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Influences of 1,2-dichlorobenzene on Bacterial Community Structure in Wetland Soil

(Pengaruh 1,2- diklorobenzena pada Struktur Komuniti Bakteria di Tanih Tanah Bencah)

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ABSTRACT

In order to explore the response of the soil microbial community to 1,2-dichlorobenzene exposure in Wetland soil, a short-term (7 weeks) mesocosm study was conducted at four 1,2-dichlorobenzene concentrations of (100, 400 and 800) μg g^{-1}. Dry soil, sterile and unsterile controls were also compared. The results obtained showed a significant effect of 1,2-dichlorobenzene on the development of bacterial populations in soils contaminated with different concentrations of 1,2-dichlorobenzene at the early time after application. In general, however, the number of populations of the same soil sample treated with the same concentration of 1,2-dichlorobenzene differed significantly with the increasing incubation time within the early 5 weeks. The scale of differences in banding patterns-showed that the microbial community structures of 1,2-dichlorobenzene-treated and non-1,2-dichlorobenzene-treated soils were not significantly different after 7 weeks of incubation. DNA in application-responsive bands from the 1,2-dichlorobenzene treatments was recovered and amplified using the universal primers. PCR products were recovered and cloned into pGEM-T Easy (Promega) and two clones were obtained. The two clones were sequenced using the automated Model 3730 DNA sequencing system. The two cloned sequences had very high similarities to an uncultured bacterium reported previously in the database of NCBI.

Keywords: Bacterial community; denaturing gradient gel electrophoresis(DGGE); wetland soil; 1,2-dichlorobenzene

ABSTRAK

Dalam usaha untuk mengkaji sambutan komuniti mikrob tanah pendedahan 1,2-diklorobenzena di tanih Tanah Bencah, satu kajian mesokosma jangka pendek (7 minggu) telah dijalankan pada empat kepekatan 1,2-diklorobenzena daripada (100, 400 dan 800) μg g -1. Tanah kering, kawalan steril dan tidak disteril juga dibandingkan. Keputusan yang diperoleh menunjukkan kesan bererti 1,2-diklorobenzena kepada pembangunan populasi bakteria dalam tanah yang tercemar dengan kepekatan 1,2-diklorobenzena yang berbeza pada awal selepas penetapan. Secara umum, bagaimanapun, bilangan populasi sampel tanah yang sama dirawat dengan kepekatan yang sama daripada 1,2-diklorobenzena berbeza jelas dengan peningkatan masa pengeraman 5 minggu pertama. Skala perbezaan pola banding menunjukkan bahawa struktur komuniti mikrob tanah 1,2-diklorobenzena dirawat dan 1,2-diklorobenzena tidak dirawat tidak berbeza secara ketara selepas 7 minggu pengeraman. DNA dalam band aplikasi responsif daripada rawatan 1,2-diklorobenzena telah pulih dan dikuatkan menggunakan primers sejagat. Produk PCR telah pulih dan diklon ke pGEM-T Mudah (Promega) dan dua klon diperoleh. Kedua-dua klon telah disusun menggunakan sistem penjujukan DNA Model automatik 3730. Kedua-dua jujukan klon mempunyai persamaan yang sangat tinggi kepada bakteria tidak berkultur yang dilaporkan sebelum ini dalam pangkalan data NCBI.

Kata kunci: Komuniti bakteria; penyahaslian kecerunan gel elektroforesis (DGGE); tanah bencah; 1,2-diklorobenzena

INTRODUCTION

The functioning of modern society is highly dependent on artificial chlorinated organic chemicals, such as chlorobenzenes, which are produced in large quantities as chemical intermediates and in the manufacture of degreasers, solvents and deodorants. Chlorobenzene compounds are widely used in various industries. During the manufacture and application, chloroaromatic compounds are inevitable to release into the environment that may cause harmful effect on the environment and human health (Bunge et al. 2003). Their prolific use inevitably results in increasing quantities being released into the environment (Thompson et al. 2009). Chlorobenzenes are chemically stable and resistant to processes such as photolysis, so their removal from the environment is dependent on the degradative activities of microbial communities. Because of the potential of microorganisms for the prevention and reversal of environmental contamination, their ability to catabolize substituted benzenes, such as 1,2-dichlorobenzene(1,2-DCB), has been extensively studied and documented (Kumagai & Matsunaga 1997; Nishino et al. 1996). However, most of these studies have investigated the interactions and degradative pathway of single organisms in flask-based liquid systems. If the potential of microbial communities to remove contaminants from soil, either

naturally or as part of a wasted contamination system, is to be realized and effectively exploited, more needs to be known about the ability of soil microbial communities to respond to a range of concentrations of common organic pollutants, such as 1,2-DCB. Furthermore, the response of soil microbial communities to pollution stress also deserves greater study, as their potential as rapid indicators of environmental contamination and ecosystem response is enormous (Thompson et al. 1999). Yancheng Wetland, famous for its large areas of marshes, is surrounded by numerous chemical plants built in Yancheng (Jiangsu Province, China). A number of plants discharge 1,2-DCB effluents of low concentration into the Yancheng Wetland and the consequences thereof are of significant interest to regulators (Chen et al. 2010; Surhio et al. 2014).

