Answers to Anonymous Referee #2

This paper deals with research on phytoremediation that is an important approach in removing of organic pollutants in soil. Although the research area in this MS is a little bit different from common papers published in BG, I do think that BG should enlarge its scope and publish such type of work to reflect the on-going development of soil pollution and its remediation. The paper consists of several experiments to analyze different influencing factors during phytoremediation of petroleum contaminated soil. These are good experiments with high-quality data, and they definitely deserve to be published. Basically, I think that the research is important in the area of geosciences as many parts of geo-system have been contaminated severely by petroleum hydrocarbons. The selected factors have also been proved to be crucial in the management of phytoremediation. However, I think the management of phytoremediation should be further discussed based on this research and some specific points should be made clear.

Answer: We would like to thank the reviewer for his/her constructive comments and suggestions on the manuscript. The modifications have been accomplished by replying to the reviewer's comments. The following is a detailed list of our responses and the changes we have made. The management of phytoremediation is also further discussed in our response to the reviewer′s comments.

In addition, we will have our paper checked by a native English speaker before submission of the revised manuscript.

In Materials and Methods, the experimental design for different influencing factors should be written in detail. For example, for the effect of fertilizer addition, when did the fertilizer was added? What indexes were tested for different experiments except petroleum content? How the EMA for bioaugmentation was prepared? What kind of PGPR bacteria was used in your experiment. How did the PGPR applied?

Answer: we replenish necessary information mentioned by the reviewer as follows: (1) the fertilizer was added at the initial time before planting. We will add the following sentence to detail the management of plant growth and addition of fertilizer in Page6, after line7. "First, a filter paper was put in the bottom of the flower pot to cover the drainage hole and TPH contaminated soil was added. For cotton planting, 20 seeds was added in each pot and arranged evenly and covered with 2-3 cm soil on the top; for tall fescue, ryegrass and alfalfa, 5 g seed was put in the soil in each pot evenly and covered with soil with 0.5-1 cm on the top. Then water was added to maintain the soil moisture to 60-90% of the maximum water holding capacity. No fertilizer was added during the remediation process, while different amount of urea was added in the soil at in experiment 2 the same time when the TPH contaminated soil was put in the flower pot and then the soil was mixed thoroughly"

We added information of analysis index and others in Page5, line17 at the end of

experiment1 as "TPH content was tested after the remediation process."; in Page5, line20 after experiment2 as "Tall fescue was used as remediation plant, TPH content was analyzed and plant biomass was weighed after 150 d of remediation"; in Page5, line23 after experiment3 as "TPH content was detected at the end of remediation."; after Page6, line2, we introduce the method of EMA preparation as "The bacteria were incubated to over 1×10^{10} cfu/g in liquid media and added to the peat at the rate of 1: 4 (w/w) to produce microbial agent." and following that we added PGPR introduction and application method as "PGPR contained mainly *Azospirillum Brasilence*, which was bought from Shanghai Pengxie Co., Ltd. Cotton seed was soaked in PGPR solution before planting. TPH content, dehydrogenase activity and PCR-DGGE analysis were carried out on the bioaugmentation process". At last, after line7 in Page6, the following sentence was added "….during the remediation process. TPH content was detected at different times of the remediation process".

Page8, line3-5, why did you select and compare the 4 different plant species. References should be given in this paragraph to explain the literature report on the selected plants for THP bioremediation.

Answer: It is important to characterize the effect of different plant species as unique rhizosphere environment can be obtained by different plant species. As phytoremediation can be affected by many environmental factors, the experiment condition differed greatly in different literature report, so we it is not a good choice to compare the result from different experiments to determine the ability of different plant on degrading TPH. By the way, as our result was conducted under the same environmental conditions, it is more reliable.

In spite of the limitation of the literature report, literature reports have given useful information on characteristics of different plant species in phytoremediation of petroleum hydrocarbons. It is important to characterize the effect of different plant species as unique rhizosphere environment can be obtained by different plant species. It was reported by Hou et al. (2001) that rooting intensity (mg root kg^{-1} soil) is the key factor leading to higher TPH loss rates and root development is crucial to evaluating the potential for phytoremediation. Ryegrass requires high levels of nitrogen for growth, where as for alfalfa no nitrogen needs to be added because rhizomes fix nitrogen in the soil (David et al., 1997). Planting both species will reduce the fertilizer requirements for each growing season. In another report by Frick et al. (1999), ryegrass, tall fescue and alfalfa were considered to be potential species for phytoremediation of petroleum hydrocarbons and alfalfa is also tolerant to petroleum contamination. From the view point of economy, cotton is a good choice as it earns money besides cleaning the petroleum hydrocarbons in the soil. Bañuelos (2006) thought that producing viable products of economical value may help sustain long-term application of field phytoremediation. In addition cotton is tolerant to salt which make it suitable to be used in saline-alkaline field (Ashraf and Ahmad, 2000).

