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Characterization of bacterial populations of 2,4,6-trinitrotoluene (TNT) contaminated soils and isolation of a *Pseudomonas aeruginosa* strain with TNT denitration activities

Thèse présentée en vue de l'obtention du grade de Docteur en sciences agronomiques et ingénierie biologique par

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Abstract

2,4,6-trinitrotoluene (TNT) is a toxic and recalcitrant pollutant contaminating soils and groundwater. Therefore, characterization of microbial populations of TNT-contaminated soils and isolation of bacteria degrading this pollutant are of primordial importance.

Comparison of hybridizations of 16S rRNA derived from uncontaminated and TNT-contaminated soil samples required the development of a functional ANOVA model. Specifically, a statistical tool was necessary to compare dissociation curves obtained from thermal dissociation analysis of RNA hybridizations to DNA microarrays, and to determine if the dissociation curves significantly differed. To test and validate the model, we used dissociation curves from in vitro transcribed 16S rRNA amplified from two environmental samples hybridized to a phylogenetic microarray. Detection and rejection of outlier curves was important for appropriate discrimination between curves. The identification of significantly different curves was more efficient with the model than approaches relying on measurements at a single temperature.

This functional ANOVA analysis was used to improve discrimination between hybridizations of two soil microbial communities. Following hybridization of in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to an oligonucleotide microarray containing group- and species-specific perfect match (PM) probes and mismatch (MM) variants, thermal dissociation was used to analyze the nucleic acid bound to each PM-MM probe set. Functional ANOVA of the dissociation curves generally discriminated PM-MM probe sets when values of T_d (temperature at 50% probe-target dissociation) could not. Maximum discrimination for many PM and MM probes often occurred at temperatures greater than T_d. Comparison of signal intensities measured prior to dissociation analysis from hybridizations of the two soil samples revealed significant differences in domain-, group-, and species-specific probes. Functional ANOVA showed significantly different dissociation curves for 11 PM probes when hybridizations from the two soil samples were compared, even though initial signal intensities for 3 of the 11 did not vary. These differences in hybridizations between the two soil samples were likely the result from the presence of TNT.

The effect of TNT on soil microbial communities was further investigated with additional uncontaminated and TNT-contaminated soil samples using 16S rRNA PCR-DGGE and cultivation-dependent techniques. In all contaminated soil samples, the amount of DNA extracted was lower than in the uncontaminated ones. Analysis of bacterial diversity by DGGE showed a predominance of *Pseudomonadaceae* and *Xanthomonadaceae* in the TNT-contaminated soil samples compared to the uncontaminated ones. *Caulobacteraceae* were also present in several contaminated soil samples. The culturable microflora of these soils was studied by plate counts on agar supplemented with dilute nutrient broth. The number of CFUs was lower in a TNT-contaminated soil inoculum than in an uncontaminated one. In the former, most of the CFUs belonged to *Pseudomonadaceae*, and to a lesser extent, to *Caulobacteraceae*. In addition to the above contaminated soil samples, a pristine soil was artificially contaminated with different concentrations of TNT and incubated for 4 months. The amount of DNA extracted decreased in the highly contaminated soil samples (1.4 and 28.5 g TNT/kg soil). After 7 days of incubation of these soil samples, there was a clear shift of their original flora to a population dominated by *Pseudomonadaceae*, *Xanthomonadaceae*, *Comamonadaceae* and *Caulobacteraceae*. When the TNT concentration was lower (140 mg TNT/kg soil), a moderate shift in the bacterial population was observed. These results indicate that TNT affects soil bacterial diversity and richness by selecting for a narrow range of bacterial species that belong mostly to *Pseudomonadaceae* and *Xanthomonadaceae*.

TNT-contaminated soil samples probably contained TNT-degrading bacteria. In order to isolate bacteria that can denitrate TNT, enrichment cultures were carried out with TNT as sole nitrogen source and in the absence of oxygen. These cultures were established starting with an uncontaminated or a TNT-contaminated soil inoculum, in the presence or absence of ferrihydrite. A significant release of nitrite was observed in the liquid culture containing TNT, ferrihydrite and inoculum from a TNT-contaminated soil. Under these conditions, *Pseudomonas aeruginosa* was the predominant bacterium in the enrichment, leading to the isolation of *P. aeruginosa* ESA-5 as a pure strain. The isolate had TNT denitration capabilities as confirmed by nitrite release in oxygen-depleted cultures containing TNT and ferrihydrite. Concomitantly, TNT-reduced compounds were detected as well as unidentified polar metabolites. The concentration of nitrite released from TNT was proportional to the concentration of ferrihydrite in the medium. The release of nitrite was lower when the concentration of initially spiked TNT was reduced by one order of magnitude. Under these conditions, the concentration of nitrite peaked and then its concentration slowly decreased and

production of ferrous ions was detected. A decrease of nitrite concentration and production of ferrous ion were observed when TNT was omitted and nitrite and ferrihydrite were provided. These results suggest that nitrite-reducing conditions were initially achieved, followed by iron-reducing conditions.

When grown aerobically on a chemically defined medium, *P. aeruginosa* strain ESA-5 produced a greenish extracellular compound. This product was identified as phenazine-1-carboxylic acid (PCA). When purified PCA was incubated with TNT in the presence of NADH, nitrite was released. The concentration of nitrite released was dependent on the concentration of NADH and PCA. Denitration also occurred with two TNT-related molecules, 2,4,6-trinitrobenzaldehyde and 2,4,6-trinitrobenzyl alcohol. The release of nitrite was coupled with the formation of two polar metabolites and mass spectrometry analyses indicated that each of these compounds had lost two nitro groups from the trinitroaromatic parent molecule. The results obtained with the PCA mediated denitration of TNT in the presence of inhibitors of oxygen reactive species suggested the involvement of superoxide ($O_2^{\cdot -}$). When exogenous PCA was added to a *P. aeruginosa* ESA-5 liquid culture containing TNT as sole nitrogen source, bacterial growth was significantly enhanced compared to cultures containing TNT without PCA.

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Useful definitions

Aquifer: a water-saturated subsurface (i.e., below the land surface) environment.

Biotransformation: modification of functional groups and/or alteration of the molecular structure of a chemical compound by microbiological catalysis. By extension, this term is generally used when the chemical transformation does not proceed up to mineralization

Beneficial degradation: in this study, beneficial degradation is defined as a catabolic reaction by which bacteria can derive carbon, nitrogen and/or energy from the substrate.

Biodegradation: a subset of biotransformation which causes simplification of an organic compound's structure by breaking intramolecular bonds. By extension, this term is generally used when mineralization can be obtained.

Bioremediation: a managed or spontaneous process in which biological, especially microbiological, catalysis acts on pollutant compounds, thereby transforming or eliminating pollutants. Specifically, three processes can be distinguished: monitoring the natural progress of degradation to ensure that the concentration of contaminant decreases with sampling time (**bioattenuation**), intentional stimulation of resident xenobiotic-degrading bacteria by providing electron acceptors or donors, water, and/or nutrients (**biostimulation**), or the addition of laboratory-grown bacteria that have appropriate degradative abilities (**bioaugmentation**).

Cometabolism: transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable substrate.

Denitration: in this study, denitration is defined as the release of nitrite from TNT, a TNT metabolite (e.g., aminodinitrotoluene compounds), or a related trinitroaromatic compound (e.g., trinitrobenzaldehyde or trinitrobenzyl alcohol).

Mineralization: conversion of an organic molecule into its inorganic constituents (e.g., CO_2, H_2O, NO_3^-, and so on).

Xenobiotic: a chemical that is only man-made, and is otherwise not found in the environment.

