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Microbial community structure during oxygen-stimulated bioremediation in phenol-contaminated groundwater

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Abstract

This research explored the changes in genetic diversity and spatial distribution of microbial communities in association with the changes in phenol concentration during a bioremediation process. Results using the traditional plate count method indicated an increase of average bacteria densities in groundwater from 10^4 to 10^7 CFU ml^{-1} initially to 10^7 to 10^9 CFU ml^{-1} after remediation. The diversity and stability of phenol-degrading bacterial communities were investigated by using single-strand-conformation polymorphism (SSCP) genetic profile analysis of 16S rDNA fragments amplified from groundwater samples. The molecular data showed a high degree of genetic similarity between communities from certain monitoring wells during the early phases of remediation, probably due to similar initial physical conditions among wells. Molecular signatures of several cultivated phenol-degrading bacterial strains could be seen in most groundwater profiles throughout the study period, suggesting that these strains were indigenous to the study site. It was also observed that the species diversity of these microbial communities increased as the phenol levels in the groundwater decreased during the 9-month study period, and recovered to the pre-treatment levels after the remediation program was completed.

Keywords: 16S rDNA; Groundwater; Microbial community; Phenol; Remediation; SSCP

1. Introduction

Due to past waste disposal practices in Taiwan, groundwater down-gradient of unlined landfills is frequently contaminated by leachate, which often leads to elevated concentrations of total phenols in the groundwater. One of the examples is the Wu-Jih site in central Taiwan, which has been designated as a groundwater contamination control site by the EPA since 2002. In March 2004, Main-Link Engineering Consultants Inc. (ML Inc.), contracted by the Taichung County Bureau of Environmental Protection, began conducting an on-site investigation which was to be followed and accompanied by an *in situ* remediation procedure. A combination of pump-and-treat fluidized bed reactors and oxygen enrichment in the monitoring well for *in situ* bioremediation was utilized. The primary objective of the treatment program was to reduce the phenol level to below 0.14 mg l^{-1} of total phenols, a level required by the regulatory guidelines to allow off-site disposal of low-level contaminated groundwater.

Phenols are common industrial pollutants generated by biodegradation of aromatic hydrocarbons under anaerobic conditions [1]. Therefore, remediation sites, contaminated with wastes such as BTEX (benzene, toluene, ethylbenzene, xylene) pose a potential threat to groundwater by producing the more soluble phenols. The relatively high water solubility of phenols also contributes to their high acute toxicity to a wide range of organisms.

Extensive research conducted in laboratory and field experiments for phenol biodegradation has shown that phenols in groundwater can be biodegraded under suitable conditions [2–4]. However, relatively little is known about the structure of a microbial community during aerobic phenol degradation. Most microorganisms in soil and groundwater samples cannot be detected by using cultivation-based methods [5,6]. As a result, conventional analyses of microorganism are unable to identify the species and the composition of the microbial community

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responsible for a successful treatment. Hence, it is necessary to utilize molecular biotechnology approaches to provide clues concerning the types of bacterial that may be involved in aerobic phenol degradation and to determine the factors affecting the growth and decline of these species.

The development of methods for exploring the microbial community dynamics during *in situ* bioremediation has received increased attention in recent years. Single-strand-conformation polymorphism (SSCP) is one of the frequently used methods, being inexpensive and highly sensitive [7,8]. In recent years SSCP has been applied extensively to detect a variety of environmental microbial communities. For instance, Stach et al. [9] demonstrated that the SSCP analysis can be used to evaluate the effectiveness of various DNA extraction and purification methods for soil samples. Balcke et al. [10] used PCR-SSCP (polymerase chain reaction) and UPGMA (unweighted pair-group method of arithmetic averages) statistics to evaluate the changes in a microbial community under alternating aerobic–anaerobic and various pH conditions. Dassonville et al. [11] used the SSCP technique to examine microbial dynamics in an anaerobic soil slurry and analyzed how the microbial dynamics depended on geochemical processes.

In this study, the surveillance data of total phenolic concentration at the Wu-Jih site, as measured by ML Inc., were compared with the results of both SSCP analyses and traditional quantitative plating studies for a better understanding of the structure and dynamics of indigenous microbial populations during the remediation treatment. The data should provide new insights into the biological processes underlying the successful removal of phenolic compounds in terms of structural evolution and adaptation of these microbial communities and their member species. These investigations also examined the feasibility of applying molecular biotechnology as a complementary measure for assessing *in situ* bioremediation in the future.

2. Materials and methods

2.1. Field site background

The phenol-contaminated site (300 m in length, 100 m in width, 12 m in depth), is located in central Taiwan as shown in Fig. 1. Situated approximately 200 m to a river, the site has a potential for discharging contaminants into the river. Having had illegally disposed waste applied to it in the past, this site has been regulated by the EPA as a groundwater contamination control site since 2002 because its total phenol concentration had exceeded the second-category groundwater quality standard ($140\,\mu g\,l^{-1}$). A slug test indicated that the velocity of groundwater flow is $0.27\,m\,day^{-1}$, the hydraulic conductivity is $1.7 \times 10^{-4}\,m\,s^{-1}$, and the hydraulic gradient is about $0.0067\,m\,m^{-1}$, flowing primarily from southeast to northwest.

