A methane-driven microbial food web in a rice field soil

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Abstract
Biological methane oxidation in wetland soils is a key process in the methane cycle, preventing large amounts of this greenhouse gas from escaping into the atmosphere. While methanotrophs are only a group of bacteria capable to oxidise and assimilate methane-C under aerobic conditions, the fate of assimilated methanotrophic biomass is largely unknown. We conducted a microcosm experiment, in which a thin layer of rice field soil was incubated under opposing gradients of oxygen and ^{13}C-labelled methane. ^{13}C-enriched "heavy" RNA could be affiliated not only to methanotrophs, but also to protozoan grazers including amoebae, ciliates, and flagellates, demonstrating a microbial food web driven by methane. The impact of protozoan grazing on methanotrophs was studied by another microcosm experiment, in which natural assemblages of bacterial community including methanotrophs retrieved from a rice field soil were re-inoculated to sterilised soils with or without protozoan isolates. Microarray analysis of *pmoA* gene showed that a group of type I methanotrophs became dramatically prominent when protozoa were absent. Protozoa isolated from the soil demonstrated selective grazing on type I methanotrophs. A series of our studies demonstrates that protozoan grazing with selectivity may have a crucial impact on the methanotrophic community in a wetland rice field soil.

Key Words
Food chain, molecular analysis, paddy field soil, predation, protists, stable isotope probing.

Introduction
Biological methane oxidation at the oxic-anoxic interface in wetland soils and sediments is a key process in methane cycling, preventing large amounts of this greenhouse gas escaping into the atmosphere (Conrad 1996). Methanotrophs are only a group of bacteria capable to oxidise and assimilate methane-C under aerobic conditions (Bowman 2000). However, methane-derived carbon may be utilised by other organisms in indirect ways. Predation on soil microbes by protozoan predators is a well-known feature (Clarholm 1994; Ekelund and Ronn 1994), but their impact on methanotrophic populations has never been studied. In this study, we report on a microbial food web driven by methane in a rice field soil. We adopted the RNA–stable isotope probing (SIP) approach (Manefield *et al.* 2002) using ^{13}C-labelled methane and universal primers for the domains Bacteria and Eukarya to follow the incorporation of methane carbon into microorganisms. The effect of protozoan grazing on methanotrophic populations was also studied through culture-dependent and independent experiments. The results demonstrate the crucial impact of selective grazing of protozoa on the methanotrophic community in the rice field soil.

Methods
Incorporation of methane carbon by prokaryotic and eukaryotic microorganisms revealed by RNA-SIP (Murase and Frenzel 2007)
Soil taken from a rice field of the Istituto Sperimentale della Risicoltura (Vercelli, Italy) in the spring of 2000 before flooding was used in this study. Microcosms made from a thin layer of water-saturated rice field soil were supported by a gas permeable membrane and supplemented with ^{13}C-methane (at 20 %[v/v] in N_2) from below and air from above, thus reproducing the oxic-anoxic interface (Figure 1A). After 20 days of incubation, RNA was extracted from soil and subjected to isopycnic centrifugation. T-RFLP (Terminal Restriction Lengths Polymorphism) and DGGE (Denaturating Gradient Gel Electrophoresis) analysis were conducted for fractionated RNA samples to study bacteria and eukarya that incorporated methane carbon.

Impact of protozoan grazing on the community composition of methanotrophs
Natural assemblages of bacterial community including methanotrophs were retrieved from the soil incubated under methane and re-inoculated to sterilised soils with or without a mixture of protozoa (1 ciliate, 3 flagellates and 4 naked amoeba) that had been isolated from the same soil. The soil was incubated in the microcosm described above. Incorporation of methane carbon into inoculated protozoa were followed by

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RNA-SIP. Community composition of methanotrophs were analysed by microarray targeting *pmoA* gene (Bodrossy *et al.* 2003).

Selectivity of protozoan grazing on methanotrophs
The number of methanotrophs-feeding protozoa in an air-dried rice field soil was estimated by determining the MPN using methanotrophs as food bacteria (Murase and Frenzel 2008). Protozoa were isolated from the positive wells inoculated with higher dilutions and their growths on different methanotrophs were tested by a cultivation method.

