LETTERS

Methanotrophic symbionts provide carbon for photosynthesis in peat bogs

Ashna A. Raghoebarsing[1], Alfons J. P. Smolders[2], Markus C. Schmid[1], W. Irene C. Rijpstra[4], Mieke Wolters-Arts[3], Jan Derksen[3], Mike S. M. Jetten[1], Stefan Schouten[4], Jaap S. Sinninghe Damsté[4], Leon P. M. Lamers[2], Jan G. M. Roelofs[2], Huub J. M. Op den Camp[1] & Marc Strous[1]

Wetlands are the largest natural source of atmospheric methane[1], the second most important greenhouse gas[2]. Methane flux to the atmosphere depends strongly on the climate[3]; however, by far the largest part of the methane formed in wetland ecosystems is recycled and does not reach the atmosphere[4,5]. The biogeochemical controls on the efficient oxidation of methane are still poorly understood. Here we show that submerged *Sphagnum* mosses, the dominant plants in some of these habitats, consume methane through symbiosis with partly endophytic methanotrophic bacteria, leading to highly effective *in situ* methane recycling. Molecular probes revealed the presence of the bacteria in the hyaline cells of the plant and on stem leaves. Incubation with ^{13}C-methane showed rapid *in situ* oxidation by these bacteria to carbon dioxide, which was subsequently fixed by *Sphagnum*, as shown by incorporation of ^{13}C-methane into plant sterols. In this way, methane acts as a significant (10–15%) carbon source for *Sphagnum*. The symbiosis explains both the efficient recycling of methane and the high organic carbon burial in these wetland ecosystems.

Peat bogs alternate between lawns and pools. Lawns are dominated by species that grow up to several decimetres above the water table. Pools are dominated by aquatic species, such as *Sphagnum cuspidatum*, that form layers of living plants below the water table. We investigated the methane-oxidizing activity of submerged *S. cuspidatum* from peat bog pools at different field locations in the Netherlands, and compared it to the activity of *S. magellanicum* and *S. papillosum* growing in lawns. The potential methane-oxidizing activity was substantially higher in the submerged mosses (Fig. 1). In control experiments with bog water, methane was not oxidized, indicating that the methanotrophic bacteria were mainly present on or in the living *Sphagnum* tissue.

The identity and location of these methanotrophs was determined in a molecular approach. Total genomic DNA from washed *Sphagnum* plants was isolated and bacterial 16S ribosomal RNA genes were amplified, cloned into *Escherichia coli*, sequenced and analysed phylogenetically. One of the 16S rRNA gene sequences of the clone library was affiliated to a cluster of type II methanotrophs that contained acidophilic methanotrophs isolated from *Sphagnum* bogs, such as *Methylocella palustris* (identity 93%)[6] and *Methylocapsa acidiphila* (identity 93%)[7].

The full 16S rRNA gene sequence was used to design two specific oligonucleotide probes for fluorescence *in situ* hybridization (FISH). FISH was combined with serial sectioning of the stems and the stem leaves of multiple individuals of submerged *S. cuspidatum*. The methanotrophic bacterium targeted by the probes was the dominant methanotroph in *S. cuspidatum* sections, accounting for over 75% of

