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Effect of soil organic matter (SOM) and soil texture on the fatality of indigenous microorganisms in intergrated ozonation and biodegradation

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Abstract

In situ ozonation has been proposed as a method to remediate soils contaminated with organic pollutants. Soil column experiments were performed on eight different soils in order to investigate the effects of soil properties, such as soil organic matter (SOM) and soil texture on the survival and regrowth of indigenous microorganisms after *in situ* ozonation. Indigenous microorganisms were found to be very sensitive to ozone in the soil column experiments. The microbial fatality revealed a linear relationship with the SOM content in the range of 1.72–2.42% of SOM content, whereas water content was poorly correlated. Four weeks of incubation of ozone-treated soil samples allowed for the regrowth of indigenous microorganisms with inverse relation to ozonation time. The regrowth was also significantly influenced by the SOM content in the same soil texture. Oxidation and removal rate of hexadecane was affected by particle size distribution. Especially, sand exhibited the highest oxidation rate of hexadecane, which resulted from having the lowest SOM content, water content, and surface area with respect to the other samples. The soil samples ozonated for 90–180 min were determined to exhibit the lowest concentration of hexadecane, with the exception of sand, after 4 weeks of incubation. This study provided insight into the influence of SOM and soil texture on indigenous microbial potential to degrade hexadecane in integrated ozonation and biodegradation.

Keywords: *In situ* ozonation; Indigenous microorganisms; SOM; Soil texture; Hexadecane

1. Introduction

In situ ozonation is an effective method for the remediation of partially saturated soil that has been contaminated with organic chemicals [1–6]. In comparison with conventional technologies, *in situ* ozonation offers many advantages, including rapid removal of contaminants via free radical reactions generated from the catalytic reactions between ozone and metal oxides on the soil surface [7]. Several successful field applications of *in situ* ozonation have been reported [8].

Gaseous ozone supplied to the subsurface during *in situ* ozonation decomposes into oxygen, which has the potential to aid in biodegradation. However, in the remedial action, not all contaminants removed are completely mineralized, and some contaminants play a role in increasing the biodegradable carbon in *in situ* ozonation of actual contaminated soil. In this regard, Miller et al. [1] reported that the increase in biodegradable carbon was greater for contaminated soil than uncontaminated soil when using chemical oxidation. They used three different soils, i.e., one silt loam and two silt clay loams, with soil organic matter (SOM) content in the range of 0.9–3.4%. Additionally, the chemical treatment of soils also causes structural changes in SOM on the surface of soil particles. To this extent, Jung and Choi [9] reported that ozone treatment of the field soil enhanced the dissolution of hydrophilic fraction of SOM into the soil water phase. Ozone also plays as a sterilizing agent for indigenous microorganisms in *in situ* ozonation. *In situ* application of ozone exponentially decreased the population of indigenous microorganisms in PAHs-contaminated soils.

Abbreviations: DNAPL, dense nonaqueous phase liquid; GC, gas chromatography; JS, commercial sand; MFC, mass flow controller; PSE, pressurized solvent extraction; SOM, soil organic matter; S-1; -2; -3; and -4, soil samples taken from Bonchon Industrial Complex; D-1; -2; and E-1, soil samples taken from GIST campus

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Integrated chemical–biological treatment has commonly been used for the remediation of soil contaminated with organic pollutants [1,2,10,11]. Nelson et al. [10] remediated groundwater contaminated with PAH and dense nonaqueous phase liquid (DNAPL) by sparging gaseous ozone. They detected super saturated concentration of dissolved oxygen (>25 mg L^{-1}) in the monitoring wells up to 180 m from the injection well. This super saturated concentration of dissolved oxygen was found to enhance the biodegradation of PAH and DNAPL in groundwater. They also concluded that natural attenuation following the pre-oxidation process significantly improved the removal of PAH and DNAPL, when compared with either chemical or biological treatment alone. Additionally, Miller et al. [1] reported that *in situ* chemical oxidation of pesticide-contaminated soils created favorable conditions for microorganisms desirable for biodegradation.

Moreover, a combined approach using ozonation and subsequent biodegradation for silt loam contaminated with PAHs and coal tar has been successfully demonstrated [1,2]. Specifically, chrysene and benzo(a)pyrene which were not degraded by the bacteria alone, were highly degraded by the combined process. They inoculated microorganisms after chemical oxidation because chemical oxidants are generally sterilizing agents, and show adverse effects on indigenous microorganisms. Nonetheless, it is more economical and convenient to carry out natural attenuation after *in situ* ozonation using indigenous microorganisms [8,11]. Supporting this method, Jung et al. [11] presented optimum operating conditions when *in situ* ozonation combined with natural attenuation is carried out with indigenous microorganisms.

In *in situ* ozonation, it is well known that soil properties, such as SOM, water content, and soil texture significantly affect not only the survival of indigenous microorganisms [10–12], but also the degradation of organic contaminants [6,7,9,11]. However, the effects of soil properties on integrated ozonation-biological process have not yet been reported. Hence, the aims of the present study are: (1) to understand the survival and regrowth of indigenous microorganisms in soils with different soil organic matter contents, water content, and textures; (2) to examine the removal rate of hexadecane in integrated ozonation and subsequent biodegradation; (3) to determine the optimal ozonation time for enhancing the biodegradation of hexadecane in various soil textures. In this study, hexadecane was selected as the model organic contaminant, because it is the most abundant organic compound in the petroleum hydrocarbons found in diesel fuel [11].