Microorganisms play an important role in the balance and function of ecosystems. Recent scientific literature reports that soil microbes respond to anthropogenic disturbances (Pankhurst et al. 1997; Wolters & Schaefer 1994). Compared with other ecosystem components, soil microbial communities change more quickly with changes in environmental conditions (Kennedy & Stubbs 2006). Thus, changes in the abundance and diversity of microorganisms can serve as sensitive indicators of disturbances in ecosystems (Doran & Zeiss 2000; Kennedy & Smith 1995).

In the present paper, the results of a mesocosm study on changes in the soil microbial community due to 1,2-DCB contamination in an artificial wetland soil were reported. Fingerprinting of 16S rDNA fragments through denaturing gradient gel electrophoresis (DGGE) shows variations in the bacterial community structure of contaminated soils (Min et al. 2001). Additional quantitative studies of bacterial communities using the enumeration method showed that the number of aerobic bacteria is also affected by pollutants (Lü et al. 2003). The bacterial community is affected by the chemical condition of the soil and is related to microbial activities (Min et al. 2001). Thus, the effect of 1,2-DCB on the biological activity (determined by monitoring polyphenol oxidase activity), abundance (measured by microbial enumeration) and diversity (examined by polymerase chain reaction-denaturing gradient gel electrophoresis, PCR-DGGE) of the soil microbial community was determined.

Materials and Methods

Soil and soil treatment

A short-term (7 weeks) mesocosm study was conducted using soils collected from the top layer in Yancheng Wetland, China. The wetland is located in a transit belt from the subtropics to a warm temperate zone, characterized by a subtropical humid monsoon climate with relatively high winter temperature. The sampling site was uncontaminated; thus, no 1,2-DCB was detected. Immediately after collection, the soils were air dried at 25°C for one week and sieved (2 mm). In order to distinguish the effects owing to 1,2-DCB alone from those owing to direct influences of the soil characteristics, a composite soil sample was prepared by thoroughly mixing 50 kg of each sample. Some basic soil properties of the soil sample are described in Table 1.

1,2-DCB used in the study was purchased from Sinopharm Chemical Reagent Co., Ltd, in Shanghai, China (purity>99%). The stock solution of 1,2-DCB was prepared at first and added to the bulk samples of soil (1.5 kg) to get the desired concentrations of 0, 100, 400 and 800 μg g^{-1} of the 1,2-DCB, respectively. In the present study on the effects of 1,2-DCB on soil microbial communities, a total of 12 mesocosms (3 replicates×4 treatments (unsterile control 100, 400 and 800 μg g^{-1} of the 1,2-DCB)) were used. The soil was incubated in the dark at 28°C±1°C. Fifty grams of soil were collected from each mesocosm using the three-point sampling method with a core sampler 1, 2, 3, 5 and 7 weeks after treatment. Immediately after sampling, the soils were homogenized, subsampled and stored at 4°C for the following analyses (Lü et al. 2003).

DNA extraction and PCR amplification

DNA was extracted from 5 g soil samples after incubation according to the method of Chen et al. (2010) and Zhou et al. (1996) with minor modification. Each about 0.30 g soil sample was vortexed at maximum speed for 15 min at room temperature with 0.15 g of glass beads, 675 μL of phosphate buffer (100 m mol L^{-1}, pH8.0) and 10 μL of lysozyme (20 mg mL^{-1}). Subsequently, 10 μL of proteinase K (10 mg mL^{-1}) and 75 μL of 20% (w/v) SDS were added to the mixtures and the samples were incubated at 65°C for 3 h with gentle mixing every 1 h. After 3 h of incubation, the samples were centrifuged at 4500 g for 10 min at room temperature. Macromolecule pellets were re-extracted with 225 μL of phosphate buffer (100 mmol L^{-1}, pH8.0) and 25 μL of 20% (w / v) SDS, vortexed for 20 s and incubated again at 65°C. After 30 min of incubation, the samples were centrifuged as above. The supernatants were pooled and mixed with an equal volume of chloroform-isoamyl alcohol (24: 1 (v/v)). The mixture was rotated gently at room temperature for 15 min. The aqueous phase was transferred to a new tube after centrifugation at 6000 g for 10 min. Absolute ethyl alcohol (1.0 mL) was added to the aqueous

