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Contribution by the Methanogenic Endosymbionts of Anaerobic Ciliates to Methane Production in Dutch Freshwater Sediments

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Summary. Biogenic methane contributes substantially to the atmospheric methane concentration and thus to global warming. This trace gas is predominantly produced by strictly anaerobic methanogenic archaea, which thrive in the most divergent ecological niches, e. g. paddy fields, sediments, landfills, and the digestive tract of various animals. Methanogenic archaea also live as endosymbionts in the cytoplasm of anaerobic protozoa. In marine sediments these endosymbionts can contribute up to 90% to the overall rate of methanogenesis, whereas their role of in freshwater sediments is largely unknown. Here we describe the results of a one year's survey of the methanogenesis by endosymbiotic methanogens in four different Dutch freshwater sediments. The abundance of anaerobic protozoa, in particular ciliates, the methane production rates by the ecosystem and by the protists, and a number of abiotic parameters were measured. A novel method (heat-shock for 5 min) for estimating the contribution by endosymbiotic methanogens was established. Our results reveal large fluctuations of ciliate abundance throughout the year, but on average, only minor contributions by methanogenic endosymbionts to the total methanogenesis in these environments.

Key words: anaerobic ciliates, endosymbionts, freshwater sediments, methanogenesis, single cell PCR, SSU rRNA.

INTRODUCTION

Methanogenic archaea account for an annual global production of some 400 million metric tons of methane (Ferry 1997). This has a profound impact on the global change, because the atmospheric methane, which originates from these biological sources, can contribute for about 20% to global warming (Lindau *et al*. 1993). Since atmospheric methane concentrations have increased steadily by about 1-2% per year through the last decades, (Ferry 1997), detailed information about the biological - but also the unconventional, i.e. plant-dependent - sources of methane production is required for an assessment of the possibilities for a global management of greenhouse gasses (Chynoweth 1996, Waßmann and Rennenberg 1996, Hansen *et al*. 2000, Frankenberg *et al*. 2005, Keppler *et al*. 2006).

Methane formation by methanogenic archaea occurs in nearly all anaerobic environments such as, for example, marine- and freshwater sediments, marshes, wetlands, and tundra's (Williams and Crawford 1984,

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Zinder 1993). Also, the gastro-intestinal tracts of arthropods and herbivorous vertebrates represent significant sources for atmospheric methane (Crutzen *et al.* 1986, Fraser *et al.* 1986, Hackstein and Stumm 1994, Hackstein *et al.* 1996, Moss *et al.* 2000). Notably, certain methanogenic archaea thrive as intracellular symbionts in the cytoplasm of anaerobic protozoa such as amoebae, flagellates and ciliates (Vogels *et al*. 1980; van Bruggen *et al.* 1983; Fenchel and Finlay 1995; van Hoek *et al.* 2000; Hackstein *et al.* 2002, 2006). The basis for these endosymbiotic, mutualistic associations between protozoa and methanogenic archaea is provided by the host's hydrogenosomes, organelles that generate hydrogen, carbon dioxide, acetate, and ATP (Müller 1993, Fenchel and Finlay 1995, Hackstein *et al*. 2001, Martin *et al*. 2001, Embley *et al*. 2003, Yarlett and Hackstein 2005). These hydrogenosomes provide the nutritional basis for the persistence of hundreds to thousands of methanogenic endosymbionts, which colonise a single host cell (Wagener *et al.*1990, Fenchel and Finlay 1992, Finlay and Fenchel 1992, Esteban *et al.* 1993, van Hoek *et al.* 2000, Hackstein *et al*. 2002).

In marine sediments, the methanogenic endosymbionts of anaerobic protozoa can contribute substantially to biogenic methane production (Fenchel 1993). Also, rumen protozoa with their episymbionts can play an important role in the methanogenesis in the gastrointestinal tract of large herbivorous animals: elimination of these protozoa from the ruminal ecosystem can result in a decrease of methane production by some 9-45% (Newbold *et al.* 1995, Tokura *et al.* 1999). Lastly, the methanogenic endosymbionts of anaerobic ciliates in cockroaches are believed to be the major source of methane in the hindgut of these insects (Gijzen *et al.* 1991, Gijzen and Barughare 1992).

In certain freshwater sediments and rice field soils anaerobic protozoa (with methanogenic endosymbionts) can be rather abundant (Finlay and Fenchel 1991; Khalil and Shearer 1993; Finlay *et al.* 1998, 1999; Schwarz and Frenzel 2005). However, only a few studies have attempted unravelling the contribution of their endosymbionts to the production of methane in these environments so far, although anoxic freshwater environments are believed to account for more than 20% to the total methane flux to the atmosphere globally. Also, seasonal changes in methane production and the abundance of ciliates did not find the necessary attention in the past. Therefore, we have monitored the abundance of anaerobic ciliates, methane production by ciliates and free-living archaea and several biotic and abiotic parameters in four Dutch freshwater sediments over a period of one year. We established a novel technique (heat-shocking) for assaying the contributions of anaerobic ciliates to the methane emissions in strictly anaerobic microcosms. With the aid of this technique, we were able to demonstrate that the contribution of the endosymbionts of anaerobic ciliates to methanogenesis in these ecosystems differs locally by at least one order of magnitude, and is subject to substantial annual fluctuations. However, on average, the contribution of anaerobic ciliates is marginal with respect to the total emissions of methane in Dutch freshwater ecosystems.

