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Interactive comment on "High diversity of nitrogen-fixing bacteria in upper reaches of Heihe River, Northwestern China" *by* X. S. Tai et al.

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Dear Tina Treude,

Interactive comment on "High diversity of nitrogen-fixing bacteria in upper reaches of Heihe River, Northwestern China" by X. S. Tai et al.

Thanks a lot for your suggestions and the Reviewer's comments. They are really helpful to improve our manuscript! I've discussed with all the other authors seriously, which cost such long time. We would like to address our response to the comments of the Reviewer as follows (We keep the original text of the Reviewer's comments and responded them seriatim.):

Main comments: 1/ One of the main weaknesses of the study is that it does not address

clear questions and does not have hypotheses. It therefore remains too descriptive and lacks of structure. It's a pity when one look at the sampling design, which I've found quite interesting: the sampling sites are located along an altitudinal gradient, the two habitat types studied are dominated by plant species that may differ in their growth strategy/ecophysiology: : : And all this may affect nitrogen cycling (see e.g. Chapman et al 2006 New Phytol). Why did the authors choose to compare these two environments? Why did they work on an altitudinal gradient? What are the challenges behind this? What were the expected results? What conclusions can be drawn about the diversity and/or functioning of these systems?

Reply: Thanks for the suggestion. As proposed by Chapman et al. (2006), plant characteristics and interactions do in fact exert strong control on N cycling processes in many settings. However, determining where and how plants influence N cycling is a challenge. In this study, the two sites studied varied in altitude and vegetation cover with the lower shrub soil being dominated by Potentilla and the higher meadow soil dominated by Carex. Plant species that fall into the conservative category (e.g. Potentilla) will more strongly regulate N cycling than plant species exhibiting extravagant N-management (e.g. Carex), as predicted by Chapman et al. (2006). Our findings may provide evidence in support of the prediction. Correlations revealed that diversity and copies of nifH gene mostly correlated with aboveground biomass in shrub soil. In meadow soil, nifH gene diversity was principally affected by altitude while copies did by soil available K. Furthermore, assessments of diversity that consider the frequency of different nifH sequences and the phylogenetically based methods of analysis (Unifrac Significance and P Test Significance) all showed that nitrogen-fixing bacterial communities beneath Potentilla were different from the ones beneath Carex. These results suggest that plant species may influence N cycling by enhancing the fitness of certain nitrogen-fixing taxa.

Reorganizing/rewriting the introduction / discussion with these questions in mind may significantly improve the manuscript quality.

Reply: This will be done in revised paper.

2/Statistical analyses: 2.1/ The number of samples/replicates used for the statistical analyses is really unclear: 5 samples/quadrats were pooled, rendering 3 composite soils / sites (one per quadrat). Then, authors performed 3 DNA extractions per "samples" (composite samples?) and 3 PCR replicates per extractions (or pools of extractions?). But at the end, there is not any information on the number of clone libraries, qPCRs, cultivation libraries, or sets of RFLP profiles obtained for each site and used for statistical analyses (ideally one per quadrat, 3 per sites). This needs to be clarified. Besides, I've noticed that SD values were absent in most tables/figures, and that no statistical tests of significance (e.g. mean comparison tests or permutations tests) were used (or at least reported: p values must be provided) to prove that shrub vs. meadow bacterial communities characteristics are indeed different or that they indeed co-vary with environmental variables.

Reply: (1) We chose 3 quadrats per site and collected 5 soil samples in each quadrat, the 5 soil samples were pooled for one. (2) We performed 3 DNA extractions per site from the 3 composite samples. The 3 DNA extractions were pooled for PCR. (3) Cultivation of nitrogen-fixing bacteria and the q-PCR run was done in triplicate. Clone libraries, cultivation libraries and sets of RFLP profiles were performed without replicate. (4) The results in Table 1 were average values. In figure 1 and 2, the numbers stand for confidence levels above 95%.

2.2/ The analyses themselves are poorly described and not always correctly performed: 2.2.1/ Phylogenetic trees: First, authors should indicate which model of DNA evolution was used to construct the trees (amongst e.g. Jukes-Cantor, Kimura, Tamura models).