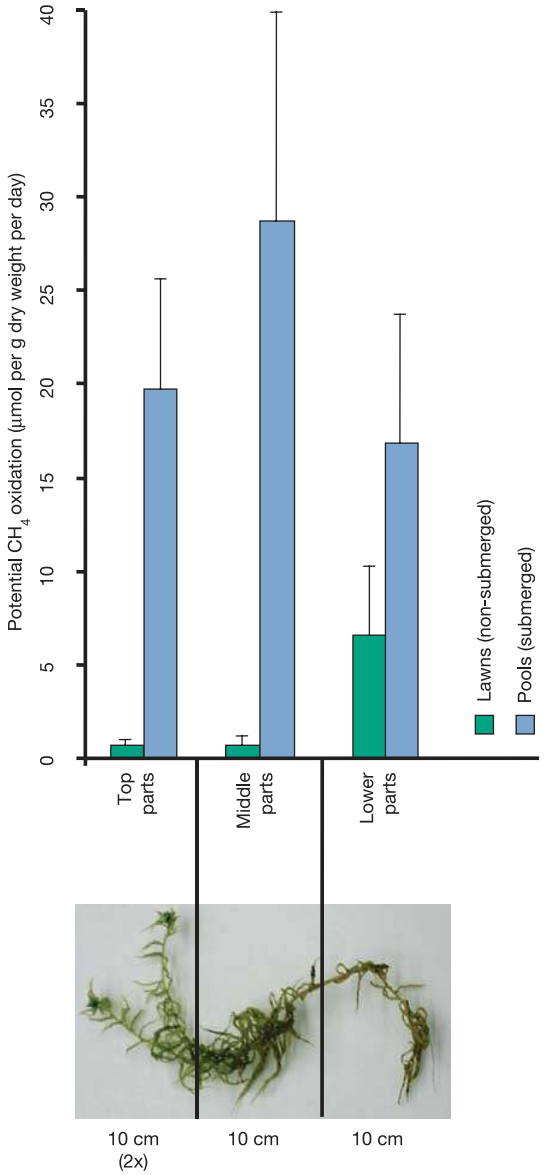

Figure 1 | Methane oxidation potential of different parts of submerged and non-submerged *Sphagnum* mosses as a measure of methanotrophs associated. Error bars indicate standard deviations of at least six independent experiments.

[1]Department of Microbiology, [2]Department of Aquatic Ecology and Environmental Biology, and [3]Department of Plant Cell Biology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands. [4]Royal Netherlands Institute for Sea Research (NIOZ), Department of Marine Biogeochemistry and Toxicology, PO Box 59, 1790 AB Den Burg, The Netherlands.