MATERIALS AND METHODS

Freshwater sediments

The samples were collected from October 1994 to September 1995 in one-month-intervals at four different locations in the vicinity of Nijmegen, The Netherlands (51°45'North 5°55'East (QRA-locator: JO21XS)), i.e. in a ditch of a minerotrophic peat-land ("de Bruuk"), a sludge backing pond of a wastewater treatment ("Dekkerswald"), an oligotrophic pond ("Plasmolen") and a low-land brook ("Tielebeek"). Sampling in 1l bottles was performed under strictly anaerobic conditions as described by Lomans *et al.* (1997). The *in situ* temperature of the sediment was measured prior to sampling. At larger intervals the alkalinity, the conductivity, the pH, and the velocity of the streaming water at the different sampling sites was measured with the aid of a YSI model 33 SCT meter.

Ciliate count

The number of the anaerobic ciliates was determined under a dissecting microscope at 50× magnification after diluting 100 µl sediment slurry with an equal amount of filter-sterilised (0.2 µm) sediment (pore)-water. The average ciliate number was determined by five independent counts using a Sedgewich rafter (Graticules LTD, Tonbridge, Kent, UK). The anaerobic ciliates were determined to the genus level with the aid of Hausmann (1985) and Patterson and Hedley (1992), which are useful for a rapid determination *in vivo*. Light- and epifluorescence microscopy of selected (living) specimen at 400× magnification was used to confirm the identity of the anaerobic ciliates and the presence endosymbiotic methanogenic archaea by their characteristic autofluorescence *in vivo* (Doddema and Vogels 1978).

Molecular identification

Single ciliates were isolated from the sample with the aid of a micropipette made from a drawn out Pasteur pipette, washed three times with sterile, anaerobic electromigration buffer (van Hoek *et al.* 1999), transferred to a Eppendorf tube and centrifuged. After addition of 50 µl of a 5% Chelex-100 suspension (Walsh *et al.* 1991) the samples were frozen and stored at -20°C. DNA extraction was performed by adding proteinase K (10 mg/ml), incubation for 3-4 h at 56°C, and subsequent heating at 95°C for 10 min. After centrifuga-

tion, the supernatant was used for PCR with primers directed against the 16S and 18S rRNA genes of the endosymbionts and the ciliate, respectively, as described earlier (van Hoek *et al.* 1998, 2000).

Phylogenetic analysis

Small ribosomal DNA sequences from the anaerobic ciliates and their methanogenic endosymbionts were aligned using the PileUp program of the Wisconsin package, version 8.1 (Wisconsin package Version 8.1, Genetics Computer Group (GCG), Madison, Wisconsin). EDNADIST (Rice *et al.* 1995) was used to calculate the sequence similarity and evolutionary distances using the Jukes and Cantor (1969) nucleotide substitution model. Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei 1987). The 18S rRNA gene sequences of the following ciliates were used for the construction of the phylogenetic trees: *Brachonella* sp. from "de Bruuk" (AJ009665), *Brachonella* sp. from "Plasmolen" (AJ009664), *Caenomorpha*-like sp. 1 from "Dekkerswald" (AJ009658), *Caenomorpha* sp. 2 from "Dekkerswald" (AJ009660), *Caenomorpha*-like sp. 4 from "Dekkerswald" (AJ009661), *Caenomorpha*-like sp. 8 from "Dekkerswald" (AJ009662), *Caenomorpha* sp. 10 from "Dekkerswald" (AJ009659), *Caenomorpha* sp. from "Plasmolen" (AJ009663), *Caenomorpha uniserialis* (U97108), *Metopus contortus* (Z29516), *Metopus palaeformis* (M86385), *Plagiopyla nasuta* (Z29442), *Plagiopyla frontata* (Z29440), *Trimyema compressum* (Z29438), *Blepharisma americanum* (M97909). The 16S rRNA gene sequences of the corresponding methanogenic endosymbionts have the following accession numbers: *Caenomorpha*-like sp. 1 from "Dekkerswald" (AJ132648), *Caenomorpha*-like sp. 4 from "Dekkerswald" (AJ132649), *Caenomorpha*-like sp. 8 from "Dekkerswald" (AJ132650), *Caenomorpha* sp. 10 from "Dekkerswald" (AJ132651), *Caenomorpha* sp. 2 from "Dekkerswald" (AJ132652), *Caenomorpha* sp from "Plasmolen" (AJ132653), *Brachonella* sp from "de Bruuk" (AJ132655), *Brachonella* sp. from "Plasmolen" (AJ132654), *Metopus contortus* (Z13957), *Metopus palaeformis* (M86386), *Plagiopyla frontata* (Z29439), *Plagiopyla nasuta* (Z29437), *Trimyema compressum* (M96976), *Methanococcus voltae* (M59290)