Reply: Distances (distance options according to the Jukes-Cantor model) and clustering with the neighbour-joining method were determined using bootstrap values based on 1000 replications (Zhang et al., 2012, J ENVIRON SCI-CHINA; Jukes and Cantor, 1969, Academic Press, New York; Saitou and Nei, 1987, MOL BIOL EVOL; Kumar et

al., 2001, BIOINFORMATICS).

Second, many branches in Fig. 4 and 5 are not very well supported (bootstrap value < 95%) to make reliable taxonomic assignments. Furthermore, these two trees were not constructed with the same references (e.g. no -proteobacteria references in Fig. 4). Making inferences on the differences between meadow and shrub communities (in terms of composition) based on such trees is not reliable (as done p. 5022 l.20ff). I would suggest constructing the phylogenetic tree (i) by using a larger number of references, encompassing a larger number nifH groups to make it more robust, and (ii) by including both meadow and shrub OTUs in the same phylogenetic tree. Authors might be interested, for instance, in the UNIFRAC website (http://bmf.colorado.edu/unifrac/), which provide statistical tools for testing differences in bacterial community composition between samples based on phylogenetic trees.

Reply: (1) We will construct the phylogenetic tree by using more references and nifH groups in revised paper as suggestion. (2) We have performed Unifrac Significance and P Test Significance tests in the UNIFRAC website with the statistical tools provided. The P-value analyzed by Unifrac Significance including all environments together was 0.03, indicating the probability that each environment has more unique branch length than expected by chance. While these statements are true based on assessments of diversity that consider the frequency of different sequences, the phylogenetically based methods of analysis did detect significant differences among communities.

2.2.2/ Clustering analysis: Which clustering method was used? (average/complete/single linkage?). Authors should also be more careful with their interpretations regarding this analysis: it shows that shrub and meadow communities display different characteristics (in terms of evenness, nifH genes abundances), not that the communities are different (as stated/suggested e.g. p.5022 l.18, p. 5024 l.8), the latter rather evoking that they harbour different taxa, an observation that is not supported by the analyses performed here (cf. 2.2.1).

Reply: (1) We performed the clustering analysis by Hierarchical Cluster with the method of average linkage between-groups (Nonnoi et al., 2012, APPL SOIL ECOL). (2) The Corrected P-value (<0.01) and Raw P-value (0.000000) which were analyzed by P Test Significance including all environments together indicated that the environments are significantly clustered on the tree. Not only did the two communities exhibit differences in diversity (based on phylotypes), but they were also phylogenetically disparate.

2.2.3/ The "Correlation" analysis, which appears to be an RDA analysis (i.e. based on linear regressions). First, authors should be careful: correlations differ from linear regressions. Second, it is unclear to me what has been really done: authors' reply to reviewer 1 suggests that RDA was chosen to find out what are the environmental parameters that may be responsible for species variations (that's sound ok for me, it is how I use it). But what I understand from the Material & Method section and Fig 6 is that community characteristics, not community composition, were used as response variables. This should be clarified.

Reply: As you say, the community characteristics, not community composition, were used as response variables.

3/ Results interpretations are not always clear: p.5024 l.8ff: Awkward: "samples spatially closer to each other, regardless of their location in the geographic range"? Ramette & Tiedje actually reported that bacterial community composition varies at small spatial scales due to environmental heterogeneity. It is not the point here: the fact that similar soils harbour communities that display similar characteristics (again, only in terms of evenness and nifH gene abundance) is to be related to their abiotic and biotic contexts, which tend to be the same within the same habitat, and which also happen to be the same for close samples (environmental context and geographic location are cofounding factors with the sampling design used by the authors). I don't believe that isolation by distance may be at play at this spatial scale.

Reply: It is a good suggestion and we will rewrite the results interpretations in revised paper.

p.5024 l.15ff: What "disturbance" stands for exactly? Freezing? Was it true during the sampling campaign? The sampling period is not indicated in the Material & Methods section.