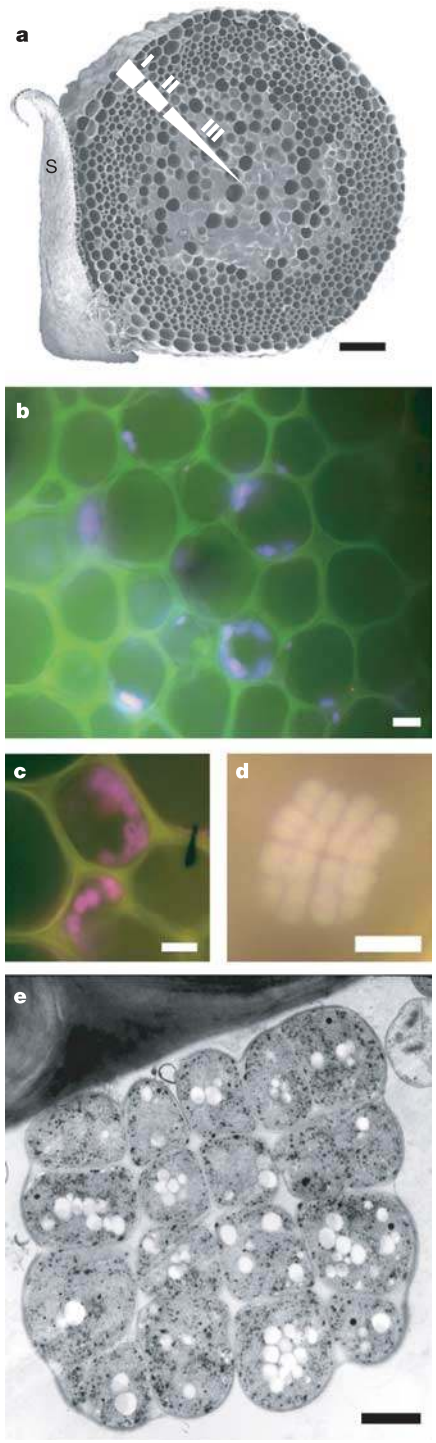

Figure 2 | *In situ* detection of the new methanotroph in *S. cuspidatum* with fluorescently labelled rRNA-targeted oligonucleotide probes. **a**, Cryo-scanning electron micrograph of a stem cross-section. S, stem leaf; I, outer cortex; II, internal cylinder; III, inner pith. Scale bar, 100 μm. **b**, **c**, Epifluorescence micrographs of the new methanotroph (purple or pink cells) in the outer cortex of *Sphagnum* stems, after a double hybridization with the specific probe S-*-18ALF-1437-a-A-18 and the general probe EUB[21]. Scale bars, 10 and 5 μm. **d**, Dense, geometric clusters of the same bacterium on a stem leaf, after a triple hybridization with the specific probe S-*-18ALF-1437-a-A-18, the general probe EUB and probe Alf968 (S-Sc-aProt-0968-a-A-18 (5′-GGTAAGGTTCTGCGCGTT-3′), specific for α-Proteobacteria). Scale bar, 5 μm. **e**, Transmission electron micrograph of a geometric cluster closely attached to a stem leaf. Scale bar, 1 μm.

Table 1 | Methane and CO_2 concentrations and $\delta^{13}C$ values in the Mariapeel bog pool

	CH_4	CO_2	Plants*
Sediment gas composition (%)	52	48	-
Bulk water concentration (μM)	50 ± 20	160 ± 30	-
$\delta^{13}C$ (‰)	−56	−14.5	−26.5

* The $\delta^{13}C$ values of growing (−26‰) and decaying (−27‰) *S. cuspidatum* were almost identical.

all α-Proteobacteria. Application of general probes showed that the α-Proteobacteria themselves made up 80% of all detected bacteria, indicating that the new methanotroph was indeed the dominant bacterium in *S. cuspidatum* sections. γ-Proteobacteria (including type I methanotrophs) were virtually absent.

In *S. cuspidatum* stems, clusters of the new methanotroph were present in the hyaline cells of the outer cortex (Fig. 2a–c; in total 10^6–10^7 methanotrophs per individual plant, total length of stem ~40 cm). Hyaline cells are dead, water-filled cells that contain pores by which solutes (and bacteria) can move in or out[8]. The presence of clusters indicated that this bacterium was actively growing inside the hyaline cells. The bacterial clusters consisted of 5–25 individual coccoid cells lying closely together in a random arrangement. On the stem leaves, the same probes hybridized with bacteria occurring as dense, geometric clusters tightly bound to the living plant cells (Fig. 2d, e, 10^5–10^6 methanotrophs per individual plant). Differences in the morphology of micro-colonies have been observed to depend on environmental conditions for other microorganisms[9]. On the basis of the measured *in vitro* methane-oxidizing capacity of *S. cuspidatum* (~20 μmol per g dry weight per day; Fig. 1) and the number of methanotrophs per plant, an activity in the order of 1–4 fmol methane $cell^{-1}\,h^{-1}$ was estimated for the associated methanotrophs. This is significantly higher than the *in vitro* methane oxidation rates reported for acidophilic methanotrophs (~0.3 fmol methane $cell^{-1}\,h^{-1}$) (ref. 6), indicating that the actual numbers of methanotrophs per *S. cuspidatum* individual might still be underestimated.

Because FISH analysis had shown that the new methanotroph was the only bacterium occurring in the characteristic geometric clusters, it was possible to identify and inspect this bacterium with transmission electron microscopy (TEM). The TEM and FISH results were consistent with respect to the localization of the methanotroph. TEM also showed that this bacterium did not contain any intracytoplasmic membranes. The absence of intracytoplasmic membranes was noted previously for the phylogenetically related type II methanotroph *M. palustris*[6]. Otherwise, intracytoplasmic membranes are a characteristic feature of methanotrophic bacteria.

