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Novel techniques for analysing microbial diversity in natural and perturbed environments

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Abstract

Molecular techniques were applied for analysing the entire bacterial community, including both the cultivated and non-cultivated part of the community. DNA was extracted from samples of soils and sediments, and a combination of different molecular methods were used to investigate community structure and diversity in these environments. Reassociation of sheared and thermally denatured DNA in solution was used to measure the total genetical diversity. PCR-denaturing gradient gel electrophoresis (DGGE) analysis of rRNA genes gave information about changes in the numerically dominating bacterial populations. Hybridisation with phylogenetic group specific probes, and sequencing provided information about the affiliation of the bacterial populations. Using DNA reassociation analysis we demonstrated that bacterial communities in pristine soil and sediments may contain more than 10000 different bacterial types. The diversity of the total soil community was at least 200 times higher than the diversity of bacterial isolates from the same soil. This indicates that the culturing conditions select for a distinct subpopulation of the bacteria present in the environment. Molecular methods were applied to monitor the effects of perturbations due to antropogenic activities and pollution on microbial communities. Our investigations show that agricultural management, fish farming and pollution may lead to profound changes in the community structure and a reduction in the bacterial diversity.

Keywords: Bacterial communities; Diversity; DNA analysis; Soil; Sediments

1. Introduction

Biodiversity has been defined as the range of significantly different types of organisms and their relative abundance in an assemblage or commu-

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nity. The diversity has also been defined according to information theory, as the amount and distribution of information in an assemblage or community (Atlas, 1984).

Traditionally, biodiversity is based upon the species as a unit. Species diversity consists of two components, species richness and species evenness (distribution). For prokaryotic organisms the species concept is obscure. This problem has been circumvented by replacing traditional identification and classification with numerical taxonomy. With this method the distances between isolates are calculated and they are then clustered into biotypes. The biotype is an operational taxonomic unit (OTU) which can be used instead of species to characterise and compare populations and communities.

One gram of soil or sediment may contain more than 10^{10} bacteria as counted in fluorescence microscope after staining with a fluorescent dye (Fægri et al., 1977; Torsvik et al., 1990a). A serious problem in microbial ecology is that the relative proportion of bacteria growing on agar plates (CFU) vary from 0.1 to 1% in pristine forest soils to 10% in environments like arable soil. This implies that investigations based on bacterial isolates may include only a minor part of the total bacterial diversity.

Molecular methods provide tools for analysing the entire bacterial community, covering also those bacteria that have not been cultured in the laboratory. Therefore, such methods are becoming increasingly important in microbial ecology (Pickup, 1991; Stackebrandt et al., 1993; Amann et al., 1995; Holben and Harris, 1995). We have applied DNA analysis at different resolution levels to analyse whole communities, bacterial isolates, and clones of specific genes. Low resolution and broad scale analysis of community DNA, like DNA-reassociation, allow assessment of the total genetical diversity of bacterial communities (Torsvik et al., 1996). PCR-denaturing gradient gel electrophoresis (DGGE) analysis of rRNA genes gives somewhat higher resolution and provides information about changes in the gross community structure (Muyzer et al., 1993). When DGGE analyses of rRNA genes are combined with hybridisation using phylogenetic probes or with sequencing, assessment of the phylogenetic affiliation of the numerically dominating members of a community can be obtained (Øvreås et al., 1997). Fluorescent in situ hybridisation (FISH) of bacterial cells with phylogenetic probes provides information about the overall taxon composition of bacterial communities or assemblies (Hahn et al., 1992; Amann et al., 1995). By cloning PCR-products from rRNA genes in whole community DNA, information about non-cultured bacteria is gained. This approach also allows comparison of the structure of the cultivated fraction of a bacterial community with the total community. To discriminate at bacterial isolate and clone levels, DNA fingerprinting and sequencing have been applied (de Bruijn, 1992; Massol-Deya et al., 1995; Stackebrandt and Rainey, 1995).

