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

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

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

Introduction

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

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

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

This study investigated NO_x^- reduction and the bacterial communities of two methanol-utilizing denitrifying bioreactors in a marine fish aquarium operating at two

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

Materials and methods

Sampling site and sampling for molecular microbiology

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

Denitrification measurements

The NO_x^- concentrations inside the reactors, near the reactor outlet ($NO_{x\text{-}out}^-$) and in the inflow water feeding the reactors ($NO_{x\text{-}inflow}^-$) were measured using Spectroquant® nitrate and nitrite test kits (Merck Millipore, Germany) with a Spectroquant® Nova 60 photometer (Merck Millipore, Germany) from both reactors 1 to 2 times per month for 2.3 years (time period 24 September 2008 to 28 December 2010). The methanol addition (Met_f, mmol/h) and water flow (W_f, L/h) rates were adjusted by the operators and for this study reported for 1.5 to 2 month periods before bacterial sampling in 2008 and 2010 (24 September to 10 November 2008 and 13 July to 8 September 2010). Hourly NO_x^- loads ($_LNO_{x\text{-}inflow}^-$ and $_LNO_{x\text{-}out}^-$, mmol N/h) were calculated using the W_f and NO_x^- concentration values for these time periods. Denitrification was then estimated indirectly as the relative NO_x^- reduction (%):

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

and as the actual NO_x^- reduction:

$$actual\ NO_{x}^{-}\ reduction = {}_{L}NO_{x\text{-}inflow}^{-} - {}_{L}NO_{x\text{-}out}^{-},$$

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

Direct denitrification measurement was performed with bottle incubation in 2010. Batches of oolitic sand (~36 mL) collected from the middle of the filter bed in AQUAR_T were put in 120 mL glass bottles (11 bottles altogether). The bottles were filled with anoxic reactor water by submerging them in the reactor, and while submerged, they were closed with caps that had butyl rubber stoppers. Each bottle was injected with ~219 µmol (~7 mg) of methanol and ~50 µmol (~0.7 mg) of NO_3^--N, which had a 2% ^{15}N isotope label, and then shaken briefly but vigorously. The NO_3^- stock solution (0.1 M NO_3^--N) was prepared from $NaNO_3$ and $K^{15}NO_3^-$ (Cambridge Isotope Laboratories, Inc., MA, USA). The total NO_3^--N concentration after the addition was ~664 µM, which is

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

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

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

Molecular microbiological analyses

DNA was extracted from 0.5–0.6 g of frozen oolitic sand from each sample using glass bead beating and phenol–chlorophorm extraction, which was followed by isopropanol-NaCl precipitation (pH 8) and dissolution of the DNA pellet in TE buffer.

PCR for the clone library analyses of the 16S rRNA genes was performed with primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) / 907R (5´-CCGTCAATTCMTTTGAGTTT-3´) as previously described [36] but using the following

program: initial denaturation at 95 °C for 5 min and 30 cycles of amplification (94 °C for 30 s, 53 °C for 1 min, 72 °C for 3 min) and final elongation at 72 °C for 15 min. The PCR products of the replicate samples were pooled for subsequent cloning and sequencing, which was carried out as previously described [36].

To get deeper insight into the bacteria inhabiting the methanol-fed denitrification reactors, the bacterial community in AQUAR_T, which used methanol as the sole carbon source, was studied with 454 pyrosequencing. Equal amounts of nucleic acid extracts from replicate samples of AQUAR_T in 2008 were pooled before the PCR reactions. PCR amplification of the 16S rRNA genes using primers 341F (5´-CCTACGGGNGGCWGCAG-3´) / 805R (5´-GACTACHVGGGTATCTAATCC-3´), subsequent purification steps and sequencing were performed as previously described [33].