2. Materials and methods

2.1. Chemicals

All chemicals used in the experiments were of analytical reagent quality. Hexadecane was purchased from Aldrich Chemical Co. ($C_{16}H_{34}$, 99%, Milwaukee, WI, USA). Acetone and methylene chloride were supplied from Fluka, and all solutions were prepared using deionized water (Ultrapure system, Barnsterd).

2.2. Soil preparation

Eight different soil samples were selected with respect to their SOM and soil texture. Four of those soil samples were collected from the Bonchon Industrial Complex (S-1, -2, -3, and -4); the geological features of the site are described elsewhere [9]. The soil samples (S-1, -2, -3, and -4) were collected directly from a core sampler at one sampling point at various core depths. The other three soil samples (D-1, D-2, and E-1) were collected from the Gwangju Institute of Science and Technology (GIST) campus. Commercial sand (JS) passed through 250–300 μm sieves was also prepared to represent soil with a low SOM content. After sampling, the soil samples were immediately transported to the laboratory for analysis and kept at 4 °C. The characteristics of the soil samples are listed in Table 1. Total carbon and nitrogen contents were determined by an element analyzer, Vario Max (Elemtar, Germany), and that particle-size analysis was done using the hydrometer method.

Table 1
Characteristics of soil samples

Soil name	Texture	Depth (cm)	Air permeability (cm^2)	pH	% (w/w) Contents of			
					Water	Carbon	Nitrogen	SOM[e]
S-1[a]	Silt loam	0–25	1.72×10^{-8}	5.2	25.3	0.446	0.056	1.76
S-2[a]	Silt loam	40–70	7.74×10^{-9}	5.1	34.7	0.662	0.072	2.17
S-3[a]	Silt loam	100–126	1.50×10^{-8}	4.9	25.1	0.938	0.059	2.42
S-4[a]	Silt loam	150–190	1.33×10^{-8}	4.6	23.3	0.927	0.063	2.31
JS[b]	255-500 μm	–[d]	–[d]	5.4	0.26	0.081	0.0026	0.42
D-1[c]	Clay loam	0–30	6.77×10^{-9}	5.2	20.7	0.305	0.045	1.73
D-2[c]	Clay loam	30–55	5.68×10^{-9}	5.1	28.5	0.296	0.042	1.78
E-1[c]	Loam	70–83	1.19×10^{-8}	4.9	22.1	0.851	0.086	2.86

[a] S-1, -2, -3, -4, and B-1 are taken at the Bonchon industrial complex in Gwangju, Korea.
[b] JS is commercial sand.
[c] D-1, D-2, and E-1 are from the GIST campus.
[d] Not determined.
[e] Determined by the Loss-On-Ignition method (Soil Science Society of America, 1996, Methods of Soil Analysis: Part 3-Chemical method); Briefly, the soil samples were baked at 400 °C for 16 h, and then the LOI content of the soil samples was calculated.

The effect of organic contaminants on indigenous microorganisms in *in situ* ozonation was also investigated. Four soil samples (JS, D-1, D-2, and E-1) were contaminated with hexadecane. Hexadecane-contaminated samples were prepared using a glass beaker containing 300 g of each soil. The beaker was spiked with the designated amount of hexadecane to adjust the hexadecane concentration to 750 mg-hexa kg-soil^{-1}. Then the soil sample was stirred for a minimum of 24 h at 65 rpm to establish a homogeneous distribution of hexadecane. The hexadecane-contaminated soils were kept in a dark room for 45 days at 20 °C before use.

2.3. Soil column experiments

Spectra Chrom HPLC glass columns (Spectrum Chromatography, Houston, TX) with a diameter of 2.5 cm and a length of 10 cm were used for the soil column experiment. The soil sample was homogeneously packed into the glass column by shaking, and porosity was kept at 0.30. Columns packed with sample soils were placed in a cyclic batch column reactor (CBCR) system, as illustrated in Fig. 1. The CBCR system consisted of an ozone generator (GL-1, PCI-WEDECO; USA), a mass flow controller (MFC; F201C-FAC-22-V, Bronkhorst Hi Tec; Netherlands), a gas washing bottle and a UV/vis spectrophotometer (Smart Plus 1900, Youngwoo Instrument Corp., Korea). The gaseous ozone was moisturized by passing it through a washing bottle, and was then injected into the UV/vis spectrophotometer at a gas flow rate of 200 mL min^{-1} using a mass flow controller (MFC). Once the ozone concentration had stabilized at 20 mg L^{-1}, the gaseous ozone was directed through the columns, from the bottom to the top. The reaction columns were sequentially sacrificed at the specified reaction-time intervals, such as 0, 30, 90, 180, and 360 min. The residual ozone in the column was purged for 1 min using nitrogen. Ozone concentration was measured and automatically recorded in real-time by a computer system connected to UV/vis spectrophotometer (Youngwoo Instrument Co., SP1900/2700, South Korea) at 290 nm. Ozone breakthrough was measured at relative ozone concentration (C/C_o) = 0.5 with the breakthrough times expressed as pore volumes. All experiments were conducted at 25 ± 2 °C.

