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Discussions

Interactive comment on "Determining the hierarchical order by which the variables of sampling season, dust outbreaks occurrence, and sampling location, can shape the airborne bacterial communities in the Mediterranean basin" *by* Riccardo Rosselli et al.

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General Remarks: The authors present the results of an interesting experiment, sampling airborne microbial communities at two locations, Sassari and Cagliari, located at the opposite ends of the Mediterranean island of Sardinia. The first facing Europe in the north, the second facing north Africa, in the south. The study of airborne microbial

communities it is of great interested nowadays as the roil of air dispersal in determining the biogeography of micro-organisms is slowly being discovered. The authors collected samples in two different seasons, at the two different locations and considering different dust events/wind direction shifts and compared the results of these variables on the sampled airborne microbial community through the use of amplicon sequencing. The idea of using these two locations is very interesting, since it can clearly capture the airmasses coming from two different continents with less interference. The results show interesting patterns, defining the season as the most important variable in defining alpha and beta diversity differences among the samples. Moreover, the location, although less clearly, seem also to influence combined with the timing of the samples used as control (before and after the dust event). In general, I think the experiment and the results are interesting but I would advise to authors to review certain sections before publication.

ANSWER: We thank the reviewer for the appreciative comments.

Specific comments: 1)The manuscript presents itself with a title that seems way more inclusive, as "Determining the hierarchical order by which the variables of sampling season, dust outbreaks occurrence, and sampling location, can shape the airborne bacterial communities in the Mediterranean basin" points to a much larger study, where the whole Mediterranean basin (and not only the island of Sardinia) is considered, and more observations and samples are available. In this case the experiment is quite small, only having two seasons, and one year time spawn, with only two locations, and it should be rather presented as a case study, since one cannot draw any certain conclusions from these observations, but only hypotheses. This is not meant in diminishing the value of the study since sampling airborne microbial communities presents technical difficulties that make the design of bigger studies quite challenging. Nevertheless, a better title should be considered.

ANSWER: We acknowledge the fact that the landing range is the Sardinia island, which spans 270 km, whose shorelines are 1849 Km-long (1/4/ of the Italian coastline total)

and which we sampled at each end of its main dimension. Nevertheless, the use of the term Mediterranean in the title is referred to the sea (medius-terrae: the sea between lands). This is because, although we used Sardinia as a mid-way catchment sink, the source of the sandy dust that is thereby discharged, encompasses the entire coastal system of the northern African continent, the Saharan atlas as well as the middle east shores and Arabian inland. Therefore, in considering the full transcontinental travel of the passively migrating microbiota, by 'Mediterranean basin' we intended the whole perimeter of origin. As regards the recommended need for more observations e.g. repeating the study in different occasions we can remind that this report is actually the companion follow-up of our prior investigation in which we had carried out the very same approach in the same two places three months earlier, and catching another 'dust vs. calm' shift of events. That one was the first outbreak of that year in the Mediterranean area and yielded the picture that the place featured at the end of the winter (Rosselli et al., 2015: Microbial immigration across the Mediterranean via airborne dust. Scientific Reports 5:16306 DOI: 10.1038/srep16306). The present work is therefore framed in a comprehensive series of analyses, as recommended.

2) The experimental design is not really clear until the results section. A table or a schematic of the design should be included at the beginning of the material and methods section.

ANSWER: We realized this lack of clarity and we found that the design had to be anticipate in the materials and methods in which we added the following description: "The experimental design involved: 2 sampling sites at the opposite corners of the Sardinia island (Sassari vs. Cagliari), 2 sampling periods (May vs. September) 2 meteorological conditions (absence vs. presence of a dust outbreak). In each of these situations, two replicate samples were taken and processed independently throughout the DNA extraction step to be pooled before sequencing. Samples were collected on Teflon filters (Sartorius Stedim Biotech) by using a Skypost Tecora apparatus (compliant to the European legislation 96/62/gmeCE) processing 39 liters of air per minute. To consti-