Sequence analysis

In the analysis of the clone library sequences, the Mothur program package [38] was used for sequence alignment, chimera-checking (chimera.uchime executable), classification of sequences into operational taxonomic units (OTUs; 97 % identity threshold) and taxonomic classification of the OTUs (using the Ribosomal Database Project database). Variations in the community structure among the samples were analyzed with hierarchical clustering (UPGMA linkage, Bray-Curtis distances) using PAST version 3.09 [13]. Representative sequences of the OTUs assigned to taxonomic groups of known methylotrophs derived from the previous literature [8, 19, 21, 22, 32, 45], in this case *Hyphomicrobiaceae* and *Methylophaga*, were subjected to phylogenetic tree analyses [neighbor-joining (NJ) method, Kimura-2 distances, pairwise exclusion of gaps] using Mega 5.05 [40].

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

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

Results

Functioning of denitrifying reactors

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

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 monitoring period (24 September 2008 to 28 December 2010, excluding the very low values caused by the pumping and carbon dosage problems on 4 November 2008 in both reactors and on 4 June 2009 in AQUAR_C; Fig. 1). When averaged over the 1.5 to 2 month period before the bacterial sampling, the average actual NO_x^- reduction rate

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

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

218
219 **Variation in microbial community structure**
220

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

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

The relative abundance of the putative methylotrophs was considerably higher in the warmer reactor AQUAR_T than in the cooler reactor AQUAR_C in both years (Table 2). In addition, methylotrophs were more abundant in 2010 than in 2008 in AQUAR_C whereas the opposite took place in AQUAR_T (Table 2). The relative abundance of *Methylophaga* was considerably higher in the warmer reactor AQUAR_T than in the cooler reactor AQUAR_C (Table 2). In AQUAR_C, *Methylophaga* were absent in 2008 and present at very low numbers in 2010. Correspondingly, there was a higher abundance of *Methylophaga* in 2010 than in 2008 in AQUAR_T (Table 2). *Methylophaga* in AQUAR_T had their closest relative in *M. nitratireducenticrescens*, whereas those in AQUAR_C were most closely related to *M. thiooxydans* (Fig. 4). Of the family *Hyphomicrobiaceae, Hyphomicrobium* were much more abundant in the warmer reactor AQUAR_T than in the cooler reactor AQUAR_C. There was a higher abundance of *Hyphomicrobium* in 2010 than in 2008 in AQUAR_C and vice versa in AQUAR_T (Table 2). In contrast, the bacteria of the Fil I cluster were much more abundant in AQUAR_C than in AQUAR_T (Table 2). In AQUAR_T, Fil I bacteria were absent in 2008 and present at very low abundance in 2010. Correspondingly, there was a higher abundance of Fil I bacteria in 2010 than in 2008 in AQUAR_C. Fil II cluster bacteria were present in low abundance in both reactors and were absent in AQUAR_T in 2010 (Table 2). *Filomicrobium* cluster bacteria were present in low abundance and only in AQUAR_T (Table 2; Fig. 3).

The relative abundance of other bacteria also varied between reactors and years and was generally higher in AQUAR_C than in AQUAR_T (Table 2). Within *Gammaproteobacteria*, there was a considerably large group of unclassified 16S rRNA gene sequences that probably represented clades without cultured representatives (Table 2). Of *Alphaproteobacteria* (other than methylotrophs), *Rhodobacteraceae* and

Phyllobacteriaceae as well as non-methylotrophic *Hyphomicrobiaceae* within *Maritalea* and Mar I clusters were detected (Table 2; Fig. 3). According to BLAST searches [1] of the NCBI nr-database, the detected *Rhodobacteraceae* were most closely related to the non-methylotrophic genus *Roseovarius*. Of the *Proteobacteria*, also *Deltaproteobacteria* were present. Other detected phyla were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Deferribacteres*, *Planctomycetes* and *Spirochaetes* (Table 2).

There were differences in the results of the clone library analysis and 454 pyrosequencing (Table 2). Most importantly, 454 pyrosequencing had lower resolution to detect *Alphaproteobacteria* or resolve different genera within *Hyphomicrobiaceae* and resulted in a higher proportion of unclassified bacterial sequences than the clone library analysis (Table 2). Since high taxonomic resolution was necessary to identify clusters especially within *Hyphomicrobiaceae*, the focus in this study was on the clone library results.