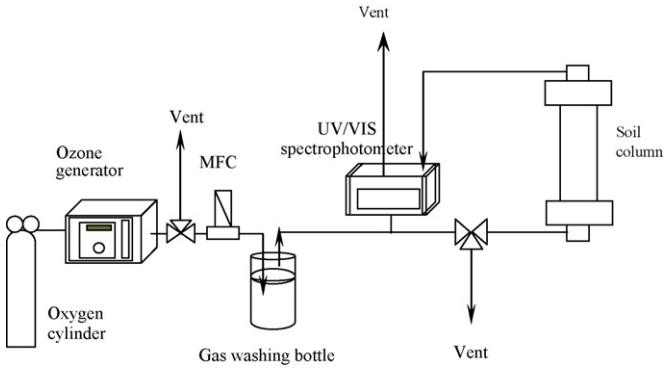

Fig. 1. Schematics of the experimental setup: cyclic batch column reactor (CBCR) system.

Ozonated soils were added to 500 mL beakers and layered to a thickness of less than 2 cm to prevent the limitation of oxygen diffusion. The beakers were then covered with polyethylene film and incubated at 20 °C for 4 weeks. The beakers were aerated every 2 or 3 days by removing the covers during incubation [13].

2.4. Extraction and analysis of hexadecane

After the ozonation experiments, hexadecane was extracted using pressurized solvent extraction (PSE, Applied Separations, Inc.; USA), which is comparable with Method 3545 (US EPA), accelerated solvent extraction [14]. To briefly summarize the procedure, 10 g of sample soil were first added to a stainless steel vessel, fitted to the PSE. Next, an extraction solvent, a 50:50 (v/v) mixture of acetone and methylene chloride, was pumped into the bottle. The temperature and pressure were increased to 100 °C and 689 kPa, respectively, and this condition was maintained for 10 min. The solvent was then discharged and fresh solvent was used to wash the vessel. The extract was analyzed for hexadecane by gas chromatography (GC) (5890 series, Hewlett-Packard; Palo Alto, CA) equipped with a flame ionization detector and an autosampler. A capillary column (Altech ECONO-CAP EC1, 30 m length × 0.32 mm ID × 0.25 μm film thickness) was used to separate hexadecane from the sample. The GC was operated with a helium-carrier-gas flow rate of 10.0 cm s^{-1}, the detector temperature of 280 °C, the inlet temperature of 270 °C, and oven temperature program starting at 40 °C (held for 10 min) and increasing at a rate of 8 °C min^{-1} to a maximum temperature of 280 °C (held 10 min). The injection volume of the extract was 1.0 μL. The hexadecane recovery rate was more than 95% for all soil samples.

Error bars in Figures and ± signs in Tables represent the standard deviation based on triplicate experiments.

2.5. Microbiological analysis

For counts of heterotrophic bacteria, 1 g of wet sample soil was added to a capped tube containing 9 mL of 0.01% sodium phosphate buffer solution (pH 7.0). The tube was vortexed for 1 min, and the supernatant was diluted and plated on Yeast Extract-Polypeptone-Glucose agar plates (per liter: 0.25 g glucose, 0.5 g polypeptone, 0.05 g yeast extract, 0.05 g ammonium nitrate, pH 7.0) [15]. The plates were then incubated at 21 °C for 4 days, and the number of colonies was counted.

3. Results and discussion

3.1. Transport of gaseous ozone through unsaturated soil columns

Ozone breakthrough was measured for various soil samples at relative ozone concentration (C/C_o) = 0.5, as shown in Fig. 2, with the breakthrough times expressed as pore volumes. The effect of SOM on ozone transport is illustrated in Fig. 2a. Note that SOM has been proposed as the highest ozone con-

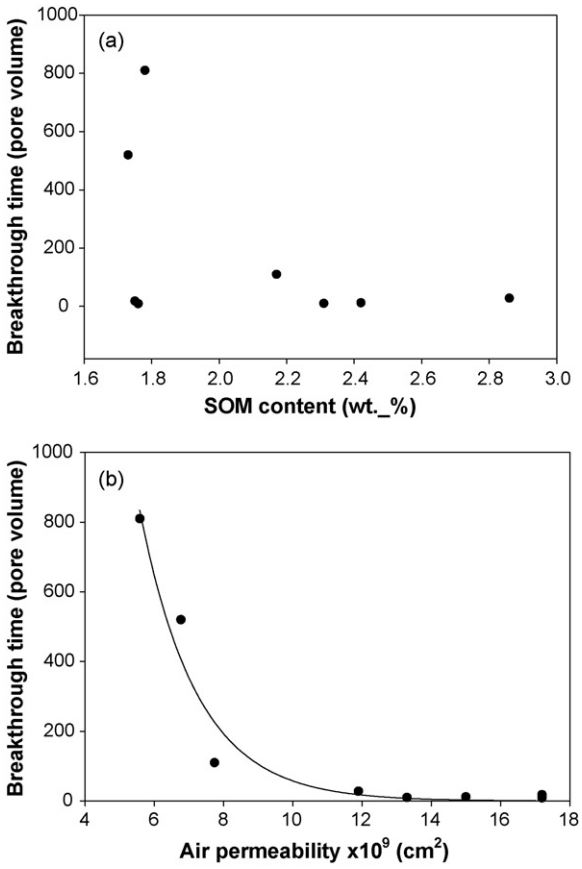

Fig. 2. Breakthrough time of gaseous ozone measured at $C/C_o = 0.5$: (a) effect of SOM; (b) effect of air permeability.

suming constituent in natural ozone consuming materials, such as SOM, soil water, and metal oxides in subsurface environments [3,4,9]. In this regard, Kim and Choi [4] demonstrated that as the SOM content increased, the time required for the gaseous ozone to completely breakthrough became longer, as noted from the onset of its appearance. However, as presented in the figure, the effect of SOM content on the transport of gaseous ozone is not clear in the soil samples used in this study. This result seems to imply that other factors not considered in previous researches seem to be affecting the transport of gaseous ozone.

