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1 **Anaerobic oxidization of methane in a minerotrophic peatland: enrichment**

2 **of nitrite-dependent methane-oxidizing bacteria**

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16 **Abstract**

17 The importance of anaerobic oxidation of methane (AOM) as methane sink in freshwater

18 systems is largely unexplored, particularly in peat ecosystems. Nitrite-dependent anaerobic

19 methane oxidation (n-damo) was recently discovered and reported to be catalyzed by the

20 bacterium 'Candidatus Methylomirabilis oxyfera' that is affiliated with the NC10 phylum. So

21 far, several M. oxyfera enrichment cultures have been obtained using a limited number of

22 fresh water sediments or wastewater treatment sludge as inoculum. In this study, using stable

23 isotope measurements and pore water profiles, we investigated the potential of n-damo in a

24 minerotrophic peat land in the south of the Netherlands that is infiltrated by nitrate-rich

25 ground water. Methane and nitrate profiles suggested that all methane produced was oxidized

26 before reaching the oxic layer, and NC10 bacteria could be active in the transition zone where

27 counter-gradients of methane and nitrate occur. Quantitative PCR showed high NC10

28 bacterial cell numbers at this methane-nitrate transition zone. This soil section was used to

29 enrich the prevalent NC10 bacteria in a continuous culture supplied with methane and nitrite

30 at an in situ pH of 6.2. An enrichment of nitrite-reducing methanotrophic NC10 bacteria was

31 successfully obtained. Phylogenetic analysis of retrieved 16S rRNA and pmoA genes showed

32 that the enriched bacteria were very similar to the ones found in situ, and constituted a new

33 branch of NC10 bacteria, with an identity percentage of less than 96% and 90% to the 16S

34 rRNA and pmoA genes of M. oxyfera, respectively. The results of this study expand our

35 knowledge of the diversity and distribution of NC10 bacteria in the environment, and

36 highlight their potential contribution to nitrogen and methane cycles.

37

38

Introduction

Wetlands are the largest single source of methane with estimated emissions of 103 Tg per year, which account for about 20% to 40% of the global annual atmospheric methane flux (1, 8, 19). It is estimated that about 50% of the methane produced in wetlands is consumed before it reaches the atmosphere; this significant microbial methane sink is usually considered to consist exclusively of aerobic methanotrophic bacteria, which degrade methane using oxygen as electron acceptor (2, 5, 19, 39). In ecosystems where oxygen is depleted but sufficient alternative electron acceptors, e.g. sulfate or nitrate are present, methane can also be converted anaerobically (25, 38). Anaerobic oxidation of methane (AOM) coupled to sulfate reduction is performed by a consortium of anaerobic methanotrophic archaea (ANME) and sulfate reducing bacteria (SRB) (25, 47). Its significance is well established for marine ecosystems, where it may consume more than 90% of the produced methane (39). In freshwater wetlands, and especially peatlands, electron acceptors are scarcer, with concentrations typically in the low µM range (37). Due to this reason, redox processes are mostly limited by electron acceptor supply, very dynamic and highly susceptible to alterations e.g. by influx of polluted groundwater and atmospheric deposition of nitrogen and sulfur species (18, 46). The influence of nitrogen pollution on methane oxidation is complex, and not all feedback loops are well understood (2, 3, 16, 29). In principal the role of the alternative electron acceptors nitrate and sulfate for diverting carbon fluxes away from methane production is better established, given that sulfate and nitrate reduction are thermodynamically more favorable than methanogenesis (17, 30, 31, 51). However, these alternative electron acceptors can in principle also enable methane oxidation (47, 54), but this topic has received only little attention with respect to methane cycling in peatlands (43).

In the meantime, for other freshwater ecosystems, more and more evidence about the occurrence of AOM coupled to sulfate (11, 40), iron(III) (42) and nitrate reduction (9, 38, 44, 50) has become available. Whereas nothing is known about the microorganisms mediating ferric iron reduction with methane, for sulfate reduction a very similar consortium of methanotrophic Archaea and SRB as in marine ecosystems is hypothesized to be responsible (11, 40). Nitrate- or nitrite-dependent AOM (n-damo), when linked to organisms, was so far always found to be performed by one bacterial species affiliated to the NC10 phylum (9, 13). Genome sequencing, expression studies and physiological experiments indicated that this bacterium, then named *Candidatus* Methylomirabilis oxyfera, is an "intra-aerobic" methanotroph that produces its own oxygen from the dismutation of nitric oxide into dinitrogen gas and oxygen. The produced oxygen is then used for canonical aerobic methane oxidation starting with the methane monooxygenase enzyme complex (12). Although 16S rRNA sequences similar to *M. oxyfera*'s were found in various environments (14), so far n-damo enrichment cultures have only been obtained from two types of ecosystems: eutrophic freshwater sediments and wastewater treatment sludge. The dominant bacteria in all described cultures were closely related ($\geq$97% identity of the 16S rRNA gene sequence) to *M. oxyfera* (13, 14, 20, 33). Currently it is unclear, however, if *M. oxyfera*-related species are the only nitrite-dependent methane oxidizing bacteria; if methane oxidation is a general feature of NC10 phylum bacteria or limited to (close relatives of) *M. oxyfera*, and how important these bacteria are for methane cycling in various ecosystems.

In this paper, we studied a minerotrophic peatland infiltrated by nitrate-containing groundwater. At the sampling site, no methane emission was detectable. Porewater profiling revealed a nitrate-methane transition zone below the oxic layer that could provide an ecological niche for n-damo microorganisms. NC10 bacteria abundance in soil cores was analyzed using quantitative PCR, and the section with highest cell numbers of *M. oxyfera*,

4

87 coinciding with the methane-nitrate transition zone, was used as inoculum for the enrichment

88 of n-damo bacteria. Mimicking field conditions as much as possible by using nitrite-amended

89 peatland water in continuous cultivation, a new cluster of *M. oxyfera*-like bacteria was

90 enriched.

91

92 **Materials and methods**

93 **Site description**. The Brunssummerheide peatland (50°55'39.63"N/5°59'50.73"E) is a small

94 (15ha) spring fen located in an oligothrophic sandy valley fed by locally upwelling, weakly

95 buffered nitrate-polluted groundwater. The peat layer is relatively thin (maximum 2.5 m) and

96 vegetation is dominated by *Sphagnum* spec., *Narthecium ossifragum* and *Molinia caerulea*.

