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Use of Plate-Wash Samples to Evaluate Bacterial Population Dynamics in Mercury- and Trichloroethylene-Contaminated Soils

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We used PCR-DGGE to analyze the effects of mercury ($HgCl_2$) and trichloroethylene (TCE) on soil bacterial communities, and we developed a method of using culturable bacteria to evaluate contaminated soils. Measurement of concentrations of total bacteria in soils contaminated with $HgCl_2$ (0, 1, 10 ppm) and TCE (0, 10, 100 ppm) revealed that bacterial concentrations remained the same regardless of type of pollutant, concentration, or sampling time. We then used PCR-DGGE to analyze the community structures of total bacteria (soil samples) and culturable bacteria (plate wash samples). Changes in band patterns in $HgCl_2$- and TCE-contaminated soil samples were small and similar, but in the plate wash samples there were greater differences, and specific bands appeared. To determine relative changes in the bacterial communities, we analyzed the DGGE profiles by nonmetric multidimensional scaling. Over 200 days, changes in the bacterial communities were small and similar in all soil samples from $HgCl_2$-and TCE-contaminated soils. In contrast, plate wash samples of the same soils showed changes in the bacterial communities; these changes were optimally detected after 50 days of culture. Unlike soil sample analysis, analysis of plate wash samples effectively clarified the effects of $HgCl_2$ and TCE on soil bacterial communities.

Key words: Soil bacteria, mercury, trichloroethylene, plate wash, PCR-DGGE

1. Introduction

Since ancient times, natural ecosystems have stayed in harmony and balance with global material cycles (carbon, nitrogen, sulfur) and energy flows. In recent years, however, various chemical substances, such as the volatile chlorinated compounds trichloroethylene (TCE)[38] and tetrachloroethylene (PCE)[27], the heavy metals mercury[15] and lead[26], and the extremely stable polychlorinated biphenyls (PCBs)[6] and polychlorinated dibenzo-*p*-dioxins (PCDDs)[12,25], were easily discarded to the environment by industrial development, and they have now become serious problems. These pollutants negatively affect living organisms and the environment: their accumulation in the environment confuses natural ecosystems, and some pollutants show hormone-like actions and carcinogenesis when they concentrate in the living body.

When nutrient sources become available in the natural environment, not only activated bacteria but also dormant and starvation forms of bacteria are swiftly activated to use the nutrients as energy sources[16]. It is thought that where various pollutants are available instead of nutrient sources and specific bacteria using these pollutants lives in contaminated soils. For example, the TCE-degrading bacteria *Methylocystis* sp. M[33] and *Mycobacterium* sp. TA27[8]; the PCE-degrading bacteria *Desulfitobacterium* sp. Y51[29] and *Methanosarcina* sp. FR[2]; the mercury-reducing bacteria *Bacillus megaterium* MB1[20] and recombinant mercury-reducing bacteria *Pseudomonas putida* PpY101/pSR134[10]; the PCB-degrading bacteria *Pseudomonas pseudoalcaligenes* KF707[30] and *Burkholderia* sp. TSN101[17]; and the PCDD-degrading bacteria *Sphingomonas wittichii* RW1[40] and *Pseudomonas resinovorans* CA10[7] have been isolated, and their metabolic pathways for the degradation of pollutants have been studied in detail.

In other studies of waste decomposition and the interaction of bacterial communities, various bacteria have been found to play important roles in the processes of decomposition in the natural environment and as field-scale composters[19,23,24]. If we are to effectively utilize such bacteria for the bioremediation of environmental pollutions, it is important that we (i) determine the characteristics of the specific bacteria that are predominate at contaminated sites; (ii) find out which bacteria coexist with these specific bacteria; and (iii) clarify the mechanisms of degradation of the pollutants at the sites of these bacterial communities. However, these pollutants consist of a number of chemical substances, and there are few data on their effects on soil bacterial communities. Recent studies have reported the effects of coal tar components such as creosote[37] and polycyclic aromatic hydrocarbons (PAHs)[1]; the effects of petroleum contamination[13]; the effects of the insecticide hexachlorocyclohexane (HCH) on microbial communities in contaminated soils[22]; and the effects of PCB contamination on plant rhizospheres[3]. The results of basic information will increase

our understanding of the effects of various pollutants on soil bacterial communities.