List of notations

Nitroaromatic compounds

TNT: 2,4,6-trinitrotoluene

[U-^{15}N]-labeled TNT: ^{15}N uniformly-labeled TNT

OHADNT: hydroxylaminodinitrotoluene

2-OHA-4,6-DNT: 2-hydroxylamino-4,6-dinitrotoluene

4-OHA-2,6-DNT: 4-hydroxylamino-2,6-dinitrotoluene

ADNT: aminodinitrotoluene

2-A-4,6-DNT: 2-amino-4,6-dinitrotoluene

4-A-2,6-DNT: 4-amino-2,6-dinitrotoluene

DANT: diaminonitrotoluene

2,4-DA-6-NT: 2,4-diamino-6-nitrotoluene

2,6-DA-4-NT: 2,6-diamino-4-nitrotoluene

2,2'-azoxy: 2,2'-azoxy-4,4',6,6'-tetranitrotoluene

4,4'-azoxy: 4,4'-azoxy-2,2',6,6'-tetranitrotoluene

TNBA: 2,4,6-trinitrobenzaldehyde

DNT: dinitrotoluene

2,4-DNT: 2,4-dinitrotoluene

2,6-DNT: 2,6-dinitrotoluene

Other chemical compounds

PCA: phenazine-1-carboxylic acid

NAD(P)H: reduced nicotinamide adenine dinucleotide (phosphate)

$OH^{\cdot}$: hydroxyl radical

$O_2^{\cdot-}$: superoxide anion radical

H_2O_2: hydrogen peroxide

$^1O_2$: singlet oxygen

Molecular biology

DGGE: denaturing gradient gel electrophoresis

TGGE: temperature gradient gel electrophoresis

RT-PCR: reverse transcription PCR

T-RFLP: terminal restriction fragment length polymorphism

16S rRNA gene: gene coding for the 16S subunit of the bacterial ribosomal RNA

PM probe: probe having a perfect-match sequence to a target nucleic acid

MM probe: probe having a mismatch sequence to a target nucleic acid

Analytical chemistry

HPLC: high performance liquid chromatography

MS: mass spectrometry

Miscellaneous

CFU: colony forming unit

ROS: reactive oxygen species

Objectives of the thesis

TNT is a xenobiotic compound associated with three main properties: (i) this compound is toxic for many living organisms, (ii) several environments, especially soils, are contaminated with this pollutant, and (iii) TNT is a persistent pollutant and therefore accumulates in contaminated environments. For these reasons, there is an urgent need to remediate soils contaminated with TNT. The use of microorganisms to bioremediate contaminated environments is seen as highly desirable because microorganisms excel at degrading a variety of organic substances. In addition, bioremediation is generally considered as a low cost and environmentally friendly method compared to physico-chemical treatments. This has resulted in a positive public perception compared to non-biological methods.

The early studies on the transformation of TNT by bacteria appeared in the late 70's. Although further research has been carried out by various groups around the world for 30 years, only a limited number of bacteria have been isolated with beneficial TNT catabolic activities (e.g., TNT-denitration activities). These activities have also been described under conditions that rarely prevail at contaminated soils (e.g., aerobic conditions). The use of these bacteria under field conditions is therefore questionable. In addition to their activities, their survival in TNT-contaminated soils was not investigated. As a result, it is important to isolate bacteria under conditions generally found in situ (e.g., anoxic and iron-reducing conditions) and determine if the isolated bacteria have any chances to perform biodegradation in situ (i.e., the bacteria need both to survive and express catalytic genes). For these reasons, an initial characterization of microbial populations in TNT-contaminated soils is a logical approach to understand how bacteria with TNT-degradation activities are likely to perform in situ. In addition, such characterization gives additional insights for the isolation of TNT-degrading bacteria from TNT-contaminated soils.

The objectives of this thesis are centered on two fundamental scientific questions resulting from the issues associated with TNT contamination (i.e., TNT is toxic and recalcitrant, and many soils are polluted) (Figure 1):

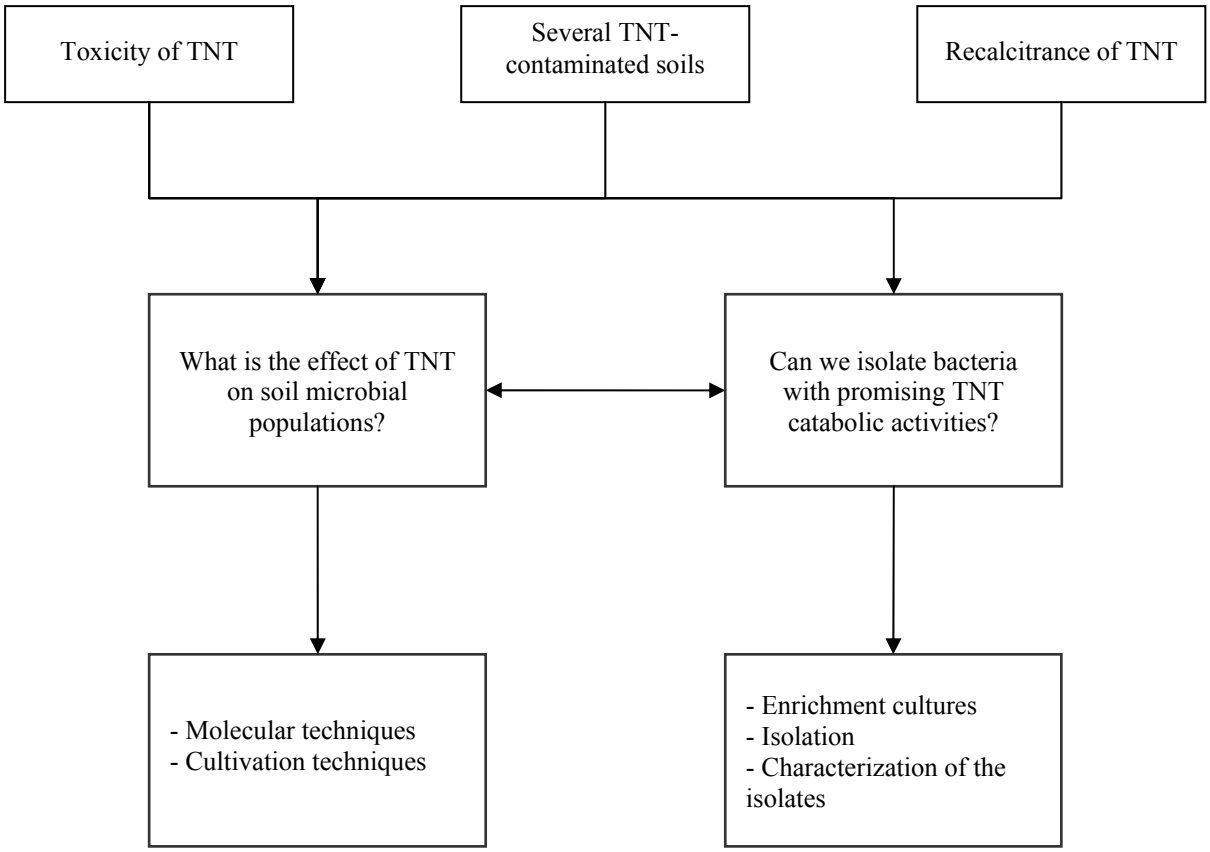


Figure 1. Objectives of the thesis and methods/experimental designs used to investigate these objectives.

OBJECTIVE 1: What is the effect of TNT on soil microbial populations? In addition to evaluating the microbial incidence of its toxicity in situ, can we gain additional insights for further isolation of TNT-degrading bacteria from these TNT-contaminated soils (i.e., by avoiding to consider TNT-contaminated soil inocula as merely microbial 'black-boxes')?

OBJECTIVE 2: Can we isolate bacteria with promising TNT catabolic activities? More specifically, can we select for versatile bacteria operating TNT degradation under conditions close to the ones prevailing in contaminated soils? Given the microbial characterization of TNT-contaminated soil samples, are the isolated bacteria likely to survive in situ?

To provide responses to these objectives, various scientific techniques and experimental designs can be used. In the case of microbial characterization of TNT-contaminated soils, diverse molecular techniques can be chosen in addition to laboratory cultivation. These molecular techniques are described in chapter 1. In the case of the isolation of TNT-degrading bacteria, enrichment culturing is a method of choice. However, various culturing design can

be used and it is therefore important to focus on *beneficial* catabolic reactions. An overview of the relevant issues of TNT contamination as well as known microbial TNT-degradation activities is provided in chapter 2, with emphasis on promising degradation activities.