Discussion

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

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

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

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

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

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

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

concentrations than the other studied species, *H. zavarzinii* and *H. denitrificans* [30]. Thus, the considerably lower N loads could explain the presence and dominance of *Hyphomicrobium* species other than *H. nitrativorans* in the AQUAR reactors. Similarly, *Filomicrobium*, Fil I and Fil II bacteria could have been favored in the lower N load conditions that prevail in the AQUAR system. Furthermore, O_2 constantly enters AQUAR reactors due to the lack of a preceding deaeration step but is rapidly consumed there based on the anoxic conditions ($[O_2] < 0.1$ mg/L, below the detection limit of the O_2 probe) that prevail inside the reactors [9]. Thus, variation in the O_2 availability between the systems can also explain the differences in the bacterial communities. Part of the *Hyphomicrobium* and *Filomicrobium* OTUs in the AQUAR reactors may have been favored by O_2. Analogously, as in aerobic methane oxidation coupled with denitrification (AME-D) [46], these bacteria could have contributed to the overall denitrification performance by consuming O_2 and by aerobically converting methanol to organic substrates utilizable by non-methylotrophic denitrifiers. However, in addition to variating NO_3^- and O_2, differences in the inocula (the original bacterial community that colonized the reactors) and in the carrier materials between the reactors as well as the amendment of the trace metal solution and the higher addition of methanol in the previously studied systems [3, 21, 22] could have also affected the differences in the bacterial communities.

The lower relative abundance of methylotrophs in the cooler reactor AQUAR_C than in AQUAR_T can be first explained by the saccharose addition in 2008 leading to over-competition of non-methylotrophic organisms in AQUAR_C. In 2010, the difference can be attributed to the lower availability of methanol (lower Met_f and methanol:$NO_{x\,inflow}^-$) in AQUAR_C. Variation in the availability of methanol can also explain the lower abundance of *Hyphomicrobium* and *Methylophaga* and the higher abundance of Fil I 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

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

Conclusions

This study confirmed that NO_3 was transformed into N_2 in a functioning saline water methanol-fed denitrification system. Combining the results of this study with those of previous studies show that *Methylophaga* and *Hyphomicrobium* can serve as target genera in monitoring the function of saline water methanol-utilizing denitrification systems. However, species-level differences among the systems, stemming from differences in the physicochemical and biological conditions, prevent the determination of individual *Methylophaga* or *Hyphomicrobium* species to be used as general target species in process monitoring. Interestingly, other bacteria with potential methylotrophic and NO_3^--reducing metabolism, within *Filomicrobium* and Fil I and Fil II clusters, were found, suggesting they also contributed to saline water methylotrophic denitrification. Whether bacteria within *Methylophaga*, *Filomicrobium*, Fil I and Fil II clusters can only reduce NO_3^- to NO_2^- or reduce NO_2^-, NO and N_2O, and whether other detected bacteria are able to couple methylotrophy with denitrification in the saline water denitrification bioreactors, is still to be resolved. These studies should employ high-throughput culturing methods to isolate target organisms, as well as metagenomics and metatranscriptomics.

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

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Figure captions:

Fig. 1 Concentration of NO_x^- in the inflow and the outflow and the relative NO_x^- reduction in the denitrification reactors, AQUAR_T and AQUAR_C of the sea water aquarium from 24 September 2008 to 28 December 2010. The sampling dates for the microbial studies (10 November 2008 and 8 September 2010) are indicated with arrows

Fig. 2 Concentrations (average +/- standard deviation) and estimated production rates of N_2 gas 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 incubation (at room temperature) of the carrier material and water from AQUAR_T with methanol and ^{15}N-labeled NO_3^-. One non-incubated water sample taken from the reactor before the incubation served as the 0 time point control (n = 1). The standard deviations of the N_2 and ^{15}N concentrations at time point 2 h are very low and masked behind the symbol