In this study, we examined the effects of inorganic mercury ($HgCl_2$; a deadly heavy metal) and trichloroethylene (TCE; a carcinogen and an industrial pollutant of volatile chlorinated compounds) on the bacterial communities of soils contaminated with these substances. We indicated the effectiveness of using plate wash samples to analyze culturable bacteria as indicators of the effects of pollutants on soil bacteria communities, and developed a method of evaluating contaminated soils by using the culturable bacteria. Furthermore, using this method, we showed the possible to obtain the culturable and specific bacterial communities in $HgCl_2$- and TCE-contaminated soils.

2. Materials and methods

2.1. Soils, and preparation of soil microcosms, and soil samples

We used alluvial soil taken from a field at the National Institute for Environmental Studies, Tsukuba, Japan. The initial water content of the soil was measured (26% [v/w]), and the soil was air-dried to about 10% water content. The dried soil was passed through a sieve (2-mm mesh) to make it a uniform size. The water content of the soil was then adjusted to 20% by adding sterile distilled water, and the soil was incubated at 10°C for 3 days in the dark. Soil microcosms were prepared in (i) 200-mL glass jars (AS ONE Corp., Osaka, Japan), each containing 100 g (dry weight) of the incubated soil and (ii) 69-mL vials (GL Sciences Inc., Tokyo, Japan), each containing 10 g (dry weight) of the incubated soil. The glass jar microcosms were contaminated with 0, 1, or 10 ppm of 2 mg/mL $HgCl_2$, and the vials were contaminated with 0, 10, or 100 ppm of 1100 mg/L TCE saturated solution. To consume a vial every sampling because TCE is a volatile compound, a large number of TCE-contaminated microcosms were prepared. All microcosms were adjusted to a 26% of final water content with sterile distilled water and were incubated statically at 20°C in the dark. Each contaminated soil in microcosm was regularly sampled and was used as a source of soil samples.

2.2. Plate culture, bacterial cell counting, and plate wash samples

Bacteria were cultured on R2A medium (yeast extract 0.5 g/L, proteose peptone 0.5 g/L, casamino acids 0.5 g/L, dextrose 0.5 g/L, soluble starch 0.5 g/L, sodium pyruvate 0.3 g/L, potassium phosphate dibasic 0.3 g/L, magnesium sulfate 0.05 g/L, agar 15 g/L, pH 7.2; Difco Laboratories, Detroit, MI, USA) at 20°C for 1 week in the dark. The number of culturable bacteria was counted by the plate culture method. Each contaminated soil sample (1 g wet weight) was dispersed in 9 mL of sterile distilled water and homogenized by vortex at top speed for 3 min. The soil solutions obtained were decimally diluted, and 200 μL of each diluted solution was mixed into 2 mL of 0.7% (w/v) agarose solution and swiftly spread onto an R2A agar plates. After cultivation, the number of bacteria in the soil sample was calculated from the number of colonies. Plate wash samples were prepared as follows: Three ml of sterile distilled water was poured on colonies on R2A agar plate, and all colonies were mixed with a disposable inoculating needle. The cell suspensions were used as the sources of plate wash samples.

2.3. DNA extraction, and PCR amplification

Total bacterial DNA from 300-mg soil samples and culturable bacterial DNA from 200-μL plate wash samples were extracted by using a Fast DNA Spin kit for soil (Qbiogene Inc., Carlsbad, CA, USA) in accordance with the manufacturer's protocol. PCR was performed with universal primers 350F-GC and 920R, located in the V3 region of the 16S rRNA genes[18], using GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) The PCR mixture contained 10 μL of template DNA solution, 1×PCR Gold buffer, 1.5 mM $MgCl_2$, 200 μM dNTP (Roche Molecular Systems, Branchburg, NJ, USA), 0.2 μM of each primer, 1.25 U AmpliTaq Gold (Roche Molecular Systems), and nuclease-free water (Promega Corp., Madison, WI, USA) in a final volume of 50 μL. Hot-start PCR was performed at 95°C for 10 min. The annealing temperature was initially set at 60°C for and was then decreased by 1°C for every two cycles for 15 s until it reached 50°C. Twenty additional cycles were carried out at 50°C. Primer extension was 10 min at 72°C. After the reaction, the PCR products were confirmed by electrophoresis in 1% (w/v) agarose gels using *i*-Mupid (Cosmo Bio Co., Tokyo, Japan).