Sediment incubations and methane measurements

After settling for at least 1 h the sediment samples were adjusted to a sediment/water ratio of 1:1 (v/v) by either removing water or sediment in an anaerobic glove box. The sediment samples were thoroughly mixed and aliquots of 20 ml were dispensed in 60 ml crimp top serum bottles. The bottles were sealed with black butyl rubber stoppers and flushed with oxygen-free nitrogen gas. Ethane was added as an internal standard for methane concentration measurements. The sediment slurries were incubated in the dark without shaking at 15°C for 24-48 h. At regular time intervals gas samples were taken from the headspace after shaking of the bottles. The gas sample was analysed on a Pye Unicam gas chromatograph equipped with a flame ionisation detector and a Porapak Q (80/100 mesh) column for the presence of methane. The methane production rate was determined from the linear increase of CH_4 in time using linear regression analysis. The measurements were not extended beyond 48 h in order to avoid excystation of ciliates from resting stages and to guarantee a survival of all ciliates present in the sample over the whole incubation period.

The dry weight of the sediment was determined after the incubation period by drying to a constant weight at 80°C. To determine the organic weight of the sediments, the dried sediment was reduced to ashes by heating to 550°C for 4 h.

Measuring the contribution to methane production by anaerobic ciliates

To estimate the contribution of anaerobic protozoa to the methane production several separation/removal methods, such as filtration over a 100 µm nylon gaze, the ice-elution method of Uhlig (Uhlig 1964), electromigration (Wagener *et al*. 1986, van Hoek *et al*. 1999), and micropipetting were tested for feasibility and reproducibility. Since all these methods were very time consuming and, in addition, led to irreproducible results, a novel method, a heat treatment of the sediment was developed. A heat-shock of 45°C for 5 min was applied to the incubation flasks in a waterbath. This treatment effectively killed all anaerobic ciliates present in the sediment.

For the determination of the contribution of the methanogenic endosymbionts, well mixed 20 ml of sediment slurries were adjusted to a sediment/water ratio of 1:1 (v/v) and dispensed into 60 ml bottles in an anaerobic glove box. Half of the bottles prepared this way were heat-shocked for 5 min at 45°C to allow determination of the methane production by free-living methanogens, the other half was not heat-shocked for a measurement of the methane production by the undisturbed sediment. The contribution of the endosymbionts was calculated from the difference between both measurements.

RESULTS

Abiotic parameters of the sampling sites

The four different sampling places near Nijmegen, The Netherlands, [51°45'N 5°55'E (QRA-locator: JO21XS)], are substantially different, but characteristic for many freshwater sediments in the Netherlands (Table 1). "de Bruuk" is a ditch in a minerotrophic peatland, "Dekkerswald" a sludge backing pond of a wastewater treatment, "Plasmolen"an oligotrophic pond in a deciduous forest, and "Tielebeek"a low-land brook. Three of the sampling places contained more or less complex communities of anaerobic ciliates; one site ("Tielebeek"), however, did not contain any anaerobic ciliates throughout the year. The yearly temperature course in the sediments followed largely the average ambient and ground temperatures as documented by the National Centre for Meteorology, the KNMI in de Bilt, The Netherlands (Fig. 1).

Seasonal variations in the abundance of anaerobic (ciliated) protozoa

The four different freshwater sediments were screened once a month for the presence of anaerobic protists as

Table 1. Features of the four Dutch freshwater sediments.

	de Bruuk	Dekkerswald	Plasmolen	Tielebeek
Characteristics	ditch of a minerotrophic peatland	sludge backing pond of a wastewater treatment	oligotrophic pond	low-land brook
Depth (m)	1.0-1.5	0.25-0.5	0.5-0.75	0.25-0.5
Alkalinity (mM)	0.4	0	0	0.01
Conductivity (µS)	430	425	380	170
pH	6.3	6.5	6.4	5.9
Velocity (m/s)	0	0	0	0.02
Organic matter[1]	15 ± 4.2	28.5 ± 6.4	58.8 ± 5.7	11.3 ± 3.3
Sediment colour	Black	green/brown	red/brown	green

[1]The organic matter was calculated as the year's average of the percentage organic weight of the dry weight of the sediment samples.

