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Effect of *Bacillus subtilis* and NTA-APG on pyrene dissipation in phytoremediation of nickel co-contaminated wetlands by *Scirpus triqueter*

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ABSTRACT

A complex mix of organic pollutants and heavy metal made the remediation of contaminated wetlands more difficult. Few research focus on the remediation for pyrene enhanced by chemical reagents and pyrene degrading bacteria in the nickel co-contaminated soil. In this paper, the effect of chemical reagents (nitrilotriacetic acid and alkyl polyglucoside) and *Bacillus subtilis* on pyrene dissipation in phytoremediation of nickel co-contaminated soil by *Scirpus triqueter* was investigated. Similar seedlings of *Scirpus triqueter* were moved to uncontaminated soil and pyrene-nickel co-contaminated soil. The pots (14.8 cm diameter and 8.8 cm height) were set up in greenhouse and treated in different ways. After 60 days, plant biomass, radial oxygen loss (ROL), soil dehydrogenase activity (DHA) and pyrene concentration in soil were determined. Results showed that ROL rate and DHA in different groups was positively correlated with pyrene dissipation from soil. In the process of remediation, chemical reagents might have an indirect slight effect on pyrene dissipation (pyrene dissipation increased 21%) by affecting DHA firstly and redistributing pyrene fractions in the presence of pyrene degrading bacteria. Pyrene degrading bacteria were likely to affect pyrene dissipation by impacting ROL rate and DHA and played a more vital role in contributing to pyrene dissipation (pyrene dissipation increased 45%) from wetland. This study demonstrated that phytoremediation for pyrene in nickel co-contaminated soil by *Scirpus triqueter* can be enhanced by the application of NTA-APG and pyrene degrading bacteria and they could be reasonably restore the ecological environment of PAH-contaminated wetlands.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) and heavy metals (HMs) contaminations are ubiquitous in soil and sediment with the rapid industrialization in many countries (Irha et al., 2003; Kołtowski et al., 2017; Sun et al., 2011). Remediation of soils co-contaminated with PAHs and HMs was extremely difficult, which has drawn much more attention (Chen et al., 2015; Wang et al., 2012). Pyrene, as a representative PAH, threats to human health. Among heavy metals, nickel is an element along with petroleum, which have caused widespread concern due to its coexistence with many PAHs (Amezcua-Allieri et al., 2005; Mielke et al., 2001). In our previous study, it was found that pyrene dissipation from soil would decrease in the nickel (Ni) co-contaminated soil compared with pyrene single contamination. In this paper, the dissipation for pyrene in phytoremediation of nickel co-contaminated soil with different treatments was investigated in pot culture experiments. Considering limited capability of plants to remove organic pollutants, inoculation of specific strains with degradation capacity was suggested as a strategy for the treatment of PAHs contaminated soil (Chaudhry et al., 2005; Tian et al., 2017). Some studies have demonstrated that degrading bacteria associated plants could significantly improve the bioremediation efficiency due to their cooperative relationship (Liu et al., 2016). For example, Gao et al. (2006) have reported that co-cultivation of PAH-degrading bacteria (*Acinetobacter* sp.) and rice greatly enhanced pyrene dissipation from waterlogged soil. Sheng and Gong (2006) found that *Pseudomonas* sp. GF3 (phenanthrene degrading bacteria) could significantly increase phenanthrene removal from the soil in the presence of wheat. As an exogenously seeded bacterium, *Bacillus subtilis* had been used for the degradation of pyrene and other PAHs, showing great potential for the application of bioremediation (Das and Mukherjee, 2007; Hunter et al., 2005). However, the remediation efficiency is also strongly linked to the availability of contaminants (Luo et al., 2018; Wang et al., 2015). PAHs due to their poor availability in soil may limit the application of bacteria assisted phytoremediation, which needed to be assisted by chemical reagents.

Alkyl polyglucoside (APG), a safe and environmentally-friendly nonionic surfactant, was reported to enhance pyrene degradation by

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increasing the bioavailability of PAHs and becoming a carbon source for microorganisms (Madsen and Kristensen, 1997). Nitrilotriacetic acid (NTA), a biodegradable chelating agent, has been proven to increase pyrene removal from soil in the presence of APG due to their synergistic effect for pyrene dissipation (Chen et al., 2016b). As we all know, the availabilities of PAHs are of importance for the plants in phytoremediation of contaminated wetland. Then, improving the accessibility of pyrene is crucial to the removal of pollutants. Nevertheless, few research focus on the effect of pyrene degrading bacteria and chemical reagent on the chemical speciation of pyrene in co-contaminated soil.

Scirpus triqueter (*S. triqueter*), with an extensive fibrous root system, was a dominant wetland species along Huangpu River. The large specific surface area of *S. triqueter* root helped to promote the efficiency of phytoextraction (Liu et al., 2013b; Zhang et al., 2011). In the roots of wetland plants, oxygen is required for their own growth, and significant amounts of oxygen supplied through their root aerenchyma may be released into the soil, which is defined as radial oxygen loss (ROL) (Armstrong, 1980; Lai et al., 2011). ROL and rhizosphere oxidation is an essential process enabling plants to detoxify phytotoxins and to tolerate an anoxic environment (Yang et al., 2014). It was reported that the flood tolerance of plants, salinity and arsenic exposure were positively correlated to ROL (Chabbi et al., 2000; Li et al., 2011; Rogers et al., 2008) Moreover, PAHs are found to be susceptible to aerobic biodegradation (He and Chi, 2016). However, the effects of ROL on the dissipation of pyrene by *S. triqueter* are uncertain, especially with the degrading bacteria inoculation or/and chemical reagents application.