Results

Incorporation of methane carbon by prokaryotic and eukaryotic microorganisms
PCR-products were obtained from the 'heavy' RNA (up to the buoyant density of 1.801 g/mL) from the $^{13}CH_4$-applied microcosm and T-RFLP patterns differed between the 'light' and 'heavy' fractions. This is well contrasted with the normal CH_4-applied microcosm, which gave PCR products only from the 'light' RNA (up to the buoyant density of 1.784 g/mL) and nearly identical T-RFLP patterns over the density gradient. A clone library of ^{13}C-labelled 'heavy' 16S rRNA included methanotroph-related sequences as a dominant group (Figure 1B) confirming the incorporation of methane-C into methanotrophic biomass. *Methylocyctis*-, *Methylobacter*- and *Micromicrobium*-related sequences were dominated in the clones from the 'heavy' RNA. The most dominant sequences next to methanotrophs could be affiliated to Myxococcales. As the case of bacterial RNA, we obtained PCR-products from the ^{13}C-labelled 18S rRNA at high buoyant densities (up to the buoyant density of 1.801 g/mL). At the same densities, we failed to obtain any product in the control treated with normal CH_4. DGGE fingerprints of the 'heavy' fractions showed distinctly different patterns from those of the 'light' fractions, indicating that a subset of the eukaryotic community had assimilated methane-C (Figure 1C). Sequences retrieved from the DGGE bands of the 'heavy' RNA fractions could be affiliated to Colpodea (Ciliophora, band A), Cercozoa (band O), Amoebozoa (bands E, L and P), and Heterolobosea (band M), suggesting that these protists grazed on methanotrophs that assimilated $^{13}CH_4$.

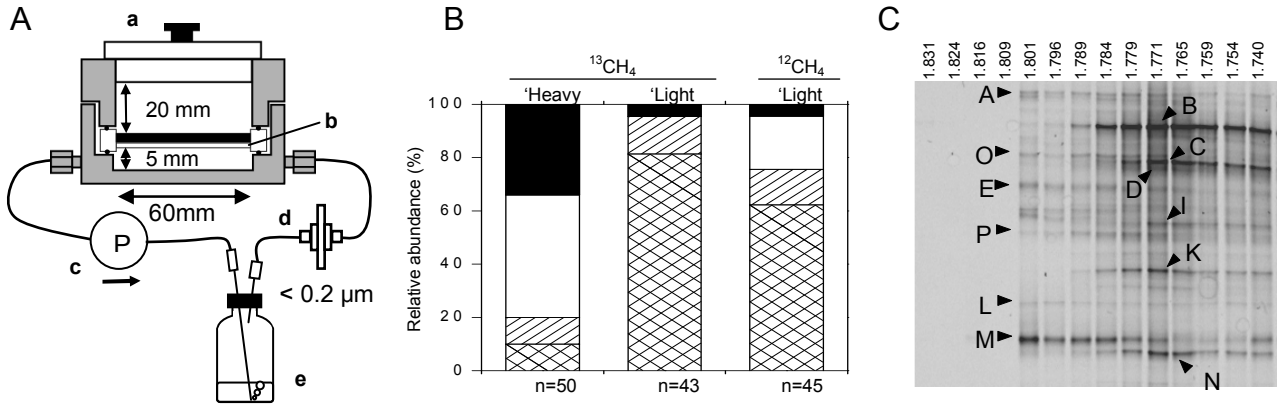

Figure 1. The gradient microcosm for the RNA-SIP experiment using ^{13}C-labelled methane. (A) A schematic drawing of the microcosm. (B) Phylogenetic affiliation and relative abundance of bacterial 16S rRNA clones from 'heavy' and 'light' RNA fractions of ^{13}C-labelled methane-treated microcosm ($^{13}CH_4$) compared to clones from the 'light' RNA fraction of the normal methane-treated microcosm ($^{12}CH_4$). Black: MOB (α-Proteobacteria); white: MOB (γ-Proteobacteria); hatched: Myxococcales; cross-hatched: others. (C) Eukaryotic DGGE fingerprints over the density-range of a fractionated rRNA centrifugation gradient for the microcosm applied with ^{13}C-labelled methane. Buoyant densities of the fractions (g/mL) are given on the lanes. Sequences retrieved from DGGE bands could be affiliated to the following taxa: bands A and B, Colpodea (Ciliophora); bands C, E, K, I, L, P, and G, Amoebozoa; bands D and O, Cercozoa; bands M and N, Heterolobosea. Redrawn from Murase and Frenzel (2007).

Impact of protozoan grazing on the community composition of methanotrophs
Inoculation of soil with protozoa enhanced methane oxidation in the initial period of incubation (up to 10 days). After 20 days of incubation, DNA and RNA were extracted from the soil to analyse the community structure of protozoa and methanotrophs. RNA-SIP (stable isotope probing) approach revealed that some of inoculated protozoa incorporated methane-^{13}C, indicating the grazing of the protozoa on methanotrophic biomass. 18S rRNA of the same protozoa were detected from the ultracentrifuged fractions with a wide range of buoyant density, suggesting that the same protozoa can have different grazing preference at the

individual level; some individuals more preferably grazed on methanotrophs and some on non-methanotrophs. Microarray analysis of pmoA gene showed that a specific group of type I methanotrophs (a subgroup of the *Methylobacter* clade) became dramatically prominent when protozoa were not inoculated (Figure 2).