Structure of the thesis

The two objectives of this thesis are described above. In order to evaluate why such objectives are important in the context of TNT bioremediation and why specific techniques/experimental designs are preferred over others, two introductory chapters are provided. The first chapter describes molecular techniques based on phylogenetic markers to analyze the bacterial community composition of contaminated environmental samples. This chapter will allow the reader to understand the importance of characterizing polluted environments from a molecular ecology point of view as well as how to characterize them. A second introductory chapter brings the reader into the general context of 2,4,6-trinitrotoluene (TNT) pollution and bioremediation strategies. More specifically, this second chapter shows that the only promising TNT catabolic pathways consist in denitration. An overview of known isolated bacteria and their TNT-denitration pathways are described. After these introductory chapters, results are provided and discussed. The first objective of this thesis was attained using two molecular methods based on the 16S rRNA gene, i.e., phylogenetic microarrays and denaturing gradient gel electrophoresis (DGGE). In order to evaluate the specificity of microarray hybridizations, thermal dissociation analysis was carried out. However, until this work was undertaken, there were no statistical tools available to analyze and compare the curves obtained from thermal dissociation. Therefore, for the correct interpretation of the curves, a statistical tool was developed and is described in chapter 3. This statistical tool was used to compare hybridizations with in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample (chapter 4). Then, DGGE was employed as a complementary method together with cultivation techniques to evaluate the microbial community structures of TNT-contaminated soils (chapter 5). The second objective of this thesis was to enrich and select for bacteria with promising TNT-denitration activities and understand their physiology. The next two chapters (chapter 6 and 7) respectively deal with the enrichment of a bacterium with unusual TNT-denitration activities, and the isolation of an extracellular metabolite involved in the denitration of TNT. Last but not least, a concluding chapter (chapter 8) is provided in order to bring into perspective the results obtained in the framework of the two initial objectives. The overall structure of this thesis is depicted in Figure 2.

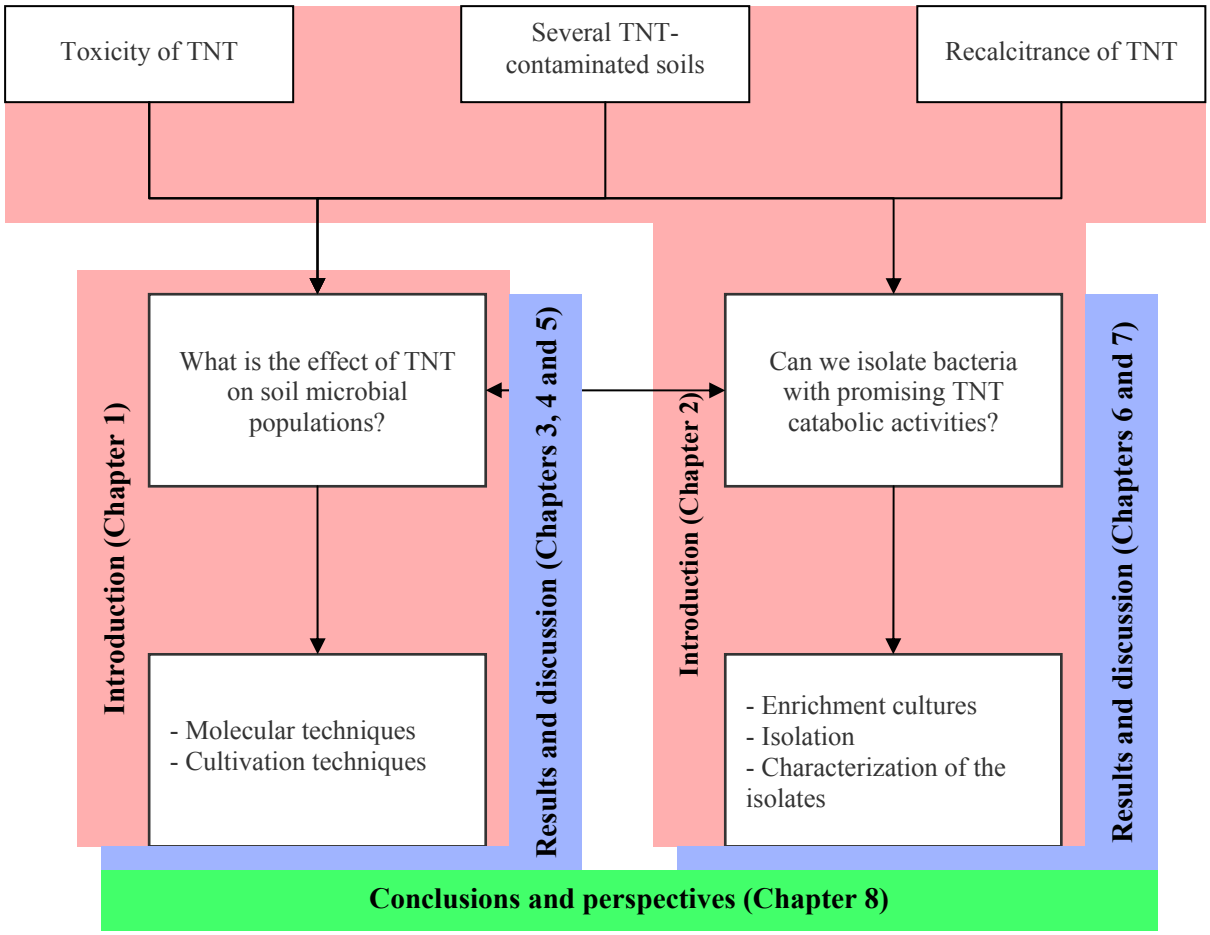


Figure 2. Structure of the thesis.

1.

Chapter 1

Characterization of microbial communities: molecular biology at the forefront

The organization of the chapters is as follows:

Chapter 1 emphasizes the characterization of microbial populations using molecular techniques based on phylogenetic markers.

Chapter 2 describes bacterial TNT transformation and shows that TNT denitration is promising for complete mineralization of the molecule.

Chapter 3 presents a functional ANOVA model to analyze dissociation curves from hybridizations to microarrays with thermal dissociation.

