

Molecular diversity in the bacterial community and the fluorescent pseudomonads group in natural and chlorobenzoate-stressed peat-forest soil

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Abstract

Bacterial community shifts in a soil microcosm spiked with 3-chlorobenzoate or 2,5-dichlorobenzoate were monitored. The V6–V8 variable regions of soil bacterial 16S rRNA and rDNA were amplified and separated by temperature gradient gel electrophoresis (TGGE) profiling. Culturing in the presence of 2.5 mM chlorinated benzoates suppressed 10 to 100 fold the total aerobic bacterial community but had no effect on the diversity within the group of fluorescent pseudomonads. In contrast, the uncultured bacterial community showed a decrease in the number of bands in the TGGE profiles of the chlorobenzoate-spiked treatments. Accordingly, the Shannon's diversity and equitability indices of these treatments reflected a decreasing trend in time. The approach allowed a direct assessment of community shifts upon contamination of soil.

Key words: TGGE – 16S rRNA – chlorobenzoate – diversity index – equitability index – soil

Introduction

Chlorinated benzoates are a group of compounds with several different agricultural and industrial applications, like insect repellents, industrial solvents, herbicides, fungicides, odorisers and dielectric fluids. As a result of

their abundant production and use, both disperse and concentrated contamination had occurred in soils, ground water, sediments and biota (Oliver and Nicol 1982; Schwarzenbach, *et al.* 1979; van Zoest and van Eck 1993). Although some of these compounds are recalcitrant in the environment, many pure and enrichment bacterial cultures belonging mainly to the genera *Pseudomonas*, *Burkholderia* and *Alcaligenes* are able to use chlorobenzoates as sole carbon and energy sources under different physiological conditions (Chaudhry and Chapalamandugu 1993; Mohon and Tiedje 1992; Haggblom 1992). However, little is known about the ecological impact of these xenobiotic compounds on soil microbial communities of natural environments.

Several concepts of species diversity have been applied to evaluate an ecosystem. Some are simply based on the number of species or operational taxonomic units (OTU) found in a given ecosystem. More complex measures, generally expressed as diversity indices, take into account the relationship between species and individuals (Lloyd *et al.* 1968). Microbial ecology studies have used cultivation methods in combination with molecular typing techniques to assess microbial diversity changes under distinct environmental conditions (Bej *et al.* 1991; Dunbar *et al.* 1999; Smit *et al.* 1997). However, these approaches are constrained to the small fraction of cultivable bacteria.

Two types of approaches have been used to address estimation of bacterial diversity by culture-independent methods. One way is to generate information on whole microbial communities by analysing the melting be-

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haviour (Torsvik *et al.* 1994), or hybridisation kinetics (Griffiths *et al.* 1996) of community-extracted DNA. These broad-scale approaches have provided crude estimates of diversity and the relative structure of soil microbial communities, but they have failed in addressing information on specific taxa. The other type of culture-independent approach mainly relies on the use of the 16S rRNA gene as phylogenetic marker. Here, basically 16S rRNA genes are amplified by PCR from DNA or reverse transcribed RNA from environmental samples. The subsequent separation or detection of specific amplicons has been achieved by different means, like cloning and sequence analysis (Barns *et al.* 1994), or quantitative hybridisation (Stahl and Amann 1991) with oligonucleotide probes designed for the detection of different phylogenetic groups (Raskin *et al.* 1994; Franks *et al.* 1998). Terminal restriction fragment length polymorphism (T-RFLP) (Wen-Tso *et al.* 1997, Sessitsch *et al.* 2001), or single strand conformation polymorphism analysis (Schwieger and Tebbe 1998; Peters *et al.* 2000) have been proposed as alternatives to resolve the amplicons and analyse microbial community diversity.

