BIOREMEDIATION OF OCDF-CONTAMINATED SOILS BY NOVEL BACTERIAL STRAIN

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Abstract. In this study, effectiveness of using *Pseudomonas mendocina* NSYSU (*P. mendocina* NSYSU) on the bioremediation of octachlorinated dibenzofuran (OCDF)-polluted soils was evaluated through microcosm experiments and gene analys is under anaerobic conditions. The major tasks were to investigate the (1) feasibility of enhancing anaerobic OCDF dechlorination by *P. mendocina* NSYSU and lecithin, and (2) functional genes for the anaerobic biodegradation of OCDF. Results show that *P. mendocina* NSYSU was able to degrade OCDF through the anaerobic dechlorinating mechanisms. Results show that lecithin was used as the carbon source and OCDF desorption additive during the dechlorination of OCDF. Lecithin supplement could enhance the biodegradation rate of OCDF. Up to 68 and 61% of OCDF was removed after a 64-day operation with lecithin and nutrient broth supplement, respectively. Results indicate that primary substrate supplement is required for the enhancement of reductive dechlorination of OCDF. Five functional genes encoding the hydrolase in *P. mendocina* NSYSU were identified. The detected specific genes played important roles in OCDF dechlorination. Results reveal that a bioremediation system using *P. mendocina* NSYSU as the inocula would be a cost-effective and acceptable remedial system to remediate furan-polluted soils.

Keywords: *microcosm; octachlorinated dibenzofuran (OCDF); Pseudomonas mendocina NSYSU; soil bioremediation; soil contamination*

Introduction

Polychlorinated dibenzofuran (PCDF) isomers, which usually produce thermal processes, have been classified as the mutagens and carcinogens (Coutinho et al., 2015; Squadrone et al., 2015). Incinerators and boilers have been considered as the major causes of PCDFs production after waste burnings (Wittsiepe et al., 2015; Pongpiachan et al., 2016; Kruse et al., 2014; Klees et al., 2015). The production of PCDFs causes ecosystem and environmental media contamination (Shin et al., 2016; Wu et al., 2014; Hoogenboom et al., 2015; Yang et al., 2015). Different furan isomers including octachlorinated dibenzofuran (OCDF, a highly chlorinated furan), have been observed in different environmental media (e.g., sediments, soils) in many industrialized areas (Urban et al., 2014; Govindan and Moon, 2015).

As a result of their hydrophobic and xenobiotic nature, OCDFs are usually very persistent in ecosystems (Liu et al., 2014; Zhao et al., 2015). Due to their highly adsorptive, less biodegradable, and highly toxic natures, the remediation of PCDF-polluted media (e.g., soils, sediments) can be a necessity but costly (Zhao et al., 2015; Anasonye et al., 2014). Compared to physical and chemical remedial methods, the

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biological method can reduce the cleanup cost for the PCDF-polluted sites if significant amounts of media need to be remediated (Vallejo et al., 2015; Tue et al., 2016). The microbial species involved in bioremediation technologies include aerobic and anaerobic processes depending on the nature of contaminant and microorganisms (Megharaj et al., 2014). Compared to aerobic process, anaerobic bioremediation of PCDF-polluted media can be more energy saving, and thus, the operation and maintenance can be reduced (Hanano et al., 2014; Chen et al., 2016; Futagami et al., 2008; Lai and Becker, 2013; Liu et al., 2013).

During the reductive dechlorinating process, the perchlorinated substances act as terminal electron acceptors, which are a process of dehalorespiration (Liu et al., 2013; Zhen et al., 2014). The perchlorinated substances are degraded to less-chlorinated byproducts by anaerobic bacteria through dechlorinating mechanisms (Liu et al., 2014; Seshadri et al., 2005; Narihiro et al., 2010; Chen et al., 2013). There are two modes of reductive dehalogenation, called hydrogenolysis and dichloroelimination, but the biological process takes place mostly as the hydrogenolytic reaction (Mohn and Tiedje, 1992; Dolfing, 2003; Hiraishi, 2008; Urbaniak, 2013; Jeon et al., 2016). Many microbial strains apply contaminants as electron acceptors during dehalorespiration mechanisms under anaerobic processes (Nam et al., 2008; Bunge and Lechner, 2009; Ahn et al., 2005).

