Effect of Rhizodegradation in Diesel-contaminated Soil under Different Soil Conditions

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Abstract: In order to develop a cultivation technique for the practical use of phytoremediation of diesel-contaminated soil, we evaluated the rhizodegradation of diesel-contaminated soil using Italian ryegrass. Experiments were conducted under two different soil conditions that were expected to reduce the influence of diesel on the plant. Under the first condition, the initial diesel concentration which is expressed in the total petroleum hydrocarbon (TPH) concentration was set to 0.80%. The concentration was almost half the upper limit for the growth of Italian ryegrass. Under the second condition, zeolite was added to the experimental soil to improve the cation exchange capacity (CEC). In 152 days experiments, we evaluated the plant growth variables, TPH concentration, soil dehydrogenase activity (DHA) that is reflective of the rhizosphere microbial activity, and the aerobic bacterial count. The results suggest that the TPH concentration in first condition (0.80%) could not bring about a significant recovery of plant growth. The plant growth observed in first condition was equal to that observed in the case of the upper limit TPH concentration used in our previous study. However, under the second condition, it is suggested that the addition of zeolite could increase plant growth, which can in turn improve the rhizodegradation effect.

Key words: Diesel-contaminated soil, Phytoremediation, Rhizodegradation, Root, Soil DHA.

Soil contamination is an important environmental problem. Remediation technologies are required to resolve the problem of soil contaminated with petroleum hydrocarbons, which are compounds used by a variety of industries. Currently, incineration and soil washing are the primary methods of choice; however, these techniques are unsatisfactory. Incineration is expensive and requires a large amount of energy. Soil washing eliminates neither the oil film nor odor, which are significant problems in the environment even at low concentrations of less than 1% (Noda et al., 2003).

Recently, phytoremediation has been identified as a potential technique for the treatment of contaminated soil. Phytoremediation is environmentally friendly and cost effective, and it produces a visually attractive outcome (Khan et al., 2000). Phytoremediation comprises five processes, namely, phytoextraction, phytovolatilization, phytodegradation, rhizodegradation, and phytostabilization (Cunningham et al., 1996).

This study focuses on the effect of rhizodegradation in diesel-contaminated soil. Rhizodegradation depends on the action of plant roots, the associated microflora, and/or their excretion products to destroy the contaminant in the root zone. A considerable amount of research has been conducted on the effect of the rhizosphere on the remediation of organic contaminants such as polyaromatic hydrocarbons (PAHs) (April and Sims, 1990; Siciliano et al., 2003), polychlorinated biphenyls (PCBs) (Donnelly et al., 1994), and hydrocarbons ( Günther et al., 1996; Banks et al., 2003; Kirk et al., 2005). However, thus far, rhizodegradation has not been sufficiently developed for widespread application. To facilitate the practical use of rhizodegradation, it is necessary to develop a cultivation technique that guarantees rhizodegradation of contaminants in soil. In a previous study, we reported that fine root growth guarantees the improved rhizodegradation activity of Italian ryegrass at the upper limit of diesel for plant growth (Kaimi et al., 2006). The microbial community in the rhizosphere is greatly influenced by roots and root exudates that vary depending on the conditions of plant growth, such as the nutrient status (Rovira, 1959; Bowen, 1969). With regard to hydrocarbon-contaminated soil, it is known that plant growth is inhibited by the toxicity of low molecular weight compounds, and that the absorption of water and nutrients by plants is limited by the hydrophobic properties of the soil (Kirk et al., 2005). To obtain the basic findings required for developing a suitable cultivation technique, we focused on the soil conditions. In the first experiment, the initial concentration of diesel which is expressed in the total petroleum hydrocarbon (TPH) in the soil was adjusted to 0.80%. It was almost half the upper limit for the growth of Italian ryegrass. We then compared the

Received 5 July 2005. Accepted 26 May 2006. Corresponding author: E. Kaimi (e.kaimi@chugai-tec.co.jp, fax+81-892-295-2240).
plant growth in the diesel-contaminated soil with that in uncontaminated soil. In the second experiment, instead of perlite, which was added to the soil in the first experiment as well as in our previous study (Kaimi et al., 2006), zeolite was added to the experimental soil to prevent the inhibition of nutrient absorption due to the hydrophobic properties of the diesel-contaminated soil.

