Environmental Toxicology

EARTHWORM TOXICITY DURING CHEMICAL OXIDATION OF DIESEL-CONTAMINATED SAND

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Abstract—An ecotoxicity test with Eisenia fetida was performed to monitor the removal of diesel and toxicity variation during the ozonation process. The three-dimensional (3-D) cell test was introduced for the monitoring of the ozonation process, and the removal rate based on total petroleum hydrocarbons (TPHs) mass was about 95% near the ozone inlet ports. This high removal rate might be caused by the low soil organic matter (SOM) content and low water content of sand. The use of a fiber-optic transfection dip probe (FOTDP) demonstrated that more than half of the injected ozone was consumed by reactions with diesel or natural ozone-consuming materials. The earthworm toxicity test using Eisenia fetida demonstrated that diesel concentrations in soil exceeding 10,000 mg/kg caused a dose-dependent weight loss in earthworms and increased mortality. Toxic effects were reduced greatly or eliminated after ozonation, and the degradation products of the ozonation were not toxic to the earthworms at the concentrations tested. One specific result was that the sublethal test on the earthworm might be more sensitive for the evaluation of the quality of contaminated soil, for some samples, which did not result in mortality and produced an adverse effect on weight.

Keywords—Earthworm Eisenia fetida Toxicity Diesel Ozonation

INTRODUCTION

The contamination of the subsurface by diesel fuel resulting from leaky fuel storage tanks, refineries, factories, oil spills, and improper waste disposal has been of concern for many decades. Considerable effort has been made to develop effective remediation technologies for subsurface areas contaminated with petroleum hydrocarbons (PHs) [1,2].

Recently, in situ ozonation technology has been employed successfully for remediation of unsaturated contaminated areas with PHs due to some advantages. First, ozone is most ideal for vadose zone treatment, compared to liquid oxidants, because it is a gas [3,4] and can be delivered readily to the contamination zone [5]. Second, ozone is generated from oxygen and degrades to oxygen. Third, metal oxides such as goethite (α-Fe₂O₃), MnO, and Al₂O₃ on the surface of sand and soil are known to accelerate ozone decomposition to generate OH⁻ via heterogeneous catalytic reactions of ozone with the metal oxides [6]. Moreover, in situ ozonation enhances the dissolution of hydrophilic organic matters from soil organic matter (SOM) [7,8]. These studies presented the considerable efficiency and feasibility of in situ ozonation for remediation of unsaturated soils contaminated with nonvolatile or semivolatile hydrocarbons.

Many studies on a combined treatment of ozonation and biodegradation have been carried out to enhance the remediation of PHs-contaminated soil [9,10]. The degradation of PHs was enhanced by biological treatment followed by ozonation in the combined treatment. However, concerns regarding toxicity change during in situ ozonation still remain. It is obvious that in situ ozonation reduces the number of indigenous microorganisms, but this does not explain clearly the change of total toxicity before/after in situ ozonation. Therefore, toxicity monitoring by established methods is required to delineate potential risk about increased toxicity by in situ ozonation.

The earthworm is one of the most favorable indicators for the ecotoxicological assessment of chemicals in the soil system. Various laboratory tests using earthworms have been conducted to investigate the toxicological effects of chemicals, such as pesticides and heavy metals, and include the simple contact test, the immersion test in diluted solutions of chemicals, and the artificially contaminated soil test [11,12]. An investigation of the earthworm’s behavioral, survival, growth, and reproductive reaction to toxic materials can help us evaluate the quality of remediated soil.

The objectives of this study were to monitor toxicity variation during the chemical oxidation process. A 3-D test cell ozonation process was used to remove the diesel component in sand, and an earthworm toxicity test was conducted at the beginning and end of ozonation. This study will support an ecotoxicological evaluation of soil quality after the use of a chemical remediation technique in both the laboratory and field.

MATERIALS AND METHODS

Three-dimensional test cell experiment

A three-dimensional (3-D) test cell with a length of 3 m, width of 2 m, and height of 2 m was constructed with stainless steel. The experimental system was composed of a liquid oxygen reservoir, an ozone generator, a flow meter, a pressure meter, a fiber-optic transfection dip probe (FOTDP) system for the measurement of ozone concentration, and the 3-D test cell.

