5.

Chapter 5

Effect of 2,4,6-trinitrotoluene on soil bacterial populations

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.

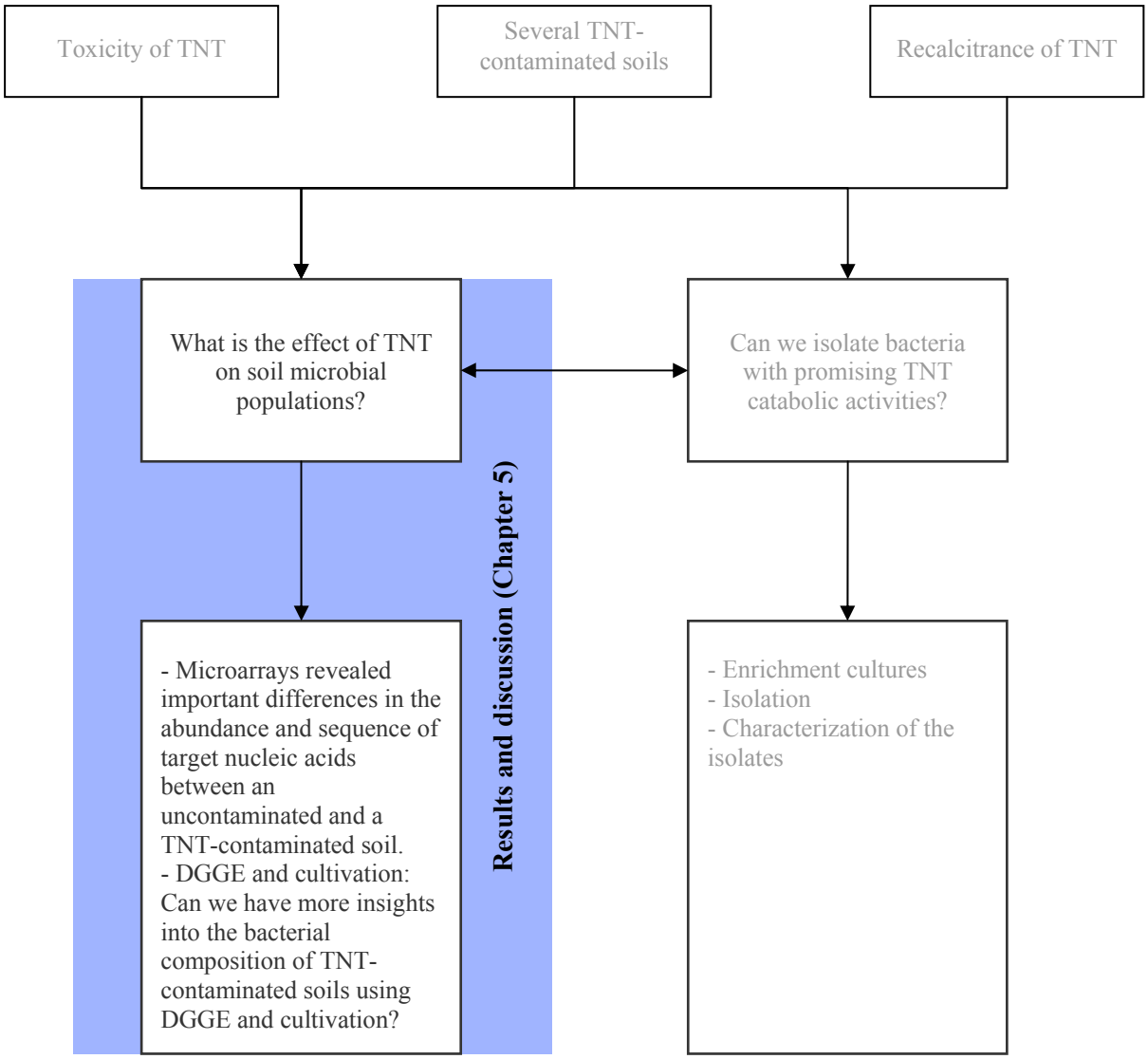
Chapter 4 compares hybridizations of in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to a phylogenetic microarray with thermal dissociation analysis.