Stimulation of intrinsic microbial consortia of dehalogenating bacteria for chlorinated compounds biodegradation is a promising technology for the remediation of chlorinated compound-contaminated soils or sediments (Fennell et al., 2011; Kuokka et al., 2014a). Microbial species, which are related to *Dehalococcoides*, could motivate the reductive dechlorinating mechanisms to biotransform chlorinated furans to less-chlorinated furans (e.g., di or monochlorinated furans) (Zhen et al., 2014; Urbaniak, 2013; May et al., 2008). Biological dechlorinating process could be a feasible mechanism to reduce the toxicity and concentration of furan isomers (Liu et al., 2014; Bunge et al., 2003). Researchers reported that the reductive dechlorination activated by specific bacteria is a potential technique to dechlorinate highly halogenated furans (Narihiro et al., 2010; Kuokka et al., 2014b). In this process, gene analysis needs to be performed to investigate the feasibility of applying reductive dechlorination for PCDF degradation via assessing existence of specific and functional genes (Hanano et al., 2014; Liu et al., 2013; Bunge et al., 2003; Yoshida et al., 2005).

Supplement of carbon sources for the use of primary substrates is necessary to enhance the reductive dechlorinating mechanisms (Liu et al., 2014; Zhen et al., 2014; Kuokka et al., 2014a). Lecithin is an amphoteric emulsifier and rhizosphere phytogenic surfactant, which has relatively lower water solubility. Lecithin can serve as emulsification reagent, and depending on environmental conditions, its phospholipids can form liposomes or micelles (Abbasi and Radi, 2016; Miller, 2016). Thus, lecithin becomes an amphipathic biosurfactant, and also a good candidate of primary substrate during the reductive dechlorination of PCDF. Lecithin could enhance the bioaffinity and bioavailability of contaminant to bacteria after injection, and thus, the contaminant biodegradation rate could be enhanced (Paria, 2008; Schacht et al., 2016).

Currently, the information related to the biodegradation of higher chlorinated PCDFs under anaerobic conditions is limited (Liu et al., 2014; Kuokka et al., 2014a; Tu et al., 2014). A pentachlorophenol (PCP)-biodegrading bacterial strain, *Pseudomonas mendocina* NSYSU (*P. mendocina* NSYSU), was isolated from PCP, dioxin and furan-contaminated soils (Kao et al., 2005). The site was also polluted by OCDF with concentrations up to 10.8 mg/kg (NSC, 2012). In this study, a biodegradation study was performed to assess if *P. mendocina* NSYSU could bioremediate OCDF-polluted soils in an anaerobic system. The major tasks of this study were as follows: (1) evaluation of the feasibility of improving OCDF biodegradation by *P. mendocina* NSYSU under anaerobic conditions, (2) evaluation of the potential of using lecithin as the primary substrate for OCDF dechlorination, and (3) assessment of the existence of functional genes for the anaerobic OCDF biodegradation.

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Materials and Methods

Incubation of P. mendocina NSYSU

P. mendocina NSYSU culture was incubated in the anaerobic nutrient broth (NB) (Difco 003-01). The components of the NB solution included the following: yeast extract 1.5 g/L, beef extract 1.5 g/L, peptic digest 5 g/L, and NaCl 5 g/L. The peptic digest and beef extract in NB contained carbon and amino acids, which could be used for microorganisms as the primary substrates. The *P. mendocina* NSYSU solution was cultured at 200 rpm for 48 h in a 50 mL flask (sealed with butyl rubber stopper) at 20°C under anaerobic conditions (purged with 100% N_2). Density of the bacteria was analyzed by the spectrophotometer (Hach Co., USA).

Batch study

The biodegradability of OCDF under anaerobic conditions was investigated in the batch microcosm study. Soils from the OCDF-polluted site were situated in southern Taiwan and *P. mendocina* NSYSU were used as the inocula. In this study, lecithin, which was used as the primary substrate and solubilisation reagent, was supplied in the microcosms. Each batch bottle contained 1 mL of lecithin (or 1 mL of mineral medium solution), 20 g of site soils, 5 mL of *P. mendocina* NSYSU solution as inocula (or 5 mL of mineral solution), and 35 mL of nutrient mineral medium (autoclaved before use) in a 70-mL serum bottle, which was sealed with Teflon-lined rubber septa. The components of nutrient mineral solution (no carbon component) were described in Tu et al. (2014). The procedures of anaerobic microcosm preparation were described in Tu et al. (2014).