Materials and Methods

1. Effect of the rhizosphere on low concentration of diesel in soil Experimental design

Three groups of 0.3-L plastic pots filled with the experimental soil were prepared. In one group, Italian ryegrass (Lolium multiflorum L.) was planted in diesel-contaminated soil. In the second group, which was the unplanted control, the pots were filled with the same soil, but Italian ryegrass was not planted to estimate the biotic loss of diesel in the absence of plants. In the third group, the pots were filled with the soil not contaminated with diesel and Italian ryegrass was planted (uncontaminated soil).

Twenty-five pots were used in the first and second groups, and 20 pots in the third group (a total of 70 pots), and samples were collected from 5 pots in groups 1 and 2, and 4 pots in group 3 at 1-mo intervals; the experiment spanned 152 d.

(1) Preparation of the experimental soil

The experimental soil was masado, which is a decomposed granite soil to which diesel, leaf mold, and perlite were added. To ensure the absorption of diesel, prior to its addition, the masado was sun-dried in a greenhouse and mixed every 3 d until the water content reached less than 1% before addition of diesel. The designated initial TPH concentration was 0.8%. However, to compensate for evaporation, absorption, etc., which may occur during the sample preparation process, the initial TPH concentration was adjusted to 1.5%. In our previous study, the initial concentration of TPH was adjusted to 1.42%, and 3.0% diesel was added (Kaimi et al., 2006). In order to obtain a homogeneous soil/contaminant mixture, the diesel was gradually sprayed into the masado in a mechanical mixer. After the designated amount of diesel was added, the mixing was continued for an additional 5 min.

In order to prevent loss by volatilization during the experiments, the masado-diesel mixture was further stabilized for 3 wk by sun drying with mixing at 4- to 5-d intervals. Commercial leaf mold and perlite were subsequently added to the soil at a concentration of 20% by volume to improve the physical characteristics of the soil. As a result of the preparation, the initial TPH concentration was 7.977 ± 146 mg kg⁻¹ (n = 5). The characteristics of the experimental soil obtained by the above procedure are summarized in Table 1.

(2) Plant material and growth conditions

Italian ryegrass seeds, a composite variety, were obtained from Yukijirushi Shubyu Co. (Sapporo, Japan). Sixteen seeds were sown in the soil in each pot. At the first-leaf developmental stage, the seedlings were thinned out, leaving 10 plants in each pot. The plants were grown in a growth chamber at 25°C and 70% RH with a photosynthetic photon flux density (PPFD) of 150 µmol m⁻² s⁻¹ (10 h d⁻¹). On alternate days, the pots were randomly rearranged in the growth chamber. At 4- or 5-d intervals, all pots were irrigated with 50 mL of water, which is 80% of the water-holding capacity, to prevent runout from the bottom of the pots.

In each pot, we added 0.14 g of a commercial inorganic fertilizer (N : P₂O₅ : K₂O = 6% : 40% : 6%). After the plants reached a height of 5 cm, a commercial liquid fertilizer (N : P₂O₅ : K₂O = 5% : 10% : 5%) that was diluted in water to 1/250 strength was supplied to the plants at 4- or 5-d intervals. All pots received identical irrigation and fertilization treatments.

(3) Procedure for sample collection from the planted soil and analysis

After measurement of the plant height, the aboveground parts of the plants were cut off and dried at 60°C for 72 h for measuring the dry weight. The soil was placed in a vat, and the roots were carefully collected by gently crushing and shaking the soil samples. The roots were washed with water, and their total length was measured. The soil was removed from the roots, collected and analyzed for TPH, dehydrogenase activity (DHA), and the aerobic bacterial count. The unplanted soils were uniformly mixed in each pot and used for analysis.