The use of denaturing gradient gel electrophoresis (DGGE) or the equivalent temperature gradient gel electrophoresis (TGGE) offers the possibility to be used as a rather broad profiling technique as well as to analyse specific bacterial groups within complex communities. Both techniques allow the separation of DNA fragments of equal size but different sequence information, therefore they are highly suitable to analyse amplicons generated from a complex mixture. Ideally, the final electrophoretic profile will reflect the relative abundance of members of a microbial community (Muyzer *et al.* 1993; Felske *et al.* 1997). Calculation of diversity by means of rarefaction curves has been applied to T-RFLP patterns of soil microbial communities (Dunbar *et al.* 2000). A more complex approach to quantify microbial diversity has been proposed by Nübel *et al.* (1999), under this approach, correlating diversity indexes were calculated based on quantification of morphotypes, carotenoids and 16S rRNA genes, the latter resolved by DGGE.

In a previous report we assessed bacterial community shifts and specifically enriched populations in a peat forest soil microcosm due to 3-chlorobenzoate (3CBA) stress (Ramírez-Saad *et al.* 2000). In the present study we have monitored the same soil system, evaluating the responses of fluorescent pseudomonads to contamination with 3CBA and 2,5DCB. TGGE was used as a community profiling technique and relative quantification of individual bands were used to determine Shannon's diversity and equitability indices (Shannon and Weaver 1963). We aimed to describe the changes in the structure of the soil bacterial community and in the

group of fluorescent pseudomonads, after addition of 2,5DCB, or 3CBA to the soil microcosm. The changes in the fluorescent pseudomonads group were evaluated with the same ecological indices, as a mean to establish whether proportional or correlative changes occurred in the total bacterial community, and a group of bacteria that are easy to isolate and screen for.

Materials and methods

Soil sampling

Soil samples were collected during the summer from a natural black alder (*Alnus glutinosa*) wet stand at the nature reserve "De Hel", Veenendaal, The Netherlands. Details about the site and properties of the peat soil were reported previously (Wolters *et al.* 1997). Soil samples free of stones and large plant material were taken in the neighbourhood of 4 black alder trees. The samples were pooled and stored at 4°C until their use, within the following week.

Microcosm experiments

The microcosm soil systems were set up as previously described (Ramírez-Saad *et al.* 2000). Three treatments were established; a) control treatment added with water at 45% (w/v), b) 2,5DCB-spiked treatment to a final concentration of 0.4 µmoles g⁻¹ wet soil, and c) 3CBA-spiked treatment with the same final concentration of 0.4 µmoles g⁻¹ wet soil. The chlorinated compounds were added to the soil in small aliquots and mixed thoroughly. Each soil-microcosm system was set up in duplicate. Samples (5 g soil) were taken at 0, 5, 10, and 15 days, replicate soil samples from each treatment were pooled together and used to extract DNA and rRNA. These same samples were also used to extract soil-bacteria for isolation of *Pseudomonas*-like and the respective fraction of fluorescent pseudomonads. For the samples taken at time 0 days, the nucleic acids extraction and the bacterial isolation procedures started 3 h after the chlorobenzoates were added to the soil.

Plate counting and isolation of fluorescent pseudomonads

Microorganisms from the original unspiked soil were extracted as previously described (Ramírez-Saad *et al.* 2000). Serial dilutions of the soil extracts were plated on tryptone-soy-broth amended with 1.5% (w/v) agar (TSA), as well as on a basal medium (BM), made as 1.8 mM CaCl₂ · 2 H₂O, 4.1 mM MgSO₄ · H₂O, 5 mM NH₄Cl, 1.4 mM K₂(NaH₂)PO₄ buffer (pH 6.9), and

trace elements solutions (Stams *et al.* 1993). The BM medium was supplemented with 5 mM glucose, or 2.5 mM of either 3CBA or 2,5DCB as sole energy and C sources, additional BM plates containing a combination of glucose and 3CBA, or glucose and 2,5DCB were also inoculated. All plates were incubated at 24 °C. TSA plates were incubated for 24 h, whereas BM plates were incubated for three days. Triplicate plates of appropriate dilutions were scored for plate counting, after respective incubation times.

Isolation of *Pseudomonas*-like and fluorescent pseudomonads was done by applying the same extraction procedure, with samples taken at days 0, 5, 10 and 15. The diluted soil extracts were first plated in TSA medium containing 50 µg ml⁻¹ cycloheximide. After one day incubation plates from each treatment, containing more than 100 colonies, but belonging to the same dilution step were taken. From those plates, single colonies were selected based on their *Pseudomonas*-like morphology (i.e. pale yellow to greenish colonies with butyrose aspect), and transferred to the differential King's medium B (KB) (King *et al.* 1954). Screening for production of fluorescent pigments was done under UV light after 2 days of incubation on KB medium at 24 °C. The numbers of *Pseudomonas*-like colonies and their respective fraction of fluorescent *Pseudomonas* were scored for each sampling time.

