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Interactive comment on "Methanotrophic activity and bacterial diversity in volcanic-geothermal soils at Pantelleria island (Italy)" *by* A. L. Gagliano et al.

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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 proteobacte-

rial 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 CH4 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.

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