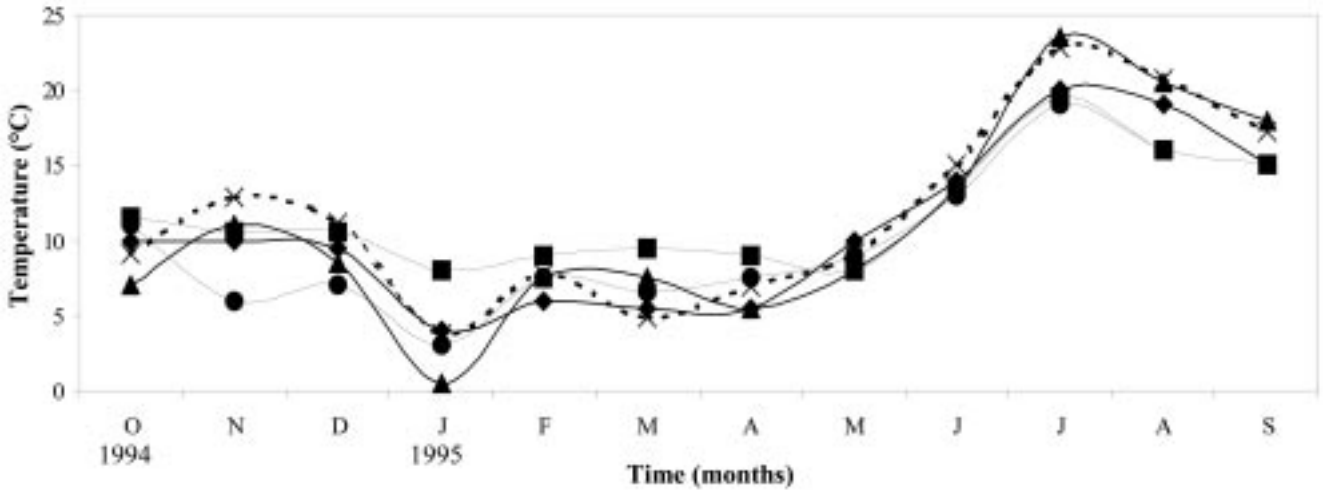
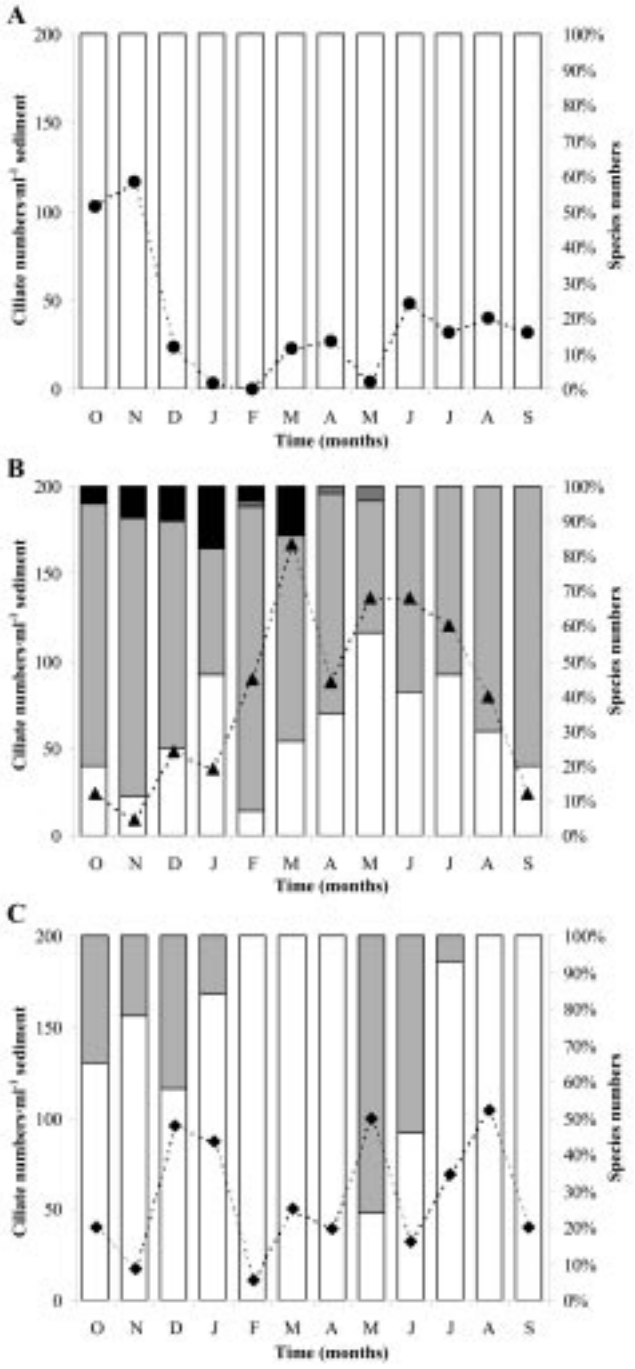


Fig. 1. *In situ* sediment temperature and average day temperature (according to the KNMI, The Netherlands) during the sampling days, from October 1994 until September 1995. "de Bruuk" sediment - filled circle, "Dekkerswald" sediment - filled triangle, "Plasmolen" sediment - filled diamond, "Tielebeek" sediment - filled square, and KNMI data - ×.

described in Materials and Methods by direct observation *in vivo*. Ciliates were the most abundant anaerobic protists in 3 out of the 4 sediments. Only a few anaerobic flagellates and amoebae were observed. Anaerobic ciliates were completely absent in the "Tielebeek" sediments over the whole one-year's survey. At the other three sampling places, the number of anaerobic ciliates exhibited a substantial variation over the year with remarkable differences between the different locations (Fig. 2). For example, the number of anaerobic ciliates exceeded 100/ml in the "de Bruuk" sediment only in the first two months of our survey (October, November), but declined to less than 50 ciliates per ml sediment for the rest of the year (Fig. 2A). The number of ciliates in "Plasmolen" sediment revealed no clear trends - during some months ciliate numbers were as low as 25 ciliates/ml sediment, whereas in other months up to 90 ciliates/ml sediment were counted (Fig. 2C). On the other hand, the number of ciliates in the "Dekkerswald" sediment exhibited a distinct peak in March with more



Figs 2A-C. The total number of anaerobic ciliates and the distribution of the species in the four different sediments; **A** - "de Bruuk" sediment; **B** - "Dekkerswald" sediment; **C** - "Plasmolen" sediment. At regular time intervals gas samples were taken from the headspace after shaking. The "Tielebeek" sediment did not contain any anaerobic ciliates throughout the screening. The circles, the triangles, and the diamonds indicate the total number of anaerobic ciliates in the three different sediments, respectively. Open bars indicate the percentage of *Brachonella* spp. of the total number of anaerobic ciliates; light-grey bars *Caenomorpha* spp.; dark-grey bars *Metopus* spp.; black bars *Plagiopyla* spp.