DNA and rRNA extraction

DNA and rRNA from soil microorganisms were simultaneously extracted as described by Felske *et al.* (1996). Briefly, 2 g (fresh weight) of soil were subjected to bead-beating to achieve cell disruption in a buffered environment, followed by differential centrifugation steps allowing separation of nucleic acids and ribosomes from the larger soil particles and cell debris. The resulting fraction was resuspended and divided in two. One fraction was phenol extracted and ethanol precipitated to obtain DNA. The other fraction, yielding rRNA was acid-phenol extracted, ethanol precipitated and DNase digested. The final pellets of both fractions were dissolved in 100 µl of an appropriate buffer. The amount of 16S and 23S rRNA obtained from each soil sample was estimated by agarose gel electrophoresis in combination with ethidium bromide staining, computer-assisted image analysis and rRNA standards (Felske *et al.* 1997).

DNA extraction of the isolated pseudomonads strains was done by aseptically picking single colonies. Each colony was resuspended in 30 µl sterile distilled water, boiled for 15 min and briefly centrifuged to pellet the cell debris. Aliquots of these crude lysates were used as template for PCR reactions.

PCR, reverse transcription (RT)-PCR and temperature gradient gel electrophoresis

PCR and RT-PCR reactions targeting the V6 to V8 variable regions (Neefs *et al.* 1990) of respectively the 16S rDNA or rRNA were set up according to Felske *et al.* (1996). In all cases 1 µl of soil-extracted DNA or rRNA was utilised as template. Similar PCR-amplifications were performed using 2–3 µl of the fluorescent *pseudomonads* cell lysates.

Amplicons obtained from the soil-extracted DNA and rRNA were separated on a TGGE system (Diagen, Düsseldorf, Germany), as described by Felske *et al.* (1998a). The high resolution achieved by TGGE was also used as an alternative and fast way to ribotype the fluorescent *Pseudomonas* isolates based on the different mobility of their respective V6 to V8 amplicons. In order to obtain a better separation of these amplicons during TGGE, the ΔT was reduced to 8 °C and a gradient ranging from 36 °C to 44 °C was used.

Estimation of Shannon's Diversity (H) and Equitability (J) indices

Species richness and evenness of total bacterial communities in control and spiked soils as well as the fluorescent pseudomonads were approached by the ecological indices of Diversity (H) and Equitability (J) proposed by Shannon and Weaver (1963), as implemented in Begon *et al.* (1990). The indices are defined by the equations: $H = -\sum P_i \ln P_i$, and $J = H/\ln S$; where P_i represents the number of individuals from one species or one OTU, divided by the total number of individuals in the community sample, and S represents the total number of OTU in the sample.

Estimation of both indices was based on the TGGE profiles shown on Fig. 1. Each band within a profile was considered as a different OTU, while bands with similar migration position in different profiles could be regarded as the same OTU. The silver stained gels were scanned in a flat bed scanner Sharp JX 330, equipped with a second film scanning unit JX-3F6 (Sharp Corp, Japan) for improved resolution. After image digitalisation, the Molecular Analyst (BioRad, Hercules, Cal., USA) software package was used to localise the bands within each profile, and to calculate band surface and intensity. The relation between band surface and the mean pixel intensity of that area was expressed as band volume values, which were taken as a surrogate measure of OTU's abundance. Band detection within a profile and the respective surface delimitation was performed by automated functions in the program. The number of OTU in a sample (S) was determined by considering only the quantifiable bands, which were those bands accounting for >2.5% of the total bands' surface in a profile.

Results and discussion

The effect of chlorinated benzoates on the bacterial community of a peat-forest soil has been assessed by both culture-dependent and culture-independent approaches. With the first approach, bacteria extracted from the original unspiked soil-sample were cultured in the presence and absence of 3CBA or 2,5DCB and glucose. In addition, soil-extracted bacteria from the treated microcosm systems were isolated, *Pseudomonas*-like colonies were picked and the fraction of fluorescent pseudomonads was scored and selected for further evaluation.

