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RESEARCH ARTICLE

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Stability of bacterial populations in tropical soil upon exposure to Lindane

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Abstract The effect of the pesticide Lindane on microbial populations was analyzed in soil with a history of contamination with various chemicals, including this pesticide. Soil microcosms were amended with 100 mg Lindane/kg soil and microbial populations were monitored for 70 days. Bacterial cell concentrations, metabolic versatility (whole community Biolog), and genetic diversity (16S rDNA/denaturing gradient gel electrophoresis) were used to monitor microbial communities. Results show the persistence of Lindane in the soil environment; at the end of the experiment, 70% of the added Lindane remained undegraded. A reduction of 50% in bacterial cell concentration was observed in Lindane-amended microcosms during the 2nd week of the experiment. This reduction was correlated with a reduction in the rate of substrate utilization as observed by Biolog. Overall, no effect of Lindane was observed on the metabolic versatility and genetic diversity in these soils, demonstrating the ability of these bacterial populations to tolerate the pressure caused by the addition of pesticides.

Keywords Lindane · Organochlorinated pesticides · Soil microbiology

Introduction

The application of pesticides is a common practice in agriculture. However, the excessive use of these chemicals may result in the loss of efficiency and the production of secondary metabolites that may affect other groups of organisms. The effects associated with the addition of pesticides include reductions in soil enzymatic activity [22] as well as in concentrations of functional groups such as nitrifiers [25]. The effect of pesticides on genetic and metabolic diversity of soil microbial populations has been reported previously [3, 4].

Lindane (1,2,3,4,5,6-γ-hexachlorocyclohexane, or gamma-HCH) is a member of the organochlorinated pesticides used extensively in agriculture worldwide during the 1970s. The use of this pesticide has been restricted in most industrialized countries; however, it continues to be used in some regions of Europe and Asia for insect control, especially in programs for the prevention of malaria [19]. Degradation of gamma-HCH has been observed in anaerobic and water-saturated soils such as rice fields [9, 11, 21]; however, few data have been reported on aerobic soils.

Genetic diversity and metabolic versatility are two factors that give microbial populations the ability to tolerate deleterious chemical exposure [1]. The genetic diversity in microbial communities can be inferred from sequence analysis of the 16S rRNA-encoding genes of members of the communities [24]. Denaturing gradient gel electrophoresis (DGGE) can be used for this objective because of its ability to detect sequence differences and separate the genes accordingly [16]. This technique has been successfully used for the characterization of microbial communities [15] and to study the effect of anthropogenic activities on microbial populations [12, 24].

Community-level carbon substrate utilization analysis has been used in previous studies to describe the metabolic versatility of microbial populations [7]. This approach involves the use of Biolog microplates.

The present study investigated the possible impact of Lindane on microbial communities in tropical soils and the tolerance of these bacterial populations to exposure. The possible effects of the pesticide on the bacterial population was analyzed by measuring bacterial concentrations, metabolic (whole community Biolog

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analysis) and genetic (16S rDNA/DGGE) profiles for a continuous 70-day period under environmental conditions.

Materials and methods

Site description

Soil samples were obtained from Frontera Creek, Puerto Rico, a site known to be contaminated with mercury and Lindane (EPA 1999, Frontera Creek NPL Site Fact Sheet; http://www.epa.gov/region02/superfund/npl/0202550c.pdf). Soil core samples were taken to a depth of 10 cm. Soil texture measurements were carried out by a commercial laboratory (Watersheds Laboratory, Little River, Ga.).

Microcosm studies

Soil samples were homogenized and sifted aseptically before the start of the experiment to remove roots and other debris. The samples were then placed in sterile Erlenmeyer flasks and covered with sterile filter paper. Prior to the start of the experiment, the microcosms were left undisturbed at room temperature for stabilization for 1 month. At time zero, Lindane (97% purity; Sigma, St. Louis, Mo.) diluted in acetone was added to the microcosms at a concentration of 100 mg/kg soil. This solution was thoroughly mixed and the acetone allowed to evaporate. Humidity was monitored gravimetrically and the loss of moisture was compensated by adding the appropriate amount of sterile deionized water. All samplings were performed destructively and the microcosms were kept at environmental temperatures in a greenhouse at the Department of Biology, University of Puerto Rico. All experiments included unamended microcosms as controls.