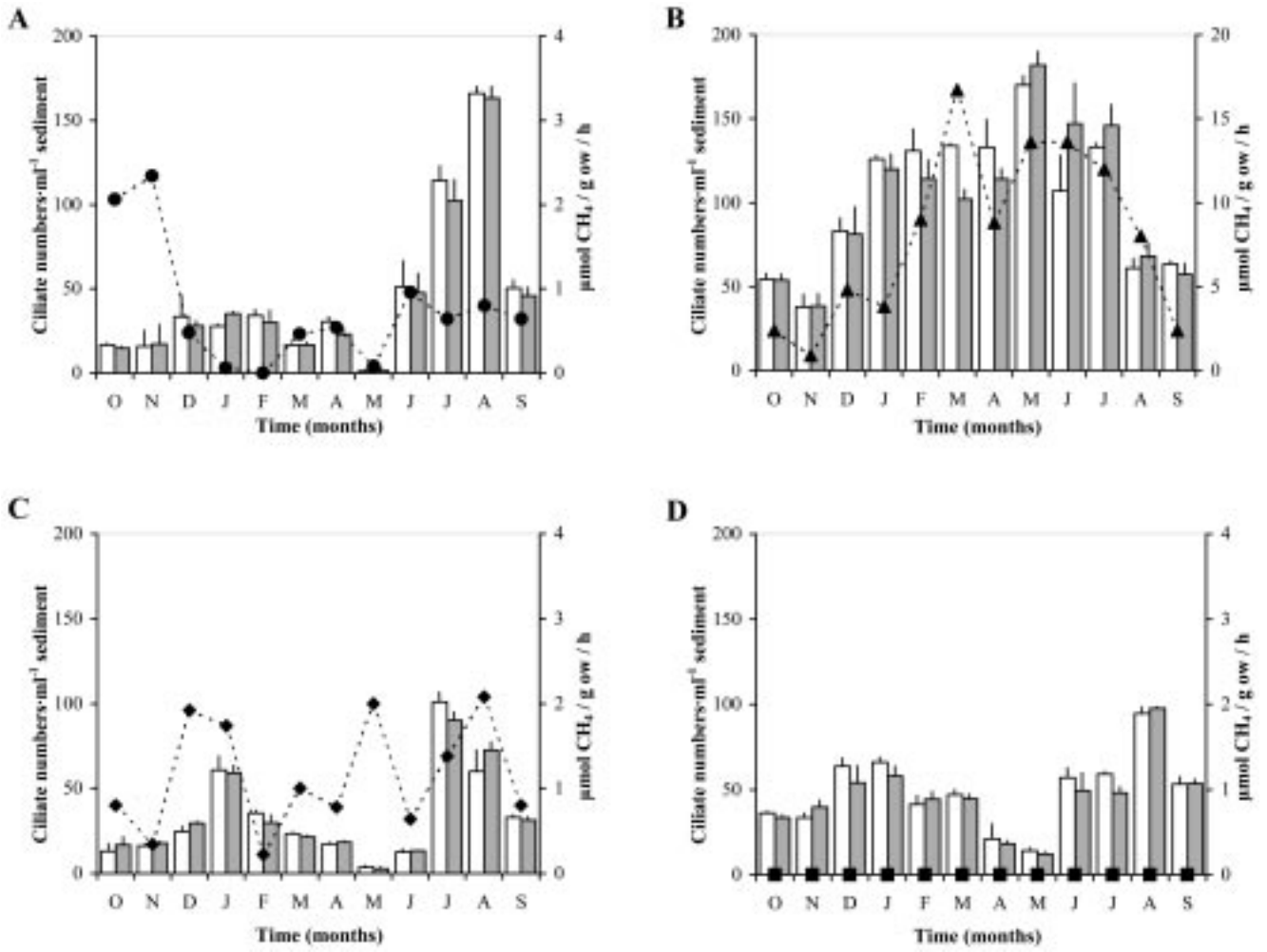
than 150 ciliates/ml, which remained at a rather high level of more than 100 ciliates /ml in the following months, with a short dip in April followed by a constant decline of ciliate numbers (Fig. 2B).

Methane production in freshwater sediments

The methane concentration in the headspace was measured in regular time intervals up to 48 h, when the ciliates were still alive. Figure 3 shows that the methane production rates in the four sampling places were different. All exhibited pronounced seasonal variations. "Dekkerswald" sediment had the highest methane production rate of all sites, up to a maximum of 17 µmol CH_4/g organic weight/h. The maximal rates of the other three sediments were 5-10 times lower; they did not exceed some 2-3 µmol CH_4/g organic weight/h. Also the seasonal pattern was different for the "Dekkerswald" sediment: the methane production rate showed an unimodal distribution from November until September of the next year with a peak in May while the other three sediments exhibited a bimodal distribution of methane production with a minimum production in May (Figs 3A, C, D).