Soil packing, contamination, and ozonation. A total of 15,330 kg of sand, which was available commercially (Doyang Science, Chumunjin, Korea), was packed in the 3-D test cell.

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Table 1. Characteristics of sand in three-dimensional test cell

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (μm)</td>
<td>300–106</td>
</tr>
<tr>
<td>Weight packed (kg)</td>
<td>15,300</td>
</tr>
<tr>
<td>Porosity</td>
<td>0.20 ± 0.506</td>
</tr>
<tr>
<td>Bulk density (g/cm³)</td>
<td>1.548</td>
</tr>
<tr>
<td>Water content (wt %)</td>
<td>8.85</td>
</tr>
<tr>
<td>Water saturation</td>
<td>0.137</td>
</tr>
<tr>
<td>SOM content (wt %)</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* SOM = soil organic matter.

in 50- to 60-cm lifts. The lifts were compacted carefully to maintain homogeneous porosity and bulk density. The metal oxidant content of the sand is described elsewhere [6], and the characteristics of the sand used are listed in Table 1. After packing, an impermeable layer was formed on the surface of the sand layer to prevent the leakage of gaseous ozone and to create a constant pressure distribution within the 3-D test cell. Four inlet and outlet ports, each with a diameter of 6 cm, were installed in the 3-D test cell. Details of the ports are illustrated in Figure 1.

Commercially available diesel fuel was purchased at a local gas station. The diesel fuel was weathered for two weeks at room temperature and then used to contaminate the 3-D test cell. To mimic the leakage of diesel fuel from a storage tank, the diesel fuel was released by gravity from the top of the 3-D test cell at 10 different points. The ozone inlet and outlet ports and the ozone monitoring points in the 3-D test cell are shown in Figure 1. When the concentration of gaseous ozone produced by the ozone generator reached 100 ± 3 mg/L, it was introduced continuously to the 3-D test cell at 11.8 L/min (~0.357 cm/min) for 20 d.

Monitoring of ozone concentration. An FOTDP system was employed for the in situ measurement of gaseous ozone concentration in the 3-D test cell. Details of the FOTDP system are described elsewhere [13]. Briefly, the system consists of a light source which produces intense and continuous ultraviolet light a bifurcated optic fiber bundle, a transfection probe, a miniature fiber-optic spectrometer, and a computer system. The incoming light interacts with the gaseous ozone at the tip of the probe and is partially reflected back into the probe. This reflected signal is transmitted through the output leg to the spectrometer and quantified. The probes were inserted into the 3-D test cell from the top.

**Extraction and analysis of diesel fuel.** Sand samples were taken at 18 different points and five different depths before ozone injection, yielding a total of 90 samples. The same number of samples also was collected after 20 d of ozone injection. Diesel components were extracted from the samples using Pressurized Solvent Extraction (Applied Separations, Allentown, PA, USA), which is comparable with Method 3545, accelerated solvent extraction (SW-846, U.S. Environmental Protection Agency, http://www.epa.gov/epaoswer/hazard/test/sw846.htm). Briefly, 10 g of sample soil were added to a stainless steel vessel fitted to the pressurized solvent extraction apparatus. The extraction solvent, which was a 50:50 (volume ratio) mixture of acetone and methylene chloride, was pumped into the bottle. Temperature and pressure were increased to 100°C and 100 psi, and these conditions were maintained for 10 min. The solvent then was discharged, and fresh solvent was used to wash the vessel again. The extract was analyzed for total petroleum hydrocarbon (TPH) by gas chromatography (5890 series, Hewlett-Packard, Palo Alto, CA, USA) equipped with a flame ionization detector and an autosampler. A capillary column (Alltech ECONO-CAP EC1, 30 m length × 0.32 mm ID × 0.25 mm film thicknesses; Sydney, Australia) was used to separate TPH, and standards were prepared using commercial diesel fuel; these were weathered for more than two weeks at room temperature. The gas chromatograph was operated with helium-carrier-gas at 10.0 cm/s with a detector temperature of 280°C and an inlet temperature of 270°C, and the oven temperature was maintained in accordance with the following program: 40°C for 10 min and increased thereafter at 8°C/min to 280°C (held 10 min).

