

ANSWER TO REVIEWERS 1 and 2 (July 2018)

We hereby present a detailed response to all the comments provided by the reviewers. We hope that the answers clarify the different points and that the main changes included in the revised version are sufficiently highlighted. To facilitate the revision, the original comments of the reviewers are written in italics. Then, the answers to the different comments are written in green under the corresponding original comment. The modifications proposed are shown in red.

Reply to comments of Shilei Sang:

General comment: *This study integrated different fields such as hydrogeology, ecology and microbiology applied to a real MAR facility, relating flow (infiltration) conditions, physicochemical water parameters, and microbial changes induced by managed recharge along vertical transect. Except for some marking errors, the scientific results and conclusions presented are clear, concise, and well-structured.*

We thank Mr. Sang for his comment. We are very pleased our work has been evaluated so positively. We really hope our multidisciplinary paper will be useful for many professionals from different fields of expertise. Any further comment or correction will be welcome.

Page 3, line 15: “Water entering” instead “water enters”

Page 3, line 19: “promoting” instead of “promote”

Page 4, line 12: “50 cm” instead of “50cm”

Page 4, line 12: “depth” instead of “dept”

Page 5, line 23: “due to” instead of “due the”

Page 5, line 28: “are displayed”

Page 6, line 28: “absent from” instead of “absent in”

Page 8, line 31: “medium-disturbance” instead of “medium-disturance”

Page 9, line 9: “In” instead of “in”

Reply to comments of Anonymous Referee #2:

General comment: *This paper studies an important problem of microbial community change under conditions of Managed Aquifer Recharges (MAR). Choosing a MAR facility located in NE Spain, the authors took water and soil samples during recharge and no recharge periods to compare the microbial community conditions. The authors reached a conclusion that the MAR is a naturally, passive, and efficient technique for biodegradation of pollutants in groundwater. I*

believe this work provides useful insights to improve our understanding of MAR in biochemical ways.

We want to acknowledge Anonymous Referee 2 for his/her evaluation of the present work. Regarding the comments of the Referee, we would like to clarify some aspects that maybe are not fully-developed in the manuscript.

Comment 1: *Does the types of water change with wet/dry scenarios (eg., Type 1water)? This seems possible.*

We expect that background conditions (represented by Type I water) may not affect too much the microbial composition in comparison with the conditions induced by recharge process. One of the evidences that supports this approach is the fact that diversity indices (mainly Shannon and Evenness) remains constant in Type I waters, and decrease accordingly to the influence of the recharge process in wet scenario. Under this assumption, we divide the samples according to the influence that they receive from the recharge process. For doing this, we relied on the information provided by temperature and conductivity profiles (Figures 2 and 3) and the knowledge provided by other studies performed in the same study zone (Valhondo et al., 2014, 2018).

Suggested changes: extension and clarification of the role of background water conditions in the study.

LINES 27-30, PAGE 7

“Groundwater is a quite stable aquatic environment (Griebler and Lueders, 2009) as it is shown by the low variability in the hydrochemical data (Table S1). One could expect that microbial communities in groundwater should also display low variability that could be reflected in the diversity indices. In this way, piezometer P1, which is unaffected by recharge, showed stable diversity indices regardless of the sampling campaign. However, disturbances produced by recharge, evidenced by temperature and conductivity gradients below the pond (Figures 2 and 3), favor the highest Shannon values for the intermediate-disturbed samples. As a result, the average of diversity indices remains constant between both scenarios but with higher standard deviation during the wet scenario. These two facts combined, suggest that perturbations caused by recharge influence much more the composition of microbial communities in groundwater than the natural variability of the background aquifer water between scenarios.”

Comment 2: *I believe the results of microbial communities in groundwater (Results section 3.1) should also be discussed in the Discussion section. Some of the results are lack of deep interpretation or further discussions.*

In our opinion, the fitting of our results in the Intermediate Disturbance Hypothesis is wide-argued in the discussion section. Furthermore, we discuss the potential role of some microbial phylotypes related to aerobic oxidation, dehalogenation and denitrification processes. Likewise, we relate such presence with the influence received of recharge process. However, it is true that the lack of information about the contaminants presents in the system do not allow us to

conclude the empirical relationship between pollutant concentrations and the presence of these phylotypes. In this way, we propose to include paragraph explaining this lack of evidence in order to propose future research work.

Suggested changes: the discussion section according to the responses included in comment 1 and comment 3 of Referee #2. The discussion about background conditions has been improved and a new section has been included in to the discussion.

Comment 3: Can results and conclusions of this research be extended to other areas?