Chapter 5 compares DNA extracted 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
What is the effect of TNT on soil microbial populations?
Can we isolate bacteria with promising TNT catabolic activities?
Results and discussion (Chapter 5)
- Microarrays revealed important differences in the abundance and sequence of target nucleic acids between an uncontaminated and a TNT-contaminated soil.
- DGGE and cultivation: Can we have more insights into the bacterial composition of TNT-contaminated soils using DGGE and cultivation?
- Enrichment cultures
- Isolation
- Characterization of the isolates

Effect of 2,4,6-trinitrotoluene on soil bacterial populations

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[1] L. Eyers characterized the soil samples (soil TNT concentration and % C), implemented the PCR and DGGE conditions, participated in the writing of the paper and reviewed it.

5.1. Abstract

To gain insights into the impact of 2,4,6-trinitrotolune (TNT) on microbial communities of relevance in polluted environments, we characterized several TNT-contaminated soil samples from two sites with different histories of contamination and concentrations of TNT. 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*.

5.2. Introduction

2,4,6-trinitrotoluene (TNT) is an environmental pollutant found at many sites associated primarily with military and, occasionally, civil activities. It is known to be toxic for various eukaryotic organisms like fungi, algae or worms (Stenuit et al. 2005 and references therein). However, few reports describe the effect of TNT on soil bacterial populations. By analyzing the phospholipid fatty acid composition of different soil samples, Wilke et al. (2004) found only minor differences between TNT-contaminated and uncontaminated soils while Fuller and Manning (1998) noticed a predominance of Gram-negative over Gram-positive bacteria in TNT-contaminated soils. Using respirometric activities, Gong et al. (2000) observed a decrease in the specific growth rate and an increase in organic matter content in soils with a high TNT concentration. A decrease in denitrification activity was also reported in TNT-contaminated soils (Siciliano et al. 2000). These results showed that TNT affected soil bacterial composition and activity. However, the bacterial composition at the group and species level was only partially resolved. This lack of information primarily resulted from the techniques used in these studies: phospholipid composition analyses and activity measurements were not specific enough to determine the bacterial composition of complex environments at the level of the species nor were they adequate to reveal microbial community structure as a function of contamination level and history.

16S rRNA and its gene are powerful markers to identify and classify bacteria (Woese et al. 1985). Various molecular methods based on the 16S rRNA gene have been developed to characterize the bacterial composition of environmental samples. Separation of PCR amplified 16S rRNA genes using denaturing gradient gel electrophoresis (DGGE) is a frequently used methodology that provides a direct fingerprint of environmental samples (Muyzer et al. 1993). In addition, bands can be excised from the gel and sequenced to identify the dominant species in the population analyzed. For instance, DGGE has been used by several research groups to assess soil microbial diversity and shifts in communities due to xenobiotic contamination (e.g., El Fantroussi et al. 1999a; El Fantroussi et al. 1999b; MacNaughton et al. 1999; Eyers et al. 2004a; Eyers et al. 2004b)

Over the past two decades, the use of cultivation-independent methods has rapidly expanded over traditional cultivation-dependent approaches. This has been primarily due to the

limitations of the cultivation approach as most of the bacteria remain uncultured using traditional laboratory techniques. The proportion of culturable bacteria has been estimated at less than 1% of soil bacteria (Torsvik & Øvreås 2002). However, recent developments in cultivation techniques have allowed the cultivation of previously unculturable microorganisms (Leadbetter 2003 and references therein, Joseph et al. 2003, Stevenson et al. 2004, Ferrari et al. 2005). For instance, the use of dilute nutrient broth solidified with agar resulted in viable cell counts that were 5.2% of the microscopically determined total cell counts (Janssen et al. 2002). Several colonies represented the first known isolates of soil bacteria belonging to novel lineages within the divisions *Actinobacteria*, *Acidobacteria*, *Proteobacteria*, and *Verrucomicrobia*. In some cases, adapted cultivation-dependent methods even proved to be superior to cultivation-independent methods. For example, Ellis et al. (2003) used cultivation-independent methods to compare the bacterial diversity of soil samples contaminated with metals. The authors found no significant difference between the contaminated soils using these methods, but considerable differences were observed with cultivation-dependent methods. Their data indicated that metal contamination did not have a significant effect on bacterial diversity, but affected physiological status.