An important part of our investigations has been to evaluate the different methods and their feasibility in monitoring changes in microbial communities due to antropogenic impact. We have mainly studied soil systems, but marine sediments have also been included. The soil environments investigated comprise soil under different agricultural management, sewage sludge amended soils with heavy metal contamination, and chemically polluted soils. The perturbed soils have been compared to unpolluted and pristine soils. The sediments investigated were polluted fish farm sediments that were compared to pristine sediments with similar amount of organic matter.

2. Methods for determination of community structure and diversity

2.1. DNA melting-profiles and reassociation analysis

The gross genetical structure and diversity in bacterial communities were assessed by measuring the base composition and complexity of total community DNA. Base composition profiles expressed as mole percent guanine + cytosine (%G + C), were determined by analysing the melting curves of DNA (Torsvik et al., 1995). The melting profiles were converted to %G + C profiles by calculating the first derivative of the melting curve (Ritz et al., 1997).

The sequence complexity of DNA was determined by measuring the reannealing (reassociation) kinetics of sheared and thermally denatured (single stranded) DNA in solution under defined conditions (Britten and Kohne, 1968). For assessment of prokaryotic diversity, the DNA used in the reassociation analysis had to be free from eukaryotic DNA. The bacteria were therefore separated from the soil by fractionated centrifugation method (Fægri et al., 1977) before lysis.

After bacterial lysis, the crude DNA extract was purified by hydroxyapatite chromatography, which gave highly purified DNA (Torsvik, 1980). For reassociation analysis the DNA was sheared to an average fragment length of 650 base pairs (bp). The reassociation of single stranded (melted) DNA in $6 \times$ SSC (standard saline citrate), 30% DMSO (dimethylsulfoxide) solutions at 25°C below the average melting temperature (T_m), was measured as the decrease in absorbance at 260 or 275 nm in a spectrophotometer (Torsvik et al., 1995). For an ideal second order reaction, the reassociation rate constant is expressed as $1/C_0t_{1/2}$, where C_0 is the molar concentration of nucleotides in single stranded DNA at the beginning of the reassociation, and $t_{1/2}$ the time in seconds for 50% reassociation. Under defined conditions $C_0t_{1/2}$ is proportional to the DNA complexity (heterogeneity; Britten and Kohne, 1968).

DNA from a bacterial community is a mixture of DNA from different bacterial types that are present in different proportions. The reassociation curve for community DNA has a flatter slope than an ideal second order reaction curve. When the number of DNA types and the degree of homology between them increases, the overall reaction deviate from an ideal second order kinetic. In such cases, when the rate is a function of a number of reassociation reactions with different reaction constants superimposed upon each other, the $C_0t_{1/2}$ does not have any precise meaning. Nevertheless it provides information about the DNA complexity. We have used $C_0t_{1/2}$ as a diversity index similar to species diversity indices (Torsvik et al., 1995) which take into account both the amount and distribution of information in a community. The $C_0t_{1/2}$ values were determined relative to DNA with known complexity like *Escherichia coli* DNA (genome size; 4.1×10^6 bp, 2.71×10^9 Dalton).

2.2. DGGE community profiling

PCR-amplified 16S rRNA genes (rDNA) were analysed by applying DGGE (Muyzer et al., 1993; Øvreås et al., 1997). Primers annealing to conserved regions of the rDNA were used to amplify the variable V3 region flanked by the primers. Different base composition in this variable region of rDNA from the total community, gave PCR products with different melting points. The PCR products were separated according to their melting point in polyacrylamide gels with 15–55% denaturing gradient (100% denaturant comprised 7 M urea and 40% formamide). The gels were run at 60°C and 200 V for 3 h. A 40-nucleotide G + C-clamp was used on one primer to prevent completely melting of the double-stranded DNA, and thereby improve banding of the PCR products.