Contribution to methane production by anaerobic ciliates

Figure 3 clearly shows that in all three ciliate containing sediments the peaks of methane production did not coincide with the maxima of the ciliate abundance. Accordingly, the removal of the ciliates in the microcosms by heat-shocking had no significant effect on the methane production in the ciliate-free slurries. Only in samples with ciliate counts above 70-80 ciliates/ml the methane production was slightly, but significantly, lowered after the removal of the ciliates (grey bars in Fig. 3). This was the case only the months February, March, and April in the "Dekkerswald" samples (Fig. 3B), and the month July in the "Plasmolen" samples. Unexpectedly, the heat-shock treatment of "Dekkerswald" samples in the months May, June, and July led to a slightly increased methane production (Fig. 3B). In all other months, the removal of ciliates by the heat-shock treatment did not cause significant changes in the methane production, confirming a minor role of the methanogenic endosymbionts in methane formation. Notably, the validity of the heat-shock technique for the removal of the ciliates was confirmed by the absence of any significant changes in methane production by the samples in 11 out of the 12 months studied from the "Tielebeek" location where ciliates were absent through-

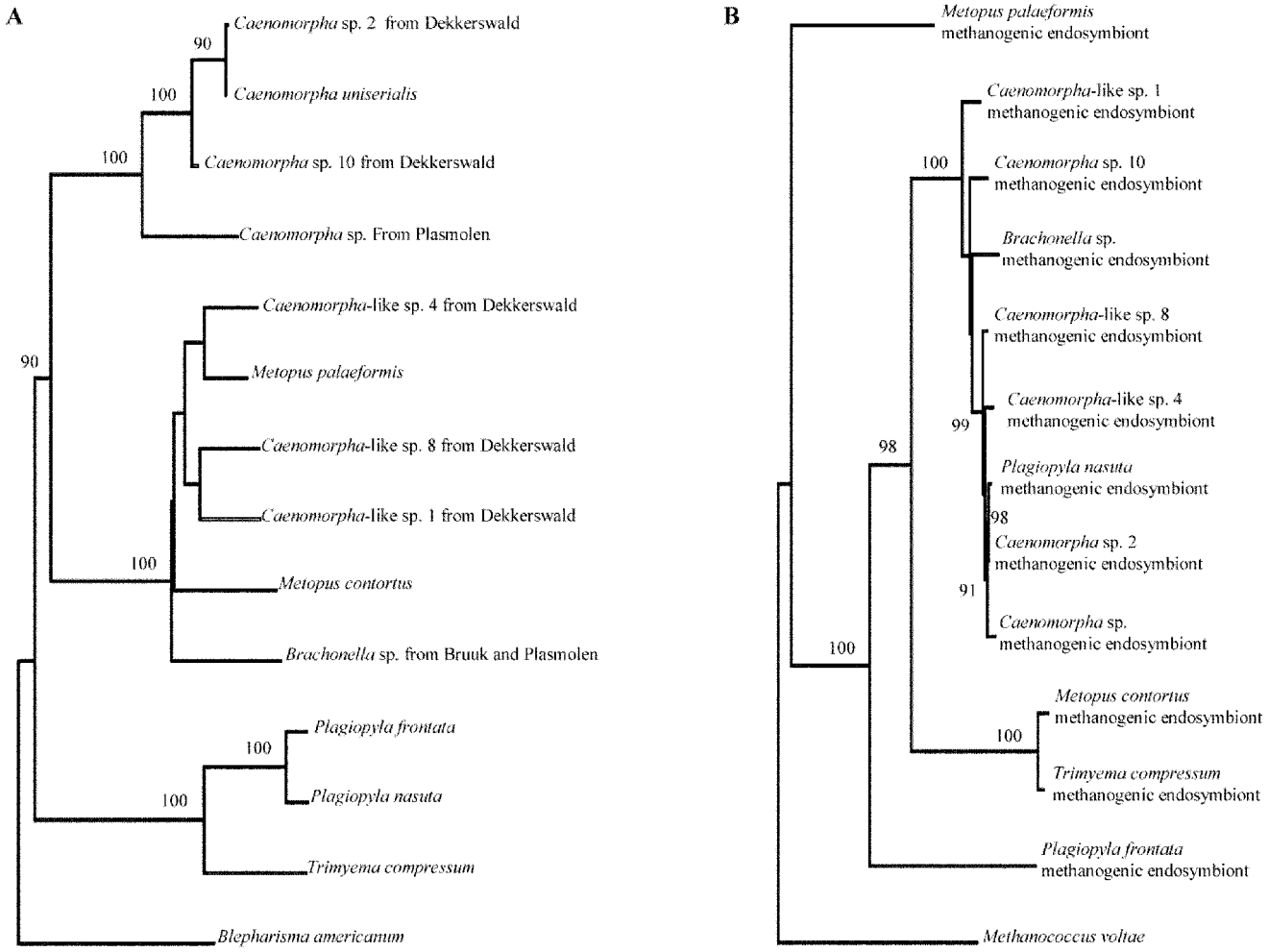


Figs 3A-D. Methane production rates of the various sediment samples; **A** - "de Bruuk" sediment; **B** - "Dekkerswald" sediment; **C** - "Plasmolen" sediment; **D** - "Tielebeek" sediment. The open bars represent untreated sediment slurries and the grey bars represent heat-shocked sediment samples (5 min 45°C). All scales are similar with the exception of the methane production of "Dekkerswald", which had to be enlarged because of the height of the production rates. The standard deviations are indicated by bars; they are based on three independent measurements. "g ow" indicates organic weight in gram. The total number of anaerobic ciliates in the four sediments is also indicated (circles, triangles, diamonds, and squares, respectively, see Fig. 2).

out the year (Fig. 3D). In 8 of such samples, heat-shock treatment caused an insignificant decrease of the methane production, whereas in 3 samples a slight, but insignificant increase could be observed.

Diversity of the anaerobic ciliates