2.4. DGGE and data analysis

DGGE was performed by the D-Code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA) with 6% (w/v) polyacrylamide gels (acrylamide to bisacrylamide ratio, 37.5:1) with a 30% to 60% (w/v) denaturing gradient (100% was defined as 7 M urea and 40% [v/v] formamide) in 0.5×TAE (pH 8.0). Electrophoresis was run at 60°C for 17 h at 70 V. After electrophoresis, the gels were stained at room temperature for 40 min with Vistra Green (Amersham Biosciences, Buckinghamshire, UK) and scanned using Typhoon 9400 (Amersham Biosciences). DGGE gel images of band positions were digitized with ImageQuant software (Amersham Biosciences). Because bacteria can have multiple copies of their rRNA gene, care should be taken with the interpretation of the number of species present in a DGGE band. Therefore, we refer to a band as a species rather than the number of bacteria. The presence or absence of a band in each lane was converted to a binary (1 or 0) matrix to make the data accessible to statistical analysis[35]. From this binary matrix, a distance matrix (anti-similarity) was calculated[21]. Then, a distance matrix was analyzed by nonmetric multidimensional scaling (NMDS) with SPSS software (SPSS Japan Inc., Tokyo, Japan). NMDS shows a map showing the relationships among samples in a Euclidean plane such that very similar

values were plotted close together. This graph of a Euclidean plane is much easier to interpret than the original distance matrix. The axes (dimensions 1 and 2) in the map have no special significance and can be rotated or mirrored without influencing the relative distances between the points. Interpretation of NMDS map can be achieved by explaining each dimension or by finding structures or patterns in multidimensional space[36]. When it was applied to DGGE data, the NMDS map shows every band pattern (a reflection of the community structure at a specific point in time) as one point, and relative changes in community structure could be visualized and interpreted by connecting consecutive points. NMDS had shown to be useful tool for analysis of genetic structures[34,36].

3. Results and Discussion

3.1. Numbers of bacteria in contaminated soils

Because distinguishing viable bacteria from dead bacteria and mineral particles is difficult, the exact numbers of bacteria in soils are not yet known. In general, it is widely recognized that several thousand millions of microorganisms exist in 1 g dry weight of environmental soils[5]. The majority of soil bacteria are heterotrophs that require organic sources for their existence, but the concentrations of these organic sources in soils are very small. Most bacteria have adapted themselves to the low concentrations of organic sources in these severe environments. When soil bacteria are cultivated, various and a large number of bacteria are more cultivated in low-concentration broth than these of high-concentration broth[9].

To reveal differences in the bacterial communities of $HgCl_2$- and TCE-contaminated soils, we therefore measured changes in the number of bacteria by the plate culture method in R2A medium (low-nutrient broth). Figures 1A (Black circles, 0 ppm; Black squares, 1 ppm; Black triangles, 10 ppm) and 1B (White circles, 0 ppm; White squares, 10 ppm; White triangles, 100 ppm) show that contamination with $HgCl_2$ or TCE had the same effect on bacterial numbers for sampling times, respectively. Furthermore, in both cases changes in the pollutant concentration had no influence on bacterial numbers, which remained almost constant for 200 days. In the case of $HgCl_2$, we considered that mercury ion (Hg^{2+}) might be strongly adsorbed onto the surfaces of the soil components and that most of the soil bacteria would be present in the interior spaces of the soils particles. The soil adsorption characteristics for heavy metals and various organic carbons are known[39]. In the case of TCE, TCE contamination at 10 ppm and 100 ppm may have been too low to influence the soil bacteria. On the other hand, Jenkinson[11] has reported that dead bacteria are immediately digested by living bacteria for survival in the soil environment. We assumed that the bacteria that used the dead bacteria might increase in number and, consequently, that the apparent number of total bacteria might be constant in contaminated soils. Furthermore, the plate culture method has some problems: for example, (i) only those bacteria that are adapted to the broth and culture conditions can be colony forming; (ii) some kinds of bacteria that are strongly influenced by the physical and chemical effects of the rapidly-formed colonies cannot form colonies; and (iii) some bacteria are extremely slow in proliferating.