As can be seen in Fig. 2b, the breakthrough time of gaseous ozone showed a non-linear relationship with air permeability in the experimental conditions. The breakthrough time was found to exponentially increase, as air permeability decreases. Air permeability is influenced by soil properties, such as soil texture, water content, and bulk density. However, although air permeability is also one of the limiting factors on the migration of gaseous ozone in field remedial action, its effect has typically been neglected in the experimental research, as most experiments were carried out with sands in small columns, not in the actual environment [3–5]. Moreover, the presence of organic compounds, such as hexadecane also decreases the transport of gaseous ozone by consuming ozone and free radicals produced by ozone decomposition in soil.

3.2. Microbial die-off in in situ ozonation

3.2.1. Effect of soil properties

Soil column experiments were performed to investigate the effect of *in situ* ozonation on the indigenous microbial population when different soil properties are taken into consideration. Gaseous ozone was injected into the column at the selected reaction time intervals of 0, 30, 90, 180, and 360 min for the soil samples. It is clearly shown that the number of heterotrophic bacteria is strongly affected by the ozonation time. For example, 30 min of ozone injection caused the fatality of more than 90% of the bacteria in all soil samples, with the exception of the D-2 soil (Fig. 3). This is in good agreement with previous reports [8,11] reporting that 60 min of ozone injection caused a decrease in total number of heterotrophic bacteria by two orders of magnitude.

In order to quantify the relationship between the fatality of heterotrophic bacteria and soil properties, such as water content and SOM content, Pearson's correlation coefficient (r) was evaluated based on the experimental results in Fig. 3a. It was found that SOM content was linearly correlated with the fatality of heterotrophic bacteria, with an r value of -0.95 in the range of 1.72–2.42% of SOM content ($p < 0.05$), whereas water content was poorly correlated with an r value of 0.23. The negative

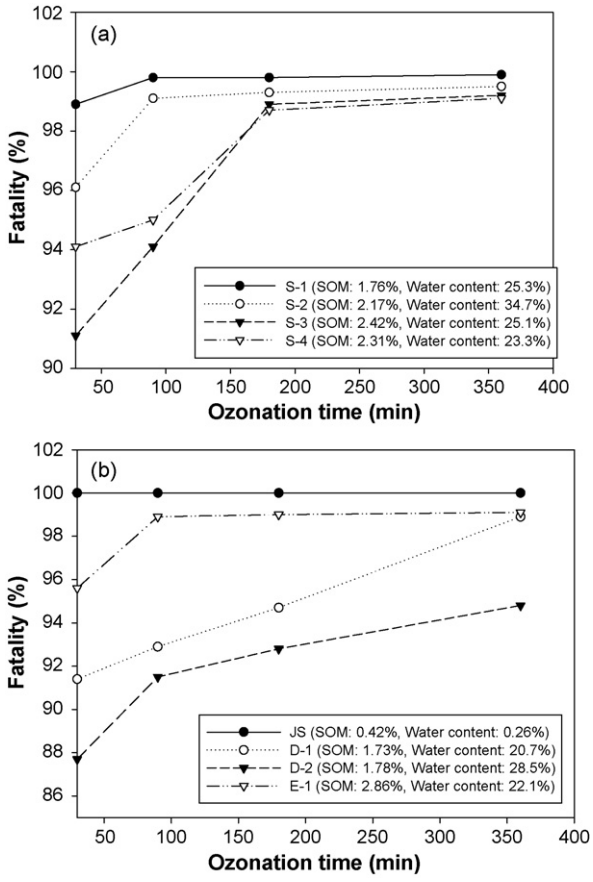

Fig. 3. Effect of ozonation time on the fatality of indigenous microorganisms according to various soil conditions: (a) the same soil texture, i.e., silt loam, with different SOM content and (b) different soil texture, i.e., sand, clay loam, and loam.

sign of r value implies that fatality is inversely proportional to SOM content. This high negative correlation between SOM content and the fatality may be attributed to the high reactivity of ozone with aromatic compounds of SOM via electrophilic reaction. SOM is known as the highest naturally occurring ozone consuming matter [9,16].

Fig. 3b presents the effect of soil texture on *in situ* ozonation. The habitats of soil microorganisms are pore spaces of various sizes and shapes that are more or less filled with water or air [17]. These pores result from the spatial arrangement of soil particles of different sizes. Hence, particle size distribution could be an important factor in determining the die-off characteristics of soil microorganisms during *in situ* ozonation. Bacterial fatality was determined to be inversely proportional to the mean particle size of the soil samples. For example, D-1 and D-2, representing clay loam, showed the lowest fatality, whereas sand exhibited the highest fatality. It is worth noting that D-1 showed lower fatality than E-1, although E-1 exhibited higher SOM and water content than D-1. Hence, the difference of soil texture between D-1 and E-1 could be a reasonable explanation for the fatality difference. The gaseous ozone may not be able to access the soil microorganisms or microcolonies in soil pores filled with soil water. Hence, clay loam, exhibiting lower porosity and surface area than loam and sand, shows correspondingly low fatality rate.