Reply: Disturbance stands for animal activity. We proceeded sampling on August 10. These information will be added in revised paper.

Furthermore, most of these statements are not supported by Table 1: meadow and shrub soils seem to display the same TN content (both 5 g.kg -1 on average), and one cannot have any idea of moisture and temperature fluctuations from the data presented here.

Reply: In spite of the high fluctuation of the TN values, average TN content was 8.4 g/kg for shrub soils and differed from that for meadow soils (5.1 g/kg). Moisture and temperature fluctuate with altitudinal gradients. In the study region, it has been reported that along the increasing altitudinal gradient from 1800 to 4500m, mean land surface temperature decreases from 33 to 15 degree centigrade, and mean monthly precipitation increases from 36 to 52mm. Both of the parameters were obtained from June to September (Jin et al., 2008, Journal of Arid Land Resources and Environment (in Chinese)). The values of moisture and temperature will be added in Table 1 in revised paper.

p. 5025 l.16: Do the authors really talk about natural selection (related to evolutionary processes, which is very unlikely: again, I don't believe that isolation by distance may be play at this spatial scale) or about habitat filtering, i.e. the fact that the abiotic/biotic conditions may enhance the fitness of certain taxa?

Reply: Thanks for the suggestion. We will rewrite it in revised paper.

4/ Finally, the literature is often cited inappropriately, mostly because the cited refer-

ences do actually already cite other references in their introduction. For instance: p. 5016 l. 26: wrong ref. The one really discussing that point is Lynch, JM, Hobbie, JE (1998) Microorganisms in Action. Blackwell Scientific Publications, Oxford p. 5017 l. 5: idem. Results from this ref do not support this statement. p. 5024 l. 5: Kizilova et al., 2012 is not a review and does not really test for primer pair taxonomic coverage. See rather Gaby and Buckley 2012 PLoS One for an in silico evalutation of nifH primer pairs.

Reply: Thanks for catching my citations! I'll be more careful. These inappropriate citations will be corrected in revised paper.

Minor comments p. 5016 l. 23: "particularly in those without any chemical fertilizer": did the authors mean low nutrient availability?

Reply: That's right. Thanks for your kind reminder! It will be changed in revised paper.

p.5020: As GenBank is constantly evolving, authors should indicate the date at which they made their BLAST analyses (or the Gen- Bank database version)

Reply: The information will be added in revised paper.

p.5021 l.6: why did the authors used the 16S gene here and not the nifH gene instead? Results obtained from those data cannot be crossed with those obtained from the clone library.

Reply: Nitrogen-fixing bacteria were screened by a typical selective medium without nitrogen source (Beauchamp et al., 2006, BIORESOURCE TECHNOL), then we chose 16S rDNA sequence analysis just for identification of these nitrogen-fixing bacterial isolates and there have been evidence confirmed the nitrogen-fixing activity of these species (data not shown).

p. 5021 l.10: redundant with p.5019 l.23

Reply: This kind of redundancy will be omitted in revised paper.

p.5023 l.12: is it copies or number of copies? To be corrected throughout the manuscript

Reply: It is number of copies and will be corrected in the revised paper.

p. 5023 l.20: qPCR instead of Q-PCR.

Reply: This will be done in revised paper.

Table 2: What are the percentages provided in the first row?

Reply: The percentages showed relative abundance of unidentified sequences from each environment (shrub soil or meadow soil). The information will be added in Table 2 in revised paper.

Fig. 6: What are the different colours for?

Reply: The red arrows and words represent environmental factors while the blue arrows indicate community characteristics. The information will be added in legend of Fig.6 in revised paper.

Thanks again for your suggestions and the Reviewer's comments! We hope that the responses answer the Reviewer's comments and we will corrected our paper seriously according to all the Reviewers' helpful suggestions. We will upload the revised paper as soon as possible before the deadline. We look forward to hearing your decision soon.

Yours sincerely, Xi Sheng Tai

Interactive comment on Biogeosciences Discuss., 10, 5015, 2013.