3.2. Effects of $HgCl_2$ and TCE on bacterial community structure

Although the total concentrations of bacteria in $HgCl_2$- and TCE-contaminated soils were constant during the sampling times, we anticipated that the diversities of bacterial communities in soil samples (included unculturable bacteria) and in plate wash samples (culturable bacteria) would be changed by the effects of pollutants. PCR-DGGE was performed to evaluate the diversity of the bacterial communities using DNA solutions extracted from soil samples and from plate wash samples.

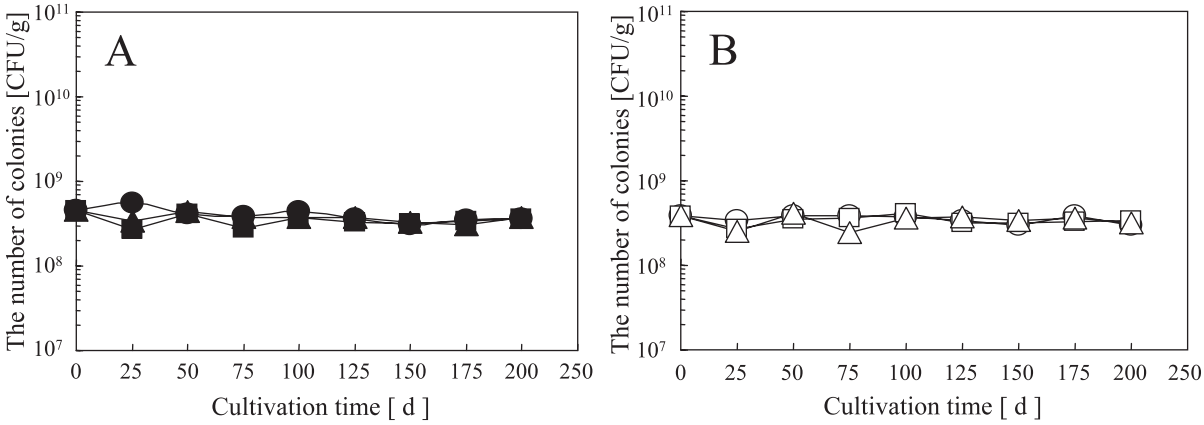

Fig. 1. Time courses of change in concentration of culturable bacteria in $HgCl_2$- and TCE-contaminated soils.
The number of colony forming units of culturable bacteria per gram of soil was counted by the plate culture method. Soils were contaminated with various concentrations of $HgCl_2$ and TCE. Diluted soil solutions were inoculated onto R2A agar plates and cultured at 20°C for 1 week in the dark. After cultivation, the number of colonies was calculated. (A) Black circles, 0 ppm; black squares, 1 ppm; Black triangles, 10 ppm; (B) White circles, 0 ppm; White squares, 10 ppm; White triangles, 100 ppm.

