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To: Dr. Tina Treude and the editorial office of Biogeosciences

Answer to Anonymous referee # 3

Manuscript: bg-2011-379, Paleoenvironmental imprint on subseafloor microbial communities in Western Mediterranean Sea Quaternary sediments

On behalf of the authors, we would like to thank the anonymous referee #3 for the interesting and constructive comments regarding the manuscript bg-2011-379.

Our responses to referee's comments are reported point by point and changes in the text are located by the number of the corresponding page and lines into the discussion paper.

This manuscript is a very interesting study on an attempt to relate paleoenvironmental changes to prokaryotic diversity in Mediterranean sediments. The paper is generally well written and the ideas and most of the methodological work are well done. However, I do have concerns about the interpretation of the PCR-DGGE data and discussions based on this part of the study. Therefore I feel that this paper warrants publication in this journal but only after some important revision and careful consideration of the points raised below.

Cell numbers in these sediments are very low, most probably due to the low TOC values within these sediments. However, because of these very low cell numbers (i.e. lower than predicted numbers based on other subsurface sediments) the authors should have taken great care over possible DNA contamination issues. These problems have been of great concern in assessing prokaryotic diversity in subsurface sediments. This is especially the case in determining bacterial diversity which is at a much greater risk from microbiological laboratory contamination.
Therefore, the authors should have included several blank DNA extractions in their studies (i.e. no sediment sample) to determine what, if any, the background contamination was from their extraction reagents/kits. Such problems become a further concern when nested PCR is used to assess microbial diversity, as was the case in this study.

As suggested by the referee, we agree that contamination is a major issue when working with low biomass samples. However, samples used in this study are not extremely low biomass as we detected abundances between 3.7 10^5 and 5.3 10^6 cells cm^{-3}. In previous studies, contaminations during DNA extraction were showed to occur when using commercial DNA extraction kits. Here, DNA was extracted under a microbiological safety cabinet, using a new cryogenic method (Alain et al., 2011) and with sterilized and filtered solutions. Blank DNA extractions on reagents and PCR negative controls were performed and resulted in any amplification of 16S bacterial/archaeal gene. This result indicates that no background contamination was present during the extraction and amplification steps. The confusion is probably due to the fact that this issue wasn't addressed in the manuscript. In order to address this issue, the following phrase "Blank DNA extractions were also performed on

reagents (no sediment sample) with the same procedure." was added in the "Materials & methods" section.

The lack of negative control DNA extractions in this study is especially concerning, as from my experience and from work by others (e.g. Tanner et al (1998) AEM 64:3110; Barton et al (2006) JMM 66:21; Webster et al (2006) FEMSEC 58:65), many of the identified bacterial types that dominate in these Mediterranean sediments are similar to those found as contaminants in DNA extractions due to lab reagents. The presence of low levels of exogenous DNA in extraction reagents also could be a reason why the authors were unable to culture Betaproteobacteria from the sediments.

Indeed, some bacterial phylotypes identified in our study belong to the same family or the same genus as contaminating sequences found in other studies. But these sequences are closely affiliated to environmental indigenous sequences detected in subseafloor sediments, rocks, aquifers, ground water, petroleum crude oil samples, etc. (e.g. Sahl et al., 2008; Wang et al., 2008; Yamane et al., 2008; Mason et al., 2010; Brazelton et al., 2012).

An answer to this issue is that the 16S rRNA gene of prokaryotes is highly conserved and does not allow discriminating between species and subspecies in certain taxonomic groups, notably among *Gammaproteobacteria*. Besides, some species such as *Ralstonia pickettii* (Yabuuchi et al., 1996) were previously named *Burkholderia pickettii* (Yabuuchi et al., 1993) or *Pseudomonas pickettii* (Ralston et al., 1973), which makes their phylogenetic affiliation more difficult to establish. In addition, most bacterial groups detected in this study are ubiquitous, extremely versatile and a phylogenetic affiliation is often not sufficient to concede the presence of contaminants. Since several assemblages detected in Western Mediterranean sediments were also detected in other marine environments, as mentioned above, it is improbable that sequences of this study are "contaminants". In addition, a recent study (Philippot et al., 2011) on two Alpine Glacier Forelands showed a significant increase in the proportion of *Betaproteobacteria* (up to sixfold) on the glaciers surface using *Betaproteobacterial* primers. In this study, the authors suggest that environmental conditions commonly present on or near glaciers select *Betaproteobacteria*. Within our study, sedimentary sequences in Ligurian Sea core are directly influenced by glaciers melting that produced important erosion. These recent results reinforce our hypothesis that *Betaproteobacteria* might be transported by rivers and deposited within marine sediments of Ligurian Sea during the LGM and the last deglaciation.