The indigenous microorganisms in sand were most sensitive to ozone in the soil samples. The initial number of the indigenous microorganisms in sand was 10^6 CFU g soil^{-1}, but this sharply decreased as soon as ozone injection was initiated. In fact, 30 min of ozonation reduced the bacteria to below the detection limit ($<2.9 \times 10^3$ CFU g soil^{-1}). This result may be due to the low surface area, low SOM content, and high porosity of sand.

3.3. Effect of hexadecane

As listed in Table 2, the initial number of indigenous microorganisms in the contaminated soils was slightly lower than in uncontaminated soils, possibly due to the toxicity of hexadecane. However, the influence of hexadecane contamination on the fatality of indigenous microorganisms is not clearly observed in this study, although Jung et al. [7] reported that the contamination of diesel fuel decreased the fatality of indigenous microorganism in *in situ* ozonation. They concluded that diesel fuel played a role as a scavenger for free radicals.

3.4. Regrowth and population dynamics of indigenous microorganisms

3.4.1. Effect of soil properties

Ozone treated soils were incubated for 4 weeks in order to investigate the effect of soil properties and texture on the regrowth characteristics of indigenous microorganisms. The microbial population increased during the incubation period; however, the increase rate was influenced by the ozonation time, as shown in Figs. 4 and 5. In the ozone-treated soil samples, approximately one order of magnitude increase in microbial population was observed after 4 weeks of incubation, with the exception of soil samples treated for 360 min. The soil samples that were treated for 360 min showed minor changes in the number of indigenous microorganisms. This limited recovery of the bacteria for all the soil samples might be caused by the discrete microhabitats of soil microorganisms in unsaturated soils and the limitation of nutrients during incubation, as there was no addition of nutrients during the experimental incubation period. This result is in good agreement with previous research by Lute et al. [12] and Jung et al. [11]. Lute et al. [12] and Jung et al. [11] reported that the incubation of ozonated soil samples without the addition of nutrients in aerobic condition exhibited only one or two orders of magnitude increase of microbial population.

The regrowth rate in the samples was also found to be affected by SOM content, as shown in Fig. 5, which shows the regrowth rate of indigenous microorganisms after 4 weeks of incubation. Note that the regrowth rate was, to some extent, linearly proportional to SOM content. For example, the Pearson's correlation coefficient (r) between the regrowth rate and SOM content was 0.97, 0.85, 0.97, and 0.98 for the soil samples ozonated for 30, 90, 180, and 360 min, respectively. The higher regrowth rate at higher SOM content may be attributed to the increased hydrophilic organics originating from SOM. This is also consistent with previous reports by Jung and Choi [9] suggesting that the ozone reaction with SOM increases the biologically degradable fraction of the water-soluble SOM in soil water. Therefore, it should be noted that SOM plays a beneficial role in the regrowth of indigenous microorganisms in ozone-treated soils.

As shown in Fig. 6, the effect of soil texture on the regrowth of indigenous microorganisms is not clearly observed and only sand showed a different regrowth pattern, although the survival of indigenous microorganisms was highly influenced by soil texture. In loam (E-1) and clay loam (D-1 and D-2), there was no relationship between average particle size and regrowth

Table 2
Population change of indigenous microorganisms (CFU g-soil^{-1})

Ozonation time (min)	Uncontaminated				Contaminated			
	JS	D-1	D-2	E-1	JS	D-1	D-2	E-1
0	7.12×10^6	9.87×10^9	1.82×10^{10}	8.84×10^8	9.93×10^5	8.16×10^9	9.43×10^9	7.16×10^8
30	ND[a]	8.49×10^8	2.24×10^9	3.89×10^7	2.41×10^4	1.0×0^9	1.47×10^9	5.41×10^7
90	ND[a]	7.01×10^8	1.55×10^9	9.72×10^6	ND[a]	7.83×10^8	1.08×10^9	3.08×10^7
180	ND[a]	5.23×10^8	1.31×10^9	8.84×10^6	ND[a]	6.45×10^8	8.30×10^8	1.58×10^7
360	ND[a]	1.09×10^8	9.46×10^8	7.96×10^6	ND[a]	2.69×10^8	6.41×10^8	9.31×10^6

[a] Not detected.