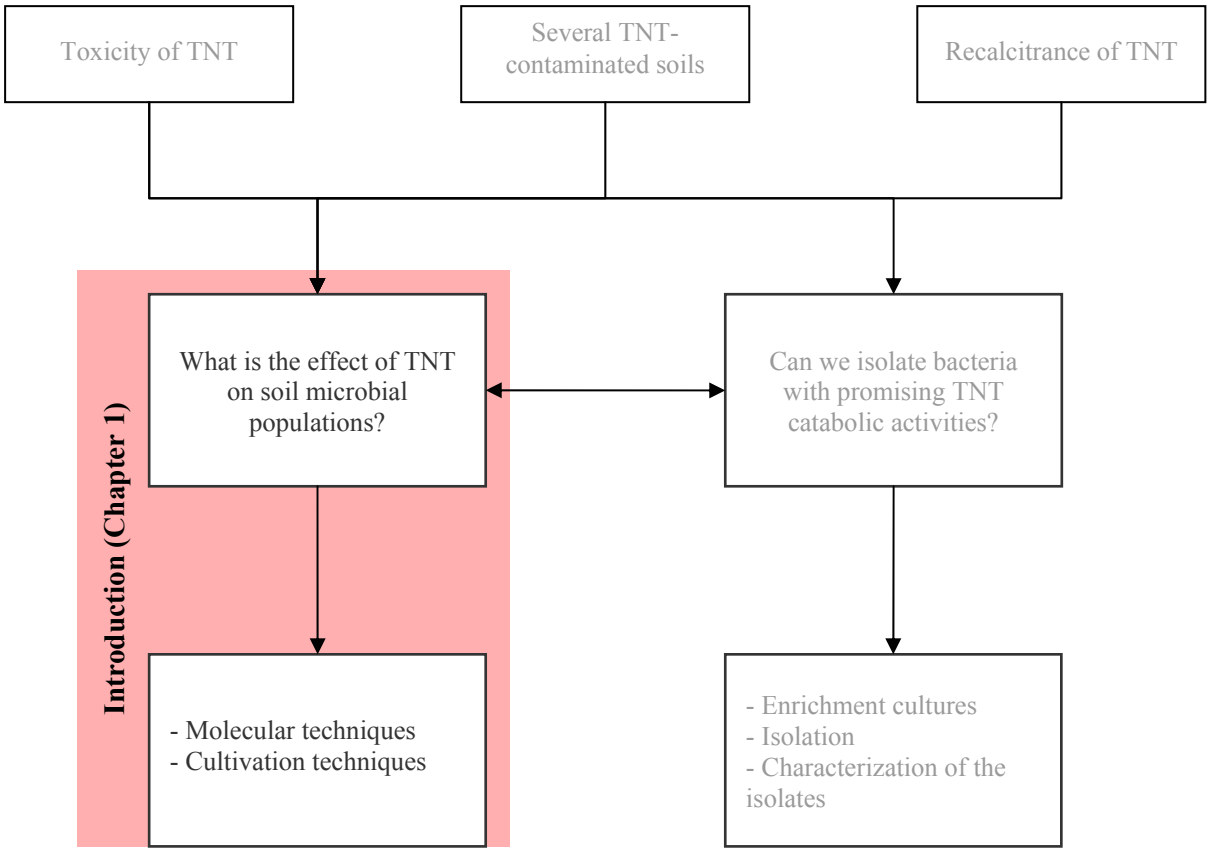
Chapter 4 compares hybridizations of in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to a phylogenetic microarray with thermal dissociation analysis.

Chapter 5 compares extracted DNA and 16S PCR-DGGE fingerprints of uncontaminated and TNT-contaminated soil samples, and of a pristine soil artificially contaminated with TNT. Plate counts on agar plates inoculated with an uncontaminated and a TNT-contaminated soil sample are also evaluated.

Chapter 6 describes the isolation and characterization of a *Pseudomonas aeruginosa* strain that denitrates TNT in the absence of oxygen and presence of ferrihydrite.

Chapter 7 shows that a phenazine molecule produced by *Pseudomonas aeruginosa* denitrates TNT.

Chapter 8 discusses the results obtained from the characterization of bacterial populations of TNT-contaminated sites and the isolation of a TNT-degrading strain.


Toxicity of TNT
Several TNT-contaminated soils
Recalcitrance of TNT
Introduction (Chapter 1)
What is the effect of TNT on soil microbial populations?
Can we isolate bacteria with promising TNT catabolic activities?
- Molecular techniques
- Cultivation techniques
- Enrichment cultures
- Isolation
- Characterization of the isolates

1.1. Phylogenetic molecular techniques[1]

Microbial characterization of polluted environments is important for several aspects. The use of microbial communities to ascertain the impact caused by anthropogenic stress in natural habitats is increasing. Each microorganisms within the contaminated ecosystem have different sensitivities to the toxicity of the pollutant, and microbial communities tend to be dominated by those organisms capable of surviving toxic contamination. For instance, members of the mycolata family of actinomycetes have mycolic acids present in their cell wall. These mycolic acids confer resistance to chemical injury and thus provide a selective advantage compared to bacteria devoid of these mycolic acids (4). The impact of the pollutant is dependent on the nature of the pollutant, its concentration and time exposure to microbial communities. Therefore, by characterizing microbial populations, one can estimate the toxicity of a compound, evaluate its concentration, and assess the time exposure needed to observe a significant effect. Also, such characterization can provide, to some extent, an indication of the nature of the pollutant. For instance, mycolata are frequently isolated from environments contaminated with polyaromatic hydrocarbons. It was found that those bacteria produced specific mycolic acids in the presence of polyaromatic hydrocarbons, probably to allow uptake of these hydrocarbons as C-source (45). Therefore, a predominance of mycolata might be correlated with the presence of polyaromatic hydrocarbons.

The need of characterizing microbial communities in contaminated ecosystems is also fuelled by the use of bacteria to remediate contaminated environments given the variety of catabolic activities that they possess to transform/mineralize pollutants. In fact, microorganisms excel at removing many contaminants from the environment by a diversity of enzymatic processes. For these reasons, there is a significant interest in microbially mediated remediation of contaminated environments because it promises to be simpler, cheaper and more environmentally friendly than physical and/or chemical treatments. Specifically, three bioremediation processes can be distinguished: monitoring the natural progress of degradation to ensure that the concentration of contaminant decreases with sampling time (bioattenuation), intentional stimulation of resident xenobiotic-degrading bacteria by providing electron acceptors or donors, water, and/or nutrients (biostimulation), or the addition of laboratory-

[1] This chapter contains information adapted from Eyers et al. (11, 12), Stenuit et al. (46). These articles are annexed to this thesis.

grown bacteria that have appropriate degradative abilities (bioaugmentation). Whatever the process considered, the assessment and the monitoring of specific pollutant-degrading bacteria and microbial communities are critical (i) to evaluate the contribution of these specific bacteria to pollutant removal and (ii) to assess interactions among microbes in a community as these interactions can alter their dynamics and the underlying microbial activities, such as catabolic reactions relevant to biotreatment operations.

An example of the importance of characterizing bacterial populations comes from operations at wastewater treatment plants. The successful operation of the conventional activated sludge process is strongly dependent on the performance of a gravity secondary clarifier that separates biomass from the effluent thanks to the formation of healthy settleable flocs. However, unsatisfactory floc formation and settling occur frequently, resulting in a bulking sludge. This is caused by the uncontrolled overproliferation of filamentous bacteria (29). Because of their open or porous structure, the flocs do not settle properly leading to the release of organic material with the treated water and a drop in the final effluent quality. In addition, biomass loss in the effluent is also triggered off by biological foaming that forms a stable scum layer on the surface of aeration basins and secondary clarifiers (42). This foam is produced mainly by filamentous bacteria and contains floating biomass that prevents an effective solid-liquid separation. Therefore, it is of major interest for wastewater treatment plant operators to rapidly detect the appearance of filamentous bacteria responsible for bulking and foaming.

Bioaugmentation strategies are also strongly dependent on the characterization of contaminated ecosystems. If one looks at previous attempts to demonstrate the potential for bioaugmentation in soils, he will see that it has resulted in both successes and failures. The indigenous community is often responsible for the rapid decline of exogenously added microorganisms because they compete for nutrients and electron acceptors. Therefore, for increased survival and long-term activity of exogenously added microorganisms, these should fill a metabolic niche that is not currently used by indigenous bacteria (8). Alternatively, indigenous bacteria themselves can be used as a bioaugmentation strategy. For instance, a selected consortium composed of four bacteria frequently found in waste metal-working fluids led to a more effective treatment than uncharacterized communities (52). Improved biodegradation capabilities can also be expected by transferring catabolic genes from a donor

strain to the already fit indigenous microflora (8). Whatever the strategy used, microbial communities (and catabolic genes) have to be identified and tracked.

In summary, enumeration and monitoring of bacterial communities and of specific xenobiotic-degrading bacteria in contaminated environments are critical to assess the impact of the pollutant to microbial populations and to prove the contribution of specific bacteria to pollutant degradation. However, the lack of sensitive, fast (i.e., 'complete in a single day') methodologies for extensive characterization of microbial communities have until recently hampered our understanding and assessment of their catabolic activities. So far, this task has been mainly approached using traditional methods that provide non-discriminating information, e.g., biochemical oxygen demand, volatile suspended solids, and so on. The use of traditional microbiological methods (e.g., enrichment cultures, plating on agar media, and so on) can take an inordinate length of time, and often underestimates microbial diversity as a result of our inability to cultivate the majority of soil organisms. Indeed, it is assumed that nearly 99% of the microorganisms present in nature cannot be isolated and cultivated using traditional techniques because of our ignorance of their physiological needs (2).