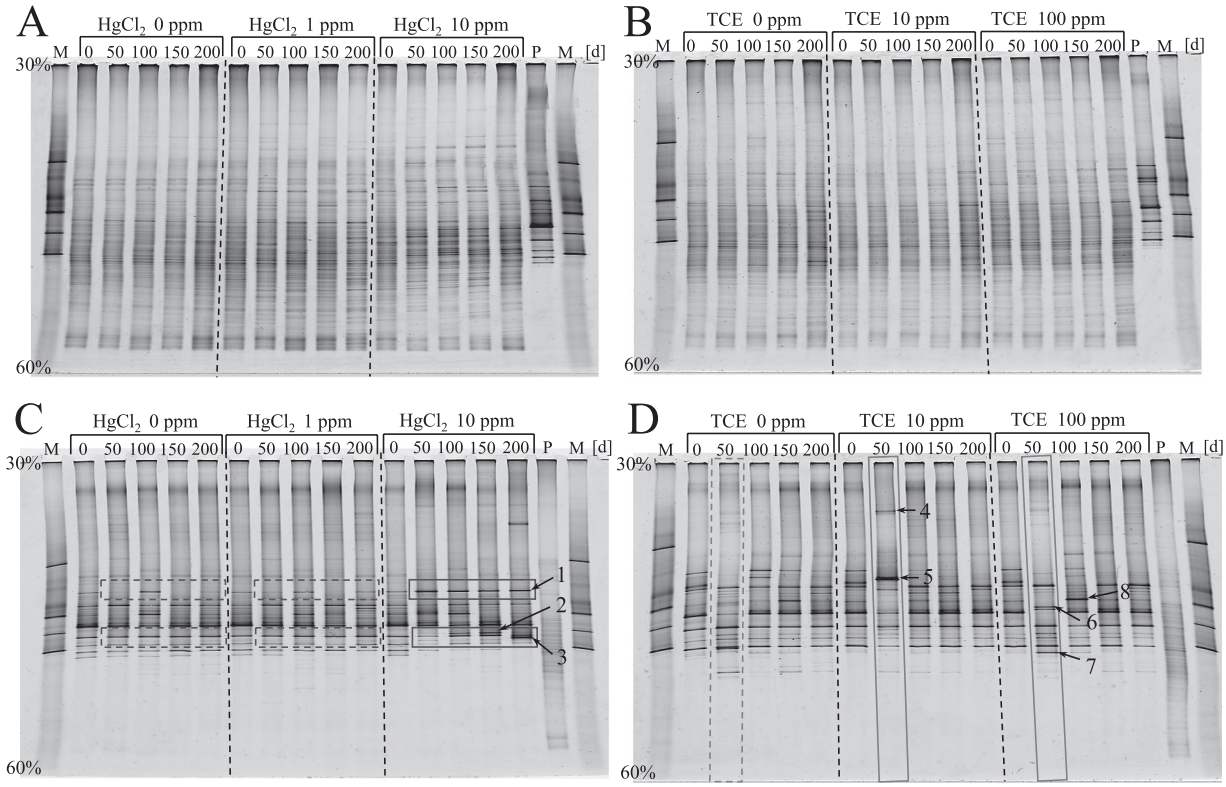

Fig. 2. DGGE analyses of diversity of total bacteria and culturable bacteria in $HgCl_2$- and TCE-contaminated soils.
Every bacterial community was analyzed by PCR-DGGE using DNA solutions extracted from soil (total bacteria) and plate wash samples (culturable bacteria). A and B are results from soil samples, and C and D are results from plate wash samples; A and C were contaminated with $HgCl_2$ and B and D with TCE. M, marker; P, positive control. (Positive controls in A and B were plate wash samples, and positive controls in C and D were soil samples).

Figures 2A and 2B show samples of soils contaminated with $HgCl_2$ or TCE, and Figures 2C and 2D shows plate wash samples of the same soils. In the soil samples (total bacteria), there were no sudden decreases in the number of bands, and large numbers of bands appeared under contaminated conditions. The band patterns produced by both contaminants were similar. The effects of changes in pollutant concentration on the bacterial community were very small, and the band patterns of the contaminated soils were almost equal to those of the control (0 ppm) at the same sampling times. In the plate wash samples (culturable bacteria), there were fewer bands than in the soil samples. Differences in the band patterns of $HgCl_2$ and TCE contamination were apparent (Figs. 2C and 2D). The band patterns at 0 ppm and 1 ppm $HgCl_2$ were similar. Two high-density bands at 10 ppm (bands 1 and 2 in the figure) appeared at all sampling times, and a specific band (band 3) appeared. Although the band 1 at 0 ppm and 1 ppm (at 0 and 100 days) appeared, they decreased at 50 and 150 days, respectively. On the other hand, the specific band patterns were shown at 10 ppm because the band 1 appeared at all sampling times (for 0 to 200 days). The bacteria represented by these bands (bands 1–3) may have been culturable mercury-resistant species. In the case of TCE, compared with the control (0 ppm), changes in the band patterns at 10 ppm and 100 ppm were increased at 50 days. High-density bands (bands 4 and 5 in the figure) appeared at 10 ppm, and specific bands (bands 6 and 7) were found at 100 ppm. Furthermore, at 100 days there was a high-density band (band 8) at 100 ppm. To notice the large change in band patterns of TCE at 50 d, the bands 4 and 5 (at 10 ppm at 50 d), bands 6 and 7 (at 100 ppm at 50 d) were not shown at 50 day at 0 ppm although the band 5 (at 100 d), bands 7 and 8 (at 150 d) appeared at 0 ppm TCE. Similarly, the band 8 (at 100 ppm at 100 d) was not shown at 100 d at 0 ppm. It is very interesting if these bands represented those bacteria were TCE-degrading bacteria. Ellis *et al.*[4] reported that the total bacterial populations in the metal-contaminated soil were largely unaffected by the metal inputs, and that the metal did not have a great effect on diversity. As the total bacterial diversity in soil is overwhelmingly high (more than 4000 species)[32], the number of bands that was detected by DGGE analysis was relatively low. Furthermore, it is known that a single DGGE band does not always represent a single bacterial strain[28]. Therefore, it is difficult to discuss for qualitative and quantitative results of band patterns and band density among soil samples from DGGE analysis, and we considered that the changes in band patterns of soil samples might appear to be very small, and that soil samples that included many kinds of bacteria would appear stable. These results indicated the effectiveness of using plate wash samples to analyze culturable bacteria as indicators of the effects of pollutants on bacterial communities.