In addition, I would have also expected the authors to have undertaken DNA extractions on a larger sample size or have pooled replicate DNA extractions due to the low biomass in these samples in order to achieve higher DNA yields to prevent further problems associated with the PCR amplification of low DNA concentrations. Further points regarding the PCR-DGGE work include, the gels shown in the suppl. data are generally quite poor in their resolution - with a lot of diffuse bands which I assume would make statistical analysis of them slightly challenging.

Several studies reported microbial diversity analyses from similar or inferior quantities of sediments (Parkes et al., 2007; Blazejak and Schippers, 2010; Webster et al., 2010; Beck et al., 2011), and several studies performed nested PCR (Fry et al., 2006; Roussel et al., 2009a; Roussel et al., 2009b).
Sometimes nested PCR is used when PCR inhibitors (such as humic acids) are coextracted during nucleic acid extraction step. We think that this might be the case here.

Statistical analyses were performed on PCR-DGGE fingerprints based on the intensity of all DGGE bands determined using ImageJ software, by removing background noise before analysis. The presence of a weak band on DGGE gels was overcome by taking into account the presence of bands with intensity superior to the background. Such treatment of fingerprint data is in accordance with the analysis of molecular fingerprints done by several authors (Schäfer and Muyzer, 2001; Fromin et

al., 2002; Fry et al., 2006; Webster et al., 2007) in marine microbial ecology. We agree that this approach is biased. It is important to notice that bacterial DGGE gels are rich and the removing background noise before analysis allowed us to overcome fussy band problem. For this reason, we chose to use the terms “relative abundances” and “trends” when quantifying the DGGE results.

Also the DGGE data shown in Figure 6 is somewhat misleading due to the authors expressing the data as percentages of each phylo group. This is incorrect as the percentages shown are only based on bands that have been excised and sequenced with a positive ID and therefore do not include the large number of unsequenced bands. These unsequenced bands should be included within the bar charts/percentages as unsequenced or other bands to help the reader understand fully the data set. e.g. 611 bands were observed by DGGE, but only 63% were identified. Also of interest and may be should be included is how many band positions does the 611 individual bands relate to, and how many of these band positions were sequenced for each site?

We agree that Fig. 6 (Fig. 3 revised manuscript) was somewhat misleading, despite the fact that all the information of interest was present. In previous version of Fig. 3, total number of bands observed on the DGGE gel for each sample, as well as total number of bands with a positive ID was already written on the figure, in brackets. Information about identified band positions on DGGE gels was also already provided in SppOS, Figures S4, S5, S6. To make our data more readable, the bar charts/percentages of figure 6 were modified to include unsequenced bands (unsequenced bands appear now in light grey on the bar charts).

I also cannot help feeling that the authors have presented a lot of unnecessary contextual data as supplementary data that is not really relevant to some of the questions they want to address.

Regarding this point, the number of figures was reduced as follows:
_Figure 1 (A,B,C,D) became Figure 1 (A,B); C,D seismic profiles were moved to SppOS.
_Figure 2 was moved to SppOS.
_Figure 3 was merged with sulfate and salinity profiles of Figure 5. Ca profile of Fig. 3 was removed. Remaining profiles of Fig. 5 were moved to SppOS.
_Figure 4 was moved to SppOS.
_Figures 6A and 6B became Figures 3A and 3B. In these novel figures, for more readability, bar charts/percentages were modified to include unsequenced bands.
_Figure 7 became Figure 4. Dendrograms were deleted.
_Figure 8 became Figure 5. In this figure, KNI23 turbidites frequencies were replaced with KESC9-30 turbidites frequencies. Relative abundances of identified *Chloroflexi* and *Betaproteobacteria* are now expressed as a function of the total number of DGGE bands (instead of the total number of identified bands).

Minor Comments

P255 L8 change to: DNA-based fingerprints showed…
As suggested by the referee, the sentence was modified. It is now written: “DNA-based fingerprints showed…”.

P255 L13 change to: ..diversity was as a result of the important…
As suggested by the referee, the sentence was modified as follows: “..diversity was as a result of the important”.

P256 L14 change to: In some sediments, the...

We modified the sentence according to the suggestion of the reviewer. It is now written: “In some sediments, the …”.

P263 L5-25 what was the gas phase in anaerobic cultures for fermenters, SRBs with VFAs?
The gas phase for fermenters, and SRBs with pyruvate/fumarate or acetate, was N_2 (100%, 1 bar).

P266 PL13-14 the authors state that 14 depths were amplified by Archaea 16S rRNA gene PCR but Fig 6 clearly shows data from only 13 depths?
The referee is right. This mistake was modified in the text: “13 depths…” instead of “14 depths…”.

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