Table 1 lists the constituents of different microcosms. Group A was dead control group containing 500 mg/L NaN_3 and 250 mg/L $HgCl_2$, and the soils were autoclaved before use. Group B was live control group containing OCDF-polluted soils and *P. mendocina* NSYSU, but no NB addition. Group C was also live control group containing OCDF-polluted soils and NB, but no *P. mendocina* NSYSU addition. Dead (Group A) and live controls (Groups B and C) were prepared to assess the effects of NB and inocula addition on OCDF removal.

Table 1. *Components of seven groups of microcosms.*

Microcosm	Inocula	Components
A (Dead control)	Sterilized soils	Sterilized OCDF-contaminated soils + nutrient medium solution + NB medium + 250 mg/L $HgCl_2$ + 500 mg/L NaN_3
B (Control-no NB)	Soils + *P. mendocina* NSYSU	OCDF-contaminated soils + nutrient medium solution + *P. mendocina* NSYSU (no NB medium)
C (Control-no strain)	Soils	OCDF-contaminated soils + nutrient medium solution + NB medium
D	Sterilized soils + *P. mendocina* NSYSU	Sterilized OCDF-contaminated soils + nutrient medium solution + *P. mendocina* NSYSU + NB medium
E	Sterilized soils + *P. mendocina* NSYSU	Sterilized OCDF-contaminated soils + nutrient medium solution + *P. mendocina* NSYSU + lecithin
F	Soils + *P. mendocina* NSYSU	OCDF-contaminated soils + nutrient medium solution + *P. mendocina* NSYSU + NB medium
G	Soils + *P. mendocina* NSYSU	OCDF-contaminated soils + *P. mendocina* NSYSU + nutrient medium solution + lecithin

Group D microcosms contained sterilized soils and *P. mendocina* NSYSU, and Group E microcosms contained sterilized (autoclaved) soils, *P. mendocina* NSYSU, and one gram of lecithin. Group F microcosms contained unsterilized soils and *P. mendocina* NSYSU, and Group G microcosms contained unsterilized soils, *P.*

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mendocina NSYSU, and one gram of lecithin. *P. mendocina* NSYSU was incubated anaerobically in NB solution and the microcosms were operated at room temperature (20ºC). Duplicate samples were analyzed for OCDF concentrations for each sampling event. The degradation efficiency of OCDF was calculated as a percentage of the concentration on day 0. The procedures for *P. mendocina* NSYSU incubation, soil extraction procedures, and OCDF analytical methods were described in Tu et al. (2014).

PCR/DGGE analysis and gene identification

Soil DNA extraction and the PCR amplification process were conducted using procedures in Baldwin et al. (2003) and Shrestha et al. (2010). Microcosm soils were applied for the PCR analyses to determine the bacteria in charge of the biodegradation of OCDF. Soil DNA extraction and the PCR amplification process were conducted using procedures in Ritalahti et al. (2006) and Shrestha et al. (2010). The primer sets were used to amplify genes encoding the dehalogenase of *P. mendocina* NSYSU (2011). The primer sets are listed in *Table 2*. The PCR-amplified products were sequenced, and the sequences were investigated by the alignment search tool for the determination of relatives in the GenBank (Yanru et al., 2005). The amplified PCR was also used for the conduction of denaturing gradient gel electrophoresis (DGGE) to evaluate the bacterial species and dominant bacteria. The DGGE procedures were described in Yanru et al. (2005).