Yes, some of them can. Of course, we present results from a field experiment, that intrinsically is not performed in controlled conditions. We think that field studies are indeed realistic but their transferability becomes more difficult. However, here we present some interpretations of our study that could be transferred to other sites/experiments: a) The diversity indices can be perfectly compared between sites, whatever it were the approach (laboratory, pilot or field studies). The methodology that we followed for the calculation of such indexes is well-reported and widely applied. b) The Intermediate Disturbance Hypothesis can be also transferred. In fact, this study evidences that this ecological hypothesis has been accomplished in the Llobregat MAR site. Why could not this approach be applied or accomplished to other impacted environments? c) We present here the results of sequencing tasks that associate some microbial species with certain environmental conditions. We want that our findings would be useful for future research in groundwater microbiology, especially in impacted zones as Llobregat River Basin. Overall, we expect our study it could be transferable to groundwater-surface interaction zones with low nutrient concentration but high amount of micropollutants, emergent organic compounds, etc (as in the case of Llobregat River or other Mediterranean high impacted rivers).

Suggested changes: creation of a new section explaining future work and extrapolation of our results to other areas

LINE 15, PAGE 10

4.3. Transferability of results and future work

The results of the present work are from a field experiment. This intrinsically means that is not performed in controlled conditions. Field studies are indeed realistic, but their transferability to other areas becomes challenging. However, some microbial and ecological approaches are comparable between experimental studies. In this way, the diversity indices are common calculations performed to assess the ecological state of a microbial community which allows comparing different communities (samples under different operational conditions in our case). Following an ecological argument, this study evidences that Intermediate Disturbance Hypothesis has been accomplished in the Llobregat MAR site. Therefore, we could expect the same behavior in other impacted areas under similar recharge conditions.

Another information that could be useful for other scientific works is the role of some microbial species in the organic matter degradation and denitrification processes, such as the role of *Vogesella sp.* and other key species discussed previously. The sequences of all species found were deposited into GenBank, so it represents a contribution to the genetic database and to the overall scientific knowledge.

Despite the novelty and transferability of this study is quite clear, future work is needed to keep evaluating the relationship between different ecological, microbial, hydrochemical and physical variables in different sites worldwide. An improvement of this multidisciplinary understanding of processes could be achieved by combining statistical techniques with process-based models. This would allow direct extension of the results from one experiment to other sites.

ANSWER TO REVIEWER 3 (December 2018)

Reply to comments of Aronne Dell'Oca:

General comment: *The present study address the impact of Managed Aquifer Recharge (MAR) activities on the features of the in situ microbial community. The latter having been characterized by analysis of both soils and (surface- ground-)water samples, during recharge and no recharge periods. The MAR facilities is located in the NE of Spain and the infiltrated water is taken from the Llobregat River, which suffers from contamination by emerging organic contaminants (ECOs) that are not fully removed during wastewater treatments. In this optic the MAR facility induces a perturbation of groundwater characteristic (e.g., organic matter, dissolved oxygen, temperature, pH) leading to the growth and stimulation of the microbial community, favoring the degradation of ECOs. The main goal of the study is that of quantify the MAR related changes on the microbial community. The Authors provide also a link with ecological principles and potential biogeochemical processes.*

I think that the paper is interesting, and I applaud the Authors for facing the challenge at a real-world field site! In my opinion the paper is worth for publication after some minor comments are addressed. Note that some of my comments are dictated by my poor background on biological community characterization methods and interpretation.

We want to acknowledge Aronne Dell'Oca for his comments of our work, especially appreciating the challenge of working at field scale in a multidisciplinary study. In the following paragraphs, we will try to clarify some aspects and add the necessary information to improve the manuscript following his recommendations.

Comment 1:

Section 2.3: Are the groundwater samples taken for the microbial analysis taken at the same location of the samples used for the hydrochemistry analysis? I imagine yes, but please make it clear in the text.

Yes, they are. We took several subsamples from each sampling site in order to characterize them hydrochemically and microbiologically.

Suggested changes: clarification of the sampling depth information

LINE 14-15, PAGE 4

“Groundwater samples were taken from -5 to 3 masl depending on the piezometers (10 samples for wet scenario and 7 for dry), at the same location as samples for the hydrochemical characterization”

Comment 2:

Section 2.3: “The first one is Richness (S), defined as the proportional number of microbial species present in a sample, i.e., equal to the total number of bands;” would it possible to further elaborate on the meaning of these bands? I am not an expert of the subject so if the Authors think that it is not needed, I agree with it, but a more detailed description of the ‘bands’ meaning would help to attract the interest of a wider audience!

Denaturing gradient gel electrophoresis (DGGE) separation of 16S rRNA gene amplification products has been thoroughly used for describing the genetic diversity of microorganisms in natural environments. The total number of bands visualized in a denaturing gel provides an estimate of the diversity within a given environment, and sequencing of selected DGGE bands enables the identification of the organisms present. For more information see the original reference: Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* 2, 317–322. So we really think we should not go further in the description of such common term in molecular microbiology.

Comment 3:

Section 2.3: The Shannon (H) and Evenness (S) are used to characterize the diversity of the microbial community. I suggest to further elaborate on their meaning right after their introduction since it would not be that clear for reader unfamiliar with entropy and measure like that. Note that I am interpreting index H as the entropy of the ‘bands spectrum’ (perhaps is not the right wording), Hmax as the entropy of a uniform bands intensity and E accordingly, e.g., $E \rightarrow 0$ the microbial community exhibit a poor variability.