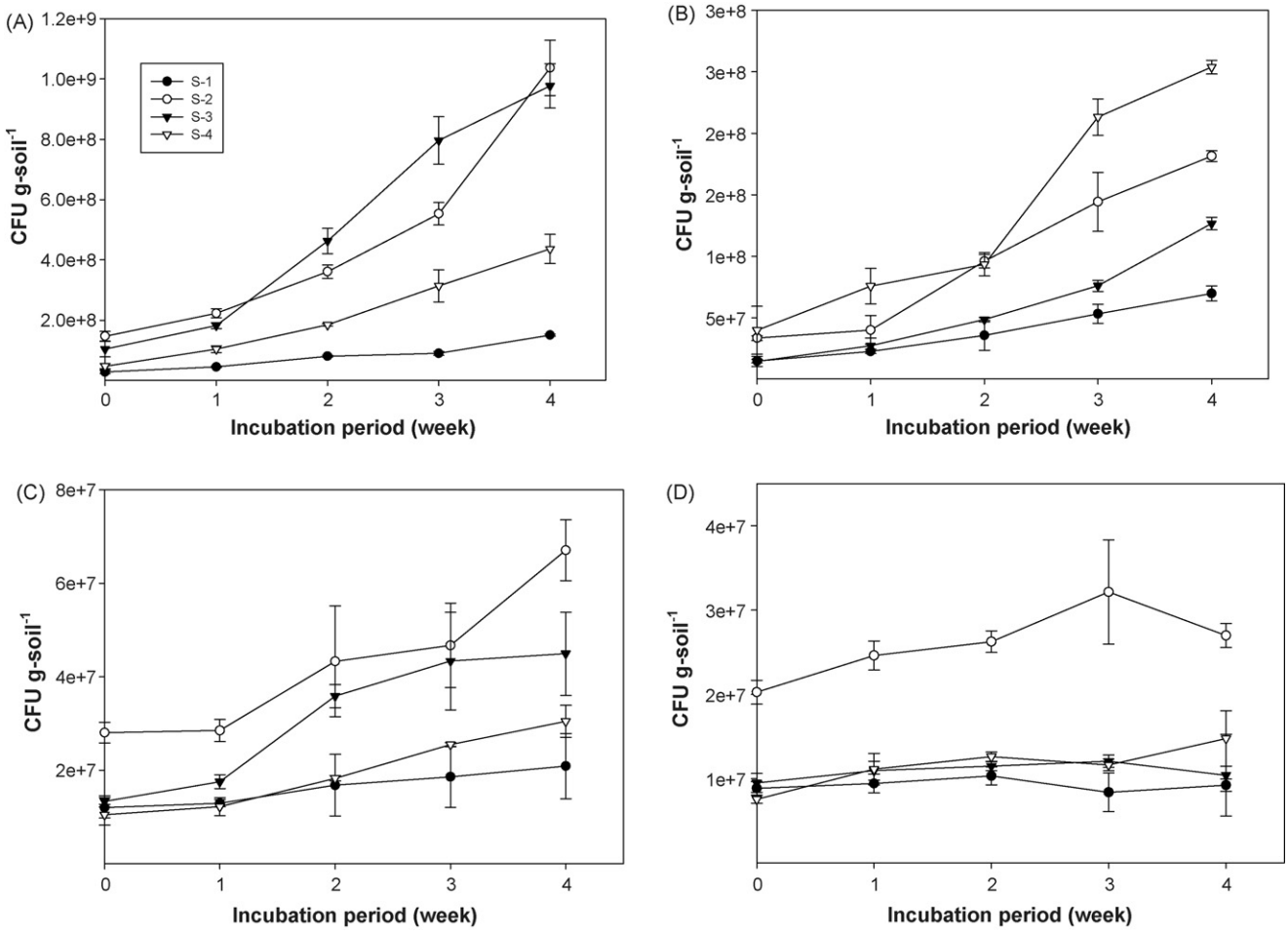

Fig. 4. Population changes of indigenous microorganisms with incubation time for the same soil texture (silt loam) treated for (A) 30 min; (B) 90 min; (C) 180 min; (D) 360 min.

rate clearly identified. For example, the D-2 soil sample (clay loam) revealed the highest regrowth rate among the soil samples ozonated for 30 min after 4 weeks of incubation (data not shown here). This result implies that the pore structure on soil surface and soil particle size may not influence the regrowth of indigenous microorganisms, because pores on soil surface filled with air or water could be possible places for indigenous microorganisms which survived after ozonation.

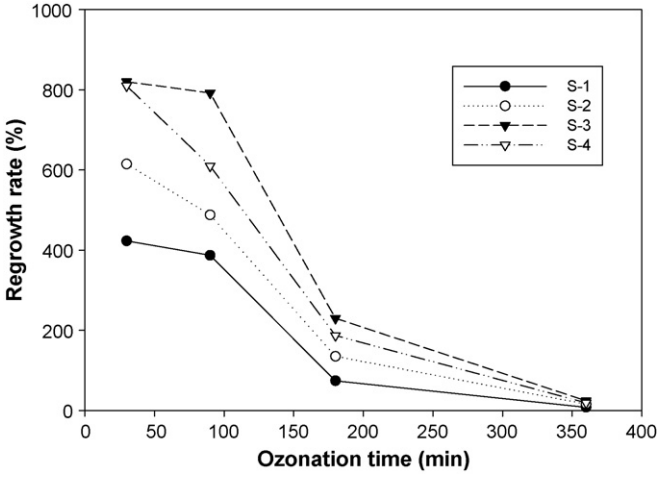

Fig. 5. Regrowth rate of indigenous microorganism after 4 weeks of incubation period in silt loam.

The number of bacteria in sand was reduced to below the detection limit after 30 min of ozone injection. Regrowth of heterotrophic bacteria was not observed in sand treated for 180 and 360 min over the entire incubation period; however, sand treated for 30 and 90 min showed regrowth of heterotrophic bacteria above the detection limit after 1 and 3 weeks, respectively. This result implies that the exposure of soils to the excess amount of ozone results in unrecoverable destroy of indigenous microorganisms in subsurface environments.

