Dear Editor,
please find submitted the new version of the following manuscript:
Title: Methanotrophic activity and bacterial diversity in volcanic-geothermal soils at Pantelleria Island (Italy)
Author(s): A.L. Gagliano et al.
MS No.: bg-2014-69
MS Type: Research Article
Iteration: Revised Submission

The discussion MS was positively evaluated by both the anonymous referees that revised it. In particular, Referee #1 judged it "*a well written article*", while Referee #2 found the paper "*well introduced and the results and discussion quite well written*".
The main weaknesses evidenced by Referee #1 were mainly related to the molecular approach and especially to the culture-dependent approach that was defined a bit too preliminary.
The weaknesses of the second referee were related to the description of methods and lack of statistics.

All the referees' comments have been addressed in the point-by point replay to the referees submitted online on 06.23.2014 (and included at the end of this letter), and most of them have been taken into consideration in the revised MS. In particular all point-by point corrections (such as measure units, experimental procedure, clarifications in the methodology, and informations lacking in the introduction) made by both the referees, have been made.
A list of all relevant changes made in the manuscript is included at the end of this letter.

Where comments could not be accepted a long and detailed explanation was given to the referee.Considering the Results, new experiments were set to satisfactorily answer to Referee #1. This led to new results that are added in the revised MS. The first new result is the sequencing and analysis of 10 other *pmoA* clones to reach a total of 26 clones. The new sequences, actually, are similar to those previously obtained confirming that all the potential diversity had already been described. The second new result is related to FIG. 5 that was substituted with a new curve of growth of strain Pan1. The new curve was constructed by increasing the inoculum concentration and measuring growth and CH_4 consumptions for a longer time lapse.

An overall revision of text and references has also been made.

Taking into account the positive opinions expressed by the referees and our effort to satisfactorily address all their comments, either by providing explanations and/or by modifying the MS, we are confident that the revised manuscript will meet the high quality standards of BG and will be accepted for publication.

Regards
Walter D'Alessandro (corresponding author)

Palermo, 2014-07-16

List of the relevant changes made in the manuscript.

General changes

1) All the measure units have been revised according to the referees' advise.

Specific changes

1) The informations on CO_2 fixation pathway by verrucomicrobial methanotrophs and related reference was added in the introduction.
2) Soil sampling and manipulations were better described in the M&M section
3) More details were added on gas sampling procedure.
4) The legend of TABLE 1 was modified to include the analytical precision of each measure.
5) In FIGURE 4 the number of clones with identical sequence was increased to include 10 new sequences.
6) FIGURE 5 A new growth curve was drawn based on a completely new experiment.
7) A comment on the factors that limit the isolation of methanotrophic verrucomicrobia was added in the Discussion together with the reference suggested by the referee.

Replay to the anonymous referee n.1

We really appreciate the comments made by the Anonymous Referee #1 and we thank him because his point of view will improve our paper; we will answer to his questions and comments point by point.

R. P. 2, line 9, p.3 line 16 and p. 3: I do not understand what the units 'kta^{-1}' and 'ta^{-1}' refer to.

A. These are tons per year and kilotons per year. These units, although not SI, are generally used for the emission of gases from volcanic or geothermal systems. We changed them in SI units (Mg a^{-1} and Gg a^{-1}) but maintain the old unit in brackets for an easy comparison with older papers.

R. P. 4, lines 23-29: The verrucomicrobial methanotrophs use the Rubisco pathway for carbon fixation (Khadem et al., 2011, J. Bacteriol. 193: 4438-4446). They do not possess the RuMP and serine pathways.

A. Thanks for the clarification. This information will be added in the revised version of the manuscript.

R. Paragraph 3.3: Why were the samples air dried? This may result in loss of activity. Why did the authors not vary the pH for activity measurements?

A. Air drying was made overnight to sieve (<2 mm) and homogenize the samples only for geochemical analyses including methane oxidation potential. To report methane oxidation potential to the dry weight of the soil subsamples of the air-dried soil were oven-dried at 105 °C. Samples for molecular and microbiological analyses were not air dried as erroneously stated in the text. We corrected and added more details in the methods' section of the revised manuscript.
As far as pH is concerned, while it is easy to control the incubation temperature for different soil samples, it is very hard to modify in a controlled manner the pH of a soil. Therefore we did not try to make consumption experiments with different pH.

R. Paragraphs 3.6, 4.6 and last part of the Discussion section: The isolation of verrucomicrobial methanotrophs is not trivial, they show hardly growth on agar plates (previously the floating filter technique was used). Not much variation was included concerning, amount of inoculum, medium composition, pH (lowering the pH to 5 would be interesting), temperatures, incubation time.