Fig. 3 Phylogenetic tree (neighbor-joining method) of *Hyphomicrobiaceae* based on a comparative analysis of 16S rRNA gene sequences in clone libraries showing the phylogenetic position of the operational taxonomic units (OTUs; at 97 % sequence similarity) in AQUAR_C and AQUAR_T in 2008 and 2010 (symbols). *Hyphomicrobium* clusters I and II were previously defined by Rainey et al. [35]. The numbers in the parentheses after the OTU number indicate the number of sequences within that OTU. The numbers at the nodes indicate the percentage of occurrence in 1000 bootstrapped trees (bootstrap values > 50 % are shown)

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

number of sequences within that OTU. The numbers at the nodes indicate the percentage of occurrence in 1000 bootstrapped trees (bootstrap values > 50 % are shown)

Online Resource figure captions

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

Table 1 Average (range) (n = 3 in both years) in the actual NO_x^- reduction rates, NO_x^- load rate ($_LNO_x^-{}_{inflow}$) and inflow methanol:NO_x^--N ratio as well as 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 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)

	AQUAR_T		AQUAR_C	
Year	2008	2010	2008	2010
Actual NO_x^- reduction (µmol N/L_{car}/h)	580[a] (60–1020)	890 (740–1090)	540[b] (290–700)	910 (880–950)
Met_f (mmol C/h)	158	98	185	85
Saccharose addition (mmol C/h)			67	
W_f (L/h)	120	70	110	93
$_LNO_x^-{}_{inflow}$ (mmol N/h)	76 (70–80)	66 (54–76)	51 (47–54)	66 (63–68)
Methanol:NO_x^--N_{inflow} (mol:mol)	2.1 (2.0–2.2)	1.5 (1.3–1.8)	3.6 (3.4–3.9)	1.3 (1.3–1.4)

[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 660–1020)

[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)

635 **Table 2** Bacterial community composition (% of sequences) in denitrifying reactors of sea water aquarium (AQUAR_T and AQUAR_C) based on 16S rRNA
636 gene clone libraries and 454 pyrosequencing. Methanol was used as the sole external carbon source except for AQUAR_C in 2008, when a mixture of
637 methanol and saccharose was used during the period before microbial sampling