2.2. Site investigation and remediation strategy

Ten groundwater monitoring wells, screened from an average of 8 to 12 m (MW1, 2, 3, 4, 5A, 6, 7, 8A, 9A, 10A) for a shallow aquifer and from an average of 26 to 32 m (MW-5B, 8B, 9B, 10B) for a deep aquifer, were installed in 2003. The total phenol concentrations and DO variations in each monitoring well during different stages of remediation are summarized in Table 1. The layout of the wells at the study site is shown in Fig. 1. It should be noted that wells IW1–IW3 are the injection wells located in the upper stream, and well RW1 is the pumping well for the remedial activities, located in the down-gradient of the groundwater.

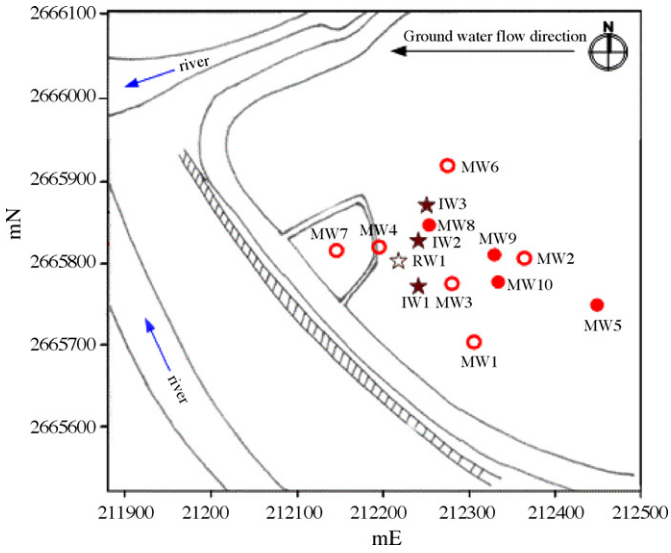


Fig. 1. Layout of study site, showing location of wells. (◯) Single monitored well; (●) double monitored wells; (□) pumping well; (⋆) injection well.

The dissolved oxygen and nutrients PO_4^{-} and NO_3^{-} are extremely low in the preliminary groundwater analysis, indicating that the groundwater is inadequate for providing the growth of indigenous microorganisms. Therefore, if *in situ* bioremediation and monitored natural attenuation (MNA) are employed as treatment technologies for the remediation of phenols, the growth of indigenous microorganisms in the groundwater may be limited not only by oxygen content but also other essential nutrients. In addition, since the sulfate concentrations are elevated, it is suspected that the major dominant microbial species is a sulfate-reducing bacterium. Since the concentrations of both O_2 and NO_3^{-} were quite low, the aerobic microorganisms and denitrifying bacteria had insufficient O_2 and NO_3^{-} to become the dominant species at the site. Moreover, a study by Broholm and Arvin [12] indicated that the biodegradation rate of a microorganism under an aerobic condition was three times greater than that in a nitrate-reducing condition. To successfully accomplish the remediation work, therefore, the selected remedial technologies must account for a sufficient oxygen content to activate the aerobic bacteria at the site.

The remediation technology operated by ML Inc., includes a two-stage system with pump-and-treat using fluidized bed reactors incorporating a Fenton-like process (i.e., Fe^{3+} and hydrogen peroxide) in the first stage and injection of pure oxygen into some of the monitoring wells in the second stage. The contaminated groundwater was pumped out from well RW1 near monitoring well MW4 as a result of its high phenol concentration, as shown in Fig. 1. Wells IW1–IW3 served as injection wells for treated and oxygen-supplied water. Moreover, the monitoring

Table 1
Total phenol and DO concentrations for monitoring wells in various remedial stages[a]

Date	Monitoring well												
	MW 1	MW 2	MW 3	MW 4	MW 5A	MW 5B	MW 6	MW 7	MW 8A[b]	MW 8B	MW 9A	MW 9B	MW 10A	MW 10B
Depth (m)	10	10	10	10	13	30	13	13	12	26.9	12	30	12	27.7
Before remediation (2004/6)[c]														
Phenol (mg l^{-1})	0.029	0.084	0.056	0.584	0.016	0.057	0.132	0.025	0.309	0.036	0.194	0.192	0.034	0.147
DO (mg l^{-1})	0.07	0.10	0.17	0.37	0.13	0.18	0.13	0.13	0.11	0.57	0.03	0.68	0.18	0.18
Latter phase of remediation (2004/12)[c]														
Phenol (mg l^{-1})	<0.001	0.129	0.057	0.018	ND[d]	0.027	ND	0.014	0.026	ND	0.005	0.027	0.007	<0.001
DO (mg l^{-1})	0.50	1	1	5.50	0.35	0.46	0.37	0.40	10.1	10.9	6.50	5.48	0.54	2.32
Aeration	N	N	N	Y	N	N	Y	N	Y	Y	Y	Y	Y	Y

[a] The groundwater quality standard for total phenols in Taiwan is 0.14 mg l^{-1}.
[b] MW8A had continued input of oxygen at 0.51 min^{-1}, and other aeration monitoring wells were aerated 5 min per 8 h.
[c] Data collected from Main-Link Engineering Corporation Inc. (2005).
[d] ND represents non-detective.

wells (MW4, 6, 8A, 8B, 9A, 9B, 10A, 10B) were periodically aerated by pure oxygen for the stimulation of indigenous contaminant-degrading bacteria to enhance the remediation efficiency. The remedial activities were started in August 2004 and completed in March 2005.