97 At the sampling site, nitrate-enriched groundwater overflows the peatland surface and

98 infiltrates into the peat layer.

99 **Porewater profile determination and soil sampling.** Nitrate and methane profiles were

100 determined by measuring the concentrations in porewater samples collected using 5 cm

101 ceramic cups (Eijkelkamp Agrisearch Equipment, Giesbeek, The Netherlands) connected to

102 Teflon tubes. Porewater samples were obtained at least in duplicate from the depth of 20 cm

103 to 220 cm at 5 or 10 cm intervals in December 2009 and June 2010. Porewater for methane

104 analyses was collected in vacuumed anaerobic glass bottles (40 ml) prefilled with 5 g sodium

105 chloride and sealed with butyl rubber stoppers. For chemical analyses, porewater was

106 collected in 60 ml syringes. Samples were transported to the laboratory within two hours in a

107 cooling box, and stored at 4 ^{o}C for maximum 14 days before analysis. Methane in the bottle

108 headspace was measured after pressure equilibration with argon using gas chromatography as

109 described previously (14). Nitrate was analyzed colorimetrically on a Traacs 800+ auto-

110 analyzer as described previously (48). Redox potential measurements were performed by

5

111   gently pushing platinum electrodes into pre-drilled holes and allowing them to equilibrate.

112   Stable readings were obtained after 30 min (15). Soil samples were obtained from 50 cm to

113   130 cm depth with a Russian peat corer, sliced into 5-20 cm intervals in the field, immediately

114   put into self-sealing plastic bags, and stored in air-tight bins with oxygen scavenger

115   (Anaerogen, Oxoid, USA), then transported to the laboratory and stored anaerobically at 4 $^{\circ}$C

116   until further analysis.

117   **Incubation.** Initially, 200 ml soil slurry of the depth layers of 80-100 cm, 100-120 cm and

118   120-135 cm (sampled in July 2009) were incubated in separate bottles (500 ml). Surface water

119   from the peatland was collected and used for medium preparation after removal of particles

120   by filtering through a hemo-filter (Hemoflow HF80S, Fresenius Medical Care, USA). The

121   medium contained: 2 mM $KHCO_3$, 0.2 mM $Na^{15}NO_2$ (99.6% ^{15}N; Isotec, USA) and 0.5 mM

122    $NaNO_3$. The bottles were made anaerobic by 6 cycles of vacuuming and gassing with Ar/CO_2

123   (75:25), followed by 5 min of flushing with Ar/CO_2. Then 10 ml $^{13}CH_4$ was injected into the

124   headspace (final concentration ca. 20%). The pH in the bottles was around 6.0 and the bottles

125   were incubated at 25 ℃, with gentle shaking at 100 rpm. The production of $^{13}CO_2$ was

126   measured by GC-MS in the headspace (see below).

127   After three months incubation, the bottle with strongest $^{13}CO_2$ production was used as

128   inoculum for continuous culturing in a 3 liter glass bioreactor (working volume 1.5 l;

129   Applikon, Schiedam, The Netherlands) that was operated in sequencing batch mode to

130   prevent biomass loss. One cycle constituted of 23 h of continuous supply of medium, 0.5 h

131   settling, finally followed by 0.5 h discharging with a level-controlled pump. To keep the

132   culture anaerobic, the reactor was continuously flushed with 20 ml min^{-1} Ar/CO_2 (95:5) and 5

133   ml min^{-1} methane. The temperature was controlled at 25 $^{\circ}$C and the pH at 6.0 to 6.2.

134   Dissolved oxygen, temperature and pH in the reactor were monitored by respective

135   electrodes. Medium was prepared as described above, except using unlabeled nitrite. The

136   nitrite concentration in the reactor was estimated daily with Merckoquant test strips (0-80 mg

137   l^{-1}; Merck, Germany), and the concentration in the medium was slowly increased from 0.2

138   mM to 2.5 mM dependent on the activity of the continuous culture. Nitrite concentrations in

139   the reactor were kept below 20 mg l^{-1} (0.44 mM). The medium loading to the reactor was

140   between 200 to 500 ml per day.

141   **Activity analysis.** Methane oxidizing activity in bottles was measured by determining the

142   amount of $^{13}CO_2$ produced from $^{13}CH_4$ oxidation with GC-MS (Agilent 5975C inert MSD;

143   Agilent, United States) as previously described (14). Activity in the reactor was tested in

144   batch experiments with the whole culture. First medium supply was stopped and unlabeled

145   nitrite was allowed to be depleted. The reactor was flushed with Ar-CO_2 (95:5) for 1 h while

146   stirring, and checked for residual methane in the headspace. When undetectable, 0.2 mM

147   $^{15}NO_2^-$ and 50ml $^{13}CH_4$ were added. 20 µl gas samples were taken every hour for $^{13}CO_2$,

148   $^{15,15}N_2$, $^{15,14}N_2$ analysis. At the same time, 1 ml culture liquid was taken and centrifuged; the

149   supernatant was kept at 4 ^{o}C for nitrite analysis. Nitrite concentrations were determined with

150   colorimetric methods as described elsewhere (23). The influence of pH on activity was

151   determined in batch incubations of 10 ml biomass in 40 ml serum bottles, buffered with MES

152   (2-(N-morpholino) ethanesulfonate, 20 mM) to pH values between 5.9 and 6.7, and with

153   MOPS (3-(N-morpholino) propanesulfonate, 20 mM) to pH values between 6.75 and 7.4

154   (measured at the end of incubation).

155   **DNA isolation.** Total DNA from soil samples was isolated with the PowerSoil$^{○,R}$ DNA

156   isolation kit (MO BIO Laboratories Inc., USA) according to the manufacturer's manual.

157   Approximately 0.3 g homogenized soil was used for DNA isolation, and two independent

158   isolations were carried out for each depth interval. DNA was eluted three times with pre-

159   warmed Milli-Q water from the column to ensure that the entire DNA had been collected.

160   DNA in the third elution was undetectable by agarose gel electrophoresis (<0.2 ng μl^{-1}). DNA

161   obtained from the same depth interval was pooled for qPCR analysis to minimize the

162   influences from soil inhomogeneities. DNA from enrichment cultures was isolated with a

163   method based on bead-beating and SDS lysis, as described previously (14). DNA quality was

164   checked on agarose gel, and concentrations were measured in triplicate with NanoDrop (ND-

165   1000, ISOGEN Life Science, The Netherlands).