Effect of 3CBA and 2,5DCB on the cultivability of soil bacteria

A high number of bacteria (4.9×10^9) could be cultivated on a rich medium with an easily degradable carbon source such as glucose (Table 1). However, when 3CBA or 2,5DCB were used as the sole carbon and energy source, less than 100 CFU g⁻¹ soil were obtained. None of these colonies were able to grow when transferred to a liquid medium containing the same components except agar. Furthermore, they did not appear when agarose or Noble agar was used instead of agar, indicating that those isolates were probably growing on some impurities present in the agar. Addition of 3CBA or 2,5DCB to the BM medium containing also glucose resulted in a 10–100 fold decrease on the amount of CFU compared to the medium containing only glucose (Table 1).

Isolation, ribotyping and diversity of fluorescent pseudomonads isolates

Isolation of the fluorescent pseudomonads was done by a two-step approach in order to facilitate UV screening and typing of single isolated colonies. First, soil-extracted bacteria were plated on rich medium (TSA), from where *Pseudomonas*-like colonies were picked and subsequently grown on KB plates, where they were screened for fluorescence. The total number of *Pseudomonas*-like isolates was not affected by amendment of 3CBA or 2,5DCB (Table 2), whereas the number of fluorescent pseudomonads was significantly reduced by addition of 2,5DCB. The compound 3CBA did not significantly reduce the fluorescent *Pseudomonas* population. In order to evaluate the differences in the ratio fluorescent *Pseudomonas*/*Pseudomonas*-like isolates, multiple χ^2 tests were performed between the treatments (Conover 1999). The comparison between the control and the 3CBA treatment showed no significant difference in their ratios at 95% confidence, while the ratio in the 2,5DCB treatment was lower and

significantly different to the other treatments. In spite of this, the estimated Shannon's diversity (H) and equitability (J) indices of all treatments showed to be roughly similar (Table 2).

Typing based on TGGE allowed grouping of the fluorescent pseudomonads in 9 different ribotypes

Table 1. Effect of the presence of chlorobenzoates in the culture medium on the number of colony forming units (CFU) obtained from unspiked peat-forest soil.

Rich medium	C. F. U. g ⁻¹ soil \pm S. D.
TSA	$>3.0 \times 10^{10}$
Basal medium (BM) supplemented with:	
[5 mM] glucose	$4.9 \times 10^9 \pm 2.0 \times 10^9$ a
[2.5 mM] 3CBA	<100 *b
[2.5 mM] 2,5DCB	<100 *b
[2.5 mM] 3CBA + [5 mM] glucose	$1.2 \times 10^8 \pm 7.2 \times 10^7$ c
[2.5 mM] 2,5DCB + [5 mM] glucose	$5.1 \times 10^7 \pm 3.6 \times 10^7$ c

* regarded as non-chlorobenzoate degraders.

Log of values followed by the same letter are non-significantly different.

Table 2. Ribotyping and ribotype abundance of the fluorescent pseudomonads isolated from the unstressed (control) and stressed (2,5DCB and 3CBA) peat soil microcosms. Shannon's diversity (H) and equitability (J) indices were estimated from the values given in the respective columns.

Ribotypes	Treatments		
	Control	2,5DCB	3CBA
A	16	6	17
B	3	4	6
C	23	13	15
D	11	4	9
V	4	3	
W		4	2
X			1
Y			1
Z	5		
Total number of fluorescent <i>Pseudomonas</i> *	62*	34	51*
Total number of <i>Pseudomonas</i> -like isolates	327	326	325
Shanon's diversity index (H)	1.55	1.64	1.57
Shanon's equitability index (J)	0.87	0.92	0.81

* the proportion of fluorescent pseudomonads/*Pseudomonas*-like isolates in the two marked treatments were not significantly different at 95% confidence.

