## Answers to Anonymous Referee #1

### General comments

Rhizoremediation of organic pollutants such as petroleum and PAHs in soil is of great interests and importance for soil remediation. In general, rhizoremediation is a very complex process because it can be affected by both plant growth and microbial community change. Until now, the mechanism of the rhizoremediation is still not clear enough. However, as one of remediation techniques, it is widely applied for soil reclamation such as in Super Fund site in USA. This paper characterized several important influencing factors during rhizoremediation of petroleum hydrocarbons, which is meaningful and might be connected directly with the field remediation process. Due to little information on how different factors influence rhizoremediation process, the main results of this paper is useful for improvement of understanding in this research area and are publishable. However, the study design should be introduced in detail and mechanism of the rhizoremediation should be further discussed.

Answers to the general comments: We appreciate 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 design of the experiment including seed planting, moisture control and fertilizer addition was further introduced and the mechanism of the rhizoremediation was further discussed. Detailed list of our responses and the changes we have made can be found in our answers to the specific comments.

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

### Specific comments

1. Page 5, please give detailed introduction on experimental design and analysis method. For example, P5, line 16-17, please detail the management of plant growth.

Answer: Different plant species need different management method. However, the management of plant species included many experiential methods, and could not be written in an easy way. So we just added information about seed planting, moisture control and fertilizer addition. We will add the following sentence to detail the management of plant growth 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 in experiment 2 at the same time when the TPH contaminated soil was put in the flower pot and then the soil was mixed

thoroughly”

2. The effect of different plant species is determined by not only the plant species, but also the growth condition of the plant. The results of different plant species should be compared to literature research to determine the ability of different plant on degrading TPH.

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 think 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. Banuelos (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.

3. Many fertilizers can be used during bioremediation process. Why the author choose urea to check the effect of fertilizer on the remediation process. How about soil P content on the plant growth and rhizoremediation process?

Answer：Urea has a higher N content and is generally used as a fertilizer. Soil P content is also important during phytoremediation process. However, our analysis result showed that P content in the test soil is high enough to sustain growth of ryegrass (Data not shown). Degradation of petroleum hydrocarbon can be achieved successfully with the N:P ratio as high as 38:1(Chavan and Mukherji, 2008). In addition, N is the most crucial factors to phytoremediation process. Excess nitrogen can depress the rate of microbial activity and petroleum degradation in contaminated soils due to osmotic soil water potential depression (Walworth et al., 2004). As proper N is important for the bioremediation process, we check solely the effect of urea addition on the remediation process. The effect of P on the phytoremediation will be analyzed in our future study.

References
[1] Walworth J, Pond A, Snape I, Rayner J, Ferguson S, Harvey P. Nitrogen requirements for maximizing petroleum bioremediation in a sub-Antarctic soil. Cold Regions Science and Technology, 2007, 48(2): 84-91.
[2] Chavan A, Mukherji S. Treatment of hydrocarbon-rich wastewater using oil degrading bacteria and phototrophic microorganisms in rotating biological contactor: Effect of N:P ratio. Journal of Hazardous Materials, 2008, 154 (1-3): 63-72.

4. P11, line22-24. “TPH concentration was the major determinant of total bacterial abundance and had positive effects on abundances of hydrocarbon degraders (Nie et

al., 2009).” Is this sentence means that higher TPH content resulted in higher bacteria and thus higher degradation rate? Please clarify it. In my opinion, at low concentration of THP, the addition of petroleum can stimulate the microbial growth. However, at high TPH concentration the microbial number will decrease with higher THP concentration as the toxicity of TPH on bacteria.