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

638 [a] Libraries generated using 454 pyrosequencing

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

673 Figure 1
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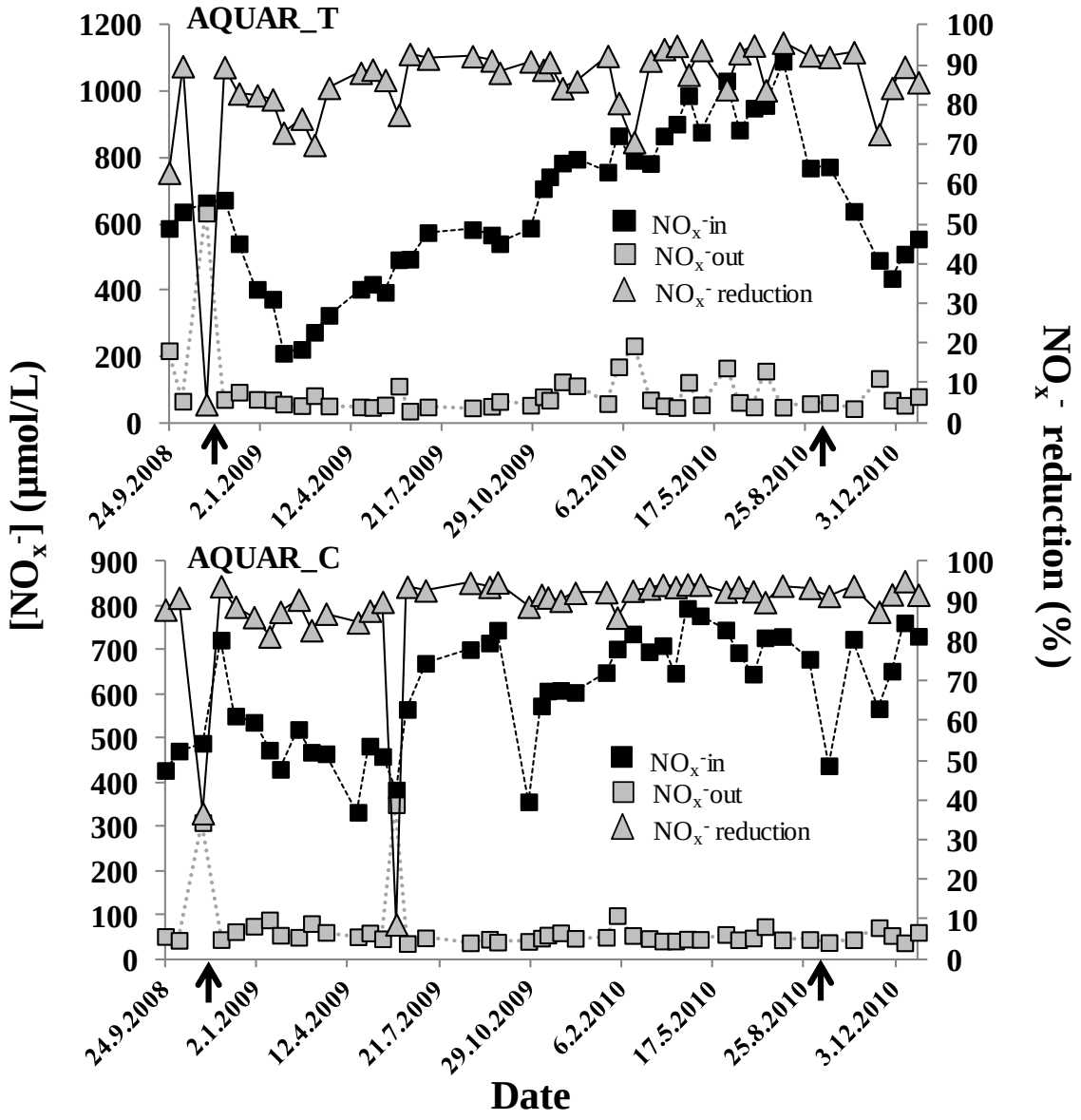

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685 Figure 2
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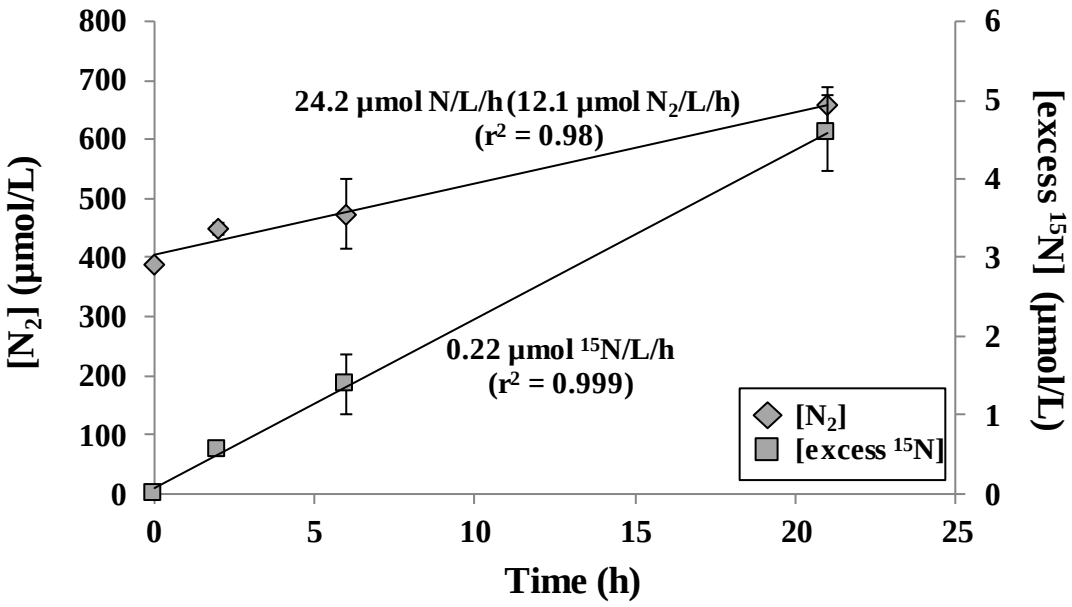