**Earthworm toxicity test**

Organism and experimental conditions. The earthworm species *E. fetida* was used as an indicator organism; specimens were purchased two weeks prior to use and stored in commercial worm bedding with worm food. The earthworms, worm bedding, and food all were purchased from Carolina Biological Supply (Burlington, NC, USA). Adult earthworms at least two months old with cuticula and possessing a weight of 0.25 to 0.35 g were selected and used in all the experiments. The experiments were conducted in a culture chamber (HB-310, Hanback, Bucheon, Korea) with constant light intensity and a temperature of 20°C. Earthworms were acclimated to the experimental conditions in the culture chamber for one week before the tests. Ten adult earthworms were placed into 200 g of soil composite (dry wt basis; refer to subsequent section concerning the soil composite), which was hydrated previously to 25% water-holding capacity in 500-ml polypropylene wide-mouth jars with loose-fitting lids (Nalgene, Rochester, NY, USA) for minimizing water evaporation and animal escape. The lids of the jars were punched with holes to allow ventilation and oxygen supply.

Toxicity tests for diesel-contaminated soils. To monitor toxicity of diesel-contaminated soil during the ozonation process, survival and growth of earthworms were observed. Soil samples were collected at several points in the 3-D test cell before and after the ozonation process was performed. These soil samples were used to make the artificial soil composite, which was prepared in accordance with Organization for Economic Co-operation and Development guideline 207 with minor modifications [11]. This soil consisted of 70% (weight/weight [w/w]) of sand, 20% (w/w) of kaolin clay [11], and 10% (w/w) of commercial peat (worm bedding, AA-14–1684) in which earthworms were shipped and stored before the experiments.

Quoted text from the content:

Fig. 1. Layout of 3-D test cell. Ozone inlet points: I-1, I-2, I-3, and I-4. Ozone outlet points: O-1, O-2, O-3, and O-4. Ozone monitoring points: B-2, B-4, B-5, and A-4. Squares on figure (A-1 through A-6, B-1 through B-6, and C-1 through C-6) represent sampling points for contaminant analysis.
In this soil composite, the sand portion represented diesel-contaminated samples. The weight of each soil composite in the experiments was 200 g (total dry wt basis). Moisture content of the soil media for all experiments was adjusted completely to 25% (w/w) using tap water that was left in an open container at least 24 h before being added to the soils. A total of 5 g of commercial worm food (AA-14–1670), consisting of proteins, fats, minerals, and carbohydrates, were added to each prepared soil sample for the duration of the experimental period.

Mortality, growth, and the behavioral response of *E. fetida* were observed after 2, 5, 7, 10, and 14 d. Additionally, control soils were included in the experimental set to confirm that uncontaminated conditions were suitable for earthworm growth. During the determination of earthworm growth, live earthworms were rinsed with cold tap water to remove adhered soil particles and blotted dry with tissue paper before each earthworm was weighed [14].

**RESULTS AND DISCUSSION**

**Removal of diesel fuel in the 3-D test cell**

Initial distribution of diesel fuel in the 3-D test cell is shown in Figure 2a. The concentration of diesel fuel was higher at the upper part and near the inlet ports than at the lower part and near the outlet ports, respectively.

The residual concentration of diesel fuel after 20 d of ozone injection is illustrated in Figure 2b. Based on TPH mass, the removal rate was calculated by the equation:

\[
\text{removal rate (\%)} = \frac{[\text{total TPH mass before ozone injection} - \text{total TPH mass after ozone injection}]}{\text{total TPH mass before ozone injection}} \times 100
\]