Groundwater samples were collected from the monitoring wells by using sterile disposable bailers. Three well volumes were purged prior to sampling. The samples were collected in dark brown bottles and kept on ice for microbiological and genetic analysis and sub-sampling for total phenols analysis. The groundwater temperature, pH, electrical conductivity, DO and ORP were measured at the field site.

2.3. DNA extraction

DNA was extracted by using an improved bead-beating method following a method described by Stach et al. [9] with modifications based on Miller et al. [13]. A groundwater sample (200 μl) was mixed with 0.8 g of 0.106 mm glass beads (Biospec Products, 11079101), 600 μl of phenol/chloroform/isoamyl alcohol (25:24:1), and 200 μl of a disrupting buffer (50 mM NaCl, 50 mM Tri–HCl pH 8, 5% SDS) in a 1.5 ml screw-cap microcentrifuge tube, which was filled with disrupting buffer to exclude air. Then, the mixture was homogenized on a Mini Bead Beater (Biospec Products, 3110BX) at 2500 rpm for 2 min. After a brief centrifugation, the supernatant was transferred to a fresh tube and extracted with 400 μl of chloroform/isoamyl alcohol (24:1). The upper aqueous phase was transferred to a fresh tube and mixed with 240 μl of isopropanol. The DNA was precipitated by centrifugation (13,500 rpm, 3 min), and washed with 240 μl of 70% ethanol. The pellet was dried by baking and then dissolved in 50 μl of TE buffer at 65 °C for 1 h. The concentration of the DNA solution was adjusted to 50 ng ml^{-1}, after which the final solution was stored at 4 °C for use as the PCR template.

2.4. Polymerase chain reaction

The microbial communities were analyzed by using the PCR-SSCP method described by Lee et al. [7] and Schwieger and Tebbe [8] with certain modifications. The V3 region of the 16S rDNA, corresponding to the nucleotide positions 334–514 of the *E. coli* gene, was amplified with the primers EUB1 (5′-CAGACTCCTACGGGAGGCAGCAG-3′) and UNV2 (5′-GTATTACCGCGGCTGCTGGCAC-3′). The PCR program included an initial denaturation of 94 °C for 5 min, and 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. The PCR products of 200 bp were verified by gel electrophoresis on 1.8% agarose gels and stored at 4 °C until further use.

2.5. SSCP gel electrophoresis

A Hoefer SE600 vertical gel electrophoresis apparatus was used for SSCP analysis. Electrophoresis was conducted in 10% polyacrylamide gel for 6 h at a constant voltage of 300 V. The gel temperature was maintained at 4 °C by using a circulating water

bath. The DNA samples were mixed with an equal volume of a denaturing solution (95% formamide, 10 mM NaOH, 0.02% bromphenol blue, 0.02% xylene cyanol, and 20 mM EDTA), heated to 95 °C for 5 min, and snap-freezed on ice before loading. The gels were visualized by using the silver-stain method, sandwiched between two pieces of Mylar membrane and dried.

2.6. Statistical comparison of SSCP pattern

The relative positions of the DNA bands in the SSCP gels were analyzed by using LabWork software. Similarities between microbial groups were calculated as Dice indices according to procedures appearing in several reports [14,15]. Dendrograms were calculated by using a clustering algorithm of a UPGMA (unweighted pair group method using arithmetic average) using cluster analysis of similarity indices, constructed by NTSYSpc software (NTSYSpc, Version 2.1e, Exeter Software, USA).

3. Results and discussion

3.1. In situ bioremediation of phenols

A preliminary site assessment was conducted to analyze 14 monitoring wells for pH, dissolved oxygen (DO), oxidation reduction potential (ORP), NO_3^-, PO_4^-, and SO_4^{2-}. Previous analyses conducted on the groundwater from the site indicated a temperature of 26 ± 1.5 °C, DO ranging from 0.30 to 0.68 mg l^{-1}, a pH of about 6.37–8.67, NO_3^- below 2.03 mg l^{-1}, PO_4^- less than 0.5 mg l^{-1}, and SO_4^{2-} ranging between 50 and 241 mg l^{-1}. The oxidation reduction potential (ORP) was consistently negative in value. Moreover, 16 organic compounds were monitored during the study. They are 1,1-dichloroethane, 1,1-dichloroethylene, 1,2-dichloroethane, 1,4-dichlorobenzene, benzene, carbon tetrachloride, chlorobenzene, chloroform, *cis*-1,2-dichloroethylene, methyl chloride, naphthalene, tetrachloroethylene, toluene, *trans*-1,2-dichloroethylene, trichloroethylene, and vinyl chloride. Of them, only toluene and chlorobenzene were detected at MW8A before remediation (June 2004), with 2.2 and 1.36 mg l^{-1}, respectively. However, toluene and chlorobenzene concentrations were dropped to 0.06 and 0.07 mg l^{-1}, and the concentrations of the remaining 14 organic compounds at all monitoring wells declined to a non-detective level after remediation (December 2004). Additional data have been described in detail by Main-Link Engineering Consultants Inc. [16].

As shown in Table 1, the phenol concentrations in monitoring wells MW4, 8A, 9A, 9B and 10B all exceeded the groundwater quality standard of 0.14 mg l^{-1} prior to remediation. However, lower phenol contents were detected in the other monitoring wells, but all below 0.14 mg l^{-1}. A comparison of phenol concentrations in shallow wells (ca. 10 m) and deep wells (ca. 30 m) indicated a higher concentration in shallow wells in MW8; whereas a lower concentration was detected in MW10.