Extraction and analysis of gamma-HCH

The concentration of gamma-HCH in the microcosms was monitored by organic solvent extraction followed by gas chromatography (GC). Methods for gamma-HCH extraction and the conditions for GC used have been described elsewhere [21].

Bacterial cell concentrations

Bacterial cells were counted by the acridine orange direct count (AODC) method. Briefly, 1 g soil was homogenized with 10 ml 0.1% pyrophosphate solution for 1 h using an orbital shaker at 200 rpm. The solution was centrifuged in sterile Oak Ridge tubes at 1,500 *g* for 5 min. A 10 µl sample of the supernatant was smeared in a toxoplasmosis slide and air-dried. The slides were filled with acridine orange solution (0.01% w/v aqueous solution) for 1 min and washed with sterile water. The slide was observed under epifluorescence (Olympus BX60) and all cells in 30 fields counted at 1,000× magnification in a serpentine fashion. The cell concentration was measured as described in [2].

Whole community Biolog analysis

Methods of cell extraction for the whole community Biolog analysis have been described previously [3]. The pellet obtained in the extraction method was suspended in 20 ml sterile 0.85% NaCl (w/v), and 150 µl was inoculated into ECOPLATES (Biolog) and incubated at 30°C in the dark. Microplates were read using a BIOLOG microstation reader. Data analysis methods for whole community Biolog have been previously described [6]. For statistical analyses, the raw difference of the OD (590 nm) reading (test well minus the blank well) was used.

DNA extraction and 16S rDNA/DGGE analysis

An Ultra Clean Soil DNA extraction kit from MoBio (Solana Beach, CA) was used as indicated by the manufacturer to isolate DNA from 0.25 g soil. PCR conditions and the sequence of 16S universal primers used are described elsewhere [15]. The PCR mixture consisted of 0.25 µM each primer, 2.5 mM Mg^{2+}, 200 µM dNTPs, 1× PCR buffer (Promega, Madison, WI), 1 U *Taq* polymerase (Promega), and 10–20 ng soil DNA in a final volume of 50 µl. PCR was run in a Mastercycler Gradient Eppendorf. DGGE was carried out using the DCODE system (Bio-Rad, Hercules, CA) using 8% polyacrylamide as the solid matrix in 1× TAE, with a range of denaturant from 40 to 70% on 16 cm×20 cm×1.0 mm plates. The 100% denaturant solution consisted of 7 M urea and 40% formamide. Electrophoresis was run at 55 V and 60°C for 16 h, gels stained with Sybr Green (Sigma) for 45 min photographed in a Gel Doc 2000 (Bio-Rad) and analyzed using Quantity One 1-D analysis software (Bio-Rad).

Results

Soil texture

The soil consisted of 25% sand, 41.4% clay, and 33.7% silt, and had an average 20% water content (v/w soil); the pH was 6.5.

Concentration of gamma-HCH in soil

Figure 1A shows data indicating that 70% (±10%) of the added Lindane remained undegraded in the microcosms for up to 70 days.

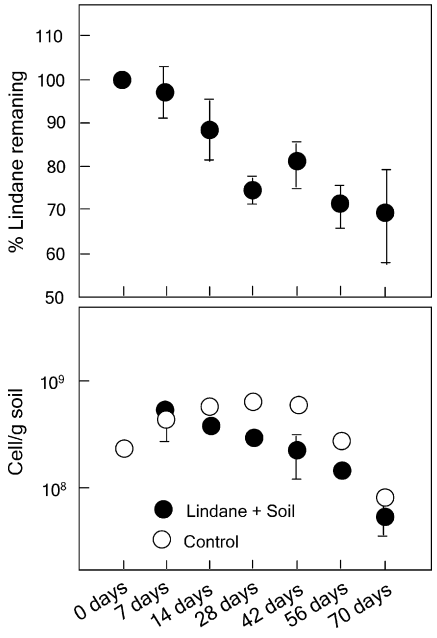

Fig. 1 Percentage (±SD) of Lindane remaining (*top*), and total cell density (±SD) of the microcosms (*bottom*) during the course of the experiment