The objective of this study was to assess the impact of TNT contamination on soil bacterial populations using both cultivation-independent and -dependent methods. DGGE analyses were carried out to provide a fingerprint of bacterial communities in TNT-contaminated soils compared to non-contaminated soils. The brighter bands were sequenced to identify the major species present in these TNT-contaminated soils. The differences between uncontaminated and TNT-contaminated soil samples were further studied by cultivation of soil bacteria on dilute nutrient broth solidified with agar. Finally, a pristine soil sample was artificially contaminated with different concentrations of TNT and changes in the bacterial community composition were monitored by DGGE.

5.3. Materials and methods

Soil samples. Two series of soil samples were investigated. The first one (samples REF, KX1, KL1, KL2, KF1, KF2, KF4, KF5, KF6, KF6b, KF6c, F1, F2, and F3) was collected at a site in Bourges, France that had been used for TNT destruction over the past 20 years. Samples were taken at different locations at the same site with a distance between them of approximately 10 meters. Uncontaminated (i.e., TNT was not detected by HPLC with a detection limit of 1 mg TNT/kg soil) soil samples were collected in an area shielded from TNT contamination by the presence of a protecting wall. A second series of soil samples were provided by Fabricaciones Extremeñas S.L., a subsidiary company of the Union Española de Explosivos (Madrid), from a site located at El Gordo, Cáceres, Spain. It was artificially contaminated with TNT for 7 months and separated in different plots. Two TNT-contaminated soil samples were taken from a contaminated plot (samples UEE and IN). Another TNT-contaminated plot was planted with corn seeds coated with *Pseudomonas putida* strain JLR11 (Esteve-Nunez et al. 1998), and samples were taken 15 (sample J15) and 29 days (sample J29) after planting. A third TNT-contaminated plot was planted with non-coated corn seeds and samples were collected 15 (sample C15) and 29 days (sample C29) after planting. One sample (sample OUT) was collected at the same site from a non-contaminated plot. For the two series, samples were collected from the top 10-cm layer of soil, except samples KF6b and KF6c which were taken at a depth of 30 cm. All samples were homogenized, sieved (2-mm mesh) to eliminate plant roots and stones, and stored at 4 °C in sterile containers.

Soil characteristics. The concentration of TNT in the soil samples was determined in triplicates as previously described (Eyers et al. 2004b). Soil pH was measured as follows: 1 g of soil was mixed with 5 g of demineralized water, blended and allowed to settle for 20 minutes three times in a row. After 1 hour of settling, the pH of the supernatant was measured. Soil texture was determined using the pipette method after removal of carbonate and organic matter with HCl and H_2O_2, respectively, and dispersion with hexametaphosphate (Kilmer & Alexander 1949). The soil carbon content was measured in triplicates by chemical oxidation with concentrated H_2SO_4 and $K_2Cr_2O_7$ (Anonymous 1972). Soil characteristics are presented in Table 1.

Cultivation experiments. One g of soil REF or KX1 was added to 100 ml of sterile dH_2O (100-fold dilution) and stirred for 2 h at 300 rpm. After settling for 1 h, the supernatant was serially diluted 10-fold in sterile dH_2O (1,000 to 100,000-fold final dilutions). Aliquots (150 µl) of each dilution were spread in three to five replicates onto agar plates supplemented with dilute nutrient broth (containing per liter: 15 g of agar (Sigma, Saint Louis, MO) and 0.065 g of nutrient broth (Oxoïd, Hampshire, UK) [i.e., 200 times less than the manufacturer's instructions]) with a sterile glass spreading rod. Plates were incubated up to 3 months to allow the emergence of slow growing bacteria. The plates were incubated in the dark at room temperature. The number of colony forming units (CFU) on each medium was counted 2, 7, 8, 14, 22, 35, 49, 63, 77, and 91 days after inoculation. For each time point, plates carrying between 50 and 250 colonies were used to calculate the number of colonies per g of soil. After 14, 35, 63 and 91 days, plates containing 50 to 250 colonies were washed with 1.5 ml of sterile dH_2O. The washing solution was centrifuged (10,000 x g for 5 min) and the pellets were used for DNA extraction. This protocol was based on the plate-wash PCR method proposed by Stevenson et al. (2004).