Fig. 2 shows the changes in phenol concentrations during the time course in certain monitoring wells. A comparison of phenol concentrations in all wells sampled in June 2004 and in March 2004 exhibited a slight increase. This elevated concentration is

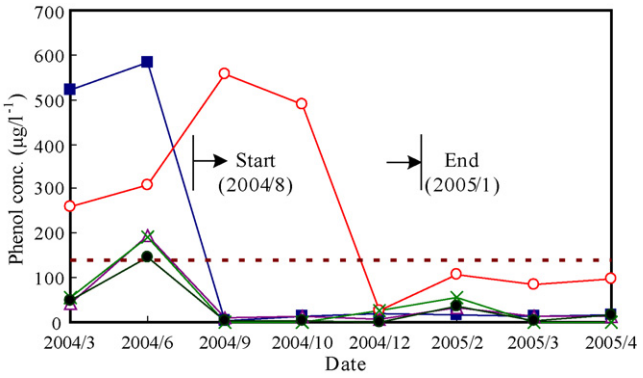


Fig. 2. Changes in phenol concentrations in monitored wells. (■) MW4; (◯) MW8A; (▲) MW9A; (×) MW9B; (●) MW10B; (- -) regulatory standard.

believed to be due to the heavy rainfall in June 2004 which persisted for 2 weeks before the sampling, thereby accelerating the downward movement of leachate from the landfill. It was also found that the highest phenol concentrations were in monitoring well MW4 (584 μg l^{-1}), dated June 2004, thus suggesting that a pumping well could be installed near MW4 to substantially reduce the spread of phenols.

Due to the action of *in situ* remediation started in August 2004, the phenol concentrations in most of the wells significantly declined to below 0.14 mg l^{-1} in the sampling data of September 2004. On the contrary, the phenol concentrations unexpectedly increased in MW8A (562 μg l^{-1}) in September 2004 (ca. 50% higher than prior to remediation) and were still high in October 2004, as shown in Fig. 2. These results are consistent with a hypothesis that the contaminant plume was narrowed and gradually moved to an area near the centre of well MW8A. Furthermore, it was found that phenol concentrations gradually decreased in most of the wells, thereby demonstrating that the treatment facilities and natural biodegradation system functioned well.

After August 2004 it was found that the phenol concentrations in well MW8A had not been reduced because of insufficient aeration. Consequently, the contaminant-degrading microorganisms failed to effectively remove phenols in groundwater concentrated and flowing from other areas. In December 2004 the results indicate that the phenol concentrations (26.3 μg l^{-1}) in MW8A were reduced to below 140 μg l^{-1} by increasing dissolved oxygen from 0.26 (October 2004) to 10.1 mg l^{-1} (December 2004) in the well.

3.2. Monitoring of microbial population

Total-count analysis aims to assess the changes in microbial populations during remedial activities. Fig. 3 displays the change in total counts in different sampling periods for each monitoring well. Prior to remediation (sampling in June 2004), the density of bacteria ranged from approximately 10^4 to 10^7 CFU ml^{-1}; however, it increased significantly to between 10^7 and 10^9 CFU ml^{-1} upon completion of remediation (data collected in March 2005). The increase was two to three orders in magnitude higher than previous results. The microbial populations increased during

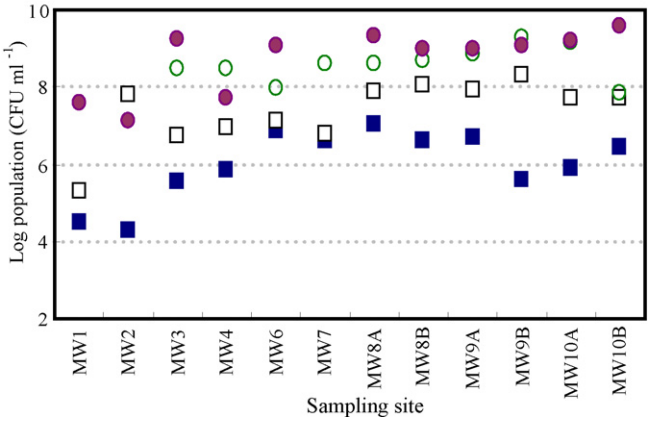


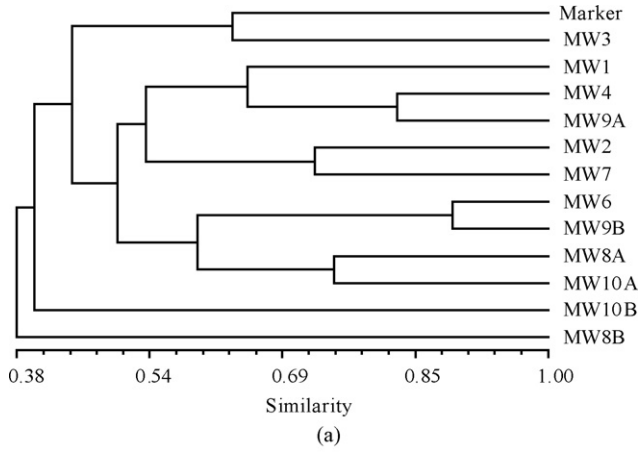
Fig. 3. Changes in total microbial counts in different sampling periods for each monitored well. (■) 2004/6; (□) 2004/9; (◯) 2004/12; (●) 2005/3.