Results and Discussion

In the batch microcosm study, OCDF-polluted soils (OCDF concentration = 10.8 mg/kg) collected from the studied site were used for the remedial investigation. *Fig. 1* presents the remained OCDF in Groups A to G microcosms during the 64-day incubation period. No significant OCDF removal [approximately 2% (Group A) to 6% (Group B) removal] was observed in control-no NB group (Group B), control-no strain (Group C), and dead-control group (Group A). The results reveal that when the soil bacteria were used as the inocula, effective OCDF biodegradation was not observed. This could be due to the fact that the furan-degrading microbial species were not the predominant bacteria in soils. Therefore, inoculation of specific bacteria would be required to improve the efficiency of OCDF biodegradation. Results show that the energy and carbon supplements were necessary to enhance the anaerobic dechlorination. Results demonstrate that OCDF could not be used as the carbon source by *P. mendocina* NSYSU or soil bacteria. Slight decrease in OCDF concentration in Group B (control-no NB) batch bottles was because natural organic carbon was consumed by *P. mendocina* NSYSU for primary substrate.

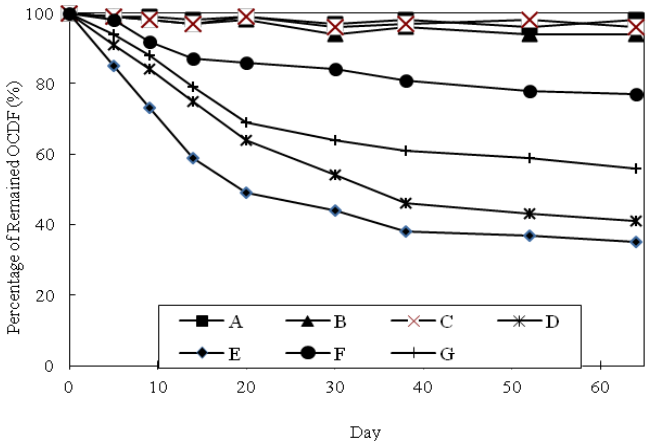

Figure 1. *The remained OCDF in microcosms during the 64-day incubation period.*

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In Group D bottles (sterilized soils with *P. mendocina* NSYSU addition), significant drop of OCDF was observed and about 59% of OCDF was degraded after 64 days. Results demonstrate that efficient OCDF biodegradation could be obtained anaerobically by *P. mendocina* NSYSU with the supplement of NB. The NB media had components of beef extract and peptic digest, which could be used as the carbon sources by *P. mendocina* NSYSU. Thus, OCDF could be dechlorinated through reductive dechlorination mechanisms using beef extract and peptic digest as the carbon sources. In Group F batch bottles (non-sterilized soils with *P. mendocina* NSYSU addition), relatively lower efficiency OCDF degradation (23%) was detected. Results might be due to the fact that indigenous bacteria competed the supplied carbon sources with *P. mendocina* NSYSU resulting in the decreased efficiency of OCDF removal.

Table 2. *Primer sets of genes encoding the dehalogenase of P. mendocina NSYSU.*

Target Gene	Primer[1]
Pmen_0263	F:5'-G GCCGGCGACGACATCATCACCGACA
	R:5'- CCCAGTCGAAGCCGGAACCACCGAC
Pmen_1621	F:5'- GCGTGCCGCGCTGCACGGATG
	R:5'- ACTCACGCAACACCGACAGCGGCTG
Pmen_3718	F:5'- AGGCTGCGGCCAAGCATGCCTGGCT
	R:5'- AGCTGACCGAGCTGCATGCGCAGGA
Pmen_4219	F:5'- GGCAAGCTGGACGTGGTGGCCTA
	R:5'- CGTTCGAGGGCGGTGGCGCGCAGTA
Pmen_4457	F:5'- CTCGCCGAGTAGCTCCTCGCGGCTA
	R:5'- ATCGACGAGCCGGATCTGACGGCGC

[1]Forward (-F) and reverse (-R) primers are indicated.

In Group E bottles (sterilized soils with *P. mendocina* NSYSU and lecithin supplement), about 65% of OCDF degradation was detected. Compared to results from Group D, lecithin supplement would improve the efficiency of OCDF degradation. The phospholipid structure in lecithin could form micelles, liposomes, and lamellar, and it is classified as amphipathic. Therefore, lecithin would enhance the affinity of OCDF to *P. mendocina* NSYSU, which results in the OCDF biodegradation efficiency.