3.4.2. *Effect of hexadecane*

Hexadecane contamination was found to significantly increase the regrowth of heterotrophic bacteria during the incubation of ozonated soil samples as shown in Table 3. This is potentially due to an increase in biodegradable carbon originating from the degradation of hexadecane. In *in situ* chemical oxidation, not all organic contaminants are completely mineralized, and some contaminants could be converted into biodegradable fractions. Hence, the treatment of organic contaminants would play a role in increasing the biodegradable

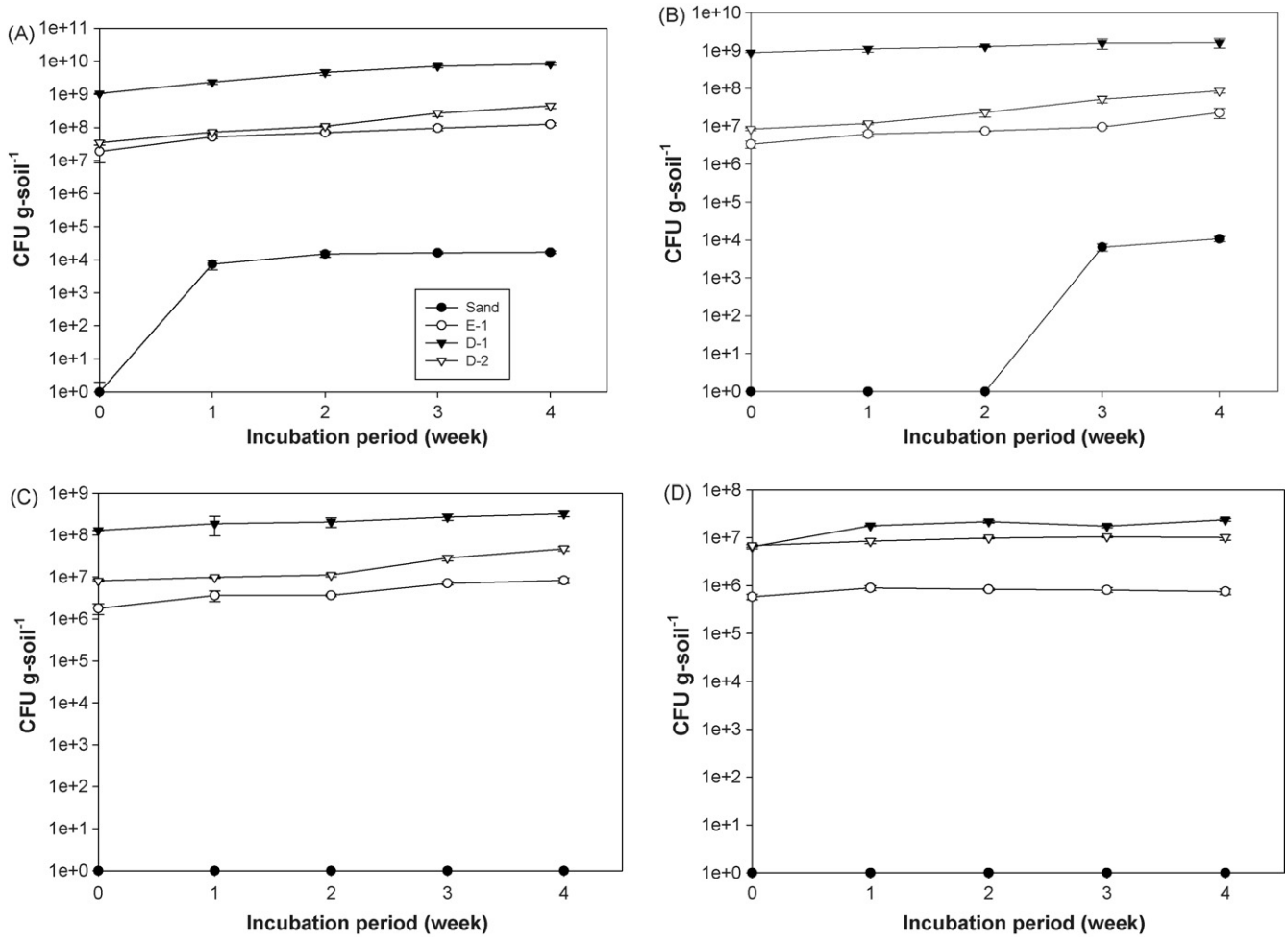

Fig. 6. Population changes of indigenous microorganisms with incubation time for different textures treated for (A) 30 min; (B) 90 min; (C) 180 min; (D) 360 min.

Table 3
Regrowth rate of indigenous microorganisms in soil samples treated contaminated with hexadecane (%)

Sample name	Ozonation time (min)	Incubation period (week)			
		1	2	3	4
JS	0	44	68	83	106
	30	190	360	445	730
	90	120	168	623	640
	180	98	115	340	414
	360	75	84	98	204
D-1	0	18	39	76	97
	30	26	48	81	115
	90	60	90	110	160
	180	104	258	352	365
	360	200	285	354	399
D-2	0	66	69	110	176
	30	163	225	1100	1820
	90	210	390	830	1680
	180	98	167	360	530
	360	84	136	166	204

carbon during *in situ* oxidation of contaminated soils. In this regard, Miller et al. [1] reported that Fenton's treatment of pendimethalin-contaminated silty loam and silty clay loam significantly increased the amount of biodegradable carbon compared to the amount in uncontaminated soil.