remediation, thus indicating that the stimulation of indigenous contaminant-degrading bacteria was functioning. Although the concentrations of viable organisms in all wells increased over their initial values, the levels of DO in MW1 and MW2 were initially lower and did not increase as much as in other wells, so that the numbers appeared to remain lower than in other better aerated wells. In contrast, the bacterial number was low (10^7 CFU ml^{-1}) in MW1 and MW2, which is interpreted as attributable to a lack of aeration in these two wells. However, it should be noted that the microbial number in MW3 and MW7 was as high as 10^8 CFU ml^{-1} without adding oxygen. A closer examination of this count reveals that these two wells were located down gradient to the oxygen-supplied monitoring wells. Hence, oxygen was not limited in MW3 and MW7. On the basis of the above-mentioned findings, the key factors affecting the decrease in phenols and increase in bacterial populations are believed to be due primarily to the oxygen supply in the groundwater. It is also likely that addition of other nutrients, e.g., nitrogen and phosphorus did not appear to limit the growth of the bacterial even though the dissolved levels of those compounds were low and they were not added. Also, these findings are consistent with a previous study by Hristova et al. [17].

3.3. Microbial community structure in monitoring wells

The DNA samples collected at all stages of the remediation process were pooled for the SSCP analysis to provide a broader perspective of the microbial diversity developed during the time span. Pair-wise similarity analyses were conducted to reveal any correlation between microbial community structure and types of pollutants as well as other environmental factors. The SSCP profiles and the results of cluster analysis are shown in Fig. 4. Fig. 4(a) indicates that some samples are highly similar to each other, for example, the sample pairs of MW6/MW9B (similarity index = 0.93) and MW4/MW9A (similarity index = 0.84). The similarity indices between other pairs of monitoring wells at the remediation site also fall within the range of 0.5–0.6.

The markers (M) in Fig. 4(b) represent a collection of four aerobic phenol-degrading strains isolated from groundwater at the study site. Three of these strains have been tentatively identified as *Pseudomonas stutzeri* (represented by bands a and c), *Acinetobacter calcoaceticus* (bands a, b, and d), and *Rhodococcus* sp. (bands c and f) based on the sequence of a ~1400 bp fragment from their 16S rDNA genes. The accession numbers of the best hit to these sequences in the GenBank as identified by the BLAST search tool, their % similarities and *E* values for the matching score (indicated in the parentheses) are AJ006103 (98%, 0.0), AY823621 (98%, 0.0), and AF420413 (98%, 0.0), respectively. SSCP analysis is based on comparing positions of single-stranded DNA bands on the gel. Each species is usually represented by two single-stranded DNA bands, but it is also possible to have bands from different species migrating to the same position. Some of the bands indicative of the presence of these cultivated strains can be seen in most of the sample profiles, thus suggesting that these strains were indigenous to the study site. In general, the similarity indices between markers (M) and monitoring wells extend up to 0.48. The process of remediation can be described as each sample site developing its own microbial community through acclimation and competition



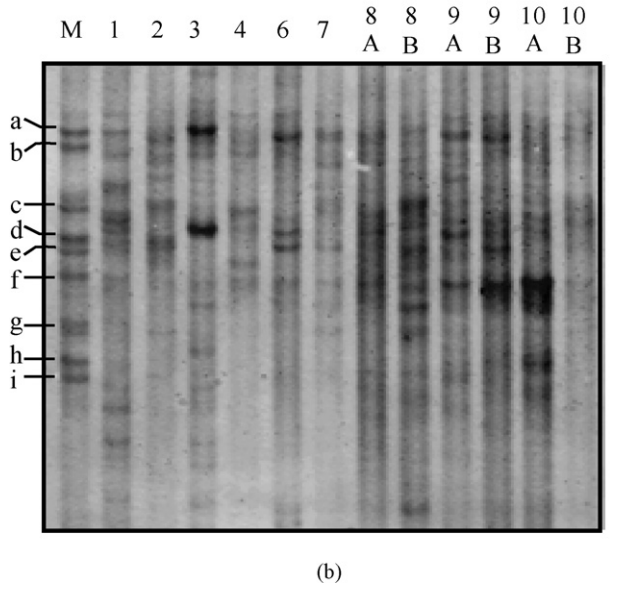


Fig. 4. Cluster analysis of SSCP community profiles of each monitored well microcosm during remediation (2004/6 to 2005/3). (a) Cluster analysis; (b) PCR-SSCP fingerprint.

among indigenous bacteria in response to the changing environments. The result is a group of highly similar communities. Such analyses combine the entire collection of species appearing at the same site, but not necessarily at the same time point during the 9-month study period; however, these analyses do not reveal any time-dependent change in the community structure, an issue addressed in the next section.

3.4. Time-course analysis of microbial communities

The structure of a microbial community can be affected by the type and concentration of pollutants, as well as other environmental factors. Changes in the community structure also reflect the effectiveness of the remediation process. Therefore, we decided to conduct a time-course study on community structure using SSCP, similarity indexing, and cluster analysis in conjunction with the traditional plate count method. We chose well MW4 and MW8A for this analysis since they contained the highest concentrations of pollutants. Well MW8A is also the one that failed to respond to the remediation treatment during the early and middle phases of the experiment, an outcome which might be attributed to a unique microbial community structure.