tute 'a sample' a continuous 24h-long air intake through the filters was performed. In the case of the dust outbreaks the 24h sampling was further divided in two periods, by considering independently the first 12 hours and the second 12 hours. The number of resulting samples was therefore 12; namely the module of three conditions: (a) Control; (b) Dust h 1-12, (c) Dust h 12-24; multiplied by 2 sampling periods and by the 2 sampling places, resulting in 3 x 2 x 2= 12 samples. As technical note, the scope and meaning of 'controls' here was that of samples that could allow to individuate the shift between one condition and its adjacent one. In our cases, catching the sudden change of wind regime by sampling immediately before or after a dust outbreak. Therefore, the controls were thus defined as single 24h time points flanking the key dust events."

3) The result section seems very messy and hard to read. First the authors introduce the readers to the microbial composition: From line 259 to 309 the authors make a great efforts in describing the different microbial taxa that compose the samples. Nevertheless, it is hard to find the logic the authors followed to write this section. Such analysis should start from a clear graph showing the different phyla composing the different samples. The graph provided (figure 3) is extremely hard to read. From there you could enter specific phyla that you want to further comment.

ANSWER: The reviewer is right, the order of presentation of the data and the quality of Fig.3 was in our view most of this problem. We have redrawn Fig. 3 and enlarged, imagery, fonts, added color-coded legend to the taxa in the picture and not in the legend; we increased its overall resolution and we placed the figure before its descriptive comments, which appear now much more sound to follow.

Then the authors present a method to identify the taxa that form a common core in the samples. The method they use, in my opinion, is not clearly explained: Line 319 to 321: "the criterion was to set a cutoff value with respect to the percent of variation occurring between the first 12 h of the collection time and the second half of it. Only the taxa which displayed a mean variation higher than 1/2 of the corresponding standard deviation were considered" So why variation WITHIN the dust event should provide this

information? Shouldn't it be better to just analyze the OTUs found in all samples? Not clear why this method was used. Generally, the results rection is hard to follow and confusing and needs a deep restructuring. Further comments below in the technical corrections.

ANSWER: all OTUs were actually taken into account as well in parallel to put in evidence any possible community variation and this is actually the way most of the results are drawn (Fig.3,4,5, S8, Tab.2,3,4). The difference in this case was to exploit an additional opportunity to detect possible dust-specific taxa. The availability of two incremental timeframes, both within the dust event, allowed to verify which ones would be enriched along with the progression of the stormy condition.

4)The discussion part is well organized. It very well explains the hypotheses of our authors to explain the results observed in relationship to the geographical and meteorological conditions. The authors could nevertheless improve the ecological value of the discussion, better linking previous experiments and observations done with microbial dust dispersal. Moreover, the discussion is mainly based on the measurements of alpha and beta diversity but does not include much of taxonomical data. So, the authors fail to discuss the results explained in the results section (259 to 309, tab 4) Only few lines are dedicated to this purpose (554-556).

ANSWER: The criticism is correct, we integrated the text as follows:

"Commenting on the taxonomical abundance shifts observed between May and September and trying to interpret the rise of some phyla and the drop of others (Fig.3; Tab. 1), a preliminary consideration needs to be recalled. The issue has to do with the distinction between actual population dynamics (ecologically-ruled) and mathematical effects of sampling from a 'ballot box' of objects, all of which compete for the constrained 100% format of results (probability-ruled). This caveat was put forward as early as statistics itself was born as a discipline (Pearson, 1897). There is in this respect a general problem in comparing communities at different time points (and even