Answer: This sentence means that addition of petroleum will stimulated the growth of hydrocarbon degrading bacteria. This has been proved by many researchers. Investigation of the factors associated with bacterial predominance revealed that, dominant culturable crude oil-degrading bacteria were better crude oil utilizers than the less frequently occurring isolates (AL-Saleh et al., 2009). Changes in the community structure of soils depended on the amount of added sludge containing TPH (Del Pann et al., 2005). However, there is no report on the maximum TPH concentration that can exert apparent toxicity on the growth of bacteria in soil environment. Bioremediation research in a field site suggested that bioremediation can be carried out successfully at TPH content level as high as 13%, this means no inhibition of bacteria growth at least at TPH concentration of 13% (Gurska et al., 2009).

We clarify the meaning of the sentence 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] AL-Saleh E, Drobiova H, Obuekwe C. Predominant culturable crude oil-degrading bacteria in the coast of Kuwait. International Biodeterioration & Biodegradation, 2009, 63(4): 400-406.

[2] 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.

[3] 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.

5. Why the authors choose cotton to study the effect of EMA and PGPR on the rhizoremediation but not ryegrass or tall fescue? In DGGE analysis result, the bands should be marked in Fig 5a and change of different bands during rhizoremediation process should be analyzed.

Answer: As combined effect of EMA with ryegrass, tall fescue or alfalfa has been studied previously (Gurska et al., 2009; Kirk et al., 2005; Huang et al., 2005), we use cotton to check the combined effect, which has not been reported until now. We also added the following sentence after Page5, line25 "…..TPH concentration of 5%. Cotton is used in this study to check the combined effect of plant and EMA as it has not been reported until now."

In Fig 5a, bands were marked as shown in the fig below. 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. ".

References

[1] 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.

[2] Kirk, J. L., Klironomos, J. N., Lee, H., and Trevors, J. T.: The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil, Environ. Pollut., 2005, 133,: 455–465.

[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.

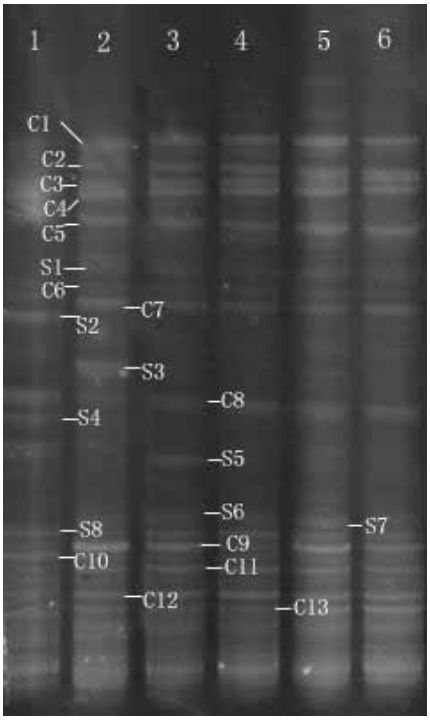


Fig5a

6. In Fig 6, it seemed that the effect of EMA and phytoremediation on TPH degradation is not so clear at initial period before 30d, please explain the reason.
Answer: At initial period, the degradation rate of TPH by EMA and phytoremediation remediation is only slight higher than that of control. However, the degradation of 20% after 15 days is very high, which suggests that native microorganism probably proliferated at the initially period of remediation. This also indicates that growth of inoculated microorganisms and plants needs a period of time. Especially, the biomass of plant is very small at 15 days time period. So the effect of planting on the TPH degradation is not so apparent. We added the following sentence in Page13, line3 as "….time of the bioremediation process. Native microorganism may also develop at initial period of bioremediation as shown from the high degradation of TPH in control of the experiment 5."

Technical corrections
1. Table 1, there is 2 "Total N", one should be changed to "Total P"
Answer: The second "N" should be changed to "P".
2. P5, line19, 50 g/m2 should be changed to 30 g/m2?
Answer: We changed 50 g/$m^2$ to 30 g/$m^2$ as the reviewer suggested.
3. P12, line3, delete "that"
Answer: We deleted "that" as the reviewer suggested.
4. Table 1, please give a foot note to "-"
Answer: We added a foot note as "- indicates under detection limit of 0.1 mg/kg"