Bacterial numbers

A reduction in cell concentration was observed in the Lindane-amended microcosms after 14 days (Fig. 1B), although after 70 days the cell concentrations had decreased to similar levels in all treatments. The mean bacterial concentration throughout the experiment was 10^8 cells/g dry soil. Two-way analysis of variance (ANOVA) found a significant difference between the treatments ($P < 0.001$) in which the Lindane-amended microcosms had the lowest concentrations.

Whole community Biolog analysis

The average concentration of cells in the inocula of the microplates was 10^7 cells/ml (as measured by AODC). The rate of average well color development (AWCD) for each sample is shown in Fig. 2. Between days 14 and 28, the AWCD was significantly lower in the Lindane microcosms at 48 h of incubation of the microplates ($P < 0.005$). The average utilization of the carboxylic acids was significantly higher ($P < 0.01$) on the amended microcosms at 28 days of experiment, although utilization of carbohydrates was significantly higher ($P < 0.01$) in the control microcosms at the same time point. A principal component analysis (PCA) of the raw differences of the OD readings was carried out with data obtained at 48 h of incubation. The first component of the PCA constitutes 28% of the variance; the second, 11.3%, and the third, 9.0%. The first component showed a negative correlation with cell concentrations ($P < 0.005$) and a positive correlation with the time of experiment ($P < 0.005$). The results of PCA are shown in Fig. 3. In this figure, showing results at 7–14 days of the experiment, the ordination of the treatments was different. The Lindane-amended microcosms are located to the left of the values of the first component. After 28 days of experiment, the treatment did not show any difference in their ordination. Using higher reading times for the PCA reduced the differences between the treatments in all samples.