To tackle these issues, different molecular techniques independent of cultivation have been developed to explore the diversity of microorganisms, cultivable or not, in natural environments. The discovery of many new bacterial lineages as well as the reassignment of the most ecologically significant groups when using these methods have led to a dramatic change in our perception of microbial diversity and the phylogenetic tree of life (2, 56). The 16S ribosomal RNA (rRNA) is a phylogenetic marker of choice to analyze bacteria because of its universal distribution among bacteria, its primary structure with conserved and variable regions allowing the design of various specific primers, and the great number of sequences stored in databases.

Application of the 16S rRNA approach to identify bacteria has resulted in interesting discoveries. In some instances, microorganisms that predominate during bioremediation were found to be closely related to organisms that have been isolated under enrichment cultures. For instance, *Geobacter* species have been shown to oxidize organic contaminants with reduction of Fe(III) oxide (25). In polluted aquifers in which microorganisms were oxidizing contaminants with concomitant reduction of Fe(III) oxides, there was a significant enrichment in microorganisms having 16S rRNA sequences related to *Geobacter* species (39, 41). In

aquifers in which the indigenous microbial community was degrading the solvent trichloroethene (TCE), 16S rRNA sequences were ~99% identical to the 16S rRNA sequence of a pure culture of the TCE-degrader *Dehalococcoides ethenogenes* (13, 37). Marine sediments with high rates of anaerobic naphthalene degradation were found to be specifically enriched in microorganisms with 16S rRNA sequences closely related to NaphS2, an anaerobic naphthalene degrader available in pure culture (14). There was a close correspondence between the potential for aerobic degradation of the fuel oxygenate methyl *tert*-butyl ether (MTBE) in groundwater and the number of organisms with 16S rRNA sequences that had more than 99% similarity to the MTBE-degrading strain PM1 available in pure culture (18).

Table 1 shows the principal techniques developed to analyze bacterial communities. Most of these techniques are based on PCR amplification of phylogenetic markers and subsequent analysis by fingerprint methods (clone libraries, RFLP, DGGE, and so on) and sequencing.

Table 1. Key molecular methods to analyze bacterial communities at the 16S rRNA level

Method	Procedure	Advantage	Disadvantage
T-RFLP	Enzymatic restriction of 16S amplicons and electrophoretic separation of the fragments	- Rapid, simple, low cost - Good reproducibility	- One pair of primers - Poor sequence resolution - Sequence information unavailable - Not quantitative
Libraries	Cloning of 16S amplicons and sequencing	- Sequencing of the amplicons - High resolution	- Labor intensive - One pair of primers
DGGE	Electrophoretic separation of 16S PCR amplicons in a gel with an increasing gradient of denaturing agents	- Rapid, simple, low cost - High resolution (1 bp difference) - Sequencing of the bands	- Bacteria with multiple copies of the 16S rRNA gene - One pair of primers - Containment of multiple amplicons in one band - Poor reproducibility - Not quantitative
Phylogenetic microarrays	Labeling of DNA or RNA fragments and hybridization on DNA probes	- High resolution (1 bp difference) - High reproducibility - Sequence information - Several probes tested at a time - High-throughput - Elimination of PCR bias	- Expensive - Early stage of development

One phylogenetic method based on the 16S rRNA consists in terminal restriction fragment length polymorphism (T-RFLP). 16S rRNA gene amplicons are cut by restriction enzymes and the fragments are visualized by electrophoresis. Amplicons that have different nucleotide sequence at the site of restriction can be differentiated, and in some cases bacteria can be phylogenetically identified based on the size of the fragments generated. T-RFLP is a simple, rapid and low-cost method, but it suffers from the limitation inherent to the use of one pair of PCR primers at a time (thus preventing, for instance, a thorough analysis of several specific sub-groups of the population). Also, the method is not quantitative. T-RFLP has a high reproducibility, but the resolution is limited to the use of specific enzymes cutting at specific sites.

Another molecular technique available consists in the construction of 16S rRNA gene libraries. Briefly, the DNA is extracted from the ecosystem under study and 16S rRNA genes are amplified by PCR, ligated into plasmid vectors, and transferred to a bacterial host. The clones are screened in order to select for the ones harboring the plasmid and its ligated 16S rRNA gene copy. Further sequencing of 16S rRNA gene fragments of the library and comparison of the sequences with other 16S rRNA sequences can provide information on the identity of the bacteria of the community. This procedure allows the isolation and sequencing of individual 16S rRNA copies from the pool of amplicons. It has a high resolution since each copy is analyzed separately. However, this technique is labor-intensive, time-consuming, quite expensive, and one pair of primers is used at a time. Furthermore, the evolution of the microbial composition over time cannot be easily monitored using this technique.

Because of the advantages associated with 16S PCR-based denaturing gradient gel electrophoresis (DGGE) and phylogenetic microarrays, as well as the complementary information that these techniques provide when environmental communities are analyzed, they are described in more details below.

1.2 Denaturing gradient gel electrophoresis

1.2.1. Principles and advantages of DGGE

Given the limitations of 16S rRNA gene clone libraries, a simple, rapid, and relatively cheap molecular method was needed. To meet these criteria, Muyzer and his co-workers (30) developed a method combining the amplification power of PCR on 16S rRNA genes of complex microbial populations together with the possibility of denaturing gradient gel electrophoresis (DGGE) to separate DNA fragments of the same size but differing according to their base-pair sequences.

In DGGE systems, double-stranded DNA fragments are run through a polyacrylamide gel containing a linear gradient of denaturing agents (a mixture of urea and formamide) at a constant temperature (generally 60°C). During the migration in the gel, the fragment remains double stranded until it reaches a concentration of denaturing agents equivalent to a melting temperature that causes the lower melting part (also called "domain") of the molecule to melt. At this stage, the fragment changes from a helical structure to a partially melted molecule, which practically halts its migration through the gel.

Two fragments in which an AT is replaced by a GC can be theoretically separated and visualized. However, it is basically estimated that only 50% of all the single-base-pair changes in a fragment of 50 to several hundred bp can be detected by DGGE (31). To overcome this limitation, the fragments to be separated can be previously amplified by PCR using a pair of "modified" primers. The modification consists in the adjunction of a GC-rich sequence (generally 40 bp) to the 5' end of one of the primers (43). This prevents the complete dissociation of the fragments during migration in the gel and therefore the loss of sequence-dependent gel migration upon complete strand separation (43). Primers are designed to specifically hybridize to conserved regions of the bacterial 16S rRNA. By using group-specific primers, it is also possible to target well-defined communities, like the *Actinomycetes* (16) or the *Archaea* (35). Even though 16S rRNA is the gene mostly targeted, other specific genes can be amplified. Accordingly, Wawer et al. (58) analyzed the sequence diversity of the [NiFe] hydrogenase gene of *Desulfovibrio* species, an important group of sulfate-reducing

bacteria. A common set of primers used to amplify the rRNA gene of bacterial and fungal communities are listed in Table 2.