166   **Quantitative PCR.** In order to quantify n-damo bacteria and all bacteria in different depths of

167   the soil cores, quantitative PCR (qPCR) targeting the 16S rRNA gene was performed. To

168   account for imperfect primer matching and known variability of results (14), two different

169   primer pairs were used for each group. For NC10 phylum bacteria, primer pairs p1F & p1R

170   and p2F & p2R, and for all bacteria, primer pairs 1100F & 1492R and 533F & 805R (Table 1)

171   were applied. All q-PCR assays were performed according to the MIQE guidelines (Minimum

172   Information for Publication of Quantitative Real-Time PCR Experiments) (4). qPCR

173   experiments were carried out with the Bio-Rad IQTM 5 cycler real-time detection system

174   using IQTM SYBR green Supermix (Bio-Rad, United States) in 25μl reaction volume as

175   previously described (14), except using 65 ^{o}C for n-damo specific primer pairs and 58 ^{o}C for

176   universal primer pairs as annealing temperature, which had been determined as most suitable

177   for the present samples by gradient PCR. The qPCR reactions were carried out in 96 well

178   plastic plates (Bio-Rad, United States) sealed with Opti-Seal Optical disposable adhesive

179   (BIOplastics, The Netherlands). Fluorescence signals were obtained at 72 ^{o}C at the end of the

180   elongation step of each cycle. PCR products obtained with n-damo specific and universal

181   bacteria primer pairs were cloned and sequenced using the vector pGEM-T Easy (Promega,

182   United states). The sequences retrieved were of the correct length (201 bp for p1F & p1R; 292

183   bp for p2F & p2R; 291 bp for 515F & 805R and 410 bp for 1100F & 1492R), and the

8

184 obtained n-damo sequences were similar (>97.2% identity) to the sequence of *M. oxyfera*

185 (accession no. FP565575). Standard curves for n-damo bacteria and general bacteria were

186 constructed with plasmids containing corresponding inserts, taking into account the molecular

187 mass of the plasmid including the insert, and the plasmid concentration. Plasmids copy

188 numbers used as standard were between 30.7 to 3.07×10^8 µl^{-1} for NC10 bacteria, 86.9 to

189 8.69×10^8 µl^{-1} for all bacteria. Two soil cores with partial overlap were analyzed. Both cores

190 were sliced in sections between 5 and 10 cm in the field (see soil sampling and DNA

191 isolation). In Fig. 1, each depth interval is represented by its average depth. DNA isolated

192 from soil of 85-90 cm depth was used to test dilution effect; 10 times and 100 times had a

193 maximum difference of 8.7%, compared with non-diluted ones. For NC10 bacteria non-

194 diluted DNA was used as templates; but for primers targeting all bacteria, 100 times diluted

195 DNA was used. PCR efficiencies calculated based on standards were between 90.6% and

196 99.2%. Both standards and samples were run in triplicates. The copy numbers in samples

197 were calculated based on comparison with the threshold cycle values of the standard curve,

198 taking into account the dilution and the amount of total DNA obtained per gram soil.

199 **Phylogenetic analysis.** PCR was performed with DNA isolated from the soil layer used as an

200 inoculum (80-100 cm depth), the enrichment culture after 3 months of incubation in bottles,

201 and the continuous culture after 1 and 17 months of enrichment in the reactor. 16S rRNA

202 sequences of n-damo bacteria were obtained with universal bacteria primer 8F or n-damo

203 specific primer 193F in combination with n-damo specific primer 1043R (Table 1). PCR

204 products of the correct size were ligated into the pGEM-T Easy cloning vector (Promega,

205 United States) and amplified in *Escherichia coli* DH5α. Plasmids were isolated from 10 to 15

206 randomly selected white colonies per library using the GeneJet miniprep kit (Fermentas,

207 Lithuania), and were sequenced at the DNA Diagnostics Center of Nijmegen University

208 Medical Center. The sequences were aligned to reference sequences with the MUSCLE

9

209 algorithm. Phylogenetic trees were constructed with MEGA5 using the neighbor-joining

210 method and the robustness of tree topology was tested by bootstrap analysis (1000 replicates).

211 With the same DNA samples also functional gene (particulate methane monooxygenase

212 subunit A, *pmoA*) clone libraries were constructed. The particulate methane monooxygenase

213 catalyzes the first step of methane oxidation and is well conserved in methane oxidizing

214 bacteria, therefore *pmoA* is widely accepted as a marker gene for assessing diversity of

215 aerobic and *M. oxyfera*-like anaerobic methanotrophs in the environment (34, 36). Two

216 different forward primers targeting either most methanotrophs (A189b) or only close relatives

217 of *M. oxyfera* (cmo182) were combined with a specific reverse primer (cmo682) (Table 1). A

218 *pmoA* phylogenetic tree based on nucleotide sequences was constructed as described above.

219 **Fluorescence in situ hybridization.** On a monthly basis, 1.5 ml biomass was harvested from

220 the reactor and forced through a 0.5 mm needle to break big cell aggregates. Then the sample

221 was centrifuged and the pellet was washed twice with 1 ml 1×PBS, and fixed with

222 paraformaldehyde on ice for three hours. Fluorescence in situ hybridization (FISH) was

223 performed as previously described (13), using 40% formamide concentration. The following

224 oligonucleotide probes were used: S-*-DBACT-0193-a-A-18 and S-*-DBACT-1027-a-A-18

225 specific for n-damo bacteria (38) and a mixture of EUB I-III and V for most Bacteria (7).

226 Images were acquired with a Zeiss Axioplan 2 epifluorescence microscope equipped with a

227 CCD camera, together with the Axiovision software package (Zeiss, Germany).

228 **Nucleotide sequences accession numbers.** Representative 16S rRNA and *pmoA* gene

229 sequences were deposited at the National Center for Biotechnology Information

230 (http://www.ncbi.nlm.nih.gov/) with the accession numbers JX262153- JX262155 (*pmoA*)

231 and JX262156-JX262161 (16S rRNA).