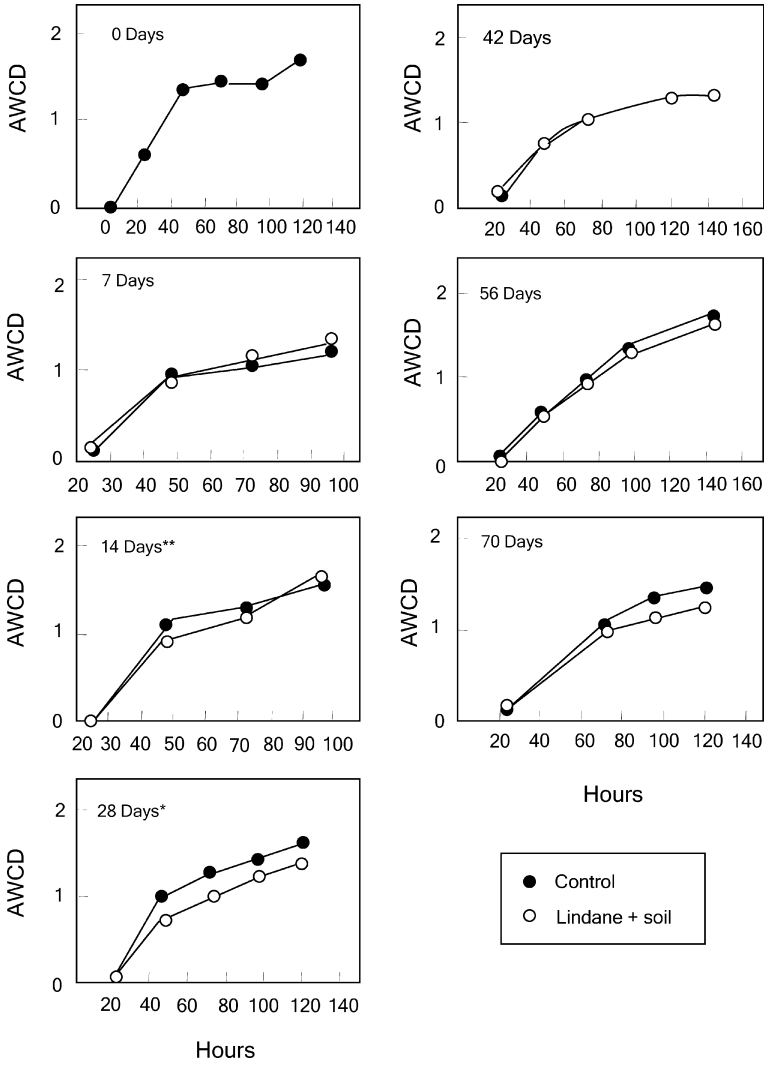

Fig. 2 Rate of average well color development (AWCD) for all sampling (±SD).
** $P < 0.001$, * $P < 0.05$

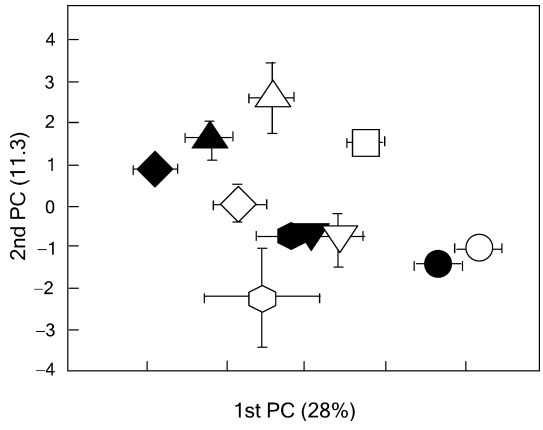

Fig. 3 First two components of the principal component analysis (PCA) of the whole community biolog analysis. *Squares* Time zero, *triangles up* 7 days, *diamonds* 14 days, *hexagons* 28 days, *triangles down* 42 days, *circles* 56 days. *White* Control soils, *black* soils with Lindane. *Bars* Standard deviation

16S rDNA/DGGE analysis

The results from the densitometric analysis of DGGE are shown in Fig. 4. No differences in band richness were observed between treatments. Densitometric readings were used for the PCA analysis, and the first component constitutes 30% of the variance; the second, 25%; and the third, 17.5%. The first component was highly correlated with the time of experiment ($P < 0.01$). Figure 5 shows the first two components (55% of the variability). The non-amended sample data between days 14 and 42 are located on the negative part of the second component. The difference between samples was reduced during the course of the 70-day experiment.

Discussion

Our results show that 70% of the Lindane added to the microcosms could be recovered after the completion of the experiment. The resilience of Lindane in the soil