Table 2. Set of primers

Pair of primers[a]	Size of the PCR product (bp)[b]	Target	Sequence	Reference
P63f-P518r	511	*Bacteria*	5'-CAGGCCTAACACATGCAAGTC-3' 5'-ATTACCGCGGCTGCTGG-3'	(10)
P338f-P518r	236	*Bacteria*	5'-ACTCCTACGGGAGGCAGCAG-3' 5'-ATTACCGCGGCTGCTGG-3'	(30)
Arch340f-Arch519r	233	*Archaea*	5'-CCCTACGGGG(C/T)GCA(G/C)CAG-3' 5'-TTACCGCGGC(G/T)GCTG-3'	(35)
Act243f-Act513r	342	*Actinomycetes*	5'-GGATGAGCCCGCGGCCTA-3' 5'-CGGCCGCGGCTGCTGGCACGTA-3'	(16)
GC clamp[c]			5'-CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGG-3'	(30)

[a] f, forward primer; r, reverse primer

[b] GC clamp included

[c] GC clamp attached to the 5' end of the reverse primer

The general strategy to obtain DGGE fingerprints is as follows (Figure 1): First of all, the DNA has to be isolated from the ecosystem, which can range from natural samples (sediments, soils, drinking water, air, and so on) to artificial systems (cultures, bioreactors, and so on). Because the DNA has to be amplified by PCR, it has to be clean enough to avoid any inhibition of the polymerase. This problem arises frequently with samples taken from soil matrices. Furthermore, the differences in cell adhesion and cell wall structure, together with the soil's characteristics, can affect the efficiency of the DNA isolation and purification. To tackle these problems, different protocols of extraction can be found in the literature, especially for soil matrices. It is recommended, once it has been experimentally confirmed that a specific protocol of extraction is efficient enough (in terms of extraction yield and purification suited for the PCR), to use the same one for all the subsequent extractions. Indeed, it has been demonstrated that different extraction protocols can give different DGGE fingerprints and therefore different apparent bacterial community structures (33).

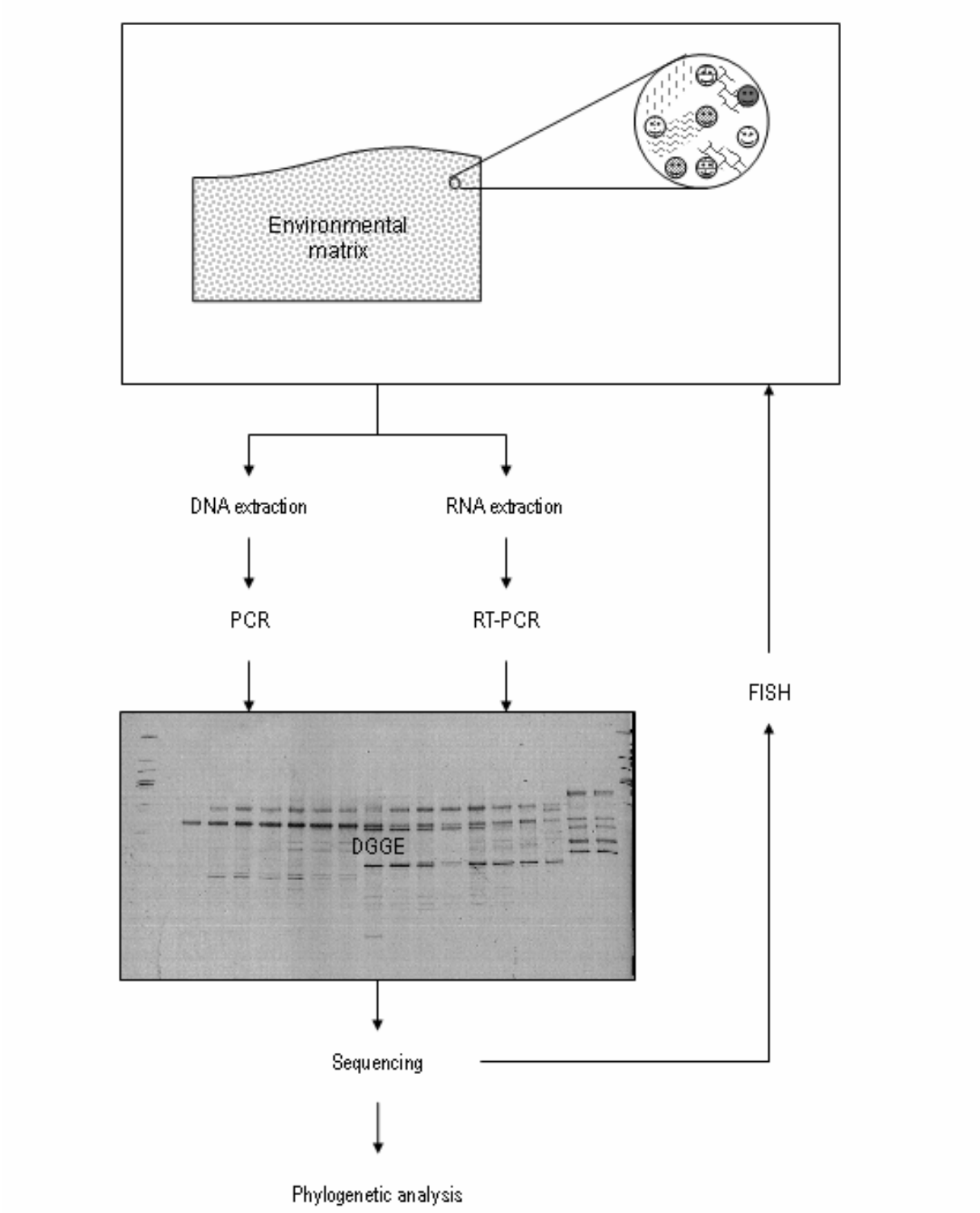


Figure 1. Flow-chart showing the successive steps required to obtain a DGGE fingerprint (sampling, nucleic acid extraction, PCR-amplification and DGGE). Each band can be sequenced for further phylogenetic analysis and/or fluorescent in situ hybridization.

Alternatively to DNA, RNA can be isolated as an indicator of the level of activity of each microorganism in the environmental matrix under study, since the number of ribosomes per cell is roughly proportional to the growth rate of bacteria (54).

Once DNA or RNA is extracted and purified, the next step is respectively the PCR or reverse transcription PCR. The primers of interest can be selected based on the list that is given in Table 1. During the PCR program, the annealing temperature has to be properly chosen to avoid the amplification of nonspecific sequences. Different increasing annealing temperatures can be evaluated until non-specific amplifications are avoided. Alternatively to this trial-and-error method, a "touchdown" PCR can be used (7), which relies on incremental annealing temperature decrease during subsequent PCR cycles. When a sufficient quantity of PCR products are available, they can be loaded on a DGGE.

The microbial identity of each DGGE band can be obtained after excision of the band of interest and further sequencing. This sequence can be compared with available 16S rRNA genes in the databases to identify the phylogenetic affiliation of each sequenced band. The Ribosomal Database Project (http://rdp.cme.msu.edu/html) offers the possibility to compare the sequence with other sequences available in the database and contains a useful package of tools for phylogenetic analysis (28) as well as probes/primer design (3). However, it has to be pointed out that these databases are incomplete and still growing. Therefore, if the sequence has a low similarity to known sequences, it is difficult to determine whether the sequence represents a novel microorganism or is part of known taxa for which no 16S rRNA gene sequences are available or of low quality (i.e., partial sequences or ambiguous sequences). Apart from this aspect of comparison, the sequence can also be used for the design of oligonucleotide probes for *in situ* monitoring of the corresponding bacterial population (fluorescent in situ hybridization [FISH] (57)).

1.2.2. Applications of DGGE

Since the first application of DGGE in natural ecosystems (30), it has found many applications for the monitoring of microbial populations in complex matrices, from natural to artificial systems. This section focuses on some of these applications.

Analysis of DGGE fingerprints is possible on a wide range of natural and artificial matrices (provided that sufficient DNA/RNA can be recovered and purified), from soils (36), wall painting (40), to wastewater systems for the bioremediation of phenol (60) or biotrickling filters containing styrene degrading biofilms (49). Furthermore, as described above, it is possible to study part of the microbial community structure by the use of primers targeting specific groups.

Thanks to the ability of this technique to analyze the evolution of microorganism community composition, MacNaughton et al. (27) evaluated the microbial population changes that occurred during bioremediation of an experimental oil spill. They discovered that the treatment of the contaminated soil promoted the growth of Gram-negative bacteria in the α-protobacteria and *Flexibacter-Cytophaga-Bacteroides* phylum. As previously mentioned, group-specific populations can be analyzed in very complex communities. In this way, Henckel et al. (15) used PCR-DGGE type I and type II methylotrophs to monitor them in rice-field soil, along with the functional genes for particulate methane monooxygenase and methanol dehydrogenase, which are important enzymes for the catabolism of all methanotrophs. They found that there was a pronounced shift in the methanotroph community when CH_4 oxidation began.