3.3. Statistical analysis by NMDS

To reveal relative changes in the bacterial communities of the band patterns in the DGGE gels, we analyzed the DGGE profiles by using NMDS. In NMDS analysis, the anti-similarities (differences) among bacterial communities are calculated on the basis of differences in the band patterns and numbers of bands in the samples compared. Consequently, every bacterial community in the sample is shown as one point on a two-dimensional display. Relative changes in the bacterial community can be shown by connecting the consecutive points, and similarities in community structures among samples can be indicated by the positions and distances of the points[14,34,36].

Figures 3A and 3B show the relative change in the bacterial communities of the $HgCl_2$- and TCE-contaminated soil, and Figures 3C and 3D show the plate wash samples. In the soil samples (total bacteria), each movement of spot was small and the large differences of spot movements were not shown among the pollutant concentrations (Figs. 3A and 3B). Although the DGGE band patterns of soil samples were very similar (Figs. 2A and 2B), all spots at 0 days in $HgCl_2$ (0, 1, 10 ppm) and TCE (0, 10, 100 ppm) in NMDS maps were shown large distance together. NMDS analyses, constructs the relative map for bacterial communities. For these reasons, we considered that the movements of spots in soil samples might be relatively magnified within narrow limits and these bacterial communities were therefore similar. On the other hand, the specific changes were shown in the plate wash samples (culturable bacteria). The movements between the spots at 0 ppm and 1 ppm in the $HgCl_2$ were small and the spots were relatively close together (Fig. 3C). In contrast, the positions of spots at 10 ppm were large distances from that of the 0 ppm and 1 ppm. The movements between the spots for 0 to 50 days were especially large at 10 ppm, the specific changes in the bacterial communities were indicated in this period. In the TCE, although the time courses of spot movements were small at 0 ppm, the spots at both 10 ppm and 100 ppm moved large distances for 0 to 50 days (Fig. 3D). As the each spot at 50-day was shown the reverse position, it was suggested the differences of bacterial communities between 10 ppm and 100 ppm. To clear the time courses relative change in the bacterial communities of plate wash samples in detail, NMDS was performed each contaminated concentration of $HgCl_2$ and TCE (Fig. 4). In the $HgCl_2$, each movement of spot at 0 ppm and 1 ppm was small over the 200-day sampling period, and all spots were generally close together (Figs. 4A and 4B). In contrast, the bacterial community at 10 ppm changed greatly between 25and 100 days, because the spot