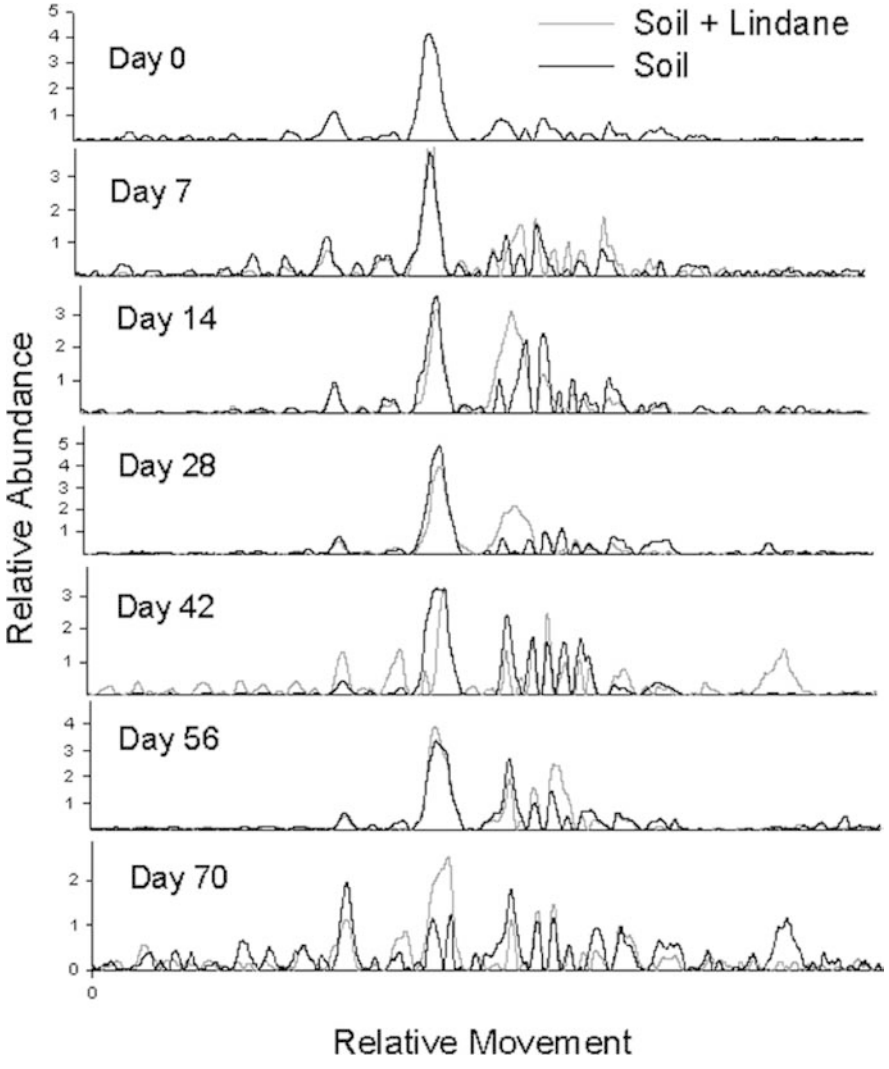

Fig. 4 Densitometric analysis of denaturing gradient gel electrophoresis (DGGE) gel with the products of amplification of 16S rDNA for Eubacteria. No difference in band richness can be observed, only differences in intensity

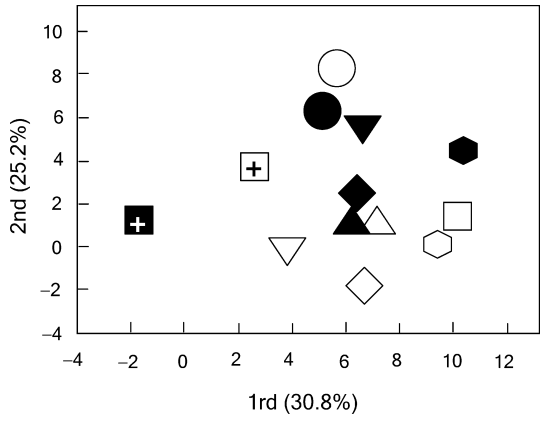

Fig. 5 First two components of the PCA of the 16S rDNA DGGE results. *Squares* Time zero, *triangles up* 7 days, *diamonds*, 14 days, *hexagons* 28 days, *triangles down* 42 days, *circles* 56 days, *square + cross* 70 days. *White* Control soils, *black* soils with Lindane

studied was similar to that previously reported in European agricultural soils [19]. The soil isolate *Sphingomonas paucimobilis* has been reported to have the ability to degrade gamma-HCH aerobically [10,13,17,18] in soils [21]. The addition of inocula (bioaugmentation) to soils where Lindane was recalcitrant resulted in the degradation of the pesticide, further indicating the role of the microbiota in pesticide degradation [26]. Lindane-degrading bacteria may not be widespread in soils. Several studies in our laboratory found that soil fungi were responsible for the 30% degradation observed (data not shown).