Soil enzymes, which can be affected by vegetation directly or indirectly, play an important role in soil biochemical process (Dick, 1994). Among soil enzymes, the dehydrogenase activity (DHA) is the catalyst for some metabolic process, such as the degradation of PAHs (Maliszewska-Kordybach and Smreczak, 2003; Zhang et al., 2014). Therefore, this study explores the effects of degrading bacteria and chemical reagents on soil enzymes by using the dehydrogenase activity (DHA) as a model.

In our previous study, the remediation efficiency of pollutants has been investigated under the combined application of chemical reagents and degrading bacteria in co-contaminated soil (Hu et al., 2017). However, there is no report showed that how pyrene degrading bacteria and chemical reagents affect pyrene dissipation during phytoremediation of co-contaminated soil and the question of the role of pyrene degrading bacteria and chemical reagents in pyrene removal from soil was thus raised.

The objectives of this study were to: (1) investigate the effect of pyrene degrading bacteria and chemical reagent on the chemical speciation of pyrene, soil dehydrogenase activities, ROL of *S. triqueter* in co-contaminated soil; (2) explore preliminarily how pyrene degrading bacteria and chemical reagents affect pyrene dissipation during phytoremediation of contaminated soil.

2. Materials and methods

2.1. Chemicals

APG1214 with a purity of 50% was bought from the China Research Institute of Daily Chemical Industry. All other reagents, analytical grade at least, which were purchased from Sinopharm.

2.2. Soil preparation

The control soil (air-dried, 2 mm sieved) gained from the topsoil (0–20 cm) without exposing previously to pyrene and Ni contamination in Shanghai University (121°42′ E, 31°18′ N) was amended with the stock solution of pyrene-acetone and the $NiCl_2$ solution, thoroughly mixed and aged for about two months after all the solvent volatilized. The final concentrations of pyrene and Ni in the soil were measured as 357 and 296 mg kg^{-1}, respectively. The concentrations were chosen according to the screening value of Ni and pyrene for soil risk assessment (Beijing Bureau of Quality and Supervision, 2011). In order to explore how pyrene degrading bacteria and chemical reagents affect pyrene dissipation under real soil conditions, the soil was not sterilized. The physicochemical characteristics of the soil were as follows: pH 8.3, organic matter 19.6 g kg^{-1}, total nitrogen 0.5 g kg^{-1}, clay 7.4%, silt 60.4%, and sand 32.2%.

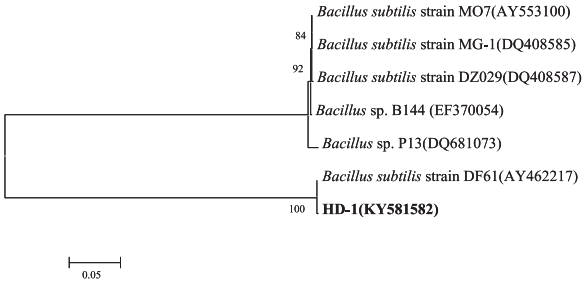

Fig. 1. Phylogenetic trees of pyrene degrading bacterium HD-1. The pattern was generated by neighbour-joining method. The Genbank accession numbers are indicated in brackets.

2.3. Preparation of bacterial suspension

A pyrene degrading bacterium named HD-1 was isolated from sludge collected in a wastewater treatment plant (121°8′ E, 31°10′ N) by using the method of restrictive substrate screening. Approximately 1500 bp of the 16S rRNA gene was amplified using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CTACGGCTACCTTGTTACGA-3′). The 16S rRNA gene sequence has been deposited in the GenBank database under the accession number KY581582. The phylogenetic tree was constructed by MEGA 6.0 and presented in Fig. 1. The 16S rRNA sequence showed high levels of sequence similarity to the species *Bacillus subtilis* (99%). Phylogenetic analysis based on 16S rRNA sequence showed that the isolate is closely related to *Bacillus subtilis* strain of DF61 with the accession number AY462217.

Bacillus subtili HD-1 was found to be resistant to nickel with a minimal inhibitory concentration of 250 μg mL^{-1} and there was a decrease in bacterial growth when the concentration of pyrene reaches 500 μg mL^{-1}. Compared with the stains isolated from rhizosphere of plants growing in the PAH-contaminated soil, *Bacillus subtili* HD-1 showed greater ability to tolerate nickel and degrade pyrene. In our previous study, results showed that the bacterial growth is greater in the medium containing pyrene using NTA-APG when the concentration below 150 mg L^{-1}. HD-1 was incubated in Luria-Bertani (LB) liquid medium at 30 °C in a rotary shaker at 150 rpm. Cells were collected by centrifugation, washed three times with sterile water and resuspended in sterile water. The cell density (the optical density at 600 nm) was adjusted to 1.0. Finally, the inoculated bacterial density in soil was 7×10^7 CFU/g.

2.4. Experimental design

The experimental design is as follows: CK (Uncontaminated soil with *S. triqueter* cultivation), P (Contaminated soil with *S. triqueter* cultivation), NP (Contaminated soil without *S. triqueter* cultivation), PB (Contaminated soil inoculated of HD-1 with *S. triqueter* cultivation), NPB (Contaminated soil inoculated of HD-1 without *S. triqueter* cultivation), PC (Contaminated soil added chemical reagents (NTA and APG) with *S. triqueter* cultivation), NPC (Contaminated soil added chemical reagents (NTA and APG) without *S. triqueter* cultivation), PBC (Contaminated soil inoculated of HD-1 and added chemical reagents (NTA and APG) of *S. triqueter* cultivation), NPBC (Contaminated soil inoculated of HD-1 and added chemical reagents (NTA and APG) without *S. triqueter* cultivation). Bacterial suspensions (10% v/w) were