232

233   **Results**

234   **Porewater profiles.** Porewater depth profiles of the Brunssummerheide sampling location

235   were determined on five occasions between July 2009 and May 2011, with an overall very

236   similar pattern. Representative winter (December 2009) and summer (June 2010) profiles are

237   shown in Fig. 1. Nitrate concentration decreased with depth, and became undetectable below

238   100 cm. No methane was detected in the upper 80 cm, but methane gradually increased below

239   the depth of 80 cm and reached the maximum concentration at around 120 centimeters (Fig.1

240   A, B). Redox data indicated that the soil was completely anoxic below 50 cm depth, and

241   living roots of vascular plants were not found below 60 cm depth. The maximum

242   concentration of nitrate (0.6 mM) in June 2010 (Fig.1 B) was about 0.2 mM higher than that

243   in December 2009 (Fig. 1. A), possibly due to relatively stronger evaporation of surface water

244   and higher groundwater influx in summer. The maximum concentration of methane remained

245   similar in both seasons, as well as the overall pattern: an opposing gradient at around 80-100

246   cm depth.

247

248   **Quantifying abundance of NC10 bacteria in different soil depths.** Total bacterial and

249   NC10 phylum abundance in different soil depths was determined in two overlapping cores by

250   qPCR using primers targeting the 16S rRNA genes. The highest cell numbers ($1.3\text{-}3.2\times10^7$ g^{-1}

251   wet soil) of NC10 bacteria were found at 80-85 cm depth (Fig. 1 C, D), coinciding with the

252   concomitant decrease of methane and nitrate (Fig. 1 A, B), and a peak in abundance of NC10

253   phylum-characteristic phospho-lipid fatty acids (Fig. 1 G; Kool et al, submitted). In contrast,

254   total bacteria cell numbers, ranging from 0.9 to 11.8×10^8 cells g^{-1} wet soil, did not show a

255   depth-related pattern (Fig. 1 E, F).

256

257 **Enrichment and activity.** Nitrite dependent methane oxidizing activity was initially

258 determined by measuring the fraction of $^{13}CO_2$ in total CO_2 after supply of $^{13}CH_4$ and nitrite

259 to three soil sections (80-100; 100-120; 120-135 cm). Despite the addition (and permanent

260 presence) of nitrate (0.5 mM), all soil cores produced some methane in the first two weeks of

261 incubation, but no methane oxidation could be detected (detection limit approximately 0.5

262 nmol d^{-1} g^{-1} soil). After about 3 month's incubation, the 80 - 100 cm section showed methane

263 oxidation activity (9.0 nmol d^{-1} g^{-1} soil, assessed as CO_2 production), and an increase in this

264 rate indicated microbial growth. This incubation (80-100 cm) was used as inoculum to start a

265 sequencing batch reactor for the enrichment of the responsible microorganism. Over the first

266 9 months of enrichment activity remained low with a nitrite reduction rate of about 50 µmol d^{-}

267 ^{1} L^{-1}, and then started to increase to about 1.0 mmol (NO_2^-) d^{-1} L^{-1} in month 15. Batch tests

268 and experience with previous NC10 bacteria enrichment cultures had indicated that nitrite was

269 preferred over nitrate; consequentially the medium, prepared with *in situ* water was not only

270 amended with nitrate, but also nitrite. To test the coupling of nitrite reduction to methane

271 oxidations, both activities were tested in batch experiments after 10 months with ^{15}N- and ^{13}C-

272 labelled substrates during the enrichment period (Fig. 2). Nitrite-N was completely recovered

273 as nitrogen gas, concomitantly methane was fully oxidized to CO_2. The ratio of $^{13}CO_2$ and

274 $^{15,15}N_2$ production was 3:4.3, similar to the theoretical stoichiometry of 3:4 (38). An activity

275 test at different pH values demonstrated that the culture preferred circumneutral conditions,

276 but was active down to the lowest tested value of 5.9 (Fig. 3).

277

278 **FISH analysis of the enriched bacteria.** FISH was performed on biomass of the enrichment

279 culture fixed every month, but no clear hybridization with NC10 specific probes was observed

280 until after 8 months of medium supply. Even though small numbers of NC10 bacteria must

281 have been present, they remained undectable at first due to strong autofluorescent background

282 and hybridization inhibiton, presumably caused by peat material. Starting at month 9, NC10

283 cells could be detected (Fig. 4A). With the progression of incubation, both total cell numbers

284 visulized by DAPI stain, and the percentage belonging to the NC10 phylum gradually

285 increased (Fig. 4 B, C) and coincided with an increased activity of the culture. At month 14

286 about 50%, and at month 19 more than 80% of the population did hybridize with the NC10

287 sepecific probes (Fig. 4).

288 **16S rRNA and *pmoA* gene phylogenetic analysis.** *M. oxyfera*-related 16S rRNA and *pmoA*

289 genes were successfully obtained from both inoculum soil and the enrichment after 1 or 17

290 months of incubation. Long (>1000 bp) 16S rRNA sequences obtained with primer 8F

291 (universal) and 1043R (NC10 specific) were used for phylogenetic analysis. Results showed

292 that the 16S rRNA sequences belong to the group A of NC10 bacteria (14), forming a cluster

293 (differences between 0.1% to 2.7%) with sequences retrieved from coal-tar contaminated

294 aquifer (AF351214, AF351217, FJ810544) and lake Constance sediment (HQ906524,

295 HQ906538) (9). These sequences share only 94.9% to 95.5% identity with *M. oxyfera* (Fig. 5

296 A).

297 The phylogenetic analysis of the *pmoA* gene showed similar results. *pmoA* sequences from

298 both soil and enrichment culture again cluster together with *pmoA* sequences retrieved from

299 Lake Constance sediment (HQ906571, HQ906568, HQ906566) (9). These *pmoA* sequences

300 had an identity with those of *M. oxyfera* of 86.2-90.9% on nucleotide level, but the 95.8-

301 97.9% on amino acid level indicated functional conservation (Fig. 5 B). No significant

302 difference could be observed between the inoculum and the 17 months old enrichment

303 culture, indicating that no population shift within the NC10 phylum had occurred. Both the

304   16S rRNA and *pmoA* genes phylogenetic results suggested that a new cluster of NC bacteria

305   had been enriched.