The removal rate was approximately 95% near the ozone inlet ports, whereas the area near ozone outlet ports showed around 80% of diesel fuel removal. An overall 90% removal rate was observed in this study. In general, the removal rate of organic compounds by in situ ozonation depends upon the experimental conditions (e.g., ozone concentration and gas flow rate), the characteristics of the soil (e.g., water content, SOM composition, and the presence of metal oxides), and contaminants. Jung et al. [10] reported that approximately 50% of the TPH were removed after 900 min of ozone injection (~1,300 pore volume) in the soil column experiments under conditions of water content of 34.2% and of SOM content of 4.99 wt-%. The relatively high removal rate in this study might be caused by the low SOM content and water content of sand. The gaseous ozone is highly reactive with SOM exhibits because of the nucleophilic characteristics of aromatic compounds, which are the main constituents of SOM. Jung et al. [15] reported that of the natural ozone-consuming materials in the subsurface system, SOM consumes the highest amount of ozone. The low concentration of SOM may enhance the direct reaction of ozone with diesel fuel. Water content also is one of the important parameters that affect the decomposition of ozone. Increased water saturation decreased gaseous ozone reaction because of the formation of water films that prevented the contact between gaseous ozone and ozone-demanding materials on soil surfaces. Jung et al. [15] pointed out that the removal rate of organic compounds is inversely proportional to water content in in situ ozonation. About a 50% removal rate was achieved from the in situ ozonation of diesel fuel–contaminated soil exhibiting a water content of 34.7% [10].

The monitoring of gaseous ozone concentration was conducted at four different points (A-4, B-2, B-4, and B-5) using the FOTDP system, with the results shown in Figure 3. Although the injected gaseous ozone concentration was maintained at 100 ± 3 mg/L during the experimental period, less than 50% of the injected ozone was detected at the test cell. This means that more than 50% of ozone was consumed by reaction with diesel fuel and/or natural ozone-consuming materials, although less than 10% of the total diesel fuel remained.

The soil pH, water content, and SOM content were measured after 20 d of ozone injection to investigate the effects of ozonation on soil properties. The pH dropped from 6.1 to 5.6, and this result is in good agreement with a previous report [7]. This pH drop is due to the oxidation of organic matter into organic acids, and generally is proportional to increases in SOM content [7]. In this research, the relatively low pH drop (0.5) may have been caused by the low organic matter content of the sample sand. Continuous injection of gaseous ozone leads to the evaporation of soil water and results in a decreased water content. As expected, the water content (wt %) dropped from 8.85 to 5.50. However, a noticeable change in SOM content was not observed.
Fig. 3. Change of gaseous ozone concentration over time at four different monitoring points.

Fig. 4. Average weight (% of initial wt) of *Eisenia fetida* in diesel-contaminated soil versus time.

Fig. 5. Average weight (% of initial wt) of *Eisenia fetida* after ozonation versus time.

Earthworm toxicity of diesel-contaminated soil

Diesel-contaminated sand samples were collected at several representative points in the 3-D test cell. Table 2 shows sampling points and diesel concentration in sand samples before and after ozonation. The results of earthworm mortality (Table 2) clearly show that ozonation reduced the lethal effect of the sand by removing the diesel component, because mortality did not occur in cases involving ozonated sand. In particular, the C-5-a sample, which showed the highest mortality (80%), showed no lethal effect after ozonation. This result might be related directly to the removal of 90% of the diesel component during ozonation. Although mortality of earthworms was found in samples with a diesel concentration over 10,000 mg/kg, differences in mortality were detected for high diesel concentrations. In particular, diesel concentration in the A-2-a sample was relatively high (14,598 mg/kg) and was associated with a mortality of only 10% during 14 d.

To detect the sublethal effect of diesel, the weight of earthworms in the toxicity test was measured and expressed as an average weight development in Figures 4 to 6. Average weight was measured as the total weight divided by the number of live earthworms and indicates the representative weight of each live earthworm [16]. The diesel concentration that began to affect the growth of earthworms was nearly 10,000 mg/kg, because a greater concentration led to a decrease of the average weight for a 14-d test. After 7 d, the average weight of earthworms diminished by 83, 76, and 86% in the B-3-a, C-2-a,