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Figure 3

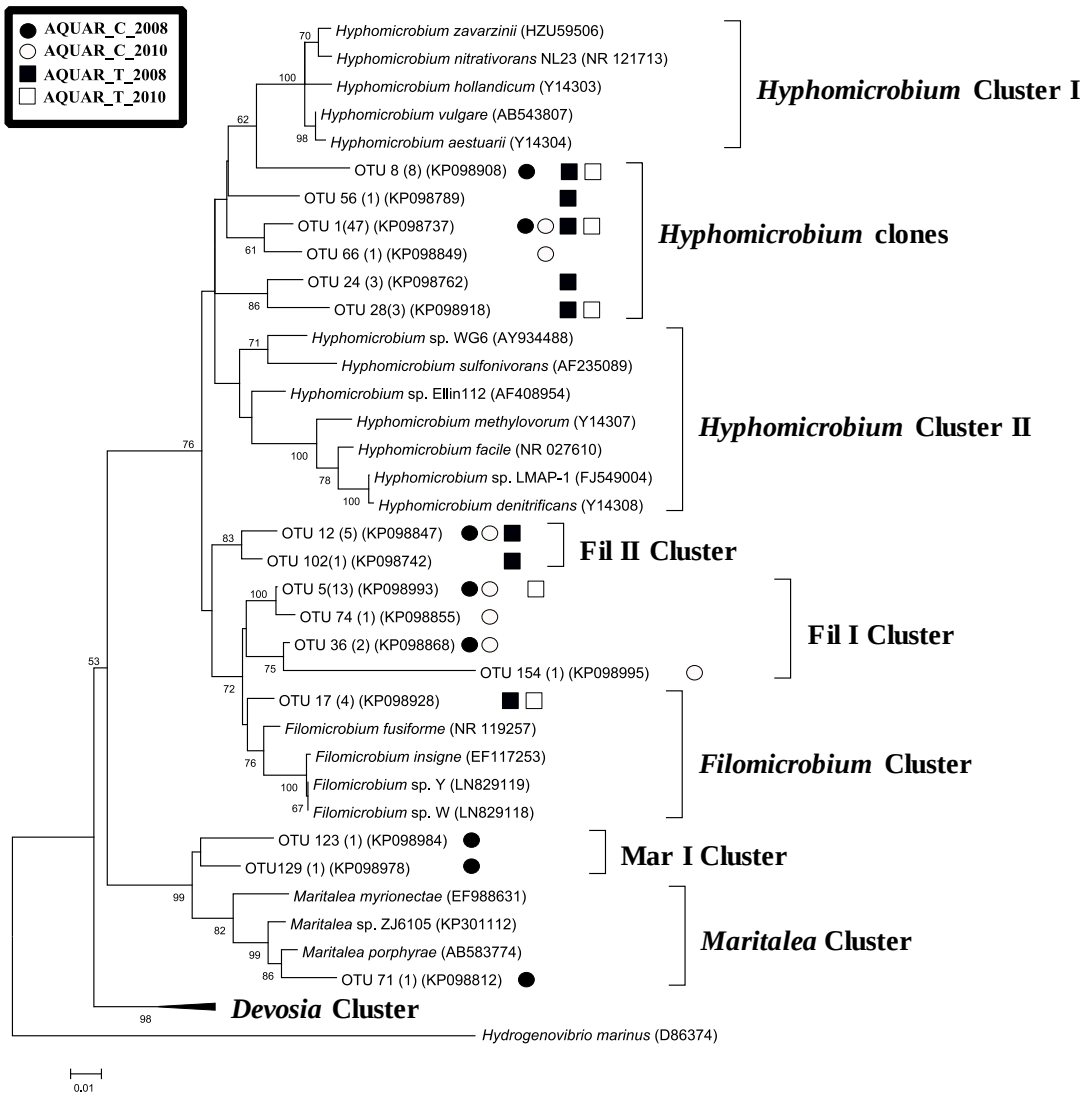
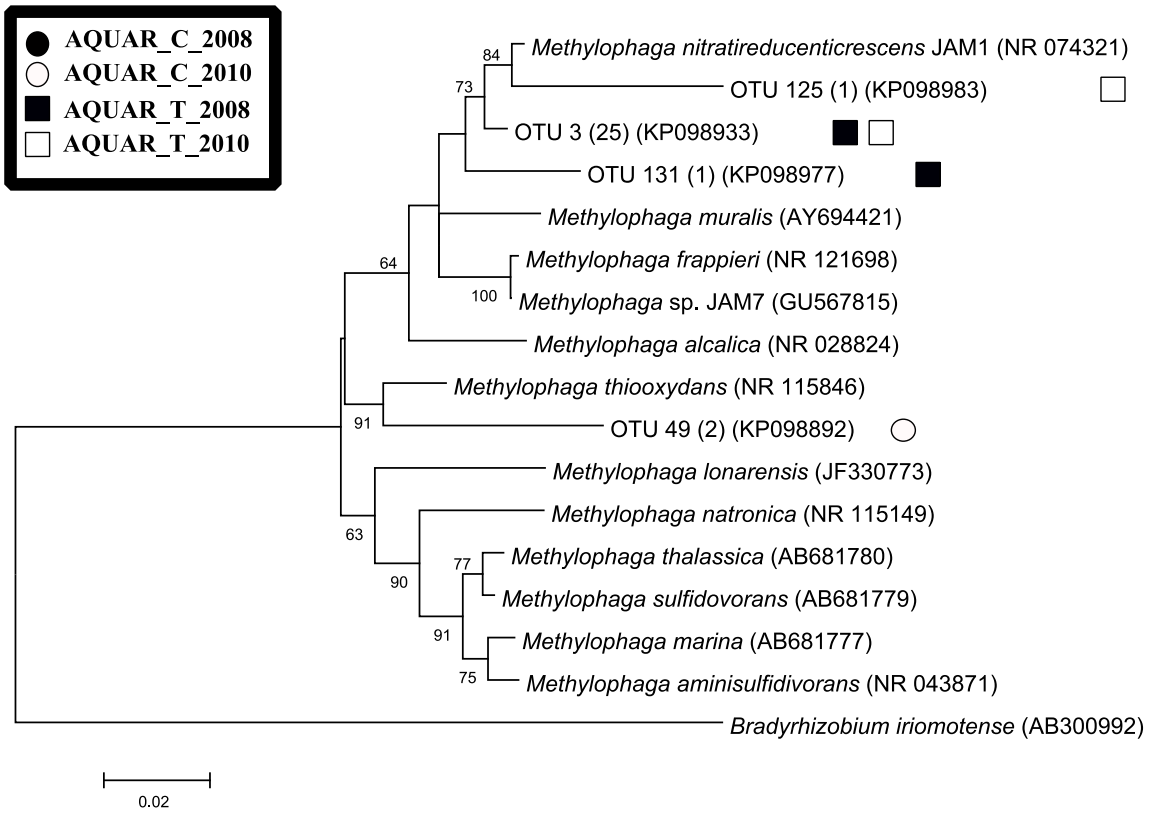