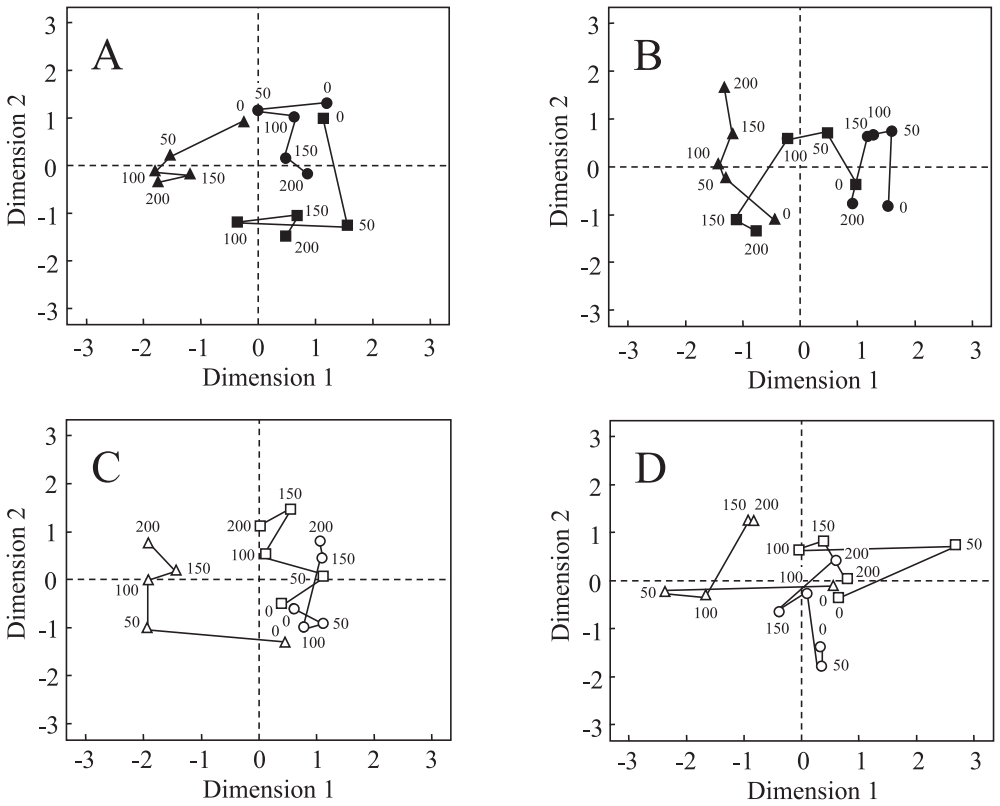

Fig. 3. NMDS analyses of relative changes in structure of bacterial communities in soil samples and plate wash samples from $HgCl_2$- and TCE-contaminated soils.
Soil sample (total bacteria) results are shown as A and B, and plate wash samples (culturable bacteria) results are shown as C and D, respectively. A and C were contaminated with $HgCl_2$ (black and white circles, 0 ppm; black and white squares, 1 ppm; black and white triangles, 10 ppm), and B and D with TCE (black and white circles, 0 ppm; black and white squares, 10 ppm; black and white triangles, 100 ppm). The number of days shown next to each spot is the sampling time.