more with DNA-based methods with fixed total DNA amount processed), whose results are based on percent values (relative abundance of taxa). In that condition the multiplication of any, determines obviously a reduction of the relative abundance of others, when those do not grow at equal or higher rate. Therefore one given group could have been increasing, but its share in the sum could appear as if it had instead decreased, if a different group has increased faster. This consideration, applies inevitably for all metagenomics/metabarcoding surveys, and should be kept in mind for all kinds of interpretations about increases and decreases which could be either real or apparent (when driven by a stronger change of a different group). As consequence, comparing different sampling points through time is linked to this inevitable constraint: the compositional nature of the datasets binds all relative frequencies to each other (Gloor et. Al., 2017). Therefore, since, as mentioned, the sum of them is bound to give always 100%, the decrease of a given species could be either apparent (driven by the increase of another), or real (due to its actual negative population dynamics). The problem is that the two causes can not be uncoupled by just comparing species frequencies at the two sampling times. Moreover, as mentioned, even an actual increase of a given species could be masked by the parallel increase of another at a faster pace (or by its net immigration into the scene). For this reason we consider with caution the possibility of looking at taxa fluctuations as indicative of their actual ecological outcomes. Having clarified that we will therefore limit to comment only the major phenomena that stand out from the comparison. The largest taxa trade off that is apparent when comparing the two periods is the decline of Proteobacteria and the parallel rise of Actinobacteria. Trying to frame this within seasonal parameters we can consider that the latter are typically relying on profuse spore formation from colonial growth, while the former are non-sporeforming bacteria either motile via flagella or gliding/swariming mechanisms. The basic life forms of the two groups predict therefore that proteobacteria would be more suited by wet seasons and vice versa. Actinobacteria have been reported by other authors to reach their peaks in fall (Glöckner, et al., 2000). Being also a group of major litter decomposers their rise along with the end of the plants' vegetative season

can be seen as compliant with their landscape and ecosystem cycles. “

Technical corrections:

Line 52: Dispersal not Dispersion. Dispersal is the act itself; dispersion is the result of dispersal.

ANSWER: Corrrection made.

Line 63: Here it seems the topic changes suddenly. Entering deep into the microbiology. I would put the paragraph ending here and not in line 60

ANSWER: Corrrection made.

Line 182: OUT

ANSWER: Corrrection made.

Figure 3: Replace it with a stacked barplot

ANSWER: Fig.3 as anticipated was completely reshaped and increased in font readability, clarity and in-picture legend for taxa colors

Line 310 to 314. This might go up in the methods. Once again, a clear section of the methods describing the methodology is needed.

ANSWER: The methods section has been implemented with the comprehensive new outline described above. One part of this sentence is however to remain in this section as, when recalling the filters operation we describe a result (the intensification of the microbial content in the filtered material during the second 12h lapse,

Line 315: OTUs or taxa? Along all the manuscript I had the feeling these words were not used consistently, please check.

ANSWER: the wording has been corrected where needed. Essentially however, the process of sequencing is yielding reads which are clustered in discrete packages of OTUs, but the subsequent annotation points for each to taxonomical names which are

the ones that we then describe. In this sense, the two terms achieve a formal equivalence. In specific cases (when one taxonomical lineage corresponds to more than one 97% identity-clustered OTUs) we find more correct to use the taxon appellative as it would encompass more than one OTU but they concur to the same best achievable name.

Tab1: It is unclear to me what you are reporting here, please explain better.

ANSWER: This addition to the text that we made will add clarity to the table :

“Besides the comparisons that included all OTUs to put in evidence community variations, in parallel we exploited an additional opportunity to detect possible dust-specific taxa. The rationale was to seek differential enrichment within the dust storm, by dissecting the process, during its progression, splitting its onset from its fully established stage. To this aim we collected separately the filters of the first 12h of the event, and replaced them with new ones that collected air during the second lapse (hours 12 to 24). Thus the availability of two timeframes, both within the dust event, allowed to verify which OTUs would be incrementally enriched along with the progression of the stormy condition. “

Furthermore, we have detailed the legend of the table (now Table 2) to clarify the content. :

” Tab. 2: Extent of OTUs change across cell harvesting time during the same dust outbreak sampling. The percent variation (either increase or decrease) of a given OTUs abundance between the values found in the community obtained by the first 12h sampling and the ones resulting from the following 12h lapse was computed. The average, minimum and maximum percent variation between counts are shown. Only taxa displaying a difference in percentages higher than half of their standard deviation were selected for the present comparison.