306

307   **Discussion**

308   The Brunssummerheide peatland is a spring fen in an oligotrophic sand valley fed by nitrate-

309   polluted groundwater, and therefore contains nitrate concentrations in the upper peat layer

310   which are uncommonly high for pristine peatlands (52). Also in contrast to many other

311   peatlands (6, 24, 26, 27), methane was not detected in the upper 70-80 cm of the depth profile

312   at 5 sampling occasions in different seasons from 2009 to 2011, even though methane was

313   produced in the deep anoxic zone (below 100 cm, Fig. 1A, B). As roots of vascular plants do

314   not reach that deep in the Brunssummerheide (maximum 60 cm), this suggested the existence

315   of an anoxic methane sink in the peat, independent from oxygen and aerenchymal transport by

316   roots, for which oxidized nitrogen species could serve as electron acceptor. The counter

317   gradient of methane and nitrate at the depth of 80 cm may provide an ideal niche for, and may

318   be at least partly created by the recently characterized n-damo bacteria. Targeting their 16S

319   rRNA gene in DNA extracts from different depths confirmed this: Highest n-damo cell

320   numbers (up to 3.2×10^7 cells g^{-1} soil) and ratios (3 to 8% of total bacterial community) were

321   observed at the depth of 80-90 cm (Fig. 1 C, D), coinciding with the methane-nitrate

322   transition zone (Fig. 1 A, B). At this depth, also a peak in abundance of phospho-lipid fatty

323   acids diagnostic for NC10 phylum bacteria was detected (Fig. 1 G; Kool et al., submitted).

324   The n-damo cell number and lipid profiles also agreed with the finding that among soil

325   samples from 80-100, 100-120 and 120-135 cm depth only the 80-100 cm sample showed

326   anaerobic methane-oxidizing activity upon incubation. Despite the relatively high numbers of

327   n-damo bacteria detected at a depth 80-90 cm, it took several months to obtain an enrichment

14

328   culture with measurable activity. Also detection by fluorescence *in situ* hybridization using

329   NC10 phylum-specific probes, hampered by a strongly auto-fluorescent background from the

330   organic-rich inoculum, was only possible after 9 months of continuous cultivation with

331   constant supply of nitrite and methane. This may be due to the "dilution" of the naturally

332   NC10 phylum-enriched soil layer with less active deeper layers (90-100 cm) in the inoculum,

333   and a very low growth rate at the prevailing conditions, especially the pH (6.0 – 6.2). The pH

334   optimum test showed that the NC10 phylum bacteria enriched in the continuous culture were

335   only acidotolerant to a certain extent, not acidiphilic. They were active down to a pH below 6,

336   but their physiological optimum was clearly higher, above 7 (Fig. 3). This is a prime example

337   for the discrepancy between physiological and ecological optimum. In contrast to previous *M.*

338   *oxyfera* enrichment cultures from neutral, eutrophic sediments (14), which had a similar

339   optimum (around 7.5), but were not active at a pH below 7 (assessed under similar conditions,

340   O. Rasigraf, MSc thesis, 2011, unpublished), a different ecotype was dominant in the more

341   acidic and low nutrient environment. According to the species delineation of 97% identity of

342   the 16S rRNA gene for bacteria in general and 93% of the *pmoA* gene diagnostic for

343   methanotrophic   bacteria   (35),   the   NC10   phylum   bacterium   dominating   the

344   Brunssummerheide enrichment culture even constitutes a new species within the genus

345   *Methylomirabilis*.

346   Like other NC10 enrichment cultures (14, 20, 33), the enrichment period was characterized by

347   a long phase without measureable activity, followed by a period of slow, but exponential

348   increase in nitrite consumption rate. In the present case, nitrite-reducing activity remained low

349   for the first 9 months, and then started to increase to about 1.0 mmol (NO_2^-) d^{-1} L^{-1} in month

350   15. After this increase it was not possible to stimulate the growth of the culture further and a

351   sort of stationary phase was reached similar to other enrichments of NC10 bacteria (14, 20,

352   22). The doubling time of the Brunssummerheide *Methylomirabilis* strain was estimated to be

353 about two months, which is 4 to 8 fold lower than the values reported before (14). It is

354 difficult to predict whether this reflects the growth rate under field conditions. On one hand

355 some factors like a higher temperature (25 ºC), the optimum temperature of methanotrophs in

356 most peat soils (19) in contrast to 10-15 ºC *in situ* and constant substrate supply may be

357 beneficial, but other factors like stirring, use of surface- instead of porewater or a decrease in

358 microbial partner communities may also be disadvantageous for growth in the laboratory.

359 However, once established, the methanotrophic community does not need to grow fast to

360 constitute a relevant methane sink in the environment. According to previous estimations,

361 *Methylomirabilis* cells in an enrichment culture have an activity of 0.1 to 0.4 fmol CH_4 cell^{-1}

362 d^{-1} (14), indicating that the Brunssummerheide soil of 80 – 85 cm depth with about 1.3 to

363 3.2×10^7 cells g^{-1} soil may convert between 1.3 and 12.8 nmol CH_4 d^{-1} g^{-1} soil. This range is at

364 the lower end of methane oxidation rates reported for aerobic methanotrophs (41) in wetlands,

365 but apparently high enough to balance the methane diffusing upwards from deeper,

366 methanogenic soil layers.

367 Nitrite is clearly the preferred electron acceptor of previously reported *M. oxyfera*

368 enrichments (13, 14, 20, 38). When nitrite was depleted in the present *Methylomirabilis*

369 enrichment culture, methane oxidizing activity in the presence of nitrate (1 mM) ceased; upon

370 addition of fresh nitrite, methane consumption started again (data not shown), demonstrating

371 that the methane-oxidizing activity of Brunssummerheide enrichment is also nitrite

372 dependent. Although nitrite was also detected in the depth profile, its concentrations were

373 much lower (max. 4.2 µM, mostly around the detection limit of the colorimetric method) than

374 those of nitrate. There was no depth-related pattern, and values were not constant over time.

375 The nitrite needed by n-damo bacteria active in the soil might be supplied by other

376 microorganisms (e.g denitrifying bacteria) or *Methylomirabilis* itself converting nitrate to

16

nitrite using organic carbon compounds other than methane. This would explain why nitrate is sufficient as an electron acceptor for methane oxidation *in situ* and in the initial batch incubations, whereas after enrichment, concomitant with a relative loss of other bacteria and a degradation of labile organic carbon, this supply path is insufficient and nitrite addition becomes mandatory for methane oxidation.