AQUAR_C_2008
AQUAR_C_2010
AQUAR_T_2008
AQUAR_T_2010
Hyphomicrobium zavarzinii (HZU59506)
Hyphomicrobium nitrativorans NL23 (NR 121713)
Hyphomicrobium hollandicum (Y14303)
Hyphomicrobium vulgare (AB543807)
Hyphomicrobium aestuarii (Y14304)
Hyphomicrobium Cluster I
OTU 8 (8) (KP098908)
OTU 56 (1) (KP098789)
OTU 1(47) (KP098737)
OTU 66 (1) (KP098849)
OTU 24 (3) (KP098762)
OTU 28(3) (KP098918)
Hyphomicrobium clones
Hyphomicrobium sp. WG6 (AY934488)
Hyphomicrobium sulfonivorans (AF235089)
Hyphomicrobium sp. Ellin112 (AF408954)
Hyphomicrobium methylovorum (Y14307)
Hyphomicrobium facile (NR 027610)
Hyphomicrobium sp. LMAP-1 (FJ549004)
Hyphomicrobium denitrificans (Y14308)
Hyphomicrobium Cluster II
OTU 12 (5) (KP098847)
OTU 102(1) (KP098742)
Fil II Cluster
OTU 5(13) (KP098993)
OTU 74 (1) (KP098855)
OTU 36 (2) (KP098868)
OTU 154 (1) (KP098995)
Fil I Cluster
OTU 17 (4) (KP098928)
Filomicrobium fusiforme (NR 119257)
Filomicrobium insigne (EF117253)
Filomicrobium sp. Y (LN829119)
Filomicrobium sp. W (LN829118)
Filomicrobium Cluster
OTU 123 (1) (KP098984)
OTU129 (1) (KP098978)
Mar I Cluster
Maritalea myrionectae (EF988631)
Maritalea sp. ZJ6105 (KP301112)
Maritalea porphyrae (AB583774)
OTU 71 (1) (KP098812)
Maritalea Cluster
Devosia Cluster
Hydrogenovibrio marinus (D86374)
0.01

Figure 4


AQUAR_C_2008
AQUAR_C_2010
AQUAR_T_2008
AQUAR_T_2010
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100
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75
Methylophaga nitratireducenticrescens JAM1 (NR 074321)
OTU 125 (1) (KP098983)
OTU 3 (25) (KP098933)
OTU 131 (1) (KP098977)
Methylophaga muralis (AY694421)
Methylophaga frappieri (NR 121698)
Methylophaga sp. JAM7 (GU567815)
Methylophaga alcalica (NR 028824)
Methylophaga thiooxydans (NR 115846)
OTU 49 (2) (KP098892)
Methylophaga lonarensis (JF330773)
Methylophaga natronica (NR 115149)
Methylophaga thalassica (AB681780)
Methylophaga sulfidovorans (AB681779)
Methylophaga marina (AB681777)
Methylophaga aminisulfidivorans (NR 043871)
Bradyrhizobium iriomotense (AB300992)
0.02

Online Resources

Journal: J Ind Microbiol Biotechnol

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

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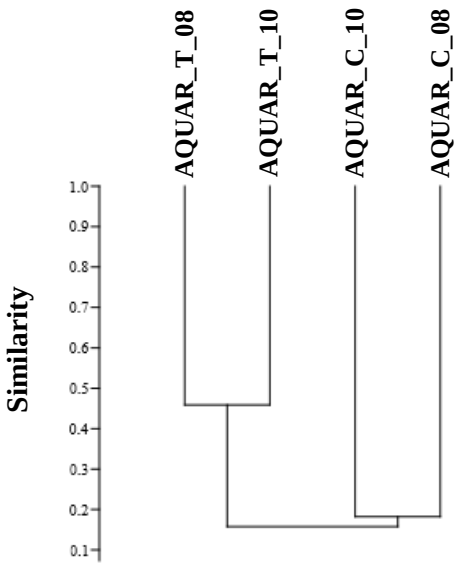
Online Resource 1



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