Response to reviewer's comments

Short comment of S. Kolb from 06.02.12

Many thanks to the reviewer for the detailed comments. Please find our answers to the specific questions below.

1) pmoA-Analyses (cloning, TRFLP) were only done in the upper 10 cm not in deeper soil layers. From my perspective it would have been mandatory to analyze depth distribution if this sampling was taken.

We agree with the reviewer that, in retrospect, it would have been useful to also collect samples from greater depths (as mentioned in the manuscript on p.1278 ln.13-16, and p.1281 ln.20-23). However, for the design of the study we initially hypothesized to primarily encounter oxidation of atmospheric methane, with rare occurrence of microsite methanogenesis (p.1263, ln.22-23). Under such assumptions, our sampling depth of 3-10 cm presumably covers the zone with highest MOB activity and abundance. Also, for locations with soil-methane concentrations below 10 µl l^{-1} throughout the profile (the majority of locations), we are confident that the sampled zone represents the active MOB community fairly well. The frequent occurrence of microsite methanogenesis and the yet unknown deep-soil methane source was discovered during the latter phase of the study, when an adaptation of the sampling scheme could not be considered anymore.
Furthermore, the study was designed to get a first overview of MOB activity and identity in a variety of glacier forefields. Time constraints would have made it rather difficult to sample full vertical profiles at all locations, taken into account the remoteness and limited accessibility of some sites. We will, however, address this issue in more depth (literally) in a follow-up study.

2) Please, comment in a revised version on how many cores were anylsed in each specific site to get methane concentration profiles.

In this study we measured in-situ methane concentration profiles by extracting soil gas at different depths, using a stainless-steel sampling rod that was gradually hammered into the soil in steps of 5 cm (p.1265, ln.1-14). No soil cores were taken.

3) The reader gets the impression that not all measured methane concentration profiles were shown in Figure 1. Please, present them all as Supplementary files.

Please note that, for the sake of readability, it was the intention of the authors to present only a selection of profiles representing all typical profile categories encountered, as stated on p.1272 ln.12-13. To prevent misunderstandings, we will expand this statement to "Profiles from four selected sites … are plotted in Fig.1". We will also present all measured profiles as Supplementary files.

4) The number of pmoA clones being sequenced is extremely low? Why? Please, coverage values at the species-level OTUs need to be documented for each library - Were they above 90%?

The aim of the molecular survey was to assess the methanotroph diversity across many different glacier forefields based on the comparative analysis of *pmoA* PCR products . In various previous studies, T-RFLP fingerprinting has proven to be an excellent tool for this type of research, but should be combined with cloning and sequencing of *pmoA*. The sequencing approach is performed to experimentally affiliate the major or dominant T-RFs with particular *pmoA* clusters or methanotroph groups, but not to exhaustively analyze methanotroph diversity. It is well known that the frequency distribution of *pmoA* phylotypes in clone libraries is affected by cloning bias (i.e., different phylotypes may be cloned in *E. coli* with different efficiency). Therefore, *pmoA* clone library composition often does not reflect the phylotype composition in the *pmoA* amplicon(s) under study. By contrast, T-RFLP fingerprinting avoids cloning and has repeatedly been shown to provide a reliable analysis of *pmoA* diversity patterns. In that respect, it is more robust than cloning/sequencing.

We observed an absolutely consistent correspondence between the phylogenetic placement of *pmoA* clones and their T-RF assignments. All the 22 *pmoA* clones having a 241-bp T-RF belonged to USCγ, while all the 59 *pmoA* sequences exhibiting a 243-bp T-RF were affiliated with *pmoA1* of *Methylocystis*. The minor T-RFs in Figure 4 could be assigned by computational analysis with high confidence to *pmoA* of a particular subgroup of USCγ (339-bp T-RF) and to *pmoA2* of *Methylocystis/Methylosinus* (350-bp T-RF), using a comprehensive *pmoA* database (Lüke and Frenzel, 2011). These *in silico* assignments were greatly supported by the consistent (strict) co-occurrence of the 241-bp and 339-bp T-RFs (both T-RFs affiliated with USCγ) and, on the other hand, the 243-bp and 350-bp T-RFs (both T-RFs affiliated with *Methylocystis*) in all the T-RFLP patterns (Figure 4).