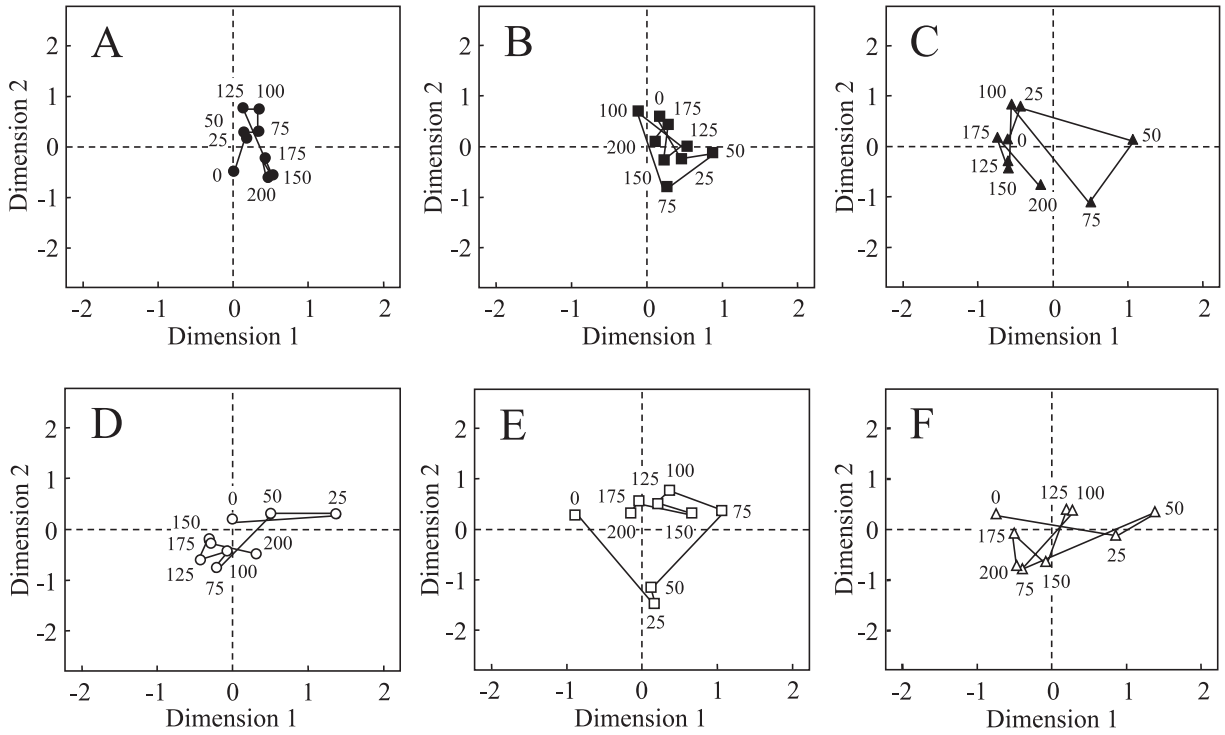

Fig. 4. NMDS analyses of relative changes in bacterial communities of culturable bacteria in $HgCl_2$- and TCE-contaminated soils.
Every bacterial community was analyzed by NMDS. A, B and C in the upper row are results of plate wash samples from $HgCl_2$-contaminated soils. D, E and F in the lower row are results of plate wash samples from TCE-contaminated soils. The number of days shown next to each spot is the sampling time. Symbols: black circles, 0 ppm; black squares, 1 ppm; black triangles, 10 ppm; white circles, 0 ppm; white squares, 10 ppm; white triangles, 100 ppm.

movement was very large (Fig. 4C). Furthermore, the spots at both 10 ppm and 100 ppm of the TCE moved large distances between 0 and 75 days (Figs. 4E and 4F). These results were consistent with Figures 3C and 3D, and indicated large changes in the bacterial communities within this period. However, the spot movements between 0 and 25 days at 0 ppm were also large (Fig. 4D). As a reason for this phenomenon, we considered that because the microcosms containing TCE, which is volatile compound, were tightly shut (0 ppm as control, 10 ppm, and 100 ppm), these airtight conditions might have affected the bacterial communities. These results suggested that the effects of $HgCl_2$ and TCE on bacterial communities could be clarified by analyzing plate wash samples taken from the microcosms after about 50 days of culture.

PCR-DGGE and NMDS analyses were performed on the plate wash samples to investigate the reproducibility of bacterial communities. We considered that there were various bacteria in the uncontaminated control microcosms. Therefore, the control microcosm soils (0 ppm) were sampled independently three times by the above-mentioned procedures. Every sample was cultured on three plates of R2A medium (total of nine plates). The plate wash samples obtained were analyzed by PCR-DGGE and NMDS. Although the densities of the bands in the DGGE gels differed among the samples, the band patterns were similar. Statistical analysis by NMDS revealed that there were no completely overlapping spots, but all the spots were gathered close together. Therefore, the bacterial communities in all samples were highly similar. Consequently, we confirmed a high rate of reproducibility using this method of analysis of plate wash samples (Data not shown). As the advantage of the plate wash samples, all the bacteria in the plate wash samples can be cultivated easily. Tamaki *et al.*[31] used gellan gum as a gelling reagent instead of agar to isolated novel microbes from freshwater sediments; they reported that they were performing continuous cultivation of these novel microbes to reveal their physiological features and functional roles in freshwater sediments. We considered developing of detection systems for $HgCl_2$- and TCE-contaminated soils using the specific bacteria as an indicator, and to utilize for practical bioremediation using the degradable metabolism of bacterial community.

In conclusion, we showed that the effects of $HgCl_2$ and TCE on bacterial communities could be clarified by the analysis of plate wash samples (culturable bacteria) taken after about 50 days of culture of the contaminated soil microcosm. As the all bacteria in plate wash samples were culturable bacteria, they should be isolated easily and it was possible to use these bacteria for bioremediation of $HgCl_2$- and TCE-contaminated soils. We consider that the use of this method might be able to reveal the effects of various pollutants on bacterial communities in contaminated soils. The use of plate wash samples is more effective than soil samples, because soil samples are apparently stable and included great kinds of bacteria.

Acknowledgments

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