The predominance of type II methanotrophs was further substantiated by the presence of bishomohopanoic acid in *Sphagnum* lipid extracts after periodic acid treatment. This compound was previously shown to form after periodic acid treatment from the C_{35} hopanetetrol derivatives, membrane rigidifiers produced by methanotrophic bacteria[10]. The natural ^{13}C contents of this compound ($\delta^{13}C = -39.8$‰) were substantially depleted relative to *Sphagnum* cell material and enriched compared to that of methane (Table 1), in accordance with its origin from serine-cycle (type II) methanotrophic bacteria[11]. Using this methanotrophic biological marker we were able to determine whether the methanotrophs associated with *Sphagnum* were actively growing. After incubating *Sphagnum* with ^{13}C-labelled methane for 5 days, isotopic analysis showed that ^{13}C-labelled methane was incorporated into this lipid in substantial amounts; nearly 50% of this lipid was synthesized from the labelled methane, indicating that the methanotrophic population had doubled over the course of the experiment.

The observed tight association of methanotrophic bacteria with *S. cuspidatum* would enable the efficient recycling into living mosses

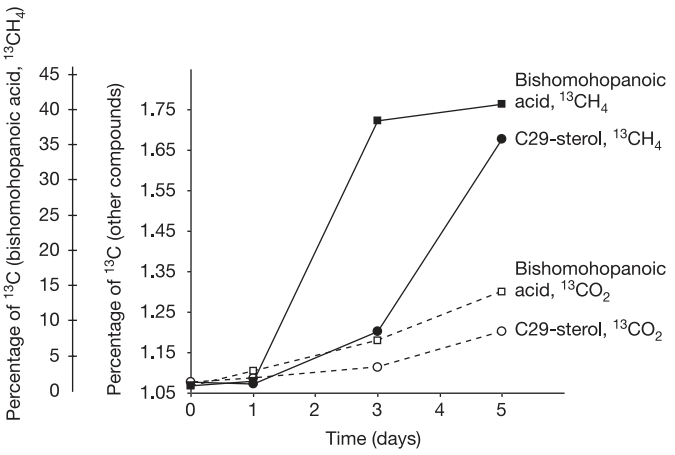

Figure 3 | Incorporation of ^{13}C label in biological markers for *Sphagnum* (circles) and methanotrophic bacteria (squares). Filled circles/solid lines show the results for labelled methane (99% ^{13}C) in the presence of unlabelled carbon dioxide; open symbols/dashed lines show the results for labelled carbon dioxide (4% ^{13}C).

of both oxygen (derived from photosynthesis) and methane (derived from decaying plants), according to the following set of equations:

$$CH_4 \text{ oxidation}: \quad CH_4 + 2\ O_2 = CO_2 + 2\ H_2O \qquad (1)$$

$$CO_2 \text{ fixation}: \quad 2\ CO_2 + 2\ H_2O = 2\ CH_2O + 2\ O_2 \qquad (2)$$

$$\text{Balance}: \quad CH_4 + CO_2 = 2\ CH_2O \qquad (3)$$

To provide experimental evidence for this scenario, the potential contribution of methane to carbon fixation by *S. cuspidatum* was investigated under conditions relevant to the field. Multiple batches of individuals of *S. cuspidatum* were incubated with ^{13}C-labelled methane in the presence of unlabelled carbon dioxide. As a control experiment, only ^{13}C-labelled carbon dioxide was supplied. Both compounds were added to a final concentration of 0.2 mM, close to the *in situ* concentrations (Table 1). Over 5 days, incorporation of the label by *S. cuspidatum* was determined via the ^{13}C incorporation into sitosterol, a *Sphagnum*-specific sterol (Fig. 3). Methane was assimilated into the sitosterol pool at a rate of 0.20 ± 0.03 μg C per g dry weight per day, compared to 1.4 ± 0.1 μg C per g dry weight per day for carbon dioxide. Thus, in the presence of carbon dioxide, at near *in situ* concentrations, the capacity of methane incorporation by *S. cuspidatum* was ~15% of the carbon dioxide assimilation capacity.