In Group G microcosms (non-sterilized group with *P. mendocina* NSYSU and lecithin supplement), about 44% of OCDF drop was detected. Although lecithin supplement could enhance the OCDF solubilization, *P. mendocina* NSYSU was not the dominant microbial species in the system, and thus, indigenous soil bacteria would complete the carbon sources with *P. mendocina* NSYSU. This would result in the decrease in OCDF degradation rate. In this study, biodegradation byproducts were not observed (data not shown), which implies that produced byproducts might be biodegraded by *P. mendocina* NSYSU or other soil bacteria in non-sterilized groups.

Fig. 2 presents the DGGE patterns for soil samples collected from Group D bottles. The DGGE patterns show that the sterilized soil contained relatively lower bacterial diversities. Results also indicate that *P. mendocina* NSYSU was the dominant bacterial species after soil sterilization. Results reveal that the supplied *P. mendocina* NSYSU played a key role in OCDF biodegradation. *Fig. 3* presents the DGGE patterns for soil samples collected from Group D bottles. The DGGE patterns show the non-sterilized soils contained higher bacterial species, and this could be because of the abundant indigenous bacterial diversities. Therefore, *P. mendocina* NSYSU was not the dominant bacterial strain in the microcosms.

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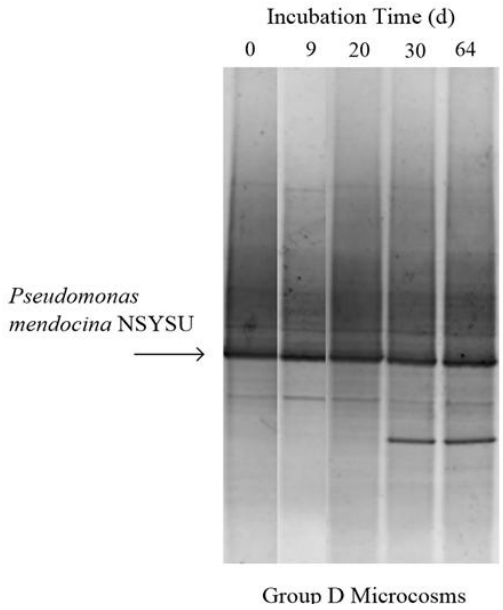

Figure 2. *DGGE patterns for soil samples collected from Group D microcosms during the 64-day incubation period.*

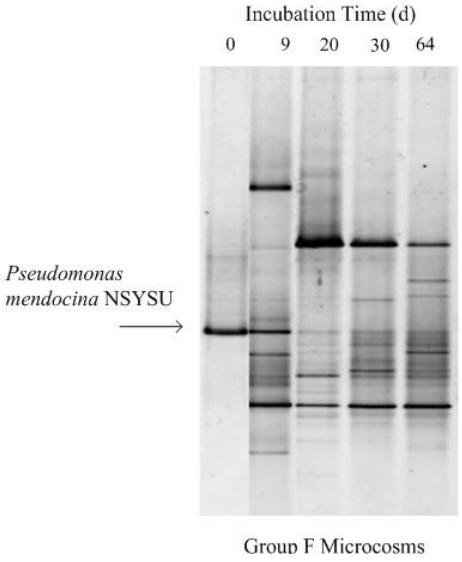

Figure 3. *DGGE patterns for soil samples collected from Group F microcosms during the 64-day incubation period.*

Results from *Figs. 2-3* show that *P. mendocina* NSYSU bands in the DGGE pattern became less notable after 40 days of operation. This resulted in the decreased OCDF removal efficiency in the latter part of the study (*Fig. 1*). Results indicate that periodical inoculation of *P. mendocina* NSYSU to maintain a higher *P. mendocina* NSYSU population is required to obtain a high OCDF removal efficiency.

The occurrence and performance of functional genes of *P. mendocina* NSYSU were investigated for furan biodegradation. Five functional genes encoding the dehalogenase in *P. mendocina* NSYSU were determined (Genes 1-5). These functional genes are

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presented in *Table 2*. Results reveal that the anaerobic soils (Group F) contained five different *P. mendocina* DNA (five HAD family hydrolases) (*Table 3*). *Fig. 4* presents the gel of PCR-amplified fragments for *P. mendocina* NSYSU and five HAD family hydrolases from Group F microcosm soils. Results demonstrate that the functional genes were in charge of biodegrading furan compounds under reductive dechlorinating mechanisms.