Sediments lacking anaerobic ciliates completely, (and significant numbers of anaerobic flagellates as well), can produce up to 2 µmol CH_4/g organic weight/h (Fig. 3D, "Tielebeek"). These methane emissions, on average, are higher than those from "Plasmolen" samples that can host up to some 90 ciliates/ml, and only slightly lower than those from "de Bruuk", where up to 150 ciliates/ml were observed. The "Dekkerswald" sediments on the other hand, have methane production rates, which are up to four times higher. In the latter sediment, the highest numbers of ciliates and the highest ciliate diversity were observed (Figs 2, 3). To rule out whether these differences correlate with ciliate diversity or not, single cell PCR was used to discriminate between identical and different ciliate species in the various sampling places and to get a preliminary overview about complexity of



Figs 4A, B. A - phylogenetic relationship of free-living anaerobic ciliates; **B** - and their methanogenic endosymbionts, based on the SSU rRNA gene. The alignments were reduced to approximately 460 (18S) and 770 (16S) positions in order to remove hypervariable regions and gaps. The trees were constructed by the neighbour-joining method. The distance data were bootstrap resampled 100 times (Felsenstein 1985). Only bootstrap values above 90% are displayed. For accession numbers of the sequences used see Materials and Methods.

the ciliate population. The isolation of DNA from single cells allows the retrieval of SSU rDNA sequences from both the ciliate and the endosymbiont, which permits an unequivocal identification of identical species (van Hoek *et al.* 2000). Therefore, the 18S rDNA of individual ciliates and the 16S rDNA of their methanogenic endosymbionts were amplified by PCR: and subjected to DNA sequence analysis. Phylogenetic analysis revealed that a distinct ciliate 18S rDNA always correlated with a specific 16S rDNA from the methanogenic endosymbiont, regardless of the sampling place. On this basis, it was possible to identify even closely related ciliates unequivocally. Figure 4 shows, that ciliates with nearly identical "ribotypes" could be discriminated by their different methanogenic endosymbionts. On this basis, in the "Dekkerswald" sediment the most diverse population of anaerobic ciliates could be identified - exceeding the expectations based on the *in vivo* identification by far (Figs 2, 4).

Especially, it revealed the existence of ciliates, which look very similar to *Caenomorpha uniserialis,* but are phylogenetically rather unrelated, i.e. the various *Caenomorpha*-likes. On the other hand, the combination of ciliate and endosymbiont SSU rRNA data also

demonstrated unequivocally that only the *Brachonella* sp. found in both "de Bruuk"- and "Plasmolen" sediments were identical (i.e. *Brachonella* sp., Fig. 4). All other anaerobic ciliates studied here were unique for their particular sampling places.