We agree that a further description of these terms should be done, especially to facilitate their interpretation.

Suggested changes: including more explanation about the meaning of the bands

LINE 26, PAGE 4

“Evenness index is proportional to the equitable distribution of bands in the gel. Shannon is an entropy index, becoming maximum (Hmax) for the most equally-distributed band patterns and with the higher value of richness.”

Comment 4:

Section 3.1.2: “The two green triangles in the center of the plot correspond to groundwater samples from P10.” and so? The fact that these two triangles of water type IV are not expected to be ‘near’ the others is not immediate to me, could please the Authors elaborate further this observation.

Furthermore, would be possible to further describe the meaning of the NMDS, its relationship with the bands and so how to read the empty and full circles? Perhaps in Appendix with other notions related with the microbial characterization or adding some reference. Once again if the Authors think that this is standard notions it would not be necessary.

Following the reviewer suggestion, we are going to improve a little bit more the explanation of NMDS plot. Respect to the second part of your comment, we want to highlight that the paramount information to describe the NMDS is present in the caption, including the meaning of empty and full circles.

Suggested changes: widen of conclusions extracted in the NMDS approach

LINE 2-3, PAGE 6

“The two green triangles in the center of the plot correspond to groundwater samples from P10, that are samples low affected by recharge. These Type IV samples remain among different sets of groundwater samples from the wet and dry scenario. It seems that the theory of P10 as a location with a high proportion of mixing is also valid for the microbial community composition behavior.”

Comment 5:

Section 3.1.2: Figure 5 in the text is Figure 6 in the figures list. Please make the piezometers identifiers more readable in the dendrogram.

We will change the order of figures in the final upload of the manuscript. Also we will increase the resolution of identifiers in the dendrograms.

Comment 6:

Section 3.1.3: I really liked Figure 6 (note that it is called fig. 5 in the list figure) and how it summarizes the lower variability in the bands for water type II an type III w.r.t. the unaffected waters, i.e., type I and IV.

Would it be possible to add the trend for the dry scenario in order to see how the H varies as a function of the recharge v.s. no-recharge conditions?

We considered that was better not to include the Shannon index values of the dry scenario to avoid confusing conclusions. In the dry scenario, Shannon indices from P8 exhibited the lowest values, indicating that some conditions did not come back after 4 months with the facility stopped (this idea is mentioned in the Section 4.1). These two samples add some noise to the tendency of the Shannon index for the rest of samples. So, the representation of dry scenario Shannon values would not provide valuable information for the IDH approach.

Comment 7:

Section 3.2: “Figures 8 and 9 show the relative abundance of bacterial phylotypes at the taxonomical level of classes for surface water and soil samples.” Should not be “Figures 8 and 9 show the relative abundance of bacterial phylotypes at the taxonomical level of classes for SOIL and WATER samples”?

“For surface water samples (Figure 9), there was a decreasing gradient in community complexity along the ponds” It would be beneficial to add something like “ see the H, S and E indices in Table 1” or add them to the Fig. 9 (the same for Fig. 8).

Indeed, there are some mistakes in the figures list. Captions in figures 8 and 9 are correct, while the uploading of figures is wrong. The figure 8 should be placed above the caption 9 and the figure 9 above the caption 8. According to this, the sentence should be written as the reviewer suggests.

On the second hand, we will add some information about diversity indices regarding surface infiltration samples. Then, the behavior of these microbial communities as a gradient, will be highlighted.

Suggested changes: rewrite sentences in section 3.2 regarding Figures 8 and 9

LINE 16-18, PAGE 7

“To study the impact of MAR on microbial community structure, soils from pre-sedimentation and infiltration basins, as well as infiltration water, were analyzed. Figures 8 and 9 show the relative abundance of bacterial phylotypes at the taxonomical level of classes for soils and surface water samples.”

LINE 23, PAGE 7

“For surface water samples (Figure 9), there was a decreasing gradient in community complexity along the ponds, as confirmed Shannon, Richness and Evenness indices (see Table 1)”.

Comment 8:

Section 4.1: I did not know the Intermediate Disturbance Hypothesis (IDH) and I really liked its application in the current study.

Line 9 pp 9: “in the latter case, values...” upper case is missing at the beginning of the sentence.

We think the referee reviewed the first version submitted by authors (version of April 2018). As the first editor indicated us, we presented a second version in July 2018 (manuscript_v2) in the “File upload” section. In this second version, the mistake was corrected.

Comment 9:

I really appreciate the fact of deal with the issues dictated by the scale of the investigated dynamics not via widespread (and so thought as unquestionably reliable at all scales) system

of partial differential equations (e.g., Darcy flow with mass continuity, advection-dispersion equation), but through a solid analysis of the available data (essentially following Information Theory metrics). This is a more common practice in the hydrology community, rather than the hydrogeology community. It remains to be seen how to move from laboratory to field scale according with this approach.