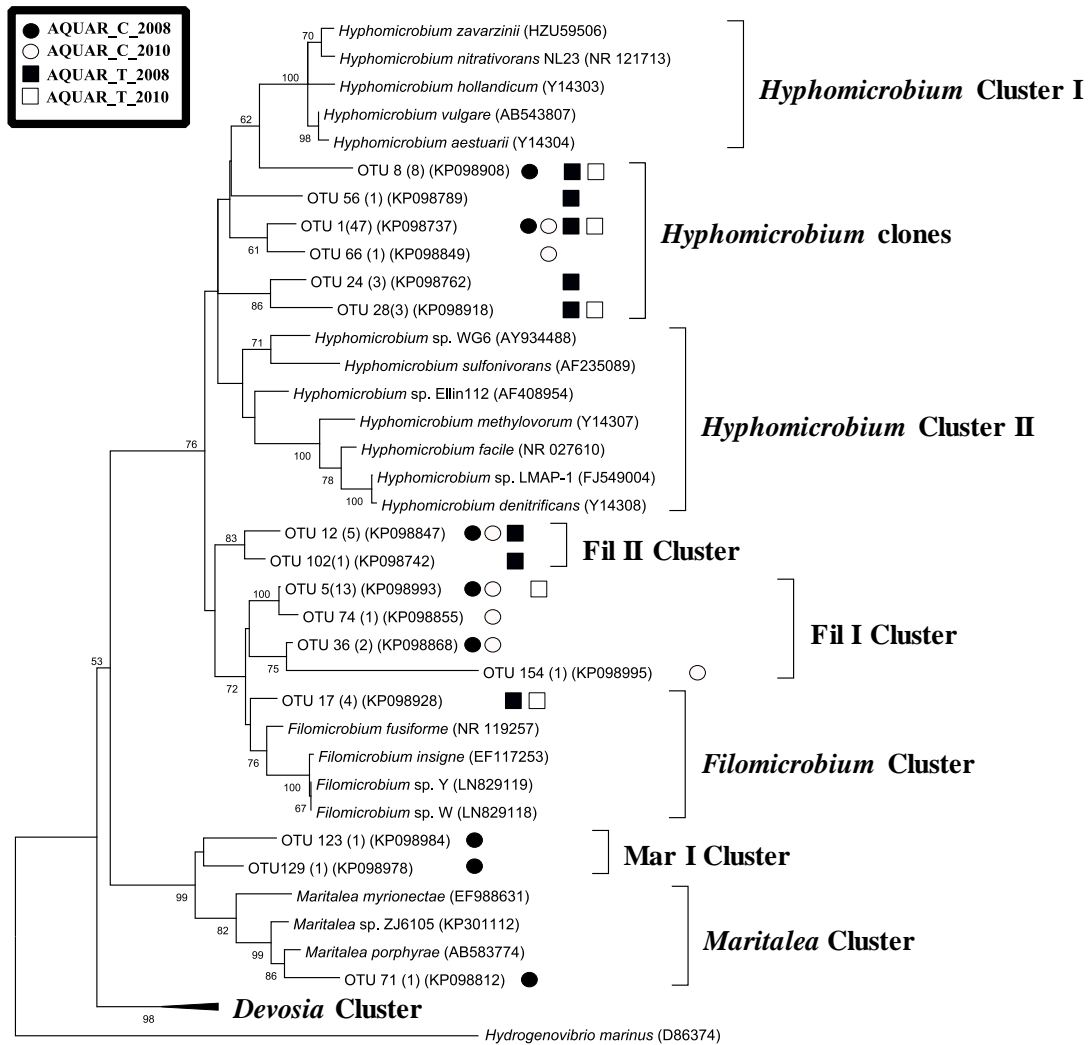
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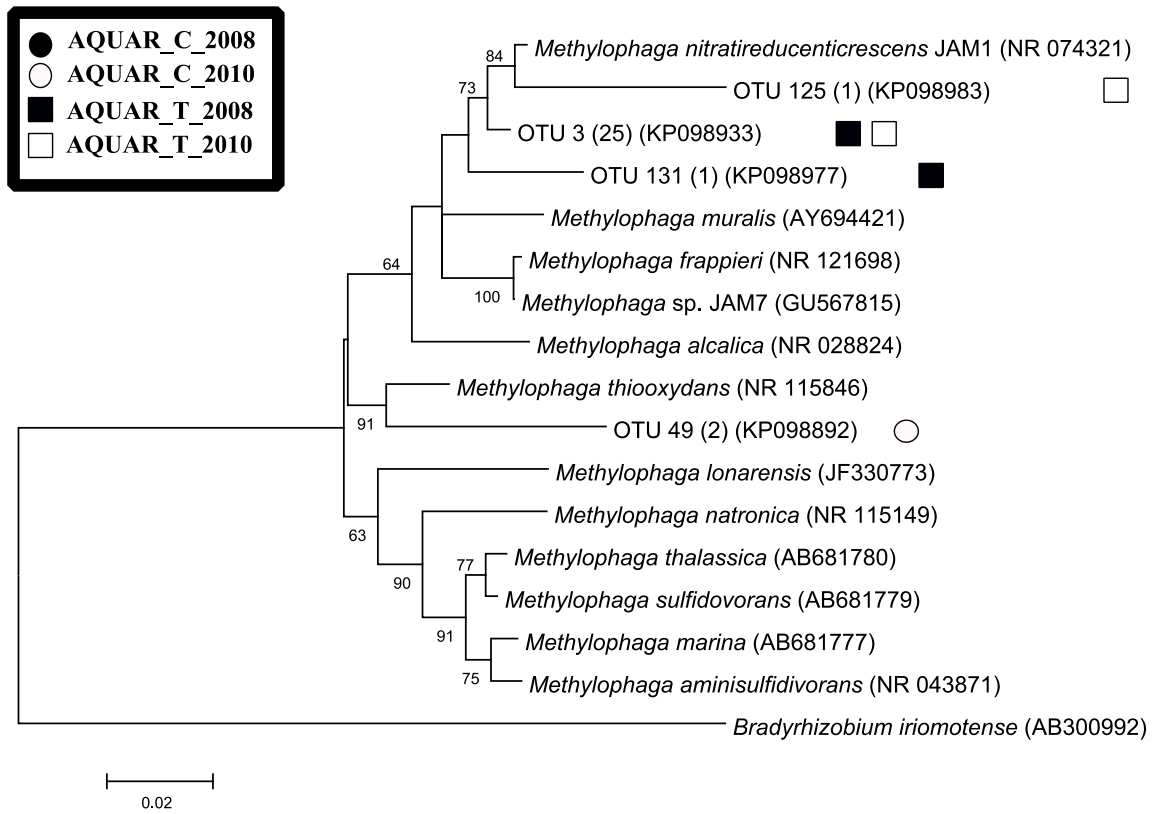
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737 Title: *Methylophaga* and *Hyphomicrobium* can be used as target genera in monitoring
738 saline water methanol-utilizing denitrification

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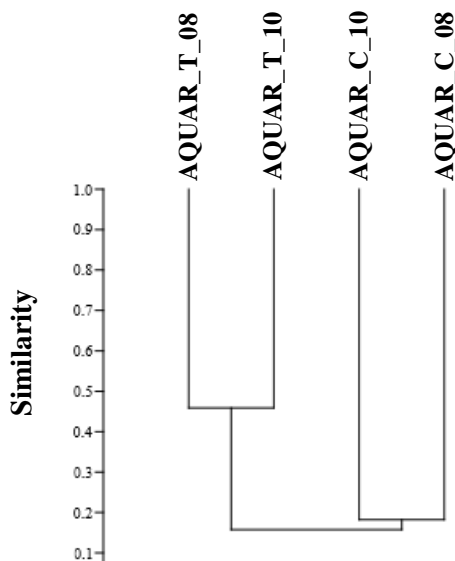
749 email-address: antti.rissanen@tut.fi

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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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