Table 1. Agronomic analysis of experimental soil.

| Analysis       | Value     | Method                        |
|----------------|-----------|-------------------------------|
| pH             | 7.2       | 1:2.5 soil/water slurry       |
| TOC (%)        | 0.9       | Flash combustion at 1000°C followed by thermic conductivity detection |
| Texture (%)    | Sandy     |                               |
| Sand           | 86.2      | Light dispersion              |
| Silt           | 9.7       | Light dispersion              |
| Clay           | 4.1       | Light dispersion              |
| T-N (mg kg⁻¹)  | 230       | Kjeldahl method               |
| T-P₂O₅ (mg kg⁻¹)| 420      | Peroxide digest               |
| Available P₂O₅ (mg kg⁻¹)| 170      | Truong method                |
| Water content (%)| 3.6      | Loss on ignition at 105°C     |
| CEC (meq100 g⁻¹)| 5.7      | NH₄⁺ saturation               |
The TPH concentration of the soil was measured according to the method published by the Ministry of the Environment of Japan (Japanese Industrial Standard Committee, 1993). The soil was first dried at room temperature, petroleum hydrocarbons were then extracted from one gram of soil by using an ultrasonic extraction method with CCl₄ as the solvent. The extract was filtered through a Florisil column and then quantified by the Fourier transform infrared (FT-IR) method. The dry weight of the soil was determined after drying the soil at 105°C; the TPH concentration in soil was shown on a dry weight basis.

(4) TPH concentration

The TPH concentration of the soil was measured according to the method published by the Ministry of the Environment of Japan (Japanese Industrial Standard Committee, 1993). The soil was first dried at room temperature, petroleum hydrocarbons were then extracted from one gram of soil by using an ultrasonic extraction method with CCl₄ as the solvent. The extract was filtered through a Florisil column and then quantified by the Fourier transform infrared (FT-IR) method. The dry weight of the soil was determined after drying the soil at 105°C; the TPH concentration in soil was shown on a dry weight basis.

(5) Soil DHA

Soil DHA was determined according to the method
described by Hayano (1997). One gram of soil was treated with 0.2 mL of 0.4% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) in 1.0 mL of 0.25 M Tris buffer containing 50 µL of 1% glucose for 6 h at 30 °C in a dark environment. The enzymically formed iodonitrotetrazolium formazan (INTF) was extracted by shaking the suspension in 10 mL of methanol vigorously for 1 min followed by filtration. The INTF was measured spectrophotometrically at 485 nm.

(6) Aerobic bacterial count
In order to enumerate the aerobic bacteria, aqueous extracts of one gram soil samples were serially diluted and plated on nutrient agar obtained from Difco Co. (catalog no. 213000). The plates were incubated for 3 d at 30°C, and the colony-forming units (cfu) were counted (Kato, 1997).
(7) Root length
The roots were removed from the soil, washed carefully, and then stained by soaking in 0.1% methylene blue for 3 min. They were then rinsed in water to remove any excess dyeing solution. The roots were spread, without any overlapping, on a transparent acryl case filled with water, and they were scanned using Epson TWAIN Pro software. The resulting image was analyzed using specialized software (Kimura and Yamasaki, 2001) to measure the root length.

(8) Statistical analysis
The data are presented as mean values and their standard deviations for each treatment. Prior to statistical analysis, the data were tested for homogeneity of variance by using Bartlett’s test. The means were compared using a one-way ANOVA followed by Bonferroni’s multiple comparisons at the 5% level of probability.

2. The effect of zeolite added to the soil
(1) Experimental design
Two groups of 0.34-L plastic pots, 16 pots each, were filled with the soil with or without 0.8% diesel added. In one group, Italian ryegrass (L. multiflorum L.) was planted in the soil to which 10% perlite or 10% zeolite had been added by volume. The second group was the control in which Italian ryegrass was planted in the soil to which 20% perlite had been added. Samples were collected from four pots at 1-mo intervals. The experiment spanned 125 d.

(2) Preparation of the experimental soil
The experimental soil was prepared in the same manner as in Experiment 1, except that zeolite or perlite was added to the soil. The initial concentration of TPH was adjusted to 1.42%, as in our previous study (Kaimi et al., 2006). To compensate for the loss of diesel by volatilization during the soil preparation process, we adjusted the concentration of diesel added to the masado soil to 3.0%. The initial TPH concentration in the soil with 10% zeolite added was 10,207 ± 834 mg kg⁻¹ (n = 5), and that in the soil with 20% perlite added was 9,533 ± 206 mg kg⁻¹ (n = 5).