TABLE 1. Main physicochemical properties of the soil tested

Organic matter ($g\ kg^{-1}$)	Total nitrogen ($g\ kg^{-1}$)	Total potassium ($g\ kg^{-1}$)	Total phosphors ($g\ kg^{-1}$)	pH
37.45±0.23	0.92±0.02	0.63±0.05	1.23±0.06	7.9±0.10

phase and the mixture was held at -20°C for 12 h. After centrifugation at 13000 g for 15 min, the pellets were washed with 70% ethanol, resuspended in 50 μL of pure water and stored at -20°C until further analysis. The extracted DNA was quantified using a spectrophotometer and analyzed by gel electrophoresis using dilutions of known amounts of k-DNA as the standard. DNA yields were determined according to DNA quantity and its weight in dried soil.

The V3 region of bacterial 16S rDNA was amplified using the primer pair described by Muyzer et al. (1993). The reactions were carried out using an Eppendorf thermocycler, applying an initial denaturation step at 94°C for 4 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 56°C for 30 s and elongation at 72°C for 45 s. The PCR mixtures (50 μL) contained 5.0 μL 10× PCR buffer (Takara, Dalian, China), 0.2 mmol L^{-1} dNTPs, 0.25 μmol L^{-1} of each primer, 1 U Ex Taq DNA polymerase (Takara, Dalian, China), and 1 μL extracted DNA solution (Chen et al. 2010).

DGGE ANALYSIS

An imaging system (Decode, Bio-rad, USA) was used for running the DGGE gels with 8% polyacrylamide in TAE buffer with a 45-60% denaturant gradient (Chen et al. 2010). In each lane of the gels, 20 μL of the amplified DNA solution was loaded. Separation of the amplicons was performed for 6 h at 60°C and 150 V. The gel was stained with AgNO3 and photographed using a transilluminator. The reamplified PCR products (-180 bp) were cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) and transformed into *Escherichia coli* JM109 as described by the manufacturer. Isolation of plasmids from *E. coli* was performed using standard protocols from the Qiagen (Valencia, Calif.) plasmid mini kit. The purified plasmids were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase, FS(Perkin-Elmer). These short fragments were compared to sequences in the Ribosomal Database Project (RDP) database.

Digitized image was processed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA) to measure relative band intensities. By carefully inspecting lane intensity curves, bands were detected manually and quantified (average peak intensity). Background intensity was subtracted (option: Rolling disc, size 10). The DGGE patterns were compared using arithmetic averages as the similarity coefficient and unpaired group method of analysis to generate the dendrogram. This method allowed the construction of dendrograms that show clustering trends among the soil samples analyzed.

RESULTS AND DISCUSSION

GENETIC FINGERPRINTING BY DGGE

PCR amplification of 16S rDNA fragments successfully generated 210 bp products visible as strong bands in the gel after electrophoresis (not shown). PCR products were separated by migration behavior in DGGE. Figure 1 shows the bacterial community structure and cluster analysis of soils amended with 1,2-DCB and non-1,2-DCB sampling after 1, 2, 3, 5 and 7 weeks, respectively. The difference in band intensity was presumed to indicate numerical differences between the target molecules. Differences in the DGGE gel patterns indicate that significant shifts in the bacterial community structure occurred in response to the perturbation. After 1,2-DCB application, the profiles of 1,2-DCB treated soils belonged to different clusters, respectively, according to the 1,2-DCB application concentration.

At the first week, the profiles of control showed 85.3% similarity with that of 100 μg 1,2-DCB g^{-1} dried soil, 69.6% similarity with that of 400 μg 1,2-DCB g^{-1} dried soil and 68.5% similarity with that of 800 μg 1,2-DCB g^{-1} dried soil (Figure 1(a)). Then two weeks later, the profiles of control showed 80.1% similarity with that of 100 μg 1,2-DCB g^{-1} dried soil, 76.2% similarity with that of 400 μg 1,2-DCB g^{-1} dried soil and 64.8% similarity with that of 800 μg 1,2-DCB g^{-1} dried soil (Figure 1(b)). The similarities between the profiles of control and the profiles of 1,2-DCB treated soil were significantly higher than that within the first week. The 1,2-DCB-treated soil profiles maintained high similarity to that representing untreated soil on the 3th week and decreased a little on the 5th week of the experiment (Figures 1(c) and 1(d)). At 7 weeks, the microbial communities in soils applied with all concentrations of 1,2-DCB were almost similar to that in the control soil, which can be seen directly without digital image analyses (Figure 1(e)).