1.2.3. Challenges and limitations of DGGE

Even if DGGE is common and one of the most widely utilized methods to analyze microbial communities, it has to be emphasized that the technique has some limitations, but also that there are solutions to overcome these limitations. First of all, the way a sample is stored can affect the microbial community. For example, anaerobic or aerobic storage or direct freezing of the samples can result in the identification of different microbial communities (38). In addition, insufficient or preferential disruption of cells can introduce a bias during subsequent DGGE analysis.

Several biases can also occur during the PCR itself (62). Suzuki and Giovannoni (48) detected a preferential amplification of rRNA genes in mixed microbial populations. The authors discovered that this preferential amplification can be limited by an appropriate choice of

primers and by decreasing the number of cycles of replication. However, they postulated that this phenomenon would be minor if the sample contains a high diversity of templates.

Another bias is the formation of chimeric molecules during PCR. Chimeric molecules are composed of parts of two sequences originating from templates with a high sequence similarity. These templates compete with primers during the annealing step of the PCR. The percentage of chimeric molecules can be considerable, depending on the number of PCR cycles and sequence similarities between templates. For example, Wang and Wang (55) observed up to 30% chimeras after 30 cycles with templates showing a 99.3% sequence similarity. They also suggested an increasing of the elongation time (2 to 5 min) and a diminution of the number of cycles of replication to reduce the formation of chimera. If chimeric molecules are still formed, the Ribosomal Database Project (28) offers the possibility to screen sequenced fragments for possible chimera.

Chimeric structures are not the only factor that can affect the interpretation of DGGE fingerprints and/or lead to an overestimation of the number of microbial species. Indeed, bacteria can have more than one 16S rRNA gene (*rrn* operons) on their chromosome. The number of *rrn* operons can differ widely, from one copy for *Bradyrhizobium japonicum* (24) to 10 copies for *Bacillus subtilis* (47). In addition, these copies can show variable sequence heterogeneities, as shown by the detection of up to 10 variants in *Paenibacillus polymyxa* when a 347-bp fragment of its 16S rRNA was amplified by PCR and then loaded on a gradient gel electrophoresis (34).

In addition to storage and PCR bias, the interpretation of DGGE fingerprints themselves can be ambiguous when very complex communities are analyzed. It is estimated that bacterial populations that constitute more than 1% of the total community can be detected by PCR-DGGE (30). This limit can be overcome by bacterial fractionation (19), differential centrifugation of extracted DNA bound to bisbenzimidazole in CsCl gradients according their G+C content (17) or by using group-specific primers.

Last but not least, one pair of primers is used at a time which does not allow extensive characterization of environmental samples. Also, experimental conditions (e.g., choice of the denaturing gradient, electrophoresis time, and so on) have to be implemented to obtain a correct separation of amplicons. For instance, a 30% to 70% range of linear gradient of

denaturing agents will be likely too broad to allow a correct separation of amplicons with similar melting domains. In this case, a narrower range of denaturing agents should be used. In addition, DGGE suffers from reproducibility problems (samples cannot be easily compared when loaded on different gels) and it is not suited for quantitative interpretation of fingerprints (5).

1.3. Phylogenetic microarrays

1.3.1. Principles and advantages of phylogenetic microarrays

An emerging technique making it possible to analyse hundreds and even thousands of genes at the same time lies in the DNA chip, thus getting away from the "one gene at a time" analysis. For these studies, extremely small amounts of biomolecules are printed at high density on solid substrata.

Microarrays (or microchips) are based on the property of a single-stranded DNA or RNA molecule ("target molecule") to hybridize to a complementary molecule ("probe") attached to a solid support (64). Compared to traditional nucleic acid membrane hybridization, microarrays present the advantage of miniaturization (thousands of probes can be spotted on a chip), higher sensitivity and rapid detection. As pointed out by Zhou and Thompson (65), microarray-based genomic technologies are bound to revolutionize the analysis of microbial community structure, function and dynamics.

DNA microarrays are coated glass microscope slides onto which thousands of target DNA samples have been spotted in a precise pattern. There are fundamental differences between microarray technologies based on the immobilization technology used to attach the probes, length and nature of the probes, and synthesis and labeling of the targets (32). The choice between the different technologies is based on parameters such as cost, probe density, specificity, sensitivity and quantification. Probes are synthesized *in situ* (e.g., "GeneChip" arrays) or spotted directly onto the solid support. GeneChip arrays are synthesized using photolithographic masks. Probes are built by repeated cycles of light-activated reactions with nucleotide monomers. In situ synthesis can also be realized by electrochemical reactions. These are by far the most efficient methods of generating high-density oligonucleotide chips, but they have practical limitations in terms of fragment length and affordability (23). On the contrary, spotted microarrays require a preliminary ex situ synthesis. Spotted microarrays are of lower cost than in situ synthesized probe microarrays as they do not require highly sophisticated equipment to synthesize probe spots. In addition, they offer a much greater flexibility since probes can be easily spotted in-house at low cost. However, they are of lower density than in situ synthesized probe microarrays. Probe material consists in synthetic

oligonucleotide sequences from 10- to 75-mer (this material is adapted for in situ synthesized and spotted microarrays) or DNA sequences such as PCR products, cDNAs or clone libraries (this material is adapted for spotted microarrays only). Longer probes offer the advantage of lower production cost and higher signal intensities. The great advantage of oligonucleotide probes is that they are more versatile and they allow the study of probe-target specificity since mismatch probes can be designed and printed on the array. Several studies have shown that perfect-match target-probe hybridizations can be differentiated from single mismatch target-probe hybridizations (e.g., 9, 50, 51). Therefore, sequence-specific signals can be distinguished from non-specific signals and sequence information of target nucleic acids can be (theoretically) deducted from hybridization events. Readout of the microarray is commonly achieved using a fluorescence signal. Fluorescent dyes can be enzymatically or chemically incorporated in the sample to be hybridized. In addition, radioactively labeled substrates can be used to provide detection of specifically labeled target molecules (i.e., the so-called "isotope arrays") (53). The resulting image of fluorescent spots is visualized by confocal laser scanning and digitized for quantitative analysis. Spot fluorescence intensity is proportional to the concentration of hybridized nucleic acids (e.g., 63). Therefore, if experimental conditions are similar, microarray experiments can be realized with good reproducibility.

A preliminary step prior to hybridization of the samples to the microarray consists in extracting DNA or RNA from pure cultures or the environment (Figure 2). A great advantage of microarrays is that PCR amplification is not necessary if sufficient material can be obtained from direct extraction. Before hybridization to target DNA spotted on the glass slide, nucleic acids are labeled with specific fluorescent molecules.

Phylogenetic microarrays consist in probes targeting phylogenetic markers such as the 16S rRNA gene. The design of probes is based on sequences retrieved from databases (e.g., GenBank database or the Ribosomal Database Project).