3.5. Decomposition of hexadecane by integrated ozonation and biodegradation

During *in situ* ozonation, the decomposition efficiency of hexadecane was evaluated for different soil textures (Fig. 7). As can be seen, a sharp decrease of hexadecane within 30 min of ozonation, followed by an asymptotic decrease was observed for all soil samples. In this regard, Jung et al. [7] previously described the decomposition of organic chemicals as a two-step process in *in situ* ozonation. The rapid reaction of ozone with soil ozone consuming materials, such as SOM and soil metal oxides, as well as the contaminant itself is responsible for the initial rapid decrease of hexadecane concentration, because catalytic reaction between ozone and ozone consuming materials produces hydroxyl radicals, which are known as stronger oxidant than ozone [3]. In addition, the heterogeneous distribution of contaminants on soil surfaces, the partitioning of contaminants into SOM, the entry of the contaminants into soil pores may also

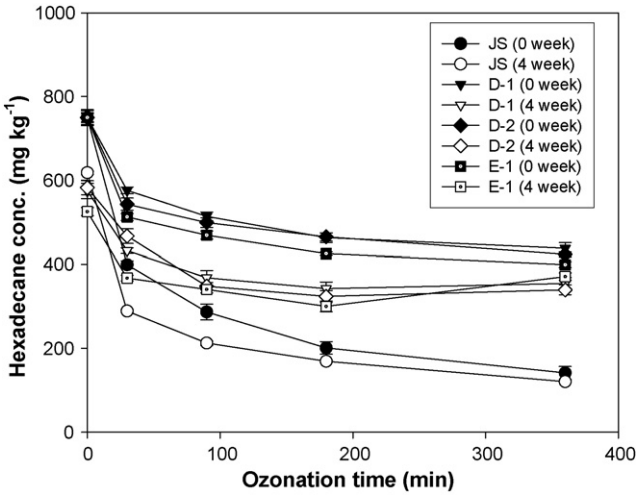

Fig. 7. Changes in hexadecane after different incubation time 0 and 4 weeks indicate the times when chemical analyses were performed during incubation.

be responsible for the asymptotical decrease of the contaminant [11].

In ozonation, the oxidation rate of hexadecane was also strongly affected by soil texture. For example, sand exhibited the highest oxidation rate of hexadecane in the soil samples, whereas D-1 and D-2 showed the lowest oxidation rate (Fig. 7). The low SOM content, surface area, and high porosity of sand seems to be responsible for the relatively high removal rate. As previously mentioned, organic contaminants trapped in pores filled with soil water and partitioned into SOM cannot be easily accessed by ozone and free radicals. Therefore, soil samples with high surface area and SOM content, and low porosity seem to show low oxidation rates of hexadecane. As such, SOM could play a significant role as a free radical scavenger.

Hexadecane concentration in the ozonated soil samples was decreased after 4 weeks of incubation (Fig. 7). This result suggests that biodegradation combined with *in situ* ozonation could enhance the removal of organic chemicals in the remediation of soils. However, the soil samples treated for 360 min showed only minor changes in the hexadecane concentration after 4 weeks of incubation, possibly due to the reduced numbers of indigenous microorganisms.

Ahn et al. [18] reported that there was an optimal ozonation time for enhancing further biodegradation. They concluded that 180 min of ozonation followed by 9 weeks of incubation for diesel-contaminated silt clay exhibited the lowest concentration of total petroleum hydrocarbon (TPH). In this study, the optimal ozonation time for each soil sample was observed in order to maximize the subsequent biodegradation. For E-1, D-1, and D-2, the optimal ozonation time was 90–180 min, consistent with the results of studies conducted by Jung et al. [11]. The samples also showed the highest regrowth rate of indigenous microorganisms during the incubation period. Therefore, it could be concluded that 90–180 min of ozonation was the optimal time for ozone injection to enhance the subsequent biodegradation in unsaturated soils with the exception of sand.

The results of this study clearly demonstrated that *in situ* ozonation followed by biodegradation increased the removal of organic contaminant, and identified the optimal ozonation time to enhance subsequent biodegradation. However, care should be taken in making the decision whether to apply *in situ* ozonation or the integrated chemical–biological process, based on the characteristics of soils; mainly SOM content and soil texture.

4. Summary and conclusions

Eight different soils were collected and treated with gaseous ozone to investigate the influence of soil properties, such as SOM and soil texture, on the survival and regrowth of indigenous microorganisms. The treated soils were incubated for 4 weeks in order to delineate the characteristics of microbial population dynamics. The following conclusions have been drawn based on the experimental results and discussion:

- Indigenous microorganisms are sensitive to ozone in the soil column experiments. The largest increase in microbial fatality was observed within the first 30 min of ozone injection. In the same soil texture, the microbial fatality showed a linear relationship with the SOM content with the Pearson's correlation coefficient (r) of -0.95, whereas water content was poorly correlated with an r value of 0.23. The effect of soil texture on the microbial fatality was also clearly observed; the microbial fatality was determined to be higher in larger mean particle size of the soil samples.
- In ozone-treated soil samples, the regrowth of indigenous microorganisms was inversely proportional to the ozonation time; the population of indigenous microorganisms for soil samples ozonated for 360 min showed a minor increase after 4 weeks of incubation, whereas around one order of magnitude increase was observed in soil samples treated for 30 min. In the same soil texture, the regrowth was significantly influenced by SOM content; however, the effect of soil texture on the regrowth of indigenous microorganisms was not clear in the experiments.
- The oxidation of hexadecane was highly affected by the particle size distribution, which represented soil texture. Sand exhibited the highest oxidation rate of hexadecane, resulting from low SOM content, water content, and surface area. It was determined that 90–180 min of ozonation was the optimal time for ozone injection that would enhance subsequent natural attenuation in unsaturated soils, with the exception of sand.

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