We agree that our statement on the unexpectedly low OTU diversity in the different sampling sites (page 18, lines 19-23) may not be fully correct. The analysis of additional *pmoA* clones may have resulted in the detection of additional species-level OTUs within, for example, USCγ. Nonetheless, it is notable that all the 22 USCγ-like *pmoA* clones belonged to only three species-level OTUs (Figure 3) and each of these three OTUs was composed of *pmoA* clones from at least two geographically different sampling sites. This finding suggests that the glacier forefields may be colonized by a few dominant methanotroph species. In a revised version, we will state our findings more precisely.

5) The TRFLP analysis has been replicated - but not the DNA-extraction which is the major source of Variance in that kind of analysis. Please, comment on that.

We used the same procedure for DNA extraction throughout the study and consistently detected USCγ across nearly all samples as the dominant methanotroph group (241-bp and 339-bp T-RFs), except for samples GRF1, GRF2, WIL1, and WIL 4 (Figure 4). These four samples were consistently dominated by *Methylocystis* (243-bp and 350-bp T-RFs). The nearly exclusive detection of *Methylocystis* in calcareous sites has a plausible explanation, being related to the fact that methane profiles from these sites revealed a substantial deep-soil CH_4 source with soil-CH_4 concentrations greater than 1000 ppmv. It should be noted that we analyzed two or three subsamples for each of the 13 different glacier forefields. The subsamples analyzed for a given glacier forefield showed identical T-RFLP patterns and can be considered true biological replicates (except for MRT, KLG, and WIL). Due to the conflicting results, the subsamples from MRT, KLG, and WIL were analyzed in triplicate, including independent DNA extraction, PCR, and T-RFLP analysis. The triplicate analysis confirmed the subsample T-RFLP patterns for MRT, KLG, and WIL, as shown in Figure 4.

6) No mRNA, which would have reflected much better the active population was analysed. Why?

We agree that the analysis of mRNA would give valuable information on the active population of MOB and will certainly be an interesting approach for follow-up studies. It was, however, the intention of this study to primarily confirm the presence of, and provide initial information on the identity of MOB in such environments, given the rather limited information available on MOB in pioneer ecosystems. Our results unambiguously show that the glacier forefields are colonized by USCγ rather than USCα. The latter group could be detected in neither single- nor second-round PCR using the primer set A189f-650r. At first glance, the dominance of USCγ may be unexpected, but strong evidence for the capability of this group to be active at low methane concentrations or even to consume atmospheric methane has already been reported by Knief *et al*. (Diversity and activity of methanotrophic bacteria in different upland soils, AEM 2003, p. 6703-6714). Nonetheless, the knowledge gained during this study will certainly be helpful for mRNA analyses on the active MOB community in future works.

The conclusion that USCgamma might be a cold ecosystem-adapted methantroph is interesting, but only a more comprehensive statistical comparison to previous studies would allow for this conclusion. Please, add a statistical comparison with soil methantroph commnuities that are subjected to similar low and higher mean annual soil temperatures, and that are as well unsaturated soils.

We agree with the reviewer that our statement regarding the widespread distribution of USCγ in cold ecosystems is misplaced in the conclusion. Our intention was to merely present a hypothesis. Indeed, for a conclusive statement we would have to do a proper comparison with other data, which would go beyond the scope of this study. Nonetheless, from our results and the study of Zheng et al. (2011) we can clearly say that some USCγ strains are indeed well adapted to cold oligotrophic environments.
Hence, we will change the statement in the conclusion on p.1282 ln.3-6 to “However, diversity of MOB was limited in both siliceous and calcareous forefields, and was strongly dominated by the USCγ group. It seemed that USCγ adapted best to the oligotrophic cold-climate conditions in the investigated pioneer ecosystem.”

comments to text:
Abstract ln 18, Please write out ’operational taxonomic units’
p.1266, ln 7, please write out ’approximately’
p. 1268, ln 27, correct ’OTU’
p. 1277, ln 22-24, This statement is only true for methane oxidation at atmospheric concetration. Please, rephrase accordingly.
p.1280, lns 3-5, This statement may also be a result of the extremely number of analysed genotypes in gene libraries. It may be that behind one TRF much more different genotypes may be hidden. Please, consider this in a revised ms version.
p. 1280, ln 11, correct ’Henneberger et al., 2012’

These issues will be addressed in the revised manuscript.