Table 2. The results of toxicity tests for diesel-contaminated sand

<table>
<thead>
<tr>
<th>Sample point</th>
<th>Before ozone injection</th>
<th>After ozone injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diesel concn. (mg/kg)</td>
<td>Mortality of earthworms (%) after 14 d</td>
</tr>
<tr>
<td>A-2-a</td>
<td>14,598</td>
<td>10</td>
</tr>
<tr>
<td>A-2-i</td>
<td>3,647</td>
<td>0</td>
</tr>
<tr>
<td>A-5-i</td>
<td>356</td>
<td>0</td>
</tr>
<tr>
<td>B-3-a</td>
<td>18,582</td>
<td>60</td>
</tr>
<tr>
<td>B-3-i</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>C-2-a</td>
<td>13,396</td>
<td>60</td>
</tr>
<tr>
<td>C-2-i</td>
<td>5,204</td>
<td>0</td>
</tr>
<tr>
<td>C-5-a</td>
<td>12,486</td>
<td>80</td>
</tr>
<tr>
<td>C-5-i</td>
<td>556</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Lowercase a and i represent a sampling depth of 50 cm and 450 cm, respectively.*
and C-5-a soil samples, respectively (Fig. 4). According to Figure 4, the data were grouped into two groups, A (diesel <
5,200 mg/kg) and B (diesel > 12,000 mg/kg). The analysis indicated that the weights of these two groups were significantly
different (paired t-test, \( p < 0.01 \)). In this study, median lethal or median effective concentrations were not calculated
because greater variation in concentrations should be tested in
order to yield reasonable median lethal concentration or me-
dian effective concentration values. One specific result of in-
terest revealed that the A-2-a sample showed a toxic effect on
the earthworm weights, even though this sample showed a
relatively low mortality when considering the high diesel con-
centration. Based on this result, the sublethal test of earth-
worms was proven to be more effective for the evaluation of
the quality of contaminated soil [16,17].

The response of earthworms after ozonation treatment also
was investigated to confirm the reduction of toxicity and to
assess the efficiency of the ozonation process. Figure 5 rep-
resents the average earthworm weights in soil samples after
ozonation. All samples showed that earthworm weight in-
creased to the same degree as that observed for uncontaminated
control sand. The t-test of weights at 7 d (median data) sug-
gested that the weights increased in soils after ozonation (\( p
< 0.05 \); comparing values before and after ozonation). In ad-
in, the Bonferroni posttest of two-way analysis of variance
indicated that the weights were significantly higher in ozonated
soils (\( p < 0.05 \)). Nevertheless, the interaction between soil
(various diesel concentration) and treatment (ozonation) was
extremely significant (\( p < 0.001 \)). Based on these analyses, it
could be said that ozonation can decrease the toxicity of diesel-
contaminated soil regarding the weights. These results may be
due to the fact that most diesel components were removed
from the soil after ozonation. In fact, ozonation removed 75
to 95% of the diesel in collected soil samples.

In the ozone process, the injection of oxidants can adversely
affect indigenous microorganisms [18], and no information is
available concerning the toxicity of byproducts of diesel ox-
idation. In Figure 6, the average weights after 7 d were plotted
against the differences between the initial and final diesel con-
centration. If a high amount of diesel reacted with ozone in
the subsurface, greater amounts of the byproduct could be
produced. If this byproduct was toxic to earthworms, the high
removal of diesel itself could not directly reduce toxicity. How-
ever, this adverse effect of the byproduct was not found in this
study. Figure 6 did not show any significant relationship, and
this indirectly indicates that a residual toxic effect of a by-
product did not exist in the subsurface.

CONCLUSION

A 3-D test cell was used to investigate the removal effi-
ciency of ozonation in diesel-contaminated soil, and earth-
worm toxicity tests were applied to confirm the reduction of
toxicity during a remediation process. The following conclu-
sions are made.

Approximately 90% of diesel was removed after 20 d of
ozone injection. This high removal rate resulted from low wa-
ter and SOM content of sample sand. However, the operation
time with other soils that contain higher SOM and/or water
contents likely is longer than ozonation time in this study to
achieve 90% removal rate.

The mortality of earthworms was decreased significantly at
the end of ozonation, indicating that the ozonation process in
fact reduced the toxicity of the sand to the earthworm.

The average weights of earthworms were influenced sig-
nificantly by diesel concentrations, and a sublethal effect (wt
development) was a more sensitive factor than the acute effect
(mortality) for the assessment of toxicity.

The byproducts of ozonation of diesel had no discernable
effect on earthworm survival or weight.

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