(3) Plant material, growth conditions, methods of sampling and statistical analysis
The procedures were the same as those in the first experiment.

Results
1. Effect of diesel a low initial concentration on plant growth
Fig. 1 shows the growth parameters of the plants grown on the soil contaminated with diesel and uncontaminated soil. The plants grown on the soil contaminated with 0.8% diesel were significantly shorter than the plants grown on the uncontaminated soil until 65 days after sowing (DAS). At 91 and 121 DAS, the plant height on the contaminated soil approached that on the uncontaminated soil. However, at 152 DAS, the plant height on the contaminated soil was significantly shorter than that on the uncontaminated soil. The amount of aboveground biomass in the contaminated soil was always lower than that in the uncontaminated soil. Until 63 DAS, the root length in the contaminated soil was shorter than that in the uncontaminated soil. At 91 DAS, the root length in the contaminated soil approached that in the uncontaminated soil. At 121 DAS, the root length in the contaminated soil exceeded that in the uncontaminated soil.

2. Biodegradation of diesel at a low concentration
Fig. 2 shows the concentration of TPH remaining in soil, soil DHA, and aerobic bacterial count of the soil. At 63 DAS, the concentration of TPH in the planted soil decreased to a level lower than that in the unplanted soil. The DHA values of the uncontaminated soils, both planted and unplanted, were significantly higher than that in the planted uncontaminated soil. At 91 DAS, the DHA of the planted contaminated soil was higher than that in the unplanted contaminated soil. At 30 DAS, the aerobic bacterial count in both the planted and unplanted contaminated soils was significantly higher than that in the uncontaminated soil. At 63 DAS, the aerobic bacterial count of the planted contaminated soil was significantly higher than that of the unplanted contaminated soil.

3. Effect of zeolite added to the soil
Fig. 3 shows the plant growth parameters of the plants grown on zeolite-added soil and perlite-added soil. The plant height on the zeolite-added soil and the perlite-added soil was not significantly different during the experiment. At 61 DAS, the aboveground biomass was greater and the root length was longer in the zeolite-added soil than in the perlite-added soil.

Fig. 4 shows the concentration of TPH remaining in the soil, soil DHA, and aerobic bacterial count of the soil. At 98 DAS, the concentration of TPH remaining in the zeolite-added soil decreased significantly to a level lower than that in the perlite-added soil. However, the differences were not significant at 125 DAS. At 28 DAS, the DHA of the zeolite-added soil was significantly higher than that in the perlite-added soil; however, at 61 DAS, the DHA of the perlite-added soil was significantly higher. From 28 DAS to 98 DAS, the aerobic bacterial counts were higher in the zeolite-added soil than in the perlite-added soil; however, difference was not significant at 125 DAS.
Discussion

1. Effect of diesel on plants

The root/shoot ratio was higher and the aboveground biomass was smaller in the plants grown on the soil contaminated with 0.8% diesel, indicating that the nutrient absorption by the plants was inhibited by diesel. The root/shoot ratio is known to increase under insufficient nutrient conditions. This is because plants allocate photosynthates to the roots rather than to the vegetative parts to obtain nutrients (Yamauchi et al., 1987). Hydrocarbons are known to inhibit plant growth. The primary inhibiting factors are considered to be the toxicity of low molecular weight compounds and the hydrophobic properties that limit the ability of plants to absorb water and nutrients (Kirk et al., 2005). Thus, it is believed that plant growth is also inhibited by diesel-contaminated soil. In addition, the plant growth parameters were almost identical to those observed in our previous study with the soil contaminated with 1.42% diesel (Kaimi et al., 2006). The dilution of diesel to half (0.8%) did not significantly improve plant growth.

Zeolite added to the soil improved the plant growth more than perlite. The cation exchange capacity (CEC) of the perlite-added soil was 5.7 meq 100 g⁻¹, but that of the zeolite-added soil was 19 meq 100 g⁻¹. It is expected that the improved CEC mitigated the damage by insufficient nutrients. The absorption of the low molecular weight component by zeolite might contribute to plant growth.