The natural carbon isotope abundances of *Sphagnum* mosses in the field ($\delta^{13}C$ −26.5‰; Table 1) are consistent with our estimate that 15% of the carbon fixed by *Sphagnum* derives from isotopically depleted methane (that is, −56‰; Table 1). *S. cuspidatum* fixes carbon dioxide via the Calvin cycle, and is able to fractionate strongly against ^{13}C (up to 29‰) at high carbon dioxide concentrations (>2 mM)[12,13]. However, unlike vascular (semi-)aquatic plants such as rice, *S. cuspidatum* does not have aerenchyma[8] that facilitate the transport of atmospheric carbon dioxide. Therefore, at lower carbon dioxide concentrations, carbon assimilation by *S. cuspidatum* is limited by mass transfer, and carbon fractionation has been reported to decrease to at most 4‰ (refs 12, 13). Because the average carbon dioxide concentration in the field was approximately 0.16 mM, a range of 4–10‰ was used as a conservative estimate for carbon fractionation by *S. cuspidatum* in the field[12,13]. With this assumption, the data from Table 1 and a simple isotopic mass balance (see Methods), we calculated that methane contributed on average between 5% and 20% to the carbon fixed by *S. cuspidatum* in the field, in good agreement with the labelling results. It is likely that variation in local conditions (water depth, exposure to wind, temperature, light availability, rates of methane ebullition compared to diffusion/advection) will affect the relative contribution of methane to the carbon uptake of *Sphagnum* mosses in space and time. This will also be determined by the location of the symbiotic methanotrophs in the plants, both in the direct vicinity of the photosynthetically active cells and in the more remote hyaline cells of the stems.

Our results show that methane is a significant and as yet overlooked supplement to the carbon intake of submerged *S. cuspidatum* in peat bogs. Peat bogs in the Northern Hemisphere store up to one-third of the carbon sequestered in soils globally[14]. This is surprising considering that the primary production is limited by the nutrient delivery through rain water and the limited delivery of carbon dioxide to the acidic waters of these ecosystems[5]. The efficient recycling of peat decomposition products (such as methane) as demonstrated here may mechanistically explain the paradox of peatlands as ecosystems with apparent low primary productivity combined with high carbon burial.

METHODS

***In situ* conditions.** In the Mariapeel nature reserve (the Netherlands: 51° 24′ 90″ N; 5° 54′ 90″ E), $\delta^{13}C$ values of *Sphagnum* mosses and material from the decaying peat were determined on freeze-dried homogenized material as described previously[15]. Concentrations and $\delta^{13}C$ values of dissolved carbon dioxide and methane were measured as described previously[16].

Methane oxidation. Potential methane oxidation of different parts of *Sphagnum* were measured by incubating 6 g of thoroughly washed *Sphagnum* in 100 ml infusion flasks sealed with airtight rubber stoppers. To prevent mass transport limitations, no additional water was added to the experiments. To each flask 1 ml of pure methane was added and methane consumption was measured every 6 h over 2 days. Methane oxidation rates were calculated by linear regression. Tenfold concentrated water samples (10^6 bacterial cells ml^{-1}) from the bog were used as controls and showed no methane oxidation. Samples were collected in the Netherlands from seven lawn locations (*S. magellanicum*, *S. papillosum*) and six bog pools (*S. cuspidatum*), one of these being the Mariapeel. Methane was measured on an HP 5890 gas chromatograph equipped with a flame ionization detector and a Porapak Q column (80/100 mesh).