By applying group specific phylogenetic probes, the affiliation of the DGGE bands to main phylogenetic subclasses of the *Bacteria*, was determined. Furthermore, this approach allowed identification of bands from the putative numerically dominant bacteria. The strongest DGGE bands were punched out from the gel, reanalysed by PCR-DGGE to ensure that they consisted of a single DNA sequence, and subjected to sequencing. The DNA sequences were aligned to those obtained from the National Center for Biotechnology Information database by the Blast program (Altschul et al., 1990) in order to assign them to bacterial subclasses and for probe designing.

2.3. DNA cloning, probing and sequencing

A clone library of PCR amplified 16S rRNA genes from DNA extracted directly from soil samples were made in pGEM-T vector (Borneman et al., 1996). Bacterial and archaeal 16S rRNA genes were amplified in separate PCR reactions using kingdom specific forward primers (bacterial-27f and archaeal-46f) and a universal reverse primer (517r) (Lane et al., 1985; Torsvik et al., 1993a).

The cloned amplicons were compared by amplified ribosomal DNA-restriction analysis (ARDRA; Vaneechoutte et al., 1992), and grouped by hybridisation with phylogenetic group specific probes. Probes targeting the following bacterial classes and subclasses were applied: *Bacteria*338, *α-Proteobacteria*, *δ-Proteobacteria*, *Cytophaga–Flexibacter–Bacteroides* (CFB) subclass (Raskin et al., 1994; Amann et al., 1995). Representatives from each group, as based on ARDRA fingerprinting and probing of the cloned amplicons, were sequenced. The phylogenetic affiliations of the clones were compared to that of 300 bacterial isolates which were hybridised with phylogenetic probes in the same manner as the clones.

3. Bacterial diversity in natural environments

The genetical diversity index $C_0t_{1/2}$, provides a measure of the total genetical complexity in a community. Like the Shannon Weaver index for species diversity it includes the amount of information in the community and how this information is distributed. The reassociation rates of bacterial community DNA from pristine soil and sediments were very slow, giving $C_0t_{1/2}$ of 4500–9000 (reassociation experiments with such complex DNA takes several weeks). In contrast the $C_0t_{1/2}$ of DNA from a mixture of 200 randomly picked bacterial isolates from the pristine soil was 28 (Torsvik et al., 1990a), corresponding to a DNA complexity of 1.4×10^8 bp. Using the genome size of *E. coli* as a unit, this complexity corresponded to approximately 30 entirely different genomes with the same size as the *E. coli* genome.

It is difficult to estimate how many bacterial types this number of genomes is derived from. The minimum number must be approximately 30 (with 0% homology). If we use the 70% homology level as a limit for 'geno-species' (Wayne et al., 1987), there would be approximately 90 'geno-species'. This is probably a maximum number. The 200 isolates were characterised by a set of morphological and physiological tests. The distances between the isolates were calculated (Simple matching coefficient) and they were clustered using Complete link, furthest distance method. When 80% similarity level was used as a limit for defining biotypes or OTUs (Torsvik et al., 1990b), we found that the 200 isolates comprised of 41 biotypes, which is comparable to the number of 'genospecies'. The complexity of total bacterial DNA is at least 200 times higher than that of DNA from the isolated bacteria. This results show that the isolated bacteria were not representative, but made up only a small and distinct subpopulation of the total bacterial community.

4. Bacterial diversity in perturbed and polluted environments

Diversity measurements have been performed in perturbed environments like marine fish farm sediments, in heavy metal polluted soils, and in model experiments with soils supplied with a sole carbon source.

The mole% G + C profile of DNA from a pristine marine sediment with high organic content (Torsvik et al., 1993b) was very broad, with a plateau ranging from 35 to 60 mole% G + C (Fig. 1). This indicates that the bacterial community comprised a wide range of bacterial groups with entirely different base composition, and that none of them was dominant. In sediment samples from fish farms the mole% G + C profiles showed a main peak with an average of 36% G + C and a smaller shoulder extending up to the same maximum mole% G + C as in the pristine sediment profile. This profile indicates a scewed bacterial distribution in the community with a dominance of bacteria having a relative low mole% G + C in contrast to the pristine sediment. In a model experiment where oxolinic acid was added to the sediment, no change in the mole% G + C could be revealed (Fig. 1). This may indicate that there were no apparent changes in the community structure, although one have to be aware of that DNA with identical mole% G + C can have very different base sequences.