A decrease in total bacterial cell concentration was observed in the Lindane-amended microcosms. Toyota et al. [25] reported a similar reduction phenomenon as a consequence of exposure to the fumigant metasodium. The decrease in cell concentration in the soil was correlated with a reduction in the rate of AWCD in the microplates of Lindane-amended microcosms. Previous work has indicated that the concentration and physiological state of the microorganisms in the inocula have a linear relationship with AWCD rates in Biolog microplates [5, 6]. In addition, the contribution of each population to the metabolic profile will be affected by the density of this population in the soil [27].

Our results show differences in the metabolic profiles for the treatments between days 7 and 28, when the data obtained after 48 h of incubation were used for statistical analyses. These results were highly influenced by cell concentration in the microcosms. During the subsequent days of the experiment, no differences were observed in either treatment. Analysis carried out with data obtained from higher reading times (96 h of incubation) resulted in a reduction in the differences observed between all samples. Our results suggest that Lindane should not affect the metabolic versatility (capability) of these microbial populations, despite the apparent effects on the physiological state and density of microbial populations exposed to this pesticide, as demonstrated by the subsequent recovery and the dynamics of the metabolic capabilities to tolerate such exposure.

Results obtained with 16S rDNA/DGGE show that the addition of Lindane does not affect the electrophoretic band richness in soil microbial populations. In addition, the differences in the relative intensities of the bands observed between treatments were reduced at the end of the experiment. Engelen et al. [3] reported that exposure to the herbicide Herbogil caused inhibition of metabolic pathways and a reduction in the mineralization of nitrogen in soil bacterial populations. They also reported enrichment of certain members of the population. Fantroussi et al. [4] reported the effects of long-term exposure to phenyl urea herbicides on soil microbiota. Some of the effects they observed were changes in metabolic and genetic profiles of the community. They also reported a decrease in culturable bacteria in the herbicide-treated soils [4]. However, subsequent recovery of the microbial population after the event of perturbation has not been reported previously.

Our results suggest that Lindane might not be accessible to microorganisms in the soil, and that the development of mechanisms to tolerate or to overcome the pressure caused by Lindane exposure is possible. Physico-chemical characteristics are useful to classify pesticides and to carry out modeling of partition tendencies in the environment [8]. Lindane was placed in a group of pesticides that tend to be easily adsorbed by the soil matrix. This strongly suggests that there is lesser bioavailability of Lindane in the soil. However, in spite of its reduced bioavailability, our results show a decrease in bacterial population density upon the addition of this pesticide to the soil.

Studies have also been conducted on the functional and genetic stability of microbial populations after exposure to chemical contamination. Previous studies have reported the ability of bacterial populations to develop a tolerance to mercury contamination after long-term exposure, in which bacterial populations recovered their metabolic versatility [14, 20]. Also, it has been reported that carbon availability is the most important factor in maintaining diversity in soil. In the case of high carbon availability, contamination with toxic compounds might not directly affect the microbial diversity of these soils [28]. Biodiversity plays an important role in maintaining the functional stability of a macro-community after the event of perturbation [23].

In conclusion, our results demonstrate that addition of Lindane to these soils did not affect the genetic and metabolic profiles of microbial populations. Although a decrease in cell concentration was observed, the effect of this reduction was overcome and it was not reflected in the final metabolic and genetic composition of these communities. This shows the ability of these populations to tolerate certain chemical pressures.