16S rRNA gene sequence analysis, FISH and electron microscopy. Total genomic DNA from *S. cuspidatum* plants containing methanotrophs, isolated with combined methods[17], was used as template for PCR amplification of 16S rRNA genes. PCR was performed with general bacterial primers[18] using a T gradient thermal cycler (Biometra), and a clone library was made as described previously[18]. Based on the obtained 16S rRNA gene sequences, two new oligonucleotide probes S-*-18ALF-0218-a-A-18 (5′-GGGCCGATCCCCC GGCGA-3′) and S-*-18ALF-1437-a-A-18 (5′-CTTGCGGTTAACAGAACG-3′) were designed using the ARB program package[19]. Apart from these species-specific probes we used group-specific probes described previously[20,21]. Fresh *S. cuspidatum* stems sectioned with a scalpel (section thickness 0.1 ± 0.05 mm) were used for FISH as described previously[22]. Formamide concentrations used in the FISH experiments varied between 10% and 20%. No signal was obtained at these formamide concentrations when testing the specificity of the probes with *Beijerinckia indica* ssp. *indica* (DSM 1715), which has the fewest mismatches of all reference organisms to the designed probes. Electron microscopy (TEM/SEM) was performed on stems and stem leaves following published protocols[6,22].

Methane incorporation measurements. *S. cuspidatum*, collected from the Mariapeel nature reserve, was washed with demineralized water and incubated in 250-ml serum bottles in 5 g wet weight aliquots with 150 ml medium as described previously[8]. ^{13}C- or ^{12}C-CH_4 or CO_2 were added to final concentrations of 200 μM as specified in the text. The bottles were shaken at 150 r.p.m. at ambient conditions and sacrificed for lipid analysis at days 0, 1, 3 and 5. Lipids were ultrasonically extracted and analysed by gas chromatography/mass spectrometry and isotope ratio gas chromatography mass spectrometry as described[23]. Hopanes were analysed by treatment of the lipid fraction with periodic acid and sodium borohydride as described previously[10,24].

Isotopic mass balancing. The measured $\delta^{13}C$ values of *S. cuspidatum* in the field (−26‰, see Table 1) resulted from assimilation of dissolved carbon dioxide (−14.5‰), respired methane (−56‰) and fractionation against ^{13}C during (mass-transfer-limited) carbon dioxide fixation[12,13] (7 ± 3‰). The following equation describes this relationship quantitatively (where *a* denotes the fraction of plant carbon derived from methane and *Ep* denotes the fractionation during

fixation):

$$\delta^{13}\mathrm{C}\ (\textit{Sphagnum}) = a\delta^{13}\mathrm{C}\ (\text{respired methane}) + (1-a)\delta^{13}\mathrm{C}\ (\text{carbon dioxide}) - Ep \quad (4)$$

Because all δ^{13}C values from equation (4) were known experimentally, it could be derived that the contribution of methane to *Sphagnum* carbon (a) was between 0.05 and 0.2 (equivalent to 5–20%).

Received 23 December 2004; accepted 9 May 2005.