Based on the above statement, we revised the manuscript and added the following

words in discussion part in Page8, after line6:

"…..saline and alkaline land. It was reported by Hou et al. (2001) that rooting intensity (mg root kg^{-1} soil) is the key factor leading to higher TPH loss rates, and root development is crucial to evaluating the potential for phytoremediation. The well developed root system of grass is its advantage in enhancing petroleum degradation, where as for alfalfa it has its potential because of its nitrogen fixation in the soil (David et al., 1997). Cotton is tolerant to salt which make it suitable to be used in saline-alkaline field (Ashraf and Ahmad, 2000). In addition, cotton is important in phytoremediation as producing viable products of economical value may help sustain long-term application of field phytoremediation (Banuelos, 2006). In general, plant species should be selected based on the soil condition and remediation purpose"

References:

[1] Hou FS, Milke MW, Leung DW, MacPherson DJ. Variations in phytoremediation performance with diesel-contaminated soil. Environ Technol. 2001, 22(2):215-22.

[2] Frick CM, Farrell RE and Germida JJ. Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. PTAC Petroleum Technology Alliance Canada, Calgary.

[3] Ashraf M, Ahmad S. Influence of sodium chloride on ion accumulation, yield components and fibre characteristics in salt-tolerant and salt-sensitive lines of cotton (Gossypium hirsutum L.). Field Crops Research, 2000, 66(2): 115-127.

[4] Bañuelos G.S. Phyto-products may be essential for sustainability and implementation of phytoremediation. Environmental Pollution, 2006, 144(1): 19-23.

[5] David J. Kelner, J. Kevin Vessey, Martin H. Entz The nitrogen dynamics of 1-, 2- and 3-year stands of alfalfa in a cropping system. Agriculture, Ecosystems & Environment, 1997, 64(1): 1-10.

It is important and interesting to test the effect of different TPH concentration on the degradation rate. You conclusion is that 2% content of TPH is suitable for phytoremediation. However, 2% TPH will exert growth inhibition on plant growth as shown by other researchers (Peng et al. 2009). So please explain why 2% can give the best remediation result in spite of the inhibition effect. This should also be further discussed in Discussion part based on literature report.

Answer: The effect of TPH content on the plant growth is different in different plant species. Peng et al. used a kind of flower (*Mirabilis Jalapa* L.) in their phytoremediation experiment. Generally flower is weak and not so tolerant compared to grass such as tall fescue which can grow well in drought and adverse environment. It is possible that 2% TPH content inhibited the growth of *Mirabilis Jalapa* L. It was report by Frick et al. (1999), ryegrass, tall fescue and alfalfa were considered to be potential species for phytoremediation of petroleum hydrocarbons and alfalfa is also tolerant to petroleum contamination. Bioremediation research in a field site suggested

that bioremediation can be carried out successfully at TPH content level as high as 13% by using ryegrass as phytoremediation plant species, this means no inhibition of bacteria growth at least at TPH concentration of 13% (Gurska et al., 2009). By using tall fescue as phytoremediation plant, Huang et al found that tall fescue grew well under TPH concentration of 5%. Based on the above analysis, we think a lower TPH content of 5% will definitely be able to enhance the degradation of TPH and possibly give the best result.

Based on the above discussion, we revised the manuscript and added the following discussion on TPH content in P11, after line24 as "….TPH concentration was the major determinant of total bacterial abundance and had positive effects on abundances of hydrocarbon degraders (Nie et al., 2009). Bioremediation of petroleum hydrocarbon could be carried out successfully under TPH concentration of 10-13% (Del Pann et al., 2005; Gurska et al., 2009), although there is no report on the maximum permissible TPH content for bioremediation. As evaluated by different organisms….."

References:

[1] Frick CM, Farrell RE and Germida JJ. Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. PTAC Petroleum Technology Alliance Canada, Calgary 1999.

[2] Gurska, J., Wang, W. X., Gerhardt, K. E., Khalid, A. M., Isherwood, D. M., Huang, X. D., Glick, B. R., and Greenberg, B. M.: Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste, Environ. Sci. Technol., 2009, 43(12): 4472–4479.

[3] Huang, X. D., El-Alawi, Y., Gurska, J., Glick, B. R., and Greenberg, B. M.: A multi-process phytoremediation system for decontamination of persistent total petroleum hydrocarbons (TPHs) from soils, Microchem. J., 2005, 81:139–147.

[4] Del Panno MT., Morelli IS., Engelen B, Berthe-Corti L. Effect of petrochemical sludge concentrations on microbial communities during soil bioremediation. FEMS Microbiology Ecology, 2005, 53(2, 1):305-316.

In Fig 5, please clarify and mark the bands that existed in the original soil, and what bands are newly developed. From DGGE result, is it possible to track the added microorganisms (EMA and PGPR)? What is the effect of added microorganisms on the native microorganisms? What do you think of your result as compared to other research result?