Tab2: Are you reporting the number of orders? If it is just the count, that is a measure

of richness, and not diversity. Also, are these results adding important information compared to the bray Curtis similarity table? It seems quite redundant and not helpful for the discussion, since the number of orders is not really helpful for any results. For quantifying community variations, Bray-Curtis is the right instrument, as used later.

ANSWER: Thank you for correcting appropriately the terminology. The term diversity was replaced. The results shown here were considered not equivalent to the Bray-Curtis values, which are relational, as those stem from pairwise operational comparisons involving two samples and expressing distance or similarity; richness is instead a property belonging to a single sample.

Tab3: Simpson and Shannon are both diversity indexes, and the use of two of them does not add much information to the discussion. Why not using richness instead of Simpson?

ANSWER: Although they are estimators of diversity, given the different formulas on which they are based, the use of both could be not redundant. For example in our prior report (Rosselli et al, 2015) on the same island, in the February dust outbreak analyzed at that time, one of the localities (Cagliari) was showing opposite results when using Shannon or Simpson indexes (increasing in one case and decreasing in the other). The richness data are actually shown for all 12 samples in the supplementary dataset at different taxonomical ranks and those regarding the number of orders, as you correctly indicated are also in Tab.2.

Tab4: should be connected to the results at line 259 to 309. But it is not.

ANSWER: The table has been appropriately moved up to the beginning of results and it is now the new Tab.1 and it is discussed along with the taxonomy part of the results

Figure 5A, the PCoA does not add any info to the study. Moreover, the dissimilarity matrix used for the PCoA should be reported.

ANSWER: We agree on this redundancy and PCoA was moved to the supplementary

material as its value was recognized not incremental compared to the PCA, discriminant analysis and the other approaches. The dissimilarity matrix is however available in the manuscript as it is actually in Table 5 (simply, the complement to 1 of those Bray-Curtis similarities, yield the distances upon which the PCoA ordination originates). The form is not that of the canonical triangular matrix as that was rearranged to suit Tab.5 purpose, but all pairwise values are inspectable from the table. Moreover each analysis can be verified and run again from the OTU table which is provided as spreadsheet in the Supplementary dataset S1.

Tab5: This is hard to read. I suggest a heatmap style table.

ANSWER: It is true and we have experimented different ways to reorganize that, including as suggested a heatmap-style arrangement with all the entries ending in a single matrix rather than three separate table sections, which however ended up more complex as it needs a tridimensional representation of the same/different season, dust/control and Cagliari/Sassari location. But what we find more useful to point at, is that the main message that this color table is meant to convey is just the fact that the low similarity pairwise comparison (conditionally formatted as red-yellow shaded cells) are almost all observed in the upper table. i.e. the between-seasons comparisons, while the green ones (higher similarities) are all distributed in the two same-period comparisons. We further stressed this aspect to direct the readers' attention to the way to get the bottom line information from this table. The message is reinforced by the other figures as mentioned but this one shows the Bray-Curtis values and allows each pair to be inspected, which is an information that the PCA, PCoA and dendrogram are not providing with such detail.

565: How was it verified? You would need additional controls after and before the dust events.

ANSWER: We agree, the sentence was actually only meant to imply that if we had observed a very similar situation between the communities of our post-dust sampling

in spring and our pre-dust sampling in fall, one could have hypothesized that the absence of those outbreaks could be conducive to airborne community stability. Such hypothesis would have required other samplings in between to be confirmed as a true stability and not cyclic recurring fluctuations, as you correctly indicate. But as the situation that we observed was in fact the opposite (deep changes occurred without the need of dust outbreaks to cause them), that hypothesis was not the standing one. We therefore limited our comments to notice that the two sites had achieved a high similarity after a dust-free period and that such situation was very different from the one they had displayed four months before. The control for comparison was in this case the last post-dust analysis available, and we stick to this in our inferences.

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