1. Hein, R., Crutzen, P. J. & Heimann, M. An inverse modeling approach to investigate the global atmospheric methane cycle. *Global Biogeochem. Cycles* **11,** 43–76 (1997).
2. Rodhe, H. A comparison of the contribution of various gases to the greenhouse effect. *Science* **248,** 1217–1219 (1990).
3. Smith, L. C. *et al.* Siberian peatlands a net carbon sink and global methane source since the early Holocene. *Science* **303,** 353–356 (2004).
4. Dedysh, S. N. *et al.* Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands. *Science* **282,** 281–284 (1998).
5. Lamers, L. P. M., Farhoush, C., Van Groenendael, J. M. & Roelofs, J. G. M. Calcareous groundwater raises bogs; the concept of ombrotrophy revisited. *J. Ecol.* **87,** 639–648 (1999).
6. Dedysh, S. N. *et al. Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int. J. Syst. Evol. Microbiol.* **50,** 955–969 (2000).
7. Dedysh, S. N. *et al. Methylocapsa acidophila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen fixing acidophilic bacterium from *Sphagnum* bog. *Int. J. Syst. Evol. Microbiol.* **52,** 251–261 (2002).
8. Rydin, H. & Clymo, R. S. Transport of carbon and phosphorus compounds about *Sphagnum*. *Proc. R. Soc. Lond.* **237,** 63–84 (1989).
9. Yao, R., Macario, A. J. L. & Conway de Macario, E. Immunochemical differences among *Methanosarcina mazei* S-6 morphologic forms. *J. Bacteriol.* **174,** 4683–4688 (1992).
10. Rohmer, M., Bisseret, P. & Neunlist, S. in *Biological Markers in Sediments and Petroleum* (eds Moldowan, J. M., Albrecht, P. & Philp, R. P.) 1–17 (Prentice Hall, London, 1992).
11. Jahnke, L. L., Summons, R. E., Hope, J. M. & Des Marais, D. J. Carbon isotopic fractionation in lipids from methanotrophic bacteria II: The effects of physiology and environmental parameters on the biosynthesis and isotopic signatures of biomarkers. *Geochim. Cosmochim. Acta* **63,** 79–93 (1999).
12. Keeley, J. E. & Sandquist, D. R. Carbon: freshwater plants. *Plant Cell Environ.* **15,** 1021–1035 (1992).
13. Smolders, A. J. P., Tomassen, H. B. M., van Mullekom, M., Lamers, L. P. M. & Roelofs, J. G. M. Mechanisms involved in the re-establishment of *Sphagnum*-dominated vegetation in rewetted bog remnants. *Wetlands Ecol. Manag.* **11,** 403–418 (2003).
14. Post, W. M., Emanuel, W. R., Zinke, P. J. & Strangenberger, A. G. Soil carbon pools and world life zones. *Nature* **298,** 156 (1982).
15. Marguillier, S., van der Velde, G., Dehairs, F., Hemminga, M. A. & Rajagopal, S. Trophic relationship in an interlinked mangrove-seagrass ecosystem as traced by δ^{13}C and δ^{15}N. *Mar. Ecol. Prog. Ser.* **151,** 115–121 (1997).
16. Smolders, A. J. P., Tomassen, H. B. M., Lamers, L. P. M., Lomans, B. P. & Roelofs, J. G. M. Peat bog restoration by floating raft formation: the effects of groundwater and peat quality. *J. Appl. Ecol.* **39,** 391–401 (2002).
17. Lomans, B. P. *et al.* Microbial populations involved in cycling of dimethyl sulfide and methanethiol in freshwater sediments. *Appl. Environ. Microbiol.* **67,** 1044–1051 (2001).
18. Juretschko, S. *et al.* Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl. Environ. Microbiol.* **64,** 3042–3051 (1998).
19. Ludwig, W. *et al.* ARB: A software environment for sequence data. *Nucleic Acids Res.* **32,** 1363–1371 (2004).
20. Amann, R. I. *et al.* Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* **56,** 1919–1925 (1990).
21. Daims, H., Bruhl, A., Amann, R., Schleifer, K. H. & Wagner, M. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst. Appl. Microbiol.* **22,** 434–444 (1999).
22. Wolters-Arts, M. *et al.* Water-conducting properties of lipids during pollen hydration. *Plant Cell Environ.* **25,** 513–519 (2002).
23. Schouten, S. *et al.* Biosynthetic effects on the stable carbon isotopic compositions of algal lipids: Implications for deciphering the carbon isotopic biomarker record. *Geochim. Cosmochim. Acta* **62,** 1397–1406 (1998).
24. Sinninghe Damsté, J. S. *et al.* The occurrence of hopanoids in planctomycetes: implications for the sedimentary biomarker record. *Organic Geochim.* **35,** 561–566 (2004).

Acknowledgements We thank K. T. van de Pas-Schoonen, A. Pol, H. P. M. Geurts, J. Eygensteyn, M. van Mullekom, J. Berk, H. Tomassen and M. M. A. van Herpen for technical support. Part of this study was supported by the Dutch Ministry of Agriculture, Nature Management and Food quality (Research Program 'Overlevingsplan Bos en Natuur').

Author Information The 16S rRNA gene sequences were deposited at GenBank under accession number AY163571. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.J.P.S. (a.smolders@science.ru.nl) or J.S.S.D. (damste@nioz.nl).