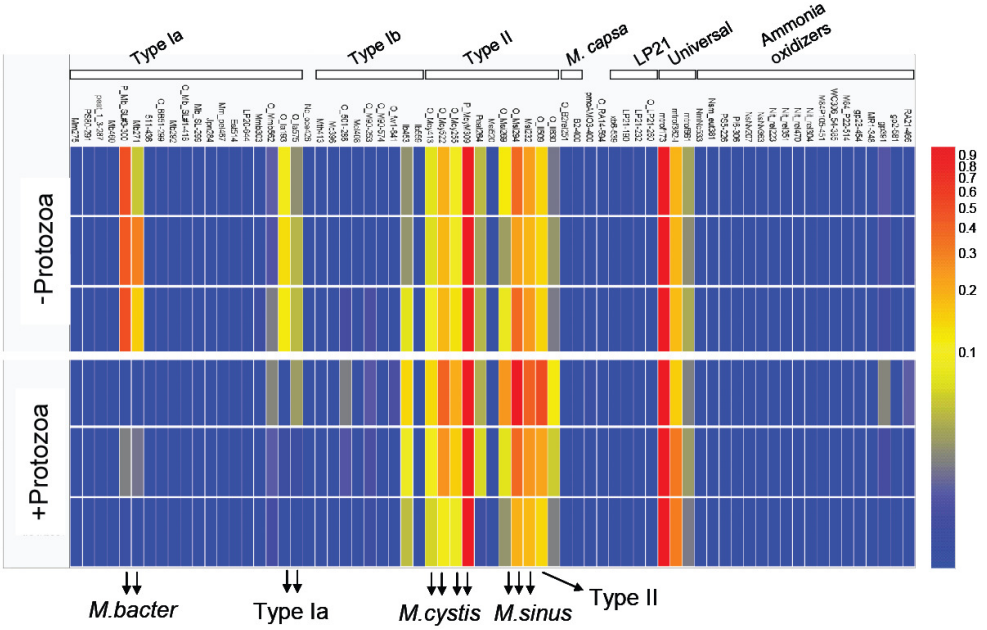

Figure 2. Microarray results showing the efficiency of hybridization of *pmoA* PCR products from soils with and without protozoa (n=3).

Selectivity of protozoan grazing on methanotrophs

Protozoa, specifically naked amoebae and flagellates, grew densely, accompanied by a decrease in the number of food bacteria in the medium. Such growth was not observed in the wells lacking food bacteria, which indicated that the protozoa fed on the food bacteria. The MPN counts (summed numbers of flagellates and amoebae) enumerated on day 7 were lower in media containing methanotrophs than in the medium containing *E. coli* (Figure 3). On day 21, seven of ten methanotrophic strains yielded protozoan MPN counts comparable with *E. coli* (10^4 MPN/[g soil dry wt.]), while three strains of *Methylocystis* spp. (strains RP1, Pi54, and LR1) yielded significantly lower numbers of protozoa (10^2–10^3 MPN/[g soil dry wt.]) than *E. coli*. The amoebae isolated from positive wells with different food bacteria generally showed a similar pattern of grazing preference on methanotrophs (Table 2); they fed on all methanotrophs except *Methylocystis* sp. strains RP1, Pi54, and LR1. The flagellate fed on *Methylocystis* sp. strain Pi54 more actively than on *Methylocystis* sp. strain H9a, and the *Hartmannella* amoeba grew as well on strain RP1 as on strain H9a.

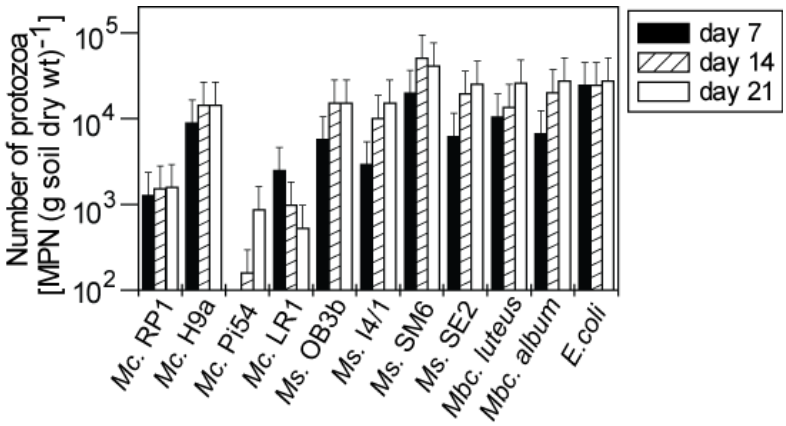

Figure 3. MPN counts of protozoa in rice field soil feeding on methanotrophs. Mc., *Methylocystis* sp.; Ms., *Methylosinus* sp.; Mbc., *Methylobacter* sp. Redrawn from Murase and Frenzel (2008).