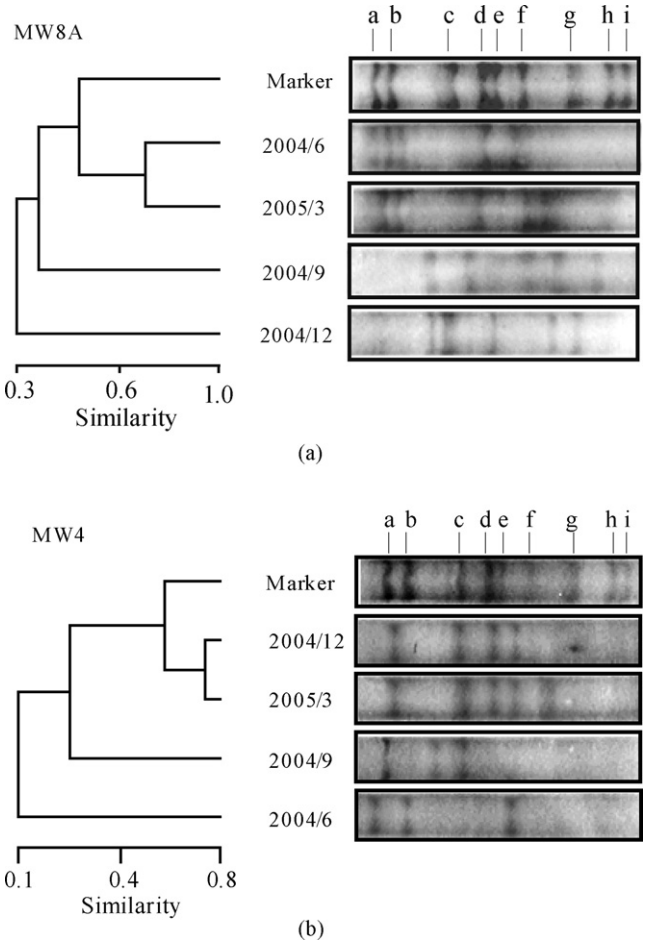


Fig. 5. Cluster analysis of SSCP community profiles of MW4 and MW8A with different sampling periods. (a) Monitored well MW4; (b) monitored well MW8A.

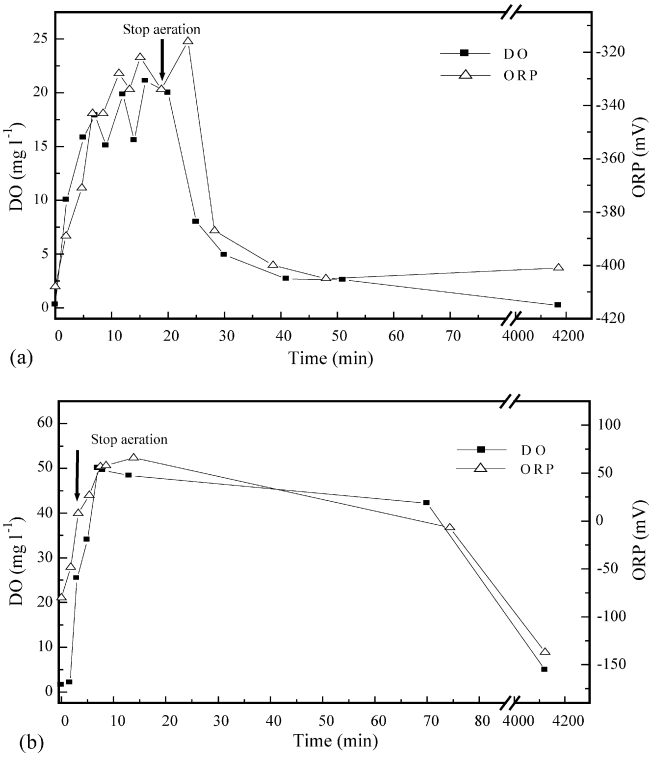


Fig. 6. DO and ORP variations with time in wells. (a) Well MW8A; (b) well MW8B.

Fig. 5(a) shows the SSCP profiles, similarity indices and the results of cluster analysis for the microbial communities from well MW4 at all stages. Two samples dated June 2004 (before treatment) and September 2004 (after treatment) are highly heterogeneous, as revealed by a similarity index of only 0.1. Similar results were obtained for well MW9A and MW9B (data not shown). In Fig. 6(a) the dissolved oxygen concentration in well MW8A rose to ca. 20 mg l^{-1} after 20 min of aeration with pure oxygen but dropped to 8 mg l^{-1} within 5 min after the cessation of aeration. A slow, steady decline was observed afterward. There are obvious differences in the results from well MW8A and well MW8B, which should relate to different water quality characteristics; however, the DO finally dropped from 50 to 5 mg l^{-1} in 3 days (Fig. 6b). As shown in Fig. 3, the total bacterial counts increased about 10–1000 times as a result of the aeration in the wells (MW4, 6, 8A, 8B, 9A, 9B, 10A, 10B). Due to DO increases in the groundwater (June–September 2004), the aerobic bacteria increased by a large amount to replace the dominant bacteria present under a less oxygen-rich environment before remediation, as indicated in Fig. 3. Subsequently, the bacterial community of MW4 developed into some of the phenol-degrading bacteria after December 2004, as aeration occurred and the phenols disappeared, the similarity in the microbial community sampled in December 2004 and March 2005 being about 0.74 (Fig. 5a). A comparison of the bacteria community with the marker during the earlier remediation periods exhibits a trend of increasing similarity to the marker during remediation, as indicated in the SSCP fingerprints of well MW4 in Fig. 5(a). Because of a continuous supply of dissolved oxygen in the groundwater,

it is reasonable to hypothesize that the aerobic bacteria became the dominant species in this area.