Table 3. *Identified genes encoding the the dehalogenase of Pseudomonas mendocina NSYSU*

Gene No.	Gene name	Gene length (bp)	Gene description	Similarity (%)
Gene 1	Pmen_0263	699	HAD family hydrolase[1]	95
Gene 2	Pmen_1621	693	HAD family hydrolase	97
Gene 3	Pmen_3718	591	HAD family hydrolase	95
Gene 4	Pmen_4219	651	HAD family hydrolase	97
Gene 5	Pmen_4457	645	HAD family hydrolase	96

[1]HAD family hydrolase: haloacid dehalogenase-like hydrolases.

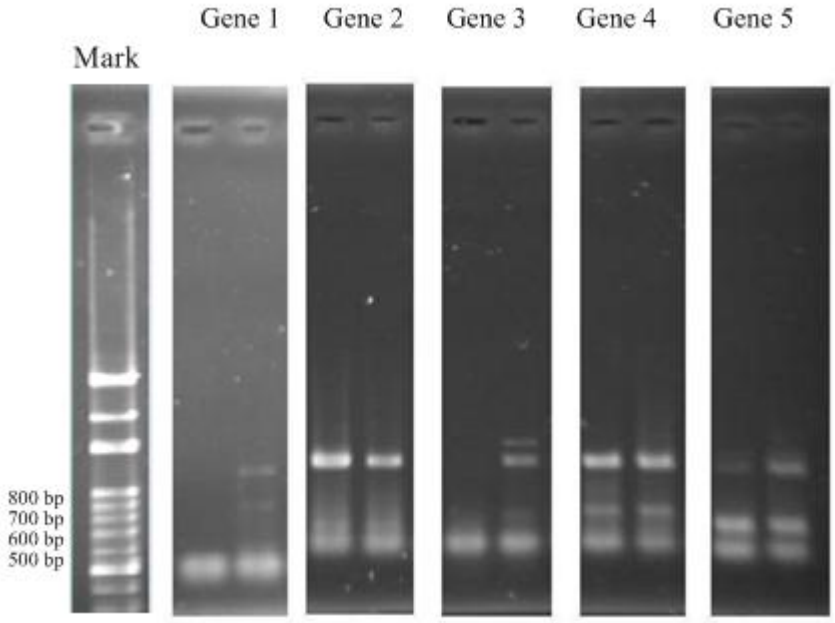

Figure 4. *Gel showing the PCR-amplified fragments for genes encoding the dehalogenase of Pseudomonas mendocina NSYSU extracted from the soil samples on day 0 and day 40; kb = kilobase*

Conclusions

This study was conducted to evaluate the capability of *P. mendocina* NSYSU on the anaerobic biodegradation of OCDF. The dehalogenase genes, which had significant contributions to the reductive dechlorination of OCDF in *P. mendocina* NSYSU were investigated. Under reductive dechlorinating conditions, *P. mendocina* NSYSU had the capability to biodegrade OCDF. Lecithin could be used as the primary substrate and

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solubilization reagent resulting in increased solubilization and anaerobic biodegradation of OCDF. The removal efficiencies for OCDF reached 65 and 59% in microcosms with and without the supplement of lecithin under anaerobic conditions, respectively.

PCR and DGGE results demonstrate that *P. mendocina* NSYSU was the dominant microbial species in sterilized soils during the operational period. Thus, *P. mendocina* NSYSU played a key role in OCDF degradation after soil sterilization. Under anaerobic conditions, five genes encoding the dehalogenase in *P. mendocina* NSYSU were identified, which were in charge of furan biodegradation. Results demonstrate that OCDF could not be used as the carbon source for *P. mendocina* NSYSU and indigenous soil bacteria under reductive dechlorinating conditions. Therefore, the addition of an appropriate substrate was required to enhance the OCDF biodegradation. Results reveal that an on-site bioreactor or in situ bioremediation using *P. mendocina* NSYSU as the inocula would be a cost-effective and acceptable remedial system to remediate furan-polluted soils.

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