The data presents an interesting conundrum about the different effects of various concentrations of 1,2-DCB. In the five-week samples the bands designated arrow are intense in the treated soils but absent in the control. This same situation also occurred in the 7-weeks samples. This result is somewhat anomalous since it might be expected that increases in the amount of 1,2-DCB would correlate in a progressive way with changes in band intensity.

It can be concluded from the anomalous banding patterns produced by different concentrations of 1,2-DCB that the microbial communities would not change proportionately with the time elapsed and concentration variation. Perhaps lower concentrations of some components of the mixture slightly inhibited the bacterial populations on the 2nd and 3rd week, while higher concentration of other components in the mixture reduced the inhibitory effect on the bacterial populations. Therefore, it seems that the bacterial populations were enhanced while a higher concentration of 1,2-DCB was applied. However, the inhibitory effect was stronger than the enhancement effect when 1,2-DCB application concentration was increased to 800 μg g^{-1} dried soil.

SPECIFIC BANDS TAPPING RECOVERY AND CLONING

In order to investigate the pattern differences in some detail, the marked bands were extracted, reamplified,

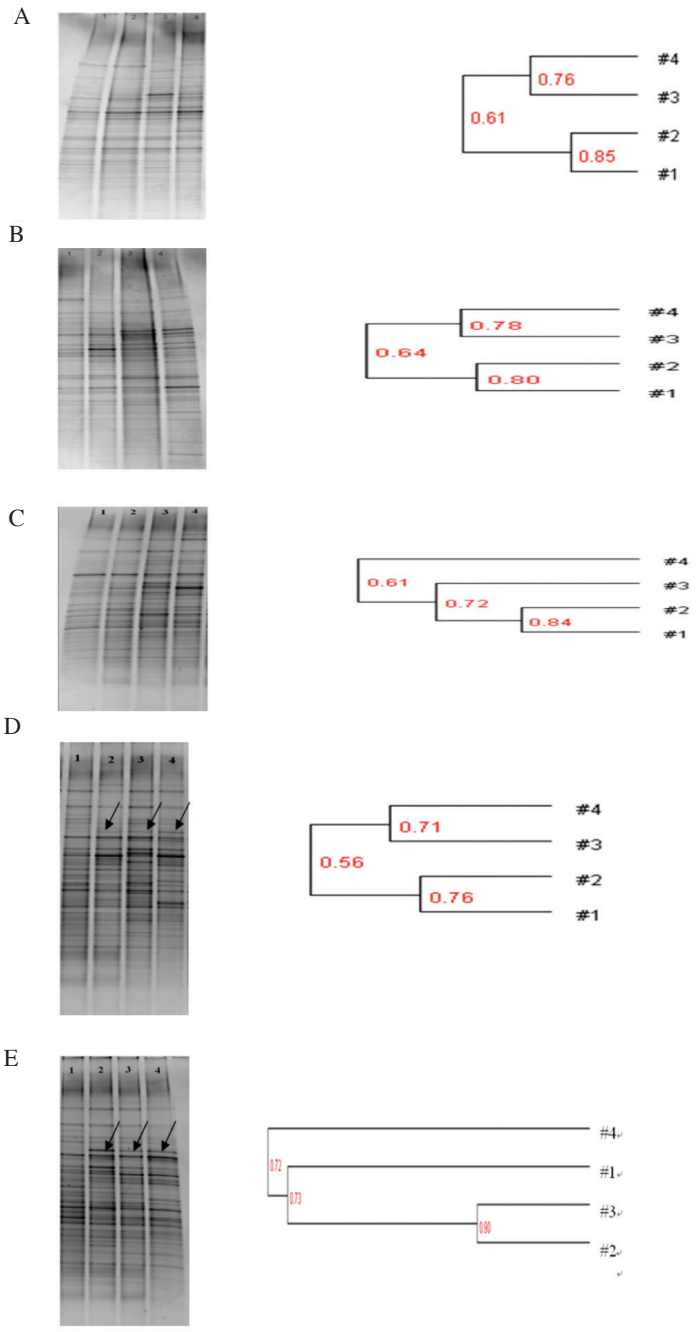

FIGURE 1. Denaturing gradient gel electrophoresis DGGE fingerprints and cluster analysis of amplified 16S rDNA gene fragments from soil treated with different concentrations of 1,2-DCB and non^{-1}, 2-DCB sampling over the course of incubation. Lanes: 1, control; 2, soil with 100 ug 1,2-DCB.g^{-1} dried soil; 3, soil with 400 ug 1,2-DCB.g^{-1} dried soil; 4, soil with 800 ug 1,2-DCB.g^{-1} dried soil. Soil measurements at A: 1 week of incubation; B: 2 weeks of incubation; C: 3 weeks of incubation; D: 5 weeks of incubation; E: 7 weeks of incubation (Qureshi et al. 2015)

cloned and sequenced as described in Materials and Methods section. The identities of cloned band were checked by DGGE separation. Sequences were identified using the BLAST database (http://www.ncbi.nlm.nih.gov).