DNA from the pristine sediment had a $C_0t_{1/2}$ of approximately 9000, corresponding to 4.8×10^{10} bp, or more than 11000 different genomes (Fig. 2). On the other hand DNA from the fish farm

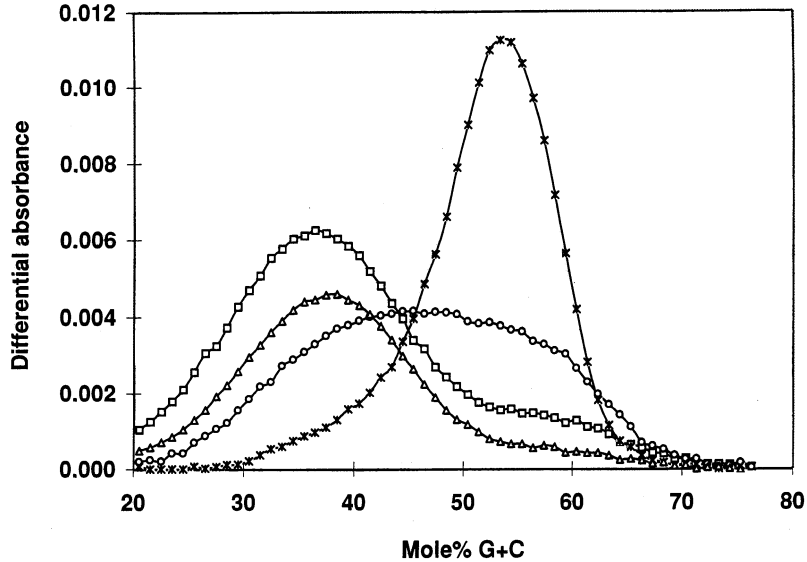

Fig. 1. Mole% G + C distribution profiles of DNA derived from the bacterial fraction of a pristine marine sediment (– ◦ –), mesocosm with fish farm sediment (– □ –), mesocosm with fishfarm sediment given 100 ppm oxolinic acid (– △ –), and *E. coli* (– × –).

sediment with accumulated organic waste had a $C_0t_{1/2}$ of 40–50. This corresponds to about 2.2–2.8×10^8 bp or about 50–70 different genomes (Torsvik et al., 1996). Thus the diversity in the organic polluted habitat was reduced approximately 250 times as compared to the pristine sediment.

In a model experiment, the effect of perturbation and oxolinic acid on bacteria in marine sediments was investigated (Hansen et al., 1992). Due to manipulation and perturbation of the sediment, the bacterial diversity was reduced to a $C_0t_{1/2}$ of approximately 10. When oxolinic acid was added to the sediment, the $C_0t_{1/2}$ was reduced to approximately 5 (Fig. 2, Table 1). In abandoned fish farms the total genetic diversity was increased again, but after 4 years the diversity was still low compared to pristine sediments (Table 1).

The community structure and diversity were also investigated in soils treated for many years with sewage sludge. The control fields had received 'uncontaminated' sewage sludge, whereas the contaminated fields received heavy metal amended sewage sludge at two rates of application (low and high metal contamination; McGrath et al., 1995). DNA was extracted from the bacterial fractions of soil samples from these fields. Applying the reassociation technique we found a $C_0t_{1/2}$ of DNA from the control soil of 7800 (Table 1). This DNA heterogeneity corresponds to a diversity of approximately 10000 different bacterial genomes with *E. coli* genome size. The diversity in the metal polluted soils were reduced and depended on the level of pollution. The $C_0t_{1/2}$ values were approximately 3700 and 1200, corresponded to 4700 and 1500 different bacterial genomes in the low and high metal polluted soils, respectively (Table 1).