Fig. 5 (a) shows the similarity in the bacterial community for water samples taken in June 2004 (before remediation), September 2004, and December 2004. The SSCP fingerprint shows that the similarity apparently declined in well MW8A and the number of DNA bands increased with the time since beginning remediation (August, 2004). A comparison of this observation with the variation in phenol concentration (Fig. 2) and total bacterial counts (Fig. 3), indicates that the low oxygen-demanding bacteria disappeared gradually or became insignificant because of an increase in dissolved oxygen and changes in phenol concentrations, etc. The bacteria became the dominant species that could utilize oxygen to degrade phenols and cause the bacterial populations to increase rapidly from 10^7 to 10^9 CFU ml^{-1}.

The similarity in the microbial community between June 2004 and September 2004 was about 0.36 in the early stage of remediation, but it increased to 0.77 when comparing June 2004 (before remediation) with March 2005 (after remediation). The number of DNA bands also diminished, thereby showing that the bacterial community became less diverse and more similar to the original situation (i.e., before remediation). This finding is consistent with a previous result obtained by Wang et al. [18], who found the composition of the bacterial community and the bacterial number became increasingly complex under different iprodione concentrations at the early stage of remediation and recovered after 23 days.

3.5. Bacterial community during different remediation stages

Fig. 7 shows the similarity of the microbial community and the SSCP fingerprints prior to remediation (June 2004). Fig. 7(a) indicates that there are two dominant groups of bacteria among the monitoring wells, wherein the similarity was greater than 0.48. Especially, high similarities of 0.90 and 0.85 were found between MW1 and 2, and between MW8B and MW10A), respectively. As shown in Fig. 7(b), some of the DNA bands are darker and more plentiful in number, thus indicating that those bacteria were relatively abundant in the community. These results are consistent with the observations in the total microbial count study (June 2004) in each monitoring well (Fig. 3). For instance, the bacterial counts are approximately 1.1×10^7, 4.2×10^6, and 4.9×10^6 CFU ml^{-1} in MW8A, 8B, and 9A, respectively.

Fig. 8(a) and (b) shows the similarity of microbial community and the SSCP fingerprints in the initial stage of remediation (September 2004). In general, there were still two major bacterial groups among the monitored wells, as shown in Fig. 6(a). It should be noted that the similarity is greater than 0.52, and

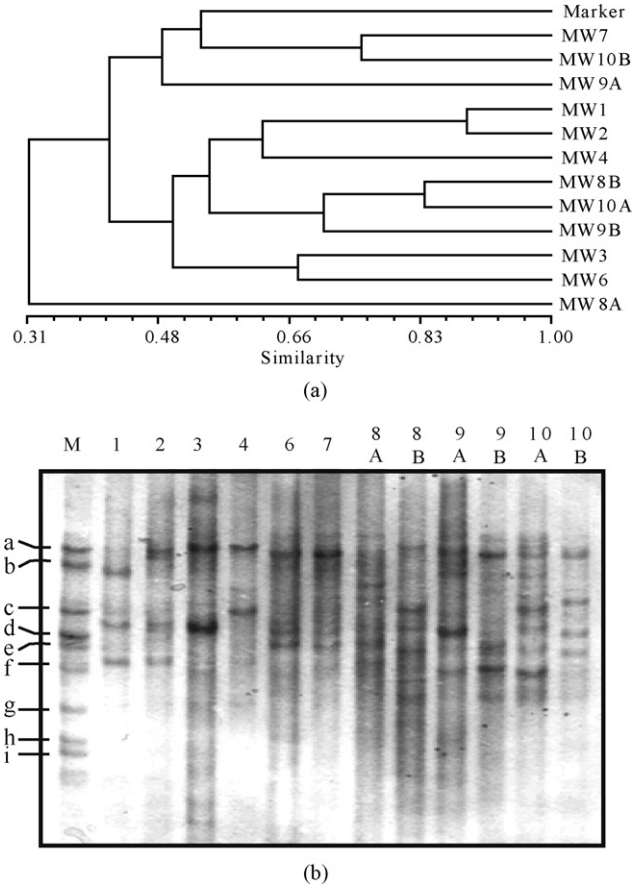


Fig. 7. Cluster analysis of SSCP profiles of microcosm in each monitored well before remediation (2004/6). (a) Cluster analysis; (b) PCR-SSCP fingerprint.