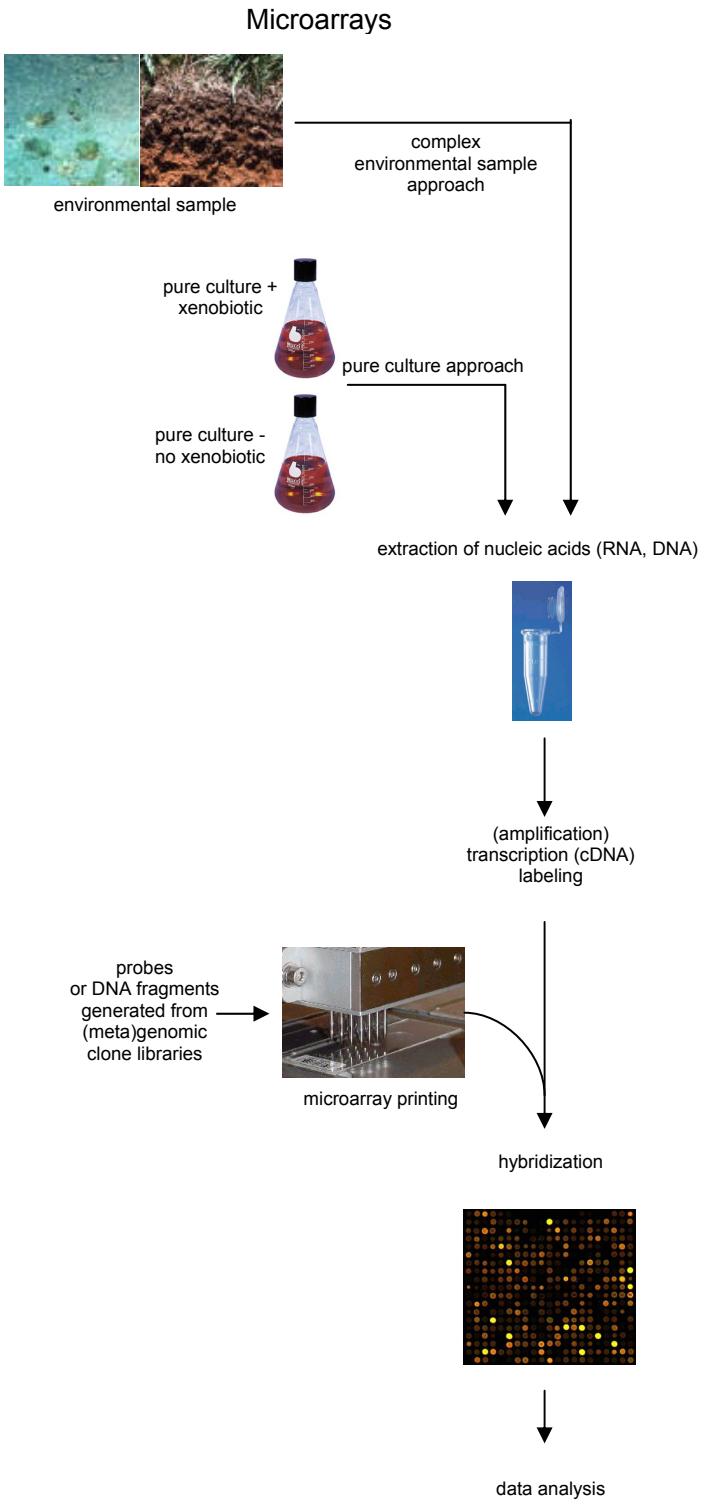


Figure 2. Construction of microarrays and hybridization with nucleic acids extracted from complex environmental samples or pure cultures[2].

[2] Adapted from (12).

1.3.2. Applications of phylogenetic microarrays

There are several examples of application of phylogenetic microarrays to analyze the microbial community structure of environmental samples. For instance, a DNA microarray containing a set of oligonucleotide probes targeting the 16S rRNAs of several groups of nitrifying bacteria has been designed for the monitoring of wastewater treatment plant samples (21). The authors demonstrated the direct detection of specific nitrifying bacteria (*Nitrosomas* spp.) in an activated sludge treatment facility without the need for prior PCR-amplification. In addition, as nitrification is brought about by a limited number of phylogenetically related bacteria, 16S rRNA-based methods can be used to provide both structural and functional information (59). For instance, an isotope array was used with a small phylogenetic microarray targeting ammonia-oxidizing bacteria (AOB) from nitrifying activated sludge samples that were incubated with [^{14}C]-bicarbonate (1). CO_2 fixation activities of the AOB populations within the complex activated-sludge communities were detectable on the microarray by ^{14}C incorporation and were confirmed independently by FISH and microautoradiography (MAR) and by control experiments where the AOB activity was inhibited.

Loy et al (26) used a 16S rRNA gene-targeted oligonucleotide microarray to specifically detect all known lineages of sulfate-reducing prokaryotes. Environmental samples were hybridized to the microarray and the results obtained were consistent with other molecular techniques. In another study, Small et al. (44) designed an oligonucleotide microarray for the direct detection (i.e., without the need for PCR amplification) of 16S rRNA extracted from soil samples. The authors were able to detect *Geobacter chapellei* directly from soil RNA extract without further purification or removal of soluble soil constituents. The detection sensitivity of *G. chapellei* in these soil extracts represented approximately $7.5*10^6$ *Geobacter* cell equivalents of RNA. In another experiment, Koizumi et al. (22) designed species-specific oligonucleotide probes based on the sequencing of dominant DGGE bands from toluene- and ethylbenzene-degrading consortia. The hybridization results obtained with the microarray and the different consortia were consistent with the DGGE results. The microarray was also used to analyze oil-contaminated sediments. However, hybridization signals were found with universal and eubacterial probes, but not with probes designed from DGGE bands.

1.3.3. Challenges and limitations of phylogenetic microarrays

Environmental application of array technology poses great challenges in terms of specificity, sensitivity and quantification (6). The specificity issue is critical, especially for 16S rRNA gene based phylogenetic microarrays since the 16 rRNA is highly conserved at the nucleotide sequence level and is present in every microorganism. In addition, the stable secondary structure of small-subunit rRNA has serious effects on hybridization specificity and sensitivity. To overcome the secondary structure problem, the target rRNA can be chemically fragmented (e.g., 20). The specificity problem can be resolved by using thermal dissociation studies. By linearly increasing the temperature of the microarray and recording signal intensities, dissociation curves are obtained. Several studies have shown that dissociation curves provide a useful tool to discriminate between perfect-match and single-mismatch probe-target duplexes (e.g., 9, 50). Another challenge is the sensitivity of microarrays. With environmental samples, the presence of various contaminants (e.g., humic acids) inhibits enzymatic reactions and generates a high signal background when environmental samples are hybridized to the microarray. Various protocols have been developed to extract RNA and DNA of sufficient purity from various environments. For instance, using bead-beating and phenol extraction, El Fantroussi et al. (9) have shown that rRNA can be extracted from sediments and used for microarray hybridization studies. In addition, this study showed that sufficient rRNA was extracted without the need for amplification prior to hybridization. Also, one should keep in mind that the sensitivity of the method is 100 to 10,000-fold lower than that of PCR (65) and this might be an issue for sequences of poor abundance. A third challenge consists in quantification. Although a promising perspective of phylogenetic microarrays lies in the possibility of determining the relative abundance of target microorganisms, quantifying microbial populations is challenging because of cross-hybridizations that may occur when dealing with complex environmental samples containing perfect and mismatch targets in unknown abundance. Again, the use of dissociation curves holds great promise for correct quantification of environmental samples (61).

1.4. Conclusions

Among the molecular techniques described above, microarrays and DGGE are two interesting and complementary techniques to assess bacterial communities. DGGE is a simple and rapid technique that provides a fingerprint of microbial populations. Different fingerprints can be easily compared on the same gel (e.g., by visual comparison). Disadvantages are the need to perform additional steps to assign a sequence to each dominant band (i.e., extraction of the band, re-amplification, and sequencing). Also, the analysis is limited to the dominant bands. Therefore, the assessment of bacterial communities in fine detail is limited unless specific primers are used. Phylogenetic microarrays are a complementary approach because hybridizations to hundreds of probes are tested at the same time, thus preventing the need of using several specific PCR primers. The sequence of the nucleic acids present in the sample can be deduced from the sequence of the probes and hybridization results (unless mismatch probe-target hybridizations occur). If phylogenetic microarrays also provide a fingerprint of microbial populations, comparison of different fingerprints is not as straightforward as in the case of DGGE. On the other hand, microarrays are suitable for quantitative comparisons of hybridization results between samples given that the relative signal intensity of each probe is proportional to the abundance of the target nucleic acids (unless mismatch probe-target hybridizations occur). Given their advantages and limitations, DGGE and microarrays are complementary techniques and provide a better characterization of environmental samples when used in parallel.

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