A. We completely agree with you, isolation of verrucomicrobia is not a trivial procedure and in fact we failed in isolating them from Pantelleria soils, although could detect them by molecular analysis. However, the isolation of verrucomicrobial methanotrophs from the Pantelleria soils was not the main objective of our study as we considered the molecular approach more informative for a first general survey to methanotrophy in this environment.
Authors supply several methods to isolate Verrucomicrobial methanotrophs; we used the method based on enrichment culture described by Islam et al. (2008), that was successful to isolate the methanotrophic strain Kam1 from an acidic hotspring of Kamchatka. The only difference with Islam's conditions is pH that was adjusted to pH 6 to mimic the natural soil conditions. Nevertheless could not isolate any verrucomicrobial methanotroph.

R. Recently it was shown that *M. fumarolicum* was dependent on addition of rare earth metals (e.g. cerium, lanthanum) to the medium (Pol et al. 2014, Environ. Microbiol. DOI: 10.1111/1462-2920.12249).

A. Studies on the relationship between Verrucomicrobia methanotrophs and rare earths are more recent than our work; this is the main reason why we did not include this aspect in our study. In any case, geothermal and volcanic soils naturally contain rare earth and in particular analysis on our soils (Gagliano A.L., PhD thesis, University of Palermo, 2014) indicated the presence of Ce and La, in soil samples used in the enrichment cultures.

R. The growth curve reported in Fig. 5 shows an increase in OD from 0.06 to 0.09, which means not even a doubling. As a rule of tumb: a consumption of 8 mmol of methane should result in an increase in OD to 2.0. Please explain. I would like to since a good growth curve from which also the doubling time could be calculated.

A. In the first experiment reported in the manuscript we probably followed the growth of strain Pant1 for a too short period of time (45 hours) in the very early stage of growth. A new growth curve was constructed where growth (measured as OD and validated with CFU/ml) and methane consumption are recorded for longer times (more than 300 hours).

R. The purity of the *Brevibacillus agri* strain (Laursen et al. 2007) is questionable and growth on methane is not well documented in this article. Apparently the isolate obtained in this study did not use methane.

A. We ourselves have considered the possibility that this strain could not be a pure culture. However there are two evidences that suggest it could be a pure culture: 1) only a single cell morphology was detected under the microscope and, more notably, 2) the 16S rDNA amplicon obtained by colony PCR (using universal primers) could be directly sequenced without cloning, demonstrating that it was a single sequence. Of course we agree that this is not enough to establish that *Brevibacillus agri* is a methane oxidizing bacterium and much more work is needed to assess it. However, faced with the choice between a) just omitting a questionable result and b) describe an interesting but still preliminary observation we opted for the last and describe nothing more that what we observed.
Note.We realised that the journal acronim of the reference Laursen et al. was wrongly reported and it will be corrected in the revised version of the manuscript.

R. Paragraph 4.3: The TTGE profiles show 4 comparable very dominant bands in samples FAV2 and FAV3. Why were these bands not sequenced in order to find out which bacterial species they represent?

A. We totally agree that TTGE results arouse the interest towards the composition of the bacterial community in Pantelleria soils but this was not the aim of our work that is focused on the methanotrophic component of the microbial community. In this work soil TGGE profiles were used to demonstrate 1) that FAV1 was not “sterile” although its harsh conditions and the absence of methanotrophic activity and 2) that the community structures of FAV2 and FAV3 were very similar as well as other physical-chemical parameters. The analysis of the total bacterial communities of FAV soils, based on a deep sequencing approach, is in progress and will be published later in a different article.

R. Paragraph 4.4 & 4.5: A total of 12 samples showed a pmoA PCR product of the right size. However only one clone library was produced and from this only 16 clones were sequenced.

A. The reviewer is right. He refers to the results of PCR amplification of proteobacterial and verrucomicrobial MMO functional genes from FAV soil DNA samples reported in Table 1.

In this study we applied a "funnel" analysis starting with the detection of methanotrophs (*pmoA* detection by PCR) in three sites FAV1, FAV2, FAV3 very close to each other. Then we decided to focus on FAV2 sample because FAV1 was not active and FAV3 was very similar to FAV2 on the basis of chemical-physical and microbiological parameters (TGGE) but less active. It is important to specify that the *pmoA* clone library was constructed on the soil sample of the first field campaign FAV2, 0-3 cm depth (Table 1).
In the second field campaign the soil samples from FAV2 profile (A to E) were collected and analysed for the presence of the functional gene and for methanotrophic activity. The highest activity was recorded in the same superficial soil layer sample FAV2A (0-2 cm) that coincided to the depth of the FAV2 single sample of the first campaign. Thus, we decided not to repeat the construction of the clone libraries for these new samples (the CH_4 consumption of the samples FAV2C, FAV2D, FAV2E is at least five-fold lower than that of the top soil) but tried to isolate the methanotrophs detected by molecular methods and measure the consumption at different temperatures. In any case, the number of sequenced clones of the FAV2 *pmoA* clone library was increased from 16 to 26. Ten new *pmoA* sequences were obtained (out of eleven clones sent for sequencing) whose sequences are identical to those that were already obtained during the first screening. Thus, in the revised version of the manuscript we will present the results of 26 *pmoA* sequences but the topology of the tree shown in figure 4 will not change.