The present study shows an additional, so far hardly investigated pathway linking the biogeochemical cycling of nitrogen and methane in peatlands. Given the world-wide increasing groundwater nitrate and atmospheric nitrogen loads (32, 45), this methane sink may become more relevant for mitigating the mobilization of carbon in the form of methane from wetlands in the future.

Table 1 Primer pairs used for qPCR analysis and clone libraries construction in this study.

		Forward primer			Reverse primer		Annealing	Target
	Designation	Sequence (5' - 3')	Reference	Designation	Sequence (5' - 3')	Reference	temp. (^{o}C)	
qPCR	p1F	GGGCTTGACATCCCACGAACCTG	(14)	p1R	CGCCTTCCTCCAGCTTGACGC	(14)	65	NC10 bact. 16S rRNA
	p2F	GGGGAACTGCCAGCGTCAAG	(14)	p2R	CTCAGCGACTTCGAGTACAG	(14)	65	NC10 bact. 16S rRNA
	533F	GTGCCAGCMGCCGCGGTAA	(49)	805R	GACTACCAGGGTATCTAATC	(28)	58	All bact. 16S rRNA
	1100F	YAACGAGCGCAACCC	(10)	1492R	GGTTACCTTGTTACGACTT	(53)	58	All bact. 16S rRNA
Clone library	8F	AGAGTTTGATYMTGGCTCAG	(21)	1043R	TCT CCA CGC TCC CTT GCG	(14)	55-65	NC10 bact. 16S rRNA
	193F	GACCAAAGGGGGCGAGCG	(14)					
	A189b	GGNGACTGGGACTTYTGG	(34)	cmo682	AAAYCCGGCRAAGAACGA	(34)	55-65	NC10 bact. *pmoA*
	cmo182	TCACGTTGACGCCGATCC	(34)					

Figure captions

Fig. 1 Depth profiles of the Brunssummerheide peatland. (A, B), Nitrate (filled square) and

methane (open circle) concentrations in porewater sampled in December 2009 (A) and in June

2010 (B). (C-F) Bacterial cell numbers (cells g^{-1} wet soil) as assessed by qPCR on DNA

extracted from two overlapping soil cores from 51 to 102 cm (open circles), and from 77 to

127 cm (open triangles). NC10 bacteria abundance was determined with primer pairs p1F &

p1R (C), andp2F & p2R (D). Total bacterial abundance was determined with primer pair 535F

& 805R (E), and 1100F & 1492R (F). (G) Relative abundance of the phospho-lipid fatty

acids 10-methyl-hexadecanoic acid (10MeC16:0, open diamonds) and 10-methyl-

hexadecanoic acid (10MeC16:1Δ7, multiplied by 3, closed squares) diagnostic of NC10

bacteria (data from Kool et al., [24A]).

Fig. 2 Activity test of the enrichment culture at month 10 with $^{15}NO_2^-$ and $^{13}CH_4$. Nitrite

(filled circle) was consumed, $^{15,15}N_2$ (filled square), $^{14,15}N_2$ and $^{13}CO_2$ (filled triangle) were

produced. The $^{13}CO_2$ production rate was 20.2 µmol d^{-1}, and the rate of $^{15,15}N_2$ production

was 29.0 µmol d^{-1}.

Fig. 3 Methane-oxidizing activity of the n-damo enrichment culture incubated at different pH

values.

Fig. 4 Fluorescence in situ hybridization of the enrichment culture at different times of

incubation. A: month 9; B: month 14; C: month 19. NC10 bacteria appear in pink, due to co-

hybridization of NC10 bacteria specific probes 193-Cy3 and 1027-Cy3 (red) and a mixture of

probes EUBI-III, IV-Cy5 (light blue) for most eubacteria and DAPI (dark blue). (Scale bars:

5µm).

Fig. 5 Phylogenetic trees of the 16S rRNA (A) and the *pmoA* genes (B, including *amoA* and

pxmA sequences) of the enrichment culture. The trees were calculated in Mega5 using the

neigbor-joining method. Bootstrap support values (1000 replicates) greater than 50% are

indicated at the nodes. The sequences obtained in this study from inoculum soil and

enrichment after 1 or 17 months of incubation are shown in bold. **References**

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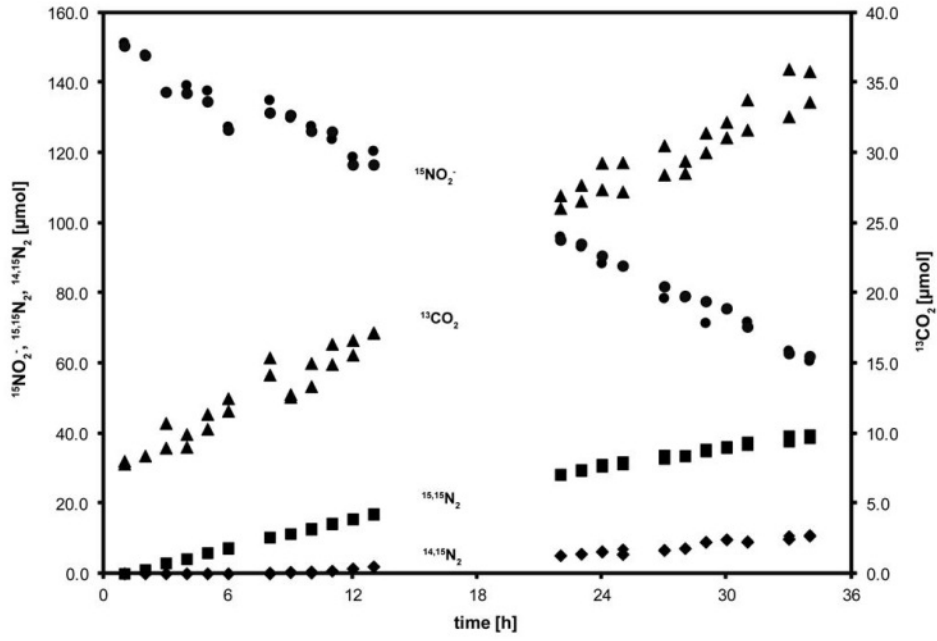477   Soil Biology and Biochemistry 43:702-717.