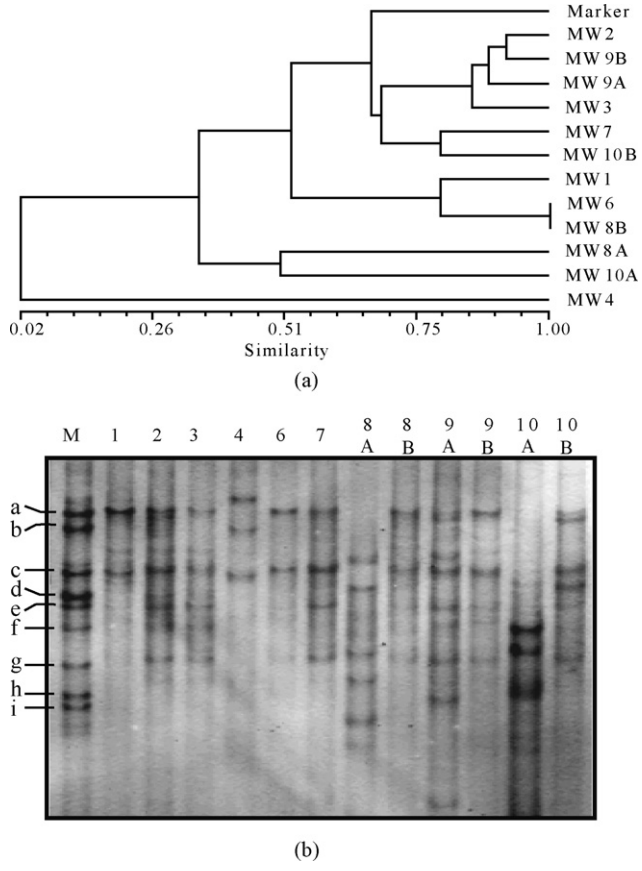


Fig. 8. Cluster analysis of SSCP profiles of microcosm in each monitored well during initial-phase remediation (2004/9). (a) Cluster analysis; (b) PCR-SSCP fingerprint.

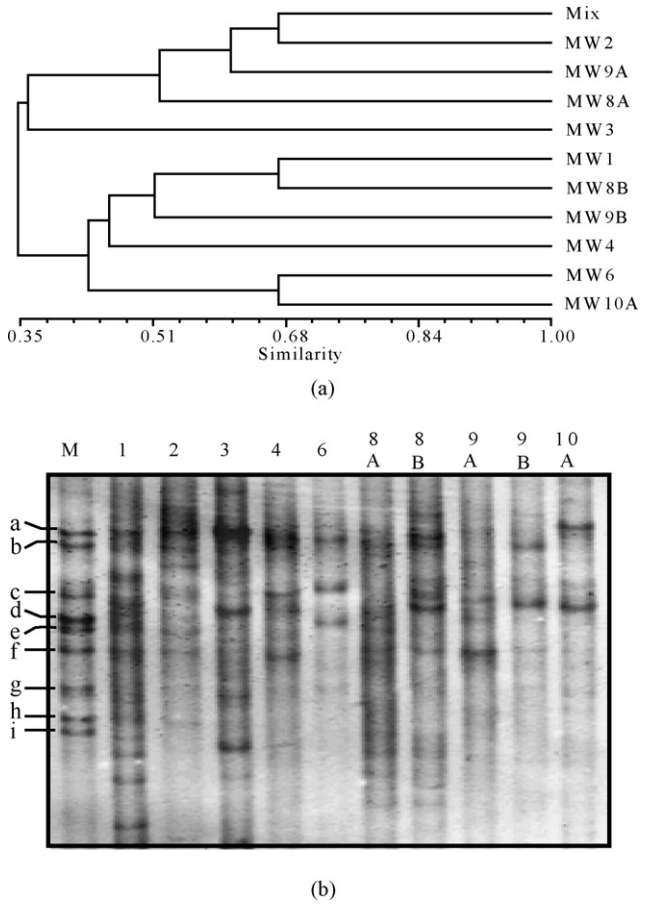


Fig. 9. Cluster analysis of SSCP profiles of microcosm in each monitored well after remediation (2005/3). (a) Cluster analysis; (b) PCR-SSCP fingerprint.

even greater than 0.80 for certain wells located in the boundary of the remediation site (MW1, 2, 6, 7) and in the wells having low phenol concentrations (MW3, 8B, 9A, 9B and 10B). The relatively greater similarity among the wells during this remediation stage compared to the pre-remediation populations indicates that environmental factors affected the composition of the microbial community. Fig. 8(a) shows that the positions of the DNA bands are similar compared with the marker, having a similarity of 0.68.

Fig. 9(a) and (b) shows the similarity of the microbial community and the SSCP fingerprints after the completion of remediation (March 2005). It was found that the similarity is less than 0.43 in most of the monitored wells, except for the similarity between MW6 and MW10A (0.67) and this result being similar to the cluster analysis in December 2004 (data not presented). The findings suggest that after removal of a dominant substrate via remediation the bacteria have begun to develop into a suitable microbial community for a less nutrient-rich environment.

4. Conclusions

The phenol concentrations were reduced below Taiwan's regulatory guidelines of 0.14 mg l^{-1} of total phenols after *in situ* remediation. The decrease in phenols and increase in bacterial populations are believed to be attributable primarily to the oxygen supply in the groundwater. In a long-term contaminated environment, microorganisms gradually developed into a unique microbial community structure. Some members of this unique community may be used as biological indicators to delineate the spatial distribution of contaminants. The SSCP technique presented in this report offers a potential alternative for determining the structure of a microbial community, having proved to be a useful tool for evaluating the success of *in situ* remediation. To further understand the relationship between microorganisms and the spatial distribution of contaminants in the future, in addition to characterizing the structure of the unique microbial community, there is a need to identify bacterial strains in order to track and observe the variations in the community.

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