The community structure in the metal contaminated soils were compared by analysing bacterial isolates and 16S rRNA clone libraries of PCR products derived from the total community DNA. Altogether, approximately 300 clones and 300 bacterial isolated from the two soils were compared. Hybridisation with phylogenetic probes revealed that the clones and the isolates gave strikingly different community composition (Table 2). Concerning the isolates, the most abundant subclass in both soils was the Gram + with low mole% G + C. This group however was by far the smallest when looking at the clones. There was also a profound discrepancy in the abun-

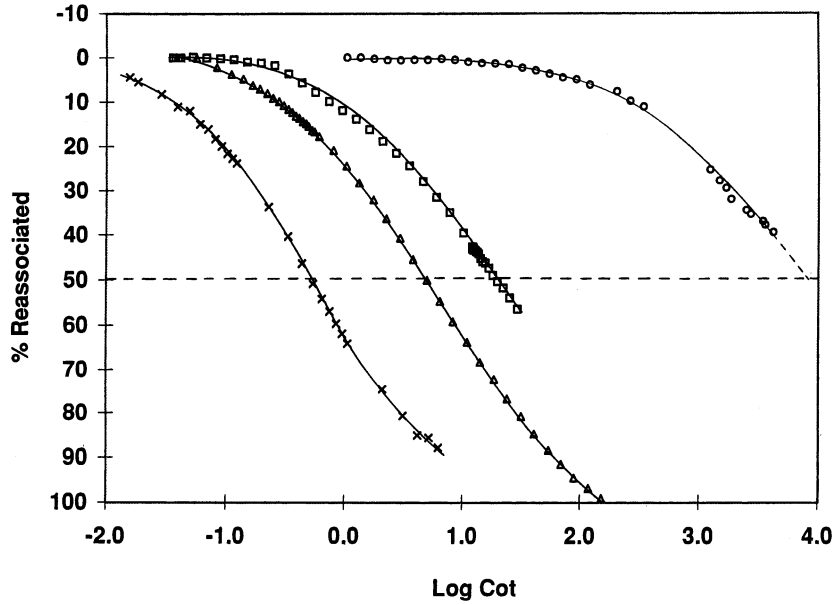

Fig. 2. Reassociation (C_0t plot) of DNA from the bacterial fraction of a pristine marine sediment (– ◦ –), mesocosm with fish farm sediment (– □ –), mesocosm with fishfarm sediment given 100 ppm oxolinic acid (– △ –), and *E. coli* (– × –). Reassociations were performed in 6 × SSC and 30% DMSO. The DNA was shared to about 420000 dalton and reassociated at 50°C. Abscissa: Log initial concentration of single stranded DNA (C_0 in moles nucleotides l^{-1}) multiplied by time in seconds. Ordinate: Percent reassociated DNA.

dance of isolates and clones belonging to the *α-Proteobacteria* subclass. The clones showed a much larger difference of community composition in the two soils than the isolates. The fraction of bacteria belonging to the *Cytophaga–Flexibacter–Bacteroides* subclass of *Bacteria* were totally dominant in the low metal contaminated soil. Here the subclass was twice as abundant as in the high metal contaminated soil. On the other hand bacteria belonging to the *α-Proteobacteria* were numerically dominant in the high metal contaminated soil. The bacterial isolates gave much smaller differences in the community structure in the two soils. The results indicate that the medium, although being a so-called non-selective medium (R2A agar; Difco Laboratories, Detroit, MI), selected for a similar subpopulation in the two soil communities. The low amount of clones belonging to the Gram + with low mole% G + C may reflect a bias in the DNA extraction from these bacteria, which may occur as endospores in the soils.

Representatives from different groups of clones, as determined by restriction analysis, were sequenced and compared to sequences in databases. Some of the sequences could be affiliated to already known bacterial genera, while others aligned in the databases with sequences from undescribed *Bacteria*.