Answer: In Fig 5a, bands were marked as shown in the fig below. S1-S7 are new bands developed during bioremediation process, and the change of different bands is analyzed as in the revised manuscript after page9, line27 "…..Some new bands developed during remediation process. Common bands C1-C13 can be found in all samples of lane 1-6. Special bands S1-S8 can only be found in certain lines, for example, S1 was found in lane2-6 but not in lane1; S2 was only found in lane1; S3

was only found in lane4, lane5; S5 was only found in lane3. ".

It is not possible to track the change of EMA and PGPR by only DGGE analysis. However, it can be detected by cutting the bands and conducting sequence analysis, then the sequence data of a certain band can be compared with the data in Genebank and microbial species can be identified.

Generally, added microorganism will grow rapidly at initial time of remediation and then compete with native microbial species and died out gradually after a long time period. In some cases, the population of introduced strains may remain stable even after 1 year (Mishra et al., 2001). Our result suggested that phytoremediation and bioaugmentation are all effective in enhancing bioremediation process. However, the combination of the two approaches will further improve the degradation effency. We think the effective microorganism will be remained active under plant growth condition and numerous bacterial straits, involving a multitude of genes, are required for effective root colonization. This may be the main mechanism of phytoremediation in enhancing TPH degradation. The same conclusion was also concluded by other researchers (Gerhardt et al, 2009; Frick et al., 1999). However, it is the first time cotton was used as phytoremediation plant, which is different from other researchers and may induce great economic value.

We revised the manuscript and added the following sentence in Page12, after line21 as "……..alters microbial community structure. In some cases, the population of introduced strains may remain stable even after 1 year (Mishra et al., 2001). However……."

We then added the following sentence to conclude the mechanism of phytoremediation in Page12 after line27 as "nutrient level and amount of inoculation. Combination of microbial degradation and phytoremediation will further improve the degradation effency. The inoculated microorganisms will remain active under plant growth condition and colonize in the root system of the plant. As microbial degradation happened"

References

[1] Mishra S, Jyot J, Kuhad RC., Lal B. Evaluation of Inoculum Addition To Stimulate In Situ Bioremediation of Oily-Sludge-Contaminated Soil. Applied and Environmental Microbiology, 2001, 67(4): 1675–1681

[2] Gerhardt, K. E., Huang, X. D., Glicka, B. R., and Greenberg, B. M.: Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges, Plant Sci., 2009, 176(5): 20–30.

[3] Frick CM, Farrell RE and Germida JJ. Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites. PTAC Petroleum Technology Alliance Canada, Calgary 1999.

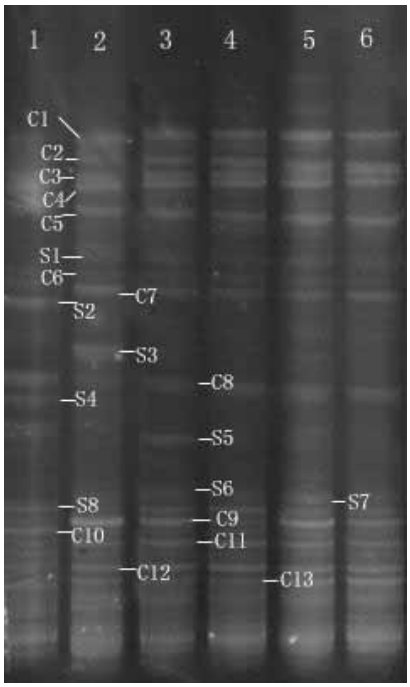

Fig5a

For the effect of remediation time on the result, I agree with you that microbial agent is generally effective at early period and plant may enhance the remediation process after certain period of growth. How can you manage both microbial agent and plant growth most effectively based on your research?

Answer: We think most important measure in enhancing TPH degradation is to regulate the soil condition such as pH, moisture content, nutrient content, which is important for the growth of native microorganisms and plants. Microbial degradation will be conducted successfully if there are enough native microorganisms for TPH degradation. As microbial degradation become stable after about one month, it is better to use plants that grow fast and become strong enough after only one month to take over the effect of inoculated micoorganisms and can maintain the activity for a long time. At this point of view, selection of plant that grow fast and has long period of growth is important in the combination of microbial remediation and phytoremediation.

So we revised the manuscript and added the following sentence in Page13, after line8 as "…..during the course of remediation. Regulation of the soil condition such as pH, moisture content, and nutrient content is the most important measure for the growth of native microorganisms and plants at initial time. It is also recommended to use plants with fast growth rate and can become strong enough after only one month to take over the effect of inoculated micoorganisms and can maintain the TPH degradation activity for a long time. At this point of view, selection of plants that grow fast and have long period of growth time is important in the combination of microbial remediation and phytoremediation. At the end time of 150 d,……"