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References

1. Atlas R (1986) Microbial diversity. Adv Microb Ecol 5:1–25
2. Bloem J, Blhuis P, Veninga M, Wieringa J (1998). Microscopic methods for counting bacteria and fungi in soil. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, New York
3. Engelen B, Meinken K, von Wintzingerode F, Heuer H, Malkomes HP, Backhaus H (1998) Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. Appl Environ Microbiol 64:2814–2821
4. Fantroussi SE, Verschuere L, Verstraete W, Top EM (1999) Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. Appl Environ Microbiol 65:982–988
5. Garland JL (1996) Analytical approach to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol Biochem 28:213–221
6. Garland J (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS Microbiol Ecol 24:289–300
7. Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microbiol 57:2351–2359
8. Gramatica P, Di Guardo A (2002) Screening of pesticides for environmental partitioning tendency. Chemosphere 47:947–956
9. Jagnow G, Haider K, Ellwardt PC (1977) Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch Microbiol 115:285–292
10. Johri AK, Dua M, Tuteja D, Saxena R, Saxena DM, Lal R (1996) Genetic manipulations of microorganisms for the degradation of hexachlorocyclohexane. FEMS Microbiol Rev 19:69–84
11. MacRea IC, Raghu K, Batista EM (1969) Anaerobic degradation of the insecticide lindane by *Clostridium* sp. Nature 221:859–860
12. McCaig AE, Glover LA, Prosser JI (2001) Numerical analysis of grassland bacterial community structure under different management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl Environ Microbiol 67:4554–4559
13. Miyauchi K, Lee HS, Fukuda M, Takagi M, Nagata Y (2002) Cloning and characterization of *linR*, involved in regulation of the downstream pathway for gamma-hexachlorocyclohexane degradation in *Sphingomonas paucimobilis* UT26. Appl Environ Microbiol 68:1803–1807
14. Muller AK, Rasmussen LD, Sorensen SJ (2001) Adaptation of the bacterial community to mercury contamination. FEMS Microbiol Lett 204:49–53
15. Muyzer G, de Waal H, Uitterlinden A (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700
16. Myers RM, Maniatis T, Lerman LS (1987) Detection and localization of single base change by denaturing gradient gel electrophoresis. Methods Enzymol 155:501–527
17. Nagata Y, Ohtomo R, Miyauchi K, Fukuda M, Yano K, Takagi M (1994) Cloning and sequencing of a 2,5-dichloro 2,5-cyclohexadiene-1,4-diol dehydrogenase gene involved in the degradation of gamma-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J Bacteriol 176:3117–3125
18. Nagata Y, Miyauchi K, Damborrsky J, Manovaa K, Ansorgova A, Takagi M (1997) Purification of a haloalkene dehalogenase of a new substrate class from a hexachlorocyclohexane-degrading bacterium, *Sphingomonas paucimobilis* UT26. Appl Environ Microbiol 63:3707–3710
19. Popp P, Bruggemann L, Keil P, Thuss U, Weiss H (2000) Chlorobenzenes and hexachlorocyclohexanes (HCHs) in the atmosphere of Bitterfeld and Leipzig (Germany). Chemosphere 41:849–855
20. Rasmussen LD, Sorensen SJ (2001) Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. FEMS Microb Ecol 36:1–9
21. Sahu SK, Patnaik KK, Bhuyan S, Sethunathan N (1993) Degradation of soil-applied isomers of hexachlorocyclohexane by a *Pseudomonas* sp. Soil Biol Biochem 25:387–391
22. Sannino F, Gianfreda L (2001) Pesticide influence on soil enzymatic activities. Chemosphere 45:417–425
23. Tilman D (1996) Biodiversity: population versus ecosystem stability. Ecology 77:350–363
24. Torsvik V, Daae FL, Sandaa RA, Ovreas L (1998) Novel techniques for analyzing microbial diversity in natural and perturbed environments. J Biotechnol 64:53–62
25. Toyota K, Ritz K, Kuninaga S, Kimura M (1999) Impacts of fumigation with meta-sodium upon soil microbial community structure in two Japanese soils. Soil Sci Plant Nutr Soil 45:207–223
26. Vogel TM, Cérémonie-Farhane H, Siminet P (2001) Bioremediation of lindane—natural microorganisms, indigenous genes and bioaugmentation. In: Proceedings of 9th International Symposium on Microbial Ecology, Amsterdam, The Netherlands. 26–31 August 2001, TU.084abstract, p 148
27. Wünsche L, Brüggemann L, Babel W (1995) Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution. FEMS Microbiol Ecol 17:295–306
28. Zhou J, Xia B, Treves DS, Wu LY, Marsh TL, O'Neill RV, Palumbo AV, Tiedje JM (2002) Spatial and resource factors influencing high microbial diversity in soil. Appl Environ Microbiol 68:326–334