Decrease in bacterial diversity as revealed by reduction in $C_0t_{1/2}$ values, may reflect reduced 'species' richness, or reduced evenness due to some bacterial types becoming dominant. In order to detect changes in the community structure and whether some bacteria became numerically dominant, DGGE analyses were performed. Examples of such analysis are shown in Fig. 3. When applying PCR-DGGE analysis on DNA from a high organic pasture soil, a very complex profile was obtained, giving numerous weak bands and few more dominant bands. In a model experiment the same soil was incubated for 3 weeks at room temperature supplied with a sole carbon source (methane gas). Such manipulation induced profound changes in the community structure and

diversity. DNA from the undisturbed control soil had a $C_0t_{1/2}$ of 5700. In the methane amended soil, the $C_0t_{1/2}$ value was reduced to 270. This corresponds to a diversity of 8000 and 380, respectively (Table 1). The DGGE analysis of methane amended soil gave a number of strong bands indicating that the community contained some numerically dominating bacteria (Fig. 3). In an enrichment culture from the same soil supplied with methane gas, the DGGE analysis revealed ten distinct bands. Two of the strongest bands could not be seen in the methane amended soil. Sequencing of these distinct bands, and alignment to a database showed that they were affiliated to methane oxidising bacteria.

Table 1
Bacterial diversity in soils and marine environments as determined by reassociation kinetics ($C_0t_{1/2}$; moles $\times 1^{-1} \times$ s at 50% reassociation), and number of 'genomes'

DNA source	$C_0t^{a}_{1/2}$	No. of genomes[b]
E. coli	0.79	1
Forest soil	4500–4700	6000
Forest soil, plate count community[c]	28	35
Pristine sediment	9000	11 400
Fish farm sediment	10	13
Fish farm sediment amended with 100 ppm oxolinic acid	5	6
Abandoned fish farm sediment	1300	1700
Sewage sludge amended soil; uncontaminated	7800	10 000
Sewage sludge amended soil; low metal contaminated	3700	4700
Sewage sludge amended soil; high metal contaminated	1200	1500
Model experiment; control agricultural soil	5700	8000
Model experiment; methane amended agricultural soil	270	380

Where nothing else is mentioned DNA were extracted from the bacterial fraction of environmental samples.
[a] In 6 × SSC, 30% DMSO.
[b] Equivalent to *E. coli* genome; 4.1×10^6 bp.
[c] DNA from a mixture of 206 isolates.

DGGE analyses were also applied in an investigation of forest podzol soils from the Kola peninsula. The soils were collected 5 and 60 km from a copper-nickel processing plant, and contained low and high heavy metal (Cu, Co and Ni) concentrations, respectively (Evdokimova, 1995). The DGGE analysis could reveal changes in the community structure and a reduction in number of bands in the high as compared to the low metal contamination (Fig. 3).

5. Concluding remarks

We have evaluated the applicability of molecular methods for analysing bacterial populations and communities. Some of the molecular fingerprinting methods (RFLP, ARDRA) were found to be too sensitive, giving too high resolution to provide reliable and robust genotypic characterisation at the community level. These methods were feasible for monitoring specific populations in microbial communities, and for assessing the diversity of bacterial isolates and cloned genes. Total DNA from complex microbial communities however, contain too much information to be analysed directly by high resolution methods.

Low resolution methods like DNA reassociation, DGGE and the use of phylogenetic gene probes were useful for monitoring changes at the community level. DNA reassociation in combination with DGGE analysis provided information on the overall diversity and changes in the community structure. The DGGE method has proved particularly useful as an initial investigation for distinguishing between communities and identifying the numerically dominating community members. The method allows a rapid screening of multiple samples, and thereby providing information about community changes and differences. Furthermore, it can be used to follow enrichment cultures and control isolation methods in order to evaluate the efficiency of these methods to comprise the numerically dominating members of communities. By Southern blot hybridisation of the DGGE gel with group specific probes and by sequencing the strongest bands, information about the affiliation of the dominating populations was gained.