R. Technical corrections: Methane concumption rates are reported in different units. This should be uniform, and would like to suggest to use 'nmol' rather that 'ng'.

A. We checked all the text but we did not find methane consumption rates expressed in units different than ng g^{-1} h^{-1}, which we preferred to maintain.

R. P.5, line 18:
replace 'cultural' with 'culture-dependent'

A. Ok, it will be replaced in the revised manuscript.

R. Fig. 5 & p. 7, line 25,p.14, line 25 and other places: What is 'umol.mol-1'?

A. It's the SI unit for gas concentration, it's equivalent to ppm in volume.

Replay to the anonymous referee n.2

Thanks for revising and appreciating our manuscript. Please find here our answers to your comments and questions.

R.: There is a more recent balance you can cite, together with Etiope 2008, of volcanic methane in the context of global CH4 budget by Stefanie Kirshke et al. 2013 Three decades of global methane sources and sinks Nature Geoscience 6, 813-823.

A.: The paper of Kirschke et al. 2013, although very interesting, does not add new data on volcanic/geothermal methane emissions. In the paper these data are included in the wider emission category of "Geological sources (including ocean)" and are mainly based on Etiope et al. 2008.

R.:The authors should explain here why are they doing this gas sampling along the profile and give more details on how are the probes done, length, for how long is the probe left on site, was it left on the site to equilibrate for how long? How much is the internal volume? It is not clear how do you sample the 20 ml of gas into the vial? I assume 20 ml are quite a big volume compared with the internal volume of the tube. Do you leave the syringe connected to the tube to equilibrate with the internal gas? Or do you use another system?

A.:The gas samples were collected simply to obtain their chemical composition and the analytical results are shown in par. 4.1 and discussed in par. 5.
For the sampling we adopted the standard procedure for soil gas sampling in volcanic/geothermal areas, i.e. putting a tube inside the soil at the desired depth and sucking out the desired gas volume with a syringe and putting the sample through a three-way valve into a sampler for the analyses in the laboratory. The main difference in our case was that we put into the soil a probe with three tubes through which, at the same site, we could collect sequentially soil gas samples from 13, 25 and 50 cm depth. The volumes can be obtained from the dimensions of the tubes given in the text and the results are between about 0.4 and 1.6 ml. Considering also the connection to the syringe the total volume never exceeds 2 ml.
For such sampling there is no need of equilibration time because the tube is driven into the soil with a core inside which is taken out before the connection to the syringe. To avoid atmospheric contamination the suction through the syringe is made very slowly (> 60 sec for 20 ml), the first stroke of the syringe is discarded and the second is collected.
We added more details in the methods' section.

R.: Is the area of probing a strong degassing area of you have mostly soil and slow degassing?

A.:The samples were all collected in an area that previous studies (D'Alessandro et al., 2009) identified as strongly degassing. This has been now specified in the manuscript

R.:Please give more details on the gas measurement and sample handling for methanotrophic activity measurements. Do you take so many samples consequently in the same vial? Or you have replicates for each sampling time? How many replicates per treatment? How much gas do you sample each sampling event? I assume 1 ml loop needs at least 3 ml to flush it if the void is not previously made in the loop.

A.:For each soil sample we made the methane consumption experiments on two soil aliquots as specified in the manuscript. Gas chromatographic analyses were made sequentially on the same vial. The introduction system of the GC (total volume about 2 ml) is evacuated with a vacuum pump before the introduction of the sample and is provided with a pressure sensor to correct small

(positive or negative) deviations from the introduction pressure (atmospheric) of the calibration standards. We added more details in the methods' section.

R.:The same needs to be done for Table 2, plus the units must be changes so to represent smaller numbers and to include the uncertainty of the estimate (st error or st deviation).

A.:Gas chromatography is a well established method for soil gas analysis and duplicate analyses are generally not made. The analytical precision of the method (±3%) has been specified in the methods' section. Furthermore, μmol mol^{-1} is one of the most used ways to report the results.