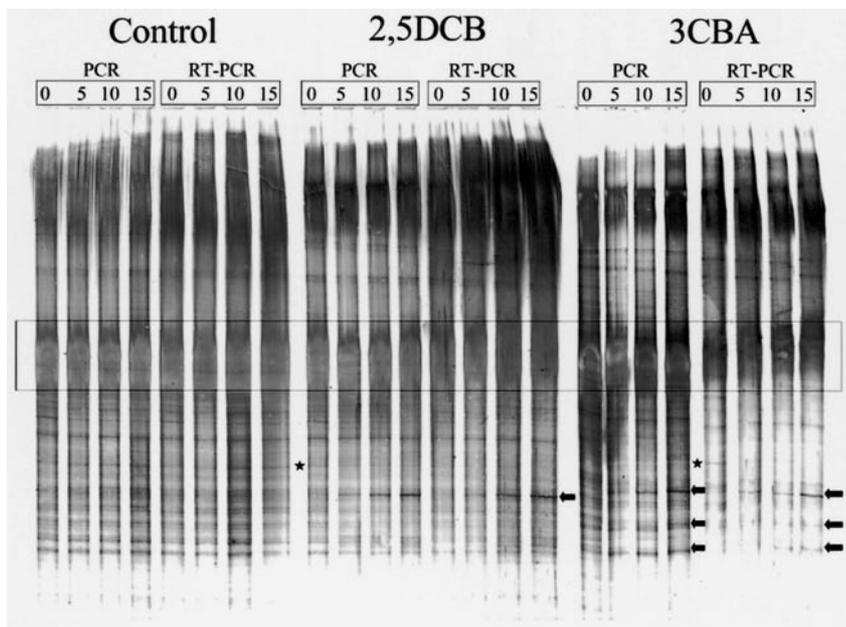


Fig. 1. DNA- and rRNA-based TGGE profiles from bacterial community in the unspiked (control) and spiked (2,5DCB and 3CBA) peat-forest soil.

(Table 2). Ribotypes A to D were found in all treatments and comprised 86% of the total fluorescent *Pseudomonas* isolates. Ribotypes V to Z were less abundant and only detected in one or two treatments. Comparison of the fluorescent *Pseudomonas* ribotype bands did not match prominent bands in the community profiles (data not shown).

TGGE-based ribotyping of the fluorescent pseudomonads has proven as fast and easy way to type a large number of isolates, resulting in single or two-band patterns determining OTU's or groups of bacteria. Furthermore, the isolates typed under this approach could be addressed in the context of the community TGGE profiles. However, the ribotype bands of the different fluorescent isolates did not have a corresponding band in the profiles, enabling the idea that this group of bacteria is a minor component of the bacterial community in our model soil. Although, it was not possible to completely ascertain this, as several ribotype bands were migrating in the same area as the single stranded DNA (marked with a rectangle in Fig. 1).

Whole bacterial community analysis

The toxic effect of 2,5DCB and 3CBA was more evident when culture independent techniques were used to address changes induced by those compounds in the soil bacterial community.

Soil-extracted DNA and rRNA were used to amplify the V6 to V8 region of 16S rDNA and rRNA, the amplicons were resolved by TGGE. Comparison of PCR-based profiles with the respective RT-PCR profiles of

the control soil did not show marked differences (Fig. 1). The numbers of scored bands (S) were quite constant in both types of profiles (Table 3). Accordingly, the values for the Shannon's Diversity (H) and Equitability (J) indices determined from the relative band volumes within each profile showed only minor variations (Table 3), indicating a stable structure and composition of the prominent bacterial community of this treatment. In contrast to the control soil, the TGGE profiles of both chlorobenzoate-spiked treatments showed some variations such as bands appearing with increasing intensity in time (marked with arrowheads in Fig. 1). Other minor bands became fainter or even disappeared probably due to the presence of chlorobenzoates (marked with star in Fig. 1). A diminishing number of quantifiable bands could be observed in spiked soils. This trend was more pronounced in 2,5DCB-treated soil, where S values decreased from 17 to 10 bands after 15 days, whereas the reduction due to 3CBA was from 12 to 10 bands (Table 3). The initial numbers of quantifiable bands in the spiked treatments were in general lower than in control soils, especially in the rRNA profiles of the 3CBA-treated soil. This could be indicating a rapid inhibitory effect on the bacterial community occurring only few hours after addition of the chlorobenzoates to the soil. The respective TGGE profiles reflected this fact with an increasing number of very faint, non-quantifiable bands, which interestingly is not reflected so markedly in the H and J values. A possible mechanism explaining the low number of detectable bands in the profiles, may be that 3CBA caused cell lysis in a fraction of the com-

Table 3. Shannon's diversity (H) and equitability (J) indices for each treatment, sampling time and target molecule (DNA or rRNA). The estimation of H and J was based on the TGGE profiles from Fig. 1. S represents the number of quantifiable bands (OTU) in each profile.