Table 2
Percent of bacterial isolates and 16S rRNA clones belonging to different phylogenetic groups in soils with low and high metal contamination

rRNA probes	Low metal contamination		High metal contamination	
	Isolates	Clones	Isolates	Clones
α-Proteobacteria	7.5	16.8	16.8	46.4
δ-Proteobacteria	0.9	8.4	6.9	8.0
CFB-group[a]	31.0	52.1	20.8	26.6
G+ with low %G+C	58.5	1.7	32.0	1.5
Others	—	22.0	—	20.5

The affiliation was determined by hybridisation with phylogenetic group specific oligonucleotide probes.
[a] CBF; Cytophaga–Flexibacter–Bacteroides group.

The diversity in bacterial communities can be expressed as the heterogeneity of community DNA. Our investigations have demonstrated that the diversity in pristine soil and sediments, and also in moderately disturbed agricultural soils are very high. The total bacterial diversity in such environments may correspond to more than 10000 bacterial types and be more than 100 times higher than the diversity of isolated bacteria from the same communities. The results from hybridisation with phylogenetic probes showed that there were great differences in the composition of cultured bacteria and cloned 16S rRNA genes derived from total community DNA. It has been shown that the bacterial populations isolated from soil are strongly dependent on the media used. With three non-selective media, only 30% of the biotypes were found on all media. This suggests that the soil contained a large number of bacterial types, and that each medium selected for only a small proportion of these biotypes (Sørheim et al., 1989).

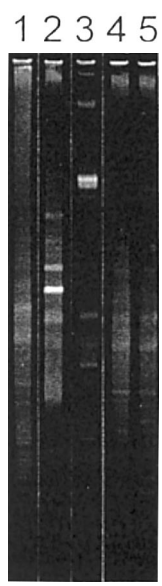

Fig. 3. DGGE analysis of PCR amplified 16S rDNA fragments from soil bacterial communities and enrichment cultures. DNA was derived from a high organic pasture soil (lane 1), the same soil incubated for 3 weeks at room temperature with methane (lane 2), an enrichment culture from the same soil supplied with methane gas (lane 3), forest podsol soils collected 5 km (lane 4) and 60 km (lane 5) from a copper-nickel processing plant.

The bacterial diversity in pristine soil and sediments may be sustained by the high physical and chemical complexity in such environments. The wide range of substrates available, physical-chemical gradients, as well as interactions between different bacterial populations (consortia) create a high number of functional niches that may change in time and space. The clonal nature of bacterial populations may also be a key to the high genetical diversity in bacterial communities. The high niche variations in heterogeneous habitats promote genetical heterogeneity and divergence of clonal cell lines in bacterial populations. Bacterial clonal lines may increase in genetical divergence even though they remain functionally related. In perturbed and polluted environments the number of functional niches apparently decrease.

In polluted areas and in fields with plant monocultures the substrate range is strongly reduced. In organic polluted environments like fish farm sediments most of the substrates are readily degradable carbohydrates, proteins and fat in contrast to pristine sediments where the major organic component is humic matter. In arable soil, management like tilling will disrupt the physical structure of the soil, and may destroy gradients and disturb many of the well balanced biological interactions. Reduction in functional niches results in a reduced bacterial diversity. Perturbations may also give a selective advantage to some bacterial populations which adapt and grow fast under new conditions. Reassociation and DGGE analysis have revealed both a reduction in diversity, and that some bacterial populations become dominant in perturbed habitats. Normally, the bacterial biomass and the overall bacterial activities like respiration are not reduced in moderately perturbed environments, they may in fact increase, but there is a smaller range of bacterial populations that is responsible for the activity. When the perturbation is removed, like in the abandoned fish farm sediment, the bacterial community seems to increase in diversity again.

In conclusion, our investigations have demonstrated that molecular methods are valuable tools for investigating the diversity and structure of bacterial communities. The combination of different methods that complemented each other is a useful strategy for monitoring changes in microbial communities and ecosystems due to perturbations like pollution.

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