2. Enhancement of biodegradation by plants

The TPH concentration in the planted soil decreased significantly accompanied with the increase in the soil DHA (Fig. 2). Similar results were obtained in our previous study (Kaimi et al., 2006). In the previous study, we revealed that the TPH concentration decreased due to the enhanced microbial activity in the rhizosphere. In this study, we analyzed the DHA of the uncontaminated soil, which was not analyzed in our previous study (Kaimi et al., 2006). The DHA of the contaminated soil was higher than that of the uncontaminated soil. This shows that soil DHA is a suitable index of the rhizodegradation activity in diesel-contaminated soil.

3. Biodegradation of TPH under different soil conditions

At 152 DAS, the initial TPH concentration of 7,977 mg kg⁻¹ in the planted soil decreased to 1,180 mg kg⁻¹, and the concentration of the remaining TPH was 14.8% (Fig. 2). In our previous study, the initial TPH concentration of 14,157 mg kg⁻¹ decreased to 1,813 mg kg⁻¹, and the concentration of the remaining TPH was 12.8% (Kaimi et al., 2006). The rate of removal of TPH was almost equal in both experiments. The soil DHA and the aerobic bacterial counts observed in this study were also almost equal to those observed in the previous study. It is considered that plant growth is necessary for rhizodegradation of diesel. The final TPH concentration observed in this study was lower than that observed in the previous study, but was proportionate to the initial concentration of TPH.

The concentration of the remaining TPH at 98 DAS was significantly lower in the zeolite-added soil than in the perlite-added soil. The aerobic bacterial count at 61 and 98 DAS was higher in the zeolite-added soil than in the perlite-added soil. It is considered that the addition of zeolite improved plant growth, which was effective in increasing the microbial count. However, at 61 DAS, the DHA of the zeolite-added soil was significantly lower than that in the perlite-added soil. Hence, it is considered that the low molecular weight component of diesel, which is much smaller than bacteria, was absorbed in the internal structure grid of zeolite (Goto, 1990). Thus, the bacteria could not access the absorbed low molecular weight component. This matter should be clarified in further study.

In an actual phytoremediation project, if the TPH concentration exceeds the upper limit for plant growth, the contaminated soil should be diluted with uncontaminated soil before the remediation. It is suggested that the rhizodegradation activity is almost equal in the soil with TPH at the upper limit for plant growth and that with half the upper limit. However, the final TPH concentration may be decreased in proportion to the degree of dilution. In addition, it is suggested that the addition of zeolite promotes the plant growth on diesel-contaminated soil, and improves the rhizodegradation effect.

4. Evaluation of the rhizosphere effect

The TPH concentration in the soil with the cultivated plant decreases through the biodegradative and non-biodegradative processes. In general, the non-biodegradative processes include leaching due to irrigation, evaporation, direct uptake by the plant, and absorption by soil or organic matter. In this study, leaching did not occur since the pots were maintained at a constant moisture. Evaporation was found to be 13% by measuring the decrease in the TPH concentration in the control soil (Kaimi et al., 2006). The loss of TPH by direct plant uptake was assumed to be negligible. Other studies have shown that mixture of polyaromatic hydrocarbons similar to diesel are not directly taken up by plants (Schwab and Banks, 1994; Reilley et al., 1996; Wetzel et al., 1997).

Petroleum contaminants may be strongly absorbed into organic matter, with a very low rate of desorption; this causes the formation of nonextractable bound residues (Kästner et al., 1995). The structure of this humic substance, which has the chemical structure of bound residues, is not completely clear; however,
it generally reduces the bioavailability, mobility, and biocidal activity of petroleum in soil (Kloskowski and Führ, 1987; Printz et al., 1995; Burauel and Führ, 2000). The formation of bound residues is promoted by adding the organic substrates that are produced by the growth and death of roots. We postulate that the formation of the bound residues contributed to the cleanup of soil contaminants. Thus, the effects of the rhizosphere may be evaluated by measuring the concentration of the TPH extractable with an organic solvent.

**Acknowledgments**

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