Conclusion

Methanotrophic biomass that assimilated methane carbon was incorporated into microbial predators such as protozoa and myxobacteria. Protozoan grazing dramatically changed the composition of the methanotrophic community with decreased dominance of Type I methanotrophs. The culture dependent study showed that

Type I methanotrophs supported the growth of soil protozoa, while some of Type II methanotrophs did not. A series of our studies is the first demonstrating the impact of protozoa on a defined group of soil bacterial population performing the same ecological functions. Protozoa are an important biotic factor shaping the methanotrophic community in situ by selective grazing. Further study is needed to understand the effect of protozoa on methane oxidation and methane cycle in a rice field soil.

Table 1. Growth of protozoa isolated from the MPN plates on different methanotrophs[1)] (Murase and Frenzel 2008).

Strain name	Food bacteria in MPN plates	Taxonomy	Food bacteria tested										
			Methanotrophs										
			Mc. RP1	*Mc.* H9a	*Mc.* Pi54	*Mc.* LR1	*Ms.* OB3b	*Ms.* I4/1	*Ms.* SM6	*Ms.* SE2	*Mb. luteus*	*Mbc. album*	*E. coli*
H9a_3E	*Mc.* H9a	Unidentified Lobosea	-	++	-	-	+++	++	+++	++	+	++	++
H9a_6E	*Mc.* H9a	*Filamoeba*	-	+++	+	-	+++	++	+++	++	++	++	++
OB3b_3A	*Ms.* OB3b	*Acanthamoeba*	+	+++	+	-	+++	+++	+++	+++	+++	+++	++
I4_6E	*Ms.* I4/1	Unidentified Lobosea	-	+++	-	-	+	++	++	++	++	++	+++
I4_5E	*Ms.* I4/1	*Filamoeba*	+	++	-	+	++	++	+++	+++	++	+++	+++
SM6_6A	*Ms.* SM6	*Acanthamoeba*	+	+++	+	-	+++	+++	+++	+++	+++	+++	+++
SE2_6F	*Ms.* SE2	*Acanthamoeba*	-	+++	+	-	+++	+++	+++	+++	+++	+++	++
Mb_5C	*Mb.* luteus	Unidentified Lobosea	+	++	+	-	+++	++	+++	++	++	++	+++
Mbc_7D	*Mbc.* album	Unidentified Lobosea	-	++	-	-	++	++	+++	+++	+++	+++	++
Mbc_3C	*Mbc.* album	*Spumella*	+	+	+++	-	++	++	+++	+	+	++	+++
Mbc_3E	*Mbc.* album	*Acanthamoeba*	+	+++	+	-	+++	++	+++	++	+++	++	++
Mbc_3H	*Mbc.* album	*Hartmannella*	++	++	-	-	+++	+++	+++	+++	+++	+++	+++
E_5F	*E. coli*	*Comandonia*	-	++	+	-	+++	++	+++	++	++	++	++
E_5C	*E. coli*	*Acanthamoeba*	-	+++	+	-	+++	+++	+++	+++	+++	+++	+++
E_5E	*E. coli*	*Comandonia*	-	+++	++	-	+++	+++	+++	+++	+++	+++	+++

1) -, no growth; +, silightly grown; ++, moderately grown; +++, actively grown. See text for the classification

References

Bodrossy L, Stralis-Pavese N, Murrell JC, Radajewski S, Weilharter A, Sessitsch A (2003) Development and validation of a diagnostic microbial microarray for methanotrophs. *Environmental Microbiology* **5**, 566-582.

Bowman J (2000) The Methanotrophs. The Families *Methylococcaceae* and *Methylocystaceae*. In 'The Prokaryotes'. (Ed. M Dworkin) (Springer: New York)

Clarholm M (1994) The microbial loop in soil. In 'Beyond the biomass'. (Eds K Ritz, J Dighton, and KE Giller) pp. 221-230. (John Wiley & Sons: Chichester)

Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). *Microbiological Reviews* 60, 609-640.

Ekelund F, Ronn R (1994) Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amebas and their ecology. *FEMS Microbiology Reviews* **15**, 321-353.

Manefield M, Whiteley AS, Griffiths RI, Bailey MJ (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Applied and Environmental Microbiology* **68**, 5367-5373.

Murase J, Frenzel P (2007) A methane-driven microbial food web in a wetland rice soil. *Environmental Microbiology* **9**, 3025-3034.

Murase J, Frenzel P (2008) Selective grazing of methanotrophs by protozoa in a rice field soil. *FEMS Microbiology Ecology* **65**, 408-414.