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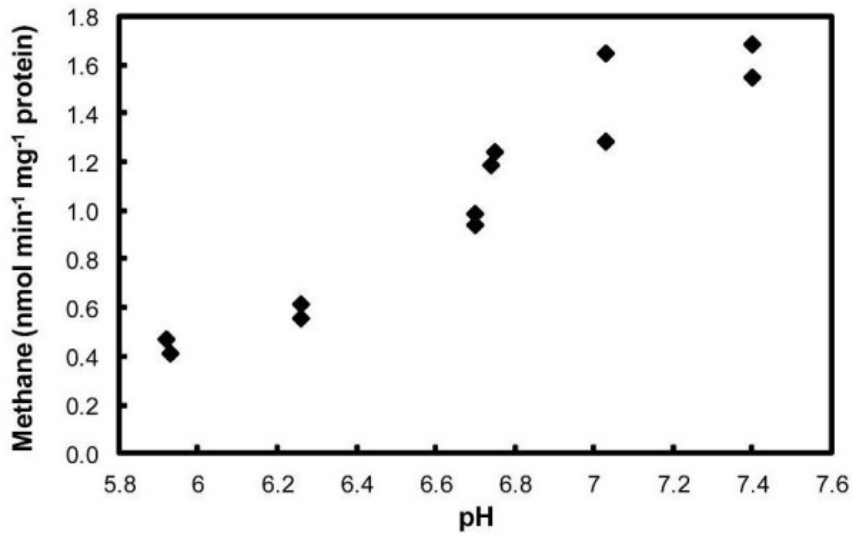562   European terrestrial habitats. Environ Pollut 159:665-76.

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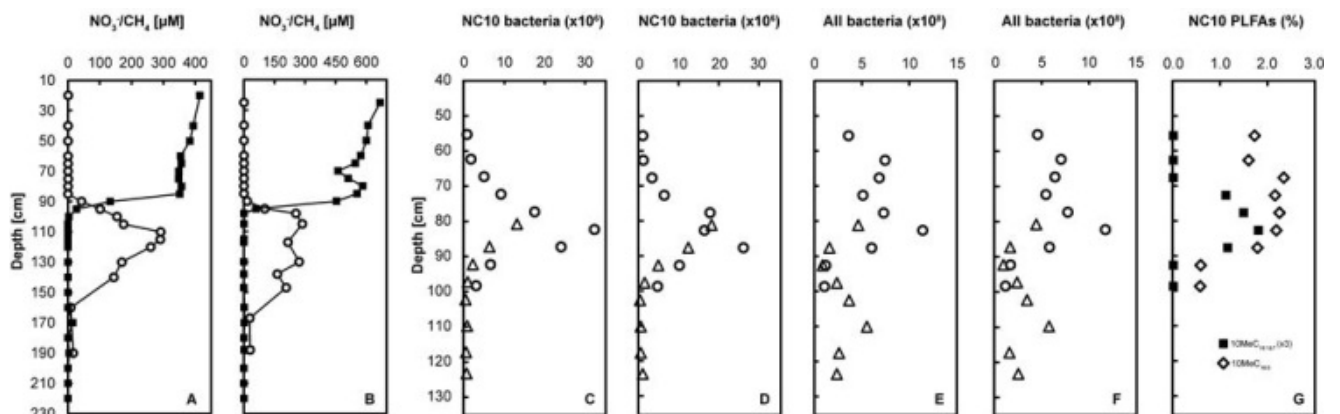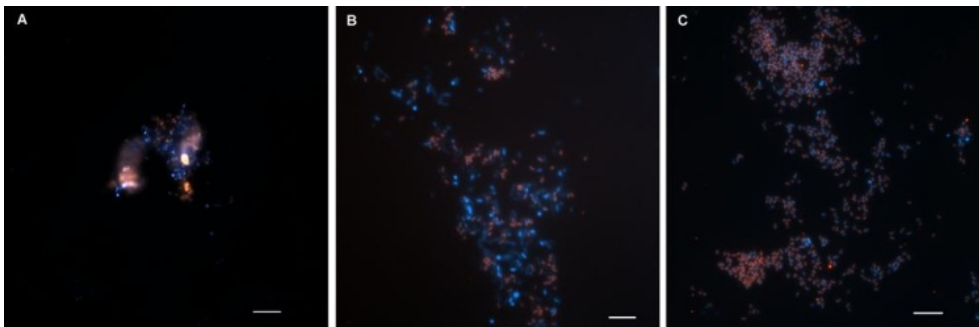
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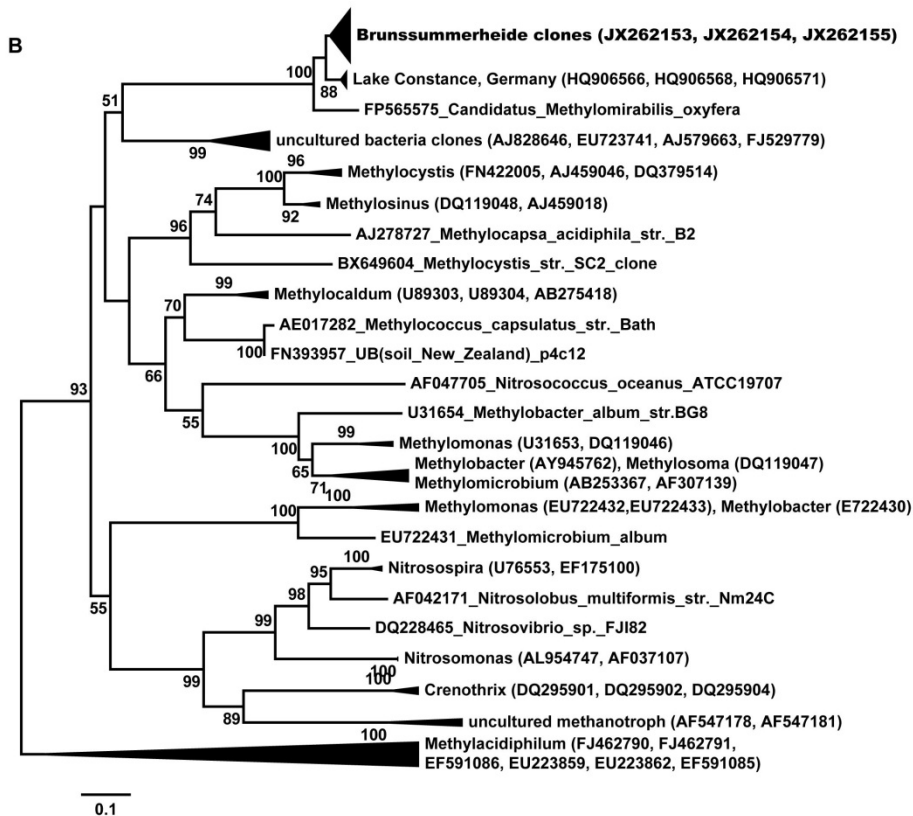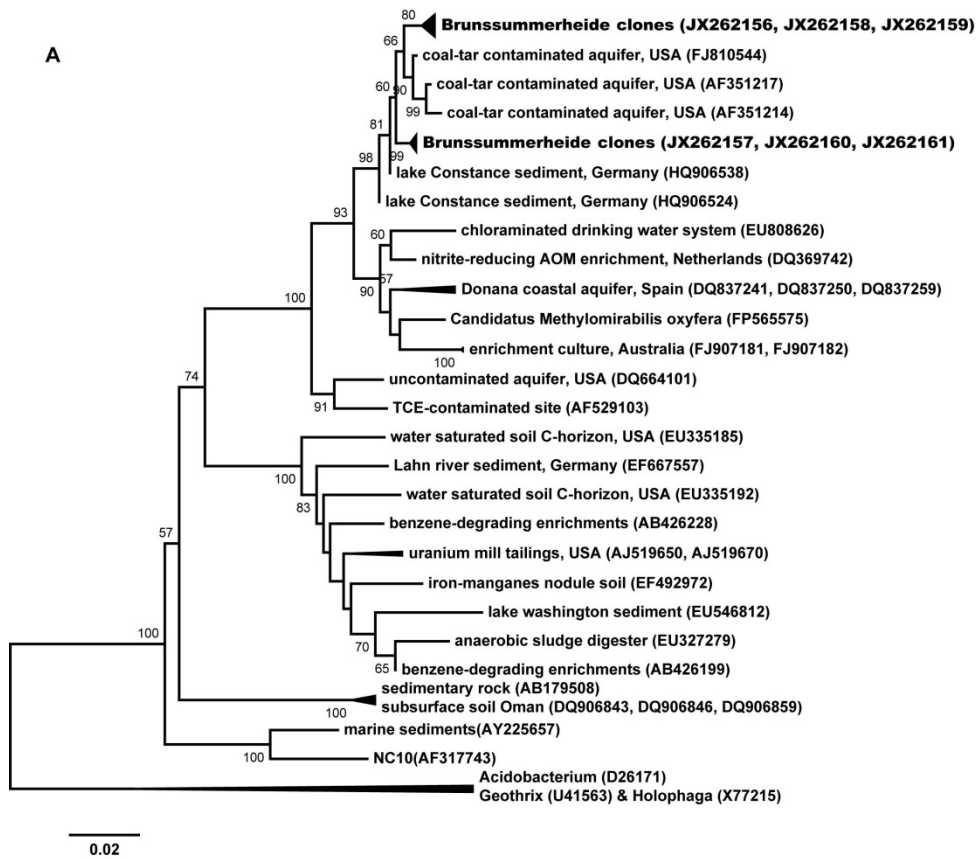
590