	Time (days)	Treatments								
		Control			2,5DCB			3CBA		
		H	J	S	H	J	S	H	J	S
DNA (PCR)	0	2.63	0.88	20	2.55	0.90	17	2.28	0.92	12
	5	2.45	0.85	18	2.45	0.90	15	1.86	0.75	12
	10	2.55	0.87	19	1.48	0.64	10	1.63	0.68	11
	15	2.46	0.85	18	1.55	0.67	10	1.48	0.65	10
rRNA (RT-PCR)	0	2.61	0.89	19	2.39	0.86	16	1.43	0.73	7
	5	2.58	0.88	19	1.78	0.86	8	0.86	0.54	5
	10	2.69	0.88	21	2.05	0.78	14	1.85	0.84	9
	15	2.68	0.89	20	1.54	0.60	13	1.60	0.77	8

munity, whose DNA was adsorbed to charged soil particles, becoming non-available for amplification processes.

Similarly, based on rRNA profiles spiked soils showed decreased S, H and J values as compared to the control soil (Table 3). However, the lowest values were found on day 5 (Table 3). The decreases of H values are mostly related to a reduction with time on the number and volume of prominent bands in the respective profiles. Decreasing equitability values (J) are reflecting unequal abundance of target molecules within the original complex mixture.

Since the estimated ecological indices for the fluorescent pseudomonads group did not correlate with chlorobenzoate induced stress, and the effect of these compounds was evident in the bacterial community by both, culture-dependent and culture independent methods, we can assume that the fluorescent pseudomonads are not a reliable indicator group for this type of stress.

TGGE and DGGE profiling have already proven to be powerful tools to analyse complex microbial communities (Muyzer *et al.* 1993, Felske *et al.* 1998a, Ramírez-Saad *et al.* 2000, Ralebitso *et al.* 2000, McCaig *et al.* 2001, Boon *et al.* 2002). The additional relative quantification of the banding patterns enabled to estimate Shannon's diversity and equitability indices providing a better insight on the changes of the community structure. The general picture of the profiling and subsequent analysis of all treatments, pointed to the detection of major changes in the structure and composition of the predominant bacterial populations of the chlorobenzoate spiked soils. We are aware that the actual bacterial soil diversity must be higher than our estimates based on TGGE profiling, this is mainly due to the constraints imposed for the relative quantification of only prominent bands. However, even this partial approach enabled us to address shifts in the presumably most

abundant, active and important fraction of the bacterial community.

Considering also minor bands in the analysis (<2.5% of total bands' area) would result in larger differences in the estimated indices, which may not be reflecting only the actual variances in diversity due to an effect of the chlorobenzoate, but also biases from the PCR, TGGE or bands detection. Certainly the whole procedure may suffer the known biases inherent to the use of PCR-based approaches when estimating microbial diversity from environmental samples [reviewed by Wintzingerode *et al.* 1997, Head *et al.* 1998, Nübel *et al.* 1999]. However, the introduced biases must be affecting our samples and estimations in an even manner, as the samples have a common origin and except for the addition of the chlorobenzoates, they were similarly treated. It has been demonstrated that the primer pair used; U968/GC and L1401 (Nübel *et al.* 1996), amplified with similar efficiency target molecules from different source (Felske *et al.* 1998b).

Application of diversity and equitability indices in combination with a community profiling technique like TGGE or DGGE is particularly useful when examining time series and population dynamics within the same community. Estimation of changes as numeric values rather than banding patterns may facilitate comparisons and prediction of trends. This approach may therefore be valuable in making decisions when addressing short and long-term responses of a bacterial community to transient environmental perturbations.

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