DISCUSSION

Analysis of four different sampling sites near Nijmegen, The Netherlands, at monthly intervals over a period of one year, clearly showed that a correlation between the methane emissions and the number of anaerobic ciliates in the particular sample did not exist. Ciliate counts of about 100 ciliates/ml can correlate with methane emission rates of less than 1 µmol CH_4/g organic weight/h, but also with emission rates of up to 15 µmol CH_4/g organic weight/h. Even sediments lacking anaerobic ciliates completely, (and significant numbers of anaerobic flagellates or amoebae with endosymbiotic methanogens as well; i.e. "Tielebeek") can produce up to 2 µmol CH_4/g organic weight/h (Fig. 3). Thus, given a comparable abundance of anaerobic ciliates in a sample, the methane emissions of these samples can vary by a factor of ten or more if normalised for organic matter in the sediments. Organic matter appeared to be the most convenient basis for comparisons between different freshwater sediments; Table 1 displays the average correlations between dry-weight and organic weight for the various sample places. Consequently, neither indirect effects by grazing nor the direct contributions to methane formation by the methanogenic endosymbionts of the anaerobic ciliates can be responsible for the observed differences in methane production in these Dutch freshwater sediments. Rather, free-living methanogens must account for the vast majority of the methane emissions in these ecosystems. Our data also reveal that even in the presence of high numbers of anaerobic ciliates in the sediments (i.e. more than 90 ciliates/ml) the endosymbionts can contribute for maximally 10% to the methane emissions of a freshwater sediment. The four Dutch freshwater sediments on average produce approximately 60 nmol methane per hour per ml of sediment. Given that a single freshwater ciliate should not produce more than 1-5 pmol methane/h, then 100 to 180 ciliates/ml, which represents the maximum abundance in the sediments studied here, could account for not more than 180-900 pmol methane/h - about 2% of the total methane production. This figure is well in the range of the 1-3 µmol methane/g organic weight/h that could be attributed to the ciliates in this study using the heat-shock technique (Fig. 3B). These 1-3 µmol correspond to 5-18% of the total methane production in these samples, but this contribution is limited to a few months of the year and to samples with the highest ciliate numbers. It is obvious that the calculations based on measurements of isolated ciliates *in vitro* (Fenchel and Finlay 1992, Schwarz and Frenzel 2005) lead to a slight underestimation of the contribution to methane production *in situ* by isolated ciliates. The composition of the ciliate community and the overall level of methane production in particular sediment seem to have a certain influence on the contribution by ciliates. Furthermore, it seems likely that grazing effects on the microbiota and differences in ciliate physiology are of greater importance than the mere number of methanogenic endosymbionts. In order to account for a contribution of 50% to the methane production in the sediments studied here, the number of ciliates in the samples must be one to two orders of magnitude higher than observed in this study. Such numbers are unlikely to be found in any Dutch freshwater sediment, but such numbers of ciliates can be reached in certain tropical environments.

The composition of the ciliate community seems to exert some influence on the methane emission. Our (preliminary) molecular analysis revealed that a meaningful assessment will require a detailed community analysis allowing identifying all changes of ciliate abundance quantitatively. Unfortunately, such a technique was not available when the survey was conducted. The increasing 18S rDNA databases of ciliates and the available molecular techniques for accessing the composition of the protozoal community in freshwater sediments will make such an analysis feasible in near future.

We have shown here, that the contribution of anaerobic ciliates to the methane production in Dutch freshwater sediments - in general - is marginal throughout the year (Fig. 3) - in clear contrast to the situation in marine sediments (Fenchel 1993), where the contribution of anaerobic ciliates to the methane production is significant. These observations principally confirm earlier speculations about a minor impact of freshwater ciliates on methane emissions (Finlay and Fenchel 1991; Khalil and Shearer 1993; Finlay *et al.* 1998, 1999) which were based on measurements of isolated ciliates under highly artificial conditions. However, the lack of suitable methods to measure the methane production of freshwater sediments after the removal of ciliates did not allow testing the validity of the extrapolations so far. Here, we

have established that heat-shocking for 5 min at 45°C allows a quantitative and efficient killing of the anaerobic ciliates in the sample without interfering significantly with the methane production by the free-living methanogens in the sample. This method requires only a short temperature pulse and avoids the difficult interpretation of results obtained after the treatment of freshwater sediments with high doses of antibiotics. All ciliates in the sample burst and become completely disintegrated within 5 min. Endosymbiotic methanogens from *Caenomorpha/Caenomorpha*-like ciliates might survive outside their hosts for some 48 h, but it is very unlikely that significant numbers of the endosymbionts of the other ciliates can survive in pore water of such a low osmotic pressure. Microscopical observations suggest that the methanogens burst easily after release from their ciliate hosts due to osmotic shock both in freshwater sediments and cockroach guts (unpublished).

Studies on several *Nyctotherus* strains from the hindgut of cockroaches have revealed substantial differences in the number of methanogenic endosymbionts between ciliates from different lines of cockroaches (van Hoek *et al.* 2000). A similar situation can be anticipated for the various anaerobic ciliates from the freshwater sediments on the basis of differences of the intensity of the autofluorescence of the ciliates (not shown). However, because of the sensitivity of the anaerobic ciliates for manipulations, and a rapid fading of the autofluorescence of the endosymbionts it is rather difficult to count the endosymbionts of the various species with the necessary accuracy. Interestingly, a simple correlation between the number of methanogenic endosymbionts and the methane production rates of the various ribotypes of cockroach-dwelling ciliates does not exist (van Hoek *et al.* 2000). Rumen ciliates produce approximately a hundred times more methane (83-250 pmol/h) - potentially due to the high numbers of methanogens, which can attach to the surface of the rumen ciliates (Kisidayova *et al.* 2000, Ushida and Jouany 1996). Nevertheless, it is possible to make some rough estimation about the methane production by the endosymbionts. Assuming that a single methanogenic bacterium can produce about 1 fmol CH_4/h (Fenchel and Finlay 1992), then a single ciliate with some 1000-5000 endosymbiotic methanogens would be able to produce 1-5 pmol methane per hour. Comparable values have been measured after incubation of anaerobic freshwater ciliates *in vitro* (Fenchel and Finlay 1992) and for *Nyctotherus ovalis* isolated from the cockroach hindgut (2.6-7.1 pmol CH_4/ciliate/h; van Hoek *et al.* 2000), but measurements *in situ*, which can take into account the real diversity of ciliates in the samples and the availability of nutrients and methanogenic substrates will allow to obtain more detailed results in the future.

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