The clones had a similarity of 96% to an uncultured bacterium sp. (FJ810734) within the 16S rDNA region analyzed. The strong similarity of the clone to the uncultured bacterium may correspond to the 1,2-DCB contaminated soil conditions, since the bacterium was one of the dominant community members for degrading hexachlorobenzene in the soil (Ibrahim et al. 2014; Tas et al. 2010). The bacteria in the soil were few in number, reach DGGE detection limit. It showed many of the bacterial communities in a 1,2-DCB-polluted soil are uncultured bacteria, which use conventional plate culture method that cannot be found. The strain was an uncultured

bacterium which can be enriched at suitable concentration of 1,2-DCB. It could be regarded as reporting gene to indicate the contamination of 1,2-DCB.

Despite the release of large quantities of pollutants into the environment, little is known of their effects of soil microbial communities, even though the productivity and sustainability of soils are dependent on their unimpaired activities. Furthermore, in the case of many organic pollutants, such as 1,2-DCB, the activities of microbial communities, represents the only means of their removal from the environment (Batool et al. 2015). There are many reports of the interaction of pollutants and exposed microbial communities; most of these have considered the effects of heavy metals on microbial processes, but there have been few investigations of the effects of organic pollutants on soil microbial diversity. Microbial communities are the most important components of soil and they are crucially involved in the fate of pollutants released into the environment (Hahn 2006). A microbial community can change both its biomass and composition in response to environmental change (Balser et al. 2002). Fingerprinting of 16S rDNA fragments through denaturing gradient gel electrophoresis shows variations in the bacterial community structure of contaminated soils (Min et al. 2001). Additional quantitative studies of bacterial communities using the enumeration method showed that the number of aerobic bacteria is also affected by pollutants (Ashraf et al. 2013; Lü et al. 2003). Bacterial community is affected by the chemical condition of the soil and is related to microbial activities (Min et al. 2001). The community diversity and metabolic potential can be used as an effective bioindicators of pollution stress and concentration effects (Thompson et al. 1999).

In one study, counts of culturable pseudomonads were actually stimulated by the midrange of 1,2-DCB concentrations and at the highest concentrations counts were similar to that of control soils (Thompson et al. 1999). The ability of microbial communities to tolerate high chlorobenzene concentration has previously been observed. In one study, concentrations of hexachlorobenzene up to 4,000 $mg.kg^{-1}$ were applied to the soil with no detectable impact on microbial biomass (Welp & Brummer 1997). Chlorobenzenes have low solubility in soil solution and their concentrations can be limited to the few micrograms per liter regardless of the total dose applied. Chemicals such as chlorobenzenes are taken up by soil microorganisms mainly from the liquid phase, so only a small proportion might have been available and in contact with soil communities and this might partially explain the high apparent tolerance to the concentrations added. Furthermore, substituted benzenes such as 1,2-DCB have strong affinities for components of the soil matrix, rendering them unavailable to extraction and to cause toxicity (Scheunert et al. 1985; Thompson et al. 1999).

Changing any of the factors that affect the microbial community induces selection pressure that, in time, changes the community. Hence, removing other factors that may affect the bacterial community in the soil is important in determining the changes caused by 1,2-DCB contamination (Zulkifley et al. 2014a, 2014b). In the present study, 12 experimental mescosms were prepared with homogenized soil, placed in the same chamber and managed under the same conditions. Hence, studying these mesocosms to examine the effect of 1,2-DCB on the abundance and diversity of microorganisms is reasonable. The conclusions drawn from the present work could reflect the actual effects of 1,2-DCB on microbial fluctuations.

Conclusion

Through different training time, different concentration on adjacent two chlorine pollution on soil microbial DNA by the wetland of genetic diversity DGGE molecular fingerprint analysis, We find that as training time changes, the treatment of soil microbial genes' diversity appear certain differences. The scale of differences in banding patterns-showed that the microbial community structures of the 1,2-dichlorobenzene-treated and non-1,2-dichlorobenzene-treated soils were not significantly different after 7 weeks of incubation.

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