591   Fig. 2

592

593

594   Fig.3

595

596

597   Fig. 4

A

Brunssummerheide clones (JX262156, JX262158, JX262159)
coal-tar contaminated aquifer, USA (FJ810544)
coal-tar contaminated aquifer, USA (AF351217)
coal-tar contaminated aquifer, USA (AF351214)
Brunssummerheide clones (JX262157, JX262160, JX262161)
lake Constance sediment, Germany (HQ906538)
lake Constance sediment, Germany (HQ906524)
chloraminated drinking water system (EU808626)
nitrite-reducing AOM enrichment, Netherlands (DQ369742)
Donana coastal aquifer, Spain (DQ837241, DQ837250, DQ837259)
Candidatus Methylomirabilis oxyfera (FP565575)
enrichment culture, Australia (FJ907181, FJ907182)
uncontaminated aquifer, USA (DQ664101)
TCE-contaminated site (AF529103)
water saturated soil C-horizon, USA (EU335185)
Lahn river sediment, Germany (EF667557)
water saturated soil C-horizon, USA (EU335192)
benzene-degrading enrichments (AB426228)
uranium mill tailings, USA (AJ519650, AJ519670)
iron-manganes nodule soil (EF492972)
lake washington sediment (EU546812)
anaerobic sludge digester (EU327279)
benzene-degrading enrichments (AB426199)
sedimentary rock (AB179508)
subsurface soil Oman (DQ906843, DQ906846, DQ906859)
marine sediments(AY225657)
NC10(AF317743)
Acidobacterium (D26171)
Geothrix (U41563) & Holophaga (X77215)

0.02

B

Brunssummerheide clones (JX262153, JX262154, JX262155)
Lake Constance, Germany (HQ906566, HQ906568, HQ906571)
FP565575_Candidatus_Methylomirabilis_oxyfera
uncultured bacteria clones (AJ828646, EU723741, AJ579663, FJ529779)
Methylocystis (FN422005, AJ459046, DQ379514)
Methylosinus (DQ119048, AJ459018)
AJ278727_Methylocapsa_acidiphila_str._B2
BX649604_Methylocystis_str._SC2_clone
Methylocaldum (U89303, U89304, AB275418)
AE017282_Methylococcus_capsulatus_str._Bath
FN393957_UB(soil_New_Zealand)_p4c12
AF047705_Nitrosococcus_oceanus_ATCC19707
U31654_Methylobacter_album_str.BG8
Methylomonas (U31653, DQ119046)
Methylobacter (AY945762), Methylosoma (DQ119047)
Methylomicrobium (AB253367, AF307139)
Methylomonas (EU722432,EU722433), Methylobacter (E722430)
EU722431_Methylomicrobium_album
Nitrosospira (U76553, EF175100)
AF042171_Nitrosolobus_multiformis_str._Nm24C
DQ228465_Nitrosovibrio_sp._FJI82
Nitrosomonas (AL954747, AF037107)
Crenothrix (DQ295901, DQ295902, DQ295904)
uncultured methanotroph (AF547178, AF547181)
Methylacidiphilum (FJ462790, FJ462791,
EF591086, EU223859, EU223862, EF591085)

0.1

598
599   Fig. 5

26