Artificial contamination of a pristine soil. A pristine soil was sampled in April 2006 at the University campus of Louvain-la-Neuve, Belgium. It was sieved on a 2-mm mesh and divided into 4 samples: a control sample (no TNT), and three samples spiked with increasing concentrations of TNT: 140 mg, 1.4 g, and 28.5 g TNT/kg of dry soil. Seven hundred and fifty g of each of these four samples were poured in 1-l beakers covered with aluminium foil and incubated in the dark at room temperature over 4 months. Each of them constituted an individual microcosm for bacterial community observations. Soil samples had an initial water content of 32.5% (measured in triplicates by drying 3 g of wet soil at 105°C up to constant weight). The water content was kept constant in the microcosms by monitoring every 15 days the percentage of humidity and readjusting it with sterile dH_2O if necessary. For each microcosm, 1 g of soil was collected for DNA extraction a few minutes after the addition of TNT (t=0), and 7, 14, 61, and 121 days after the addition of TNT.

DNA extraction. Soil DNA was extracted using the Ultra-Clean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. The DNA of plate washed CFUs was extracted from the pellets using the same kit, except that the bead-beating solution was supplemented with a 0.22 µm-filtered solution of lysozyme (5 mg/ml final concentration) and the pellet was incubated in this solution overnight at 37°C. Moreover,

the IRS solution (Inhibitor Removal Solution, designed to precipitate humic acids) provided by the manufacturer was not used. The quantity and quality of the DNA extracted was evaluated by gel electrophoresis on 1% agarose gels stained with ethidium bromide. DNA was quantified on these gels using the free software Image J (available at http://rsb.info.nih.gov/ij/).

PCR amplification. 16S rRNA genes were amplified using primers BACT63F (5'-CAGGCCTAACACATGCAAGTC-3', forward) (Marchesi et al. 1998) and UNIV518R (5'-ATTACCGCGGCTGCTGG-3', reverse) (Muyzer et al. 1993) with a 40 bp GC-clamp attached on the forward primer. BACT63F complements a conserved region in the domain *Bacteria* whereas UNIV518R complements a universally conserved region. The PCR mix (50 µl) contained 25 µl of Red'y'star Mix (Eurogentec, Seraing, Belgium), 0.25 µM of each primer and approximately 2 to 10 ng of target DNA. For some soil samples with low amounts of DNA extracted, its quantification was not possible using gel electrophoresis. Therefore, different volumes of DNA extracted were tested, and in all cases 1 to 2 µl gave the best PCR signals. PCR was performed using a Biometra TGradient cycler (Biometra, Goettingen, Germany) under the following conditions: 95°C for 5 min, followed by 30 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min), with a final extension at 72°C for 10 min. PCR products were visualized by gel electrophoresis on 1.5% agarose gels stained with ethidium bromide and quantified with the Image J software.

DGGE analysis and sequencing of DGGE fragments. DGGE analysis was carried out with the DCode universal mutation detection system (Bio-Rad, Hercules, CA). PCR products were loaded onto 6% (wt/vol) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels with a linear denaturing gradient from 35 to 55% (where 100% contains 7M urea and 40% (vol/vol) deionized formamide) in 0.5 X TAE buffer (20 mM Tris base, 10 mM acetate, 0.5 mM EDTA [pH 8]). Three hundred to 650 ng of PCR products were loaded onto the gel, except for samples KF6b and KF6c. For the latter, PCR amplification gave low yields, and the largest volume of PCR product was loaded onto the DGGE gel, which corresponded to 150 ng of PCR product. The electrophoresis was run at 60°C and 185V for 5h30. Gels were stained with ethidium bromide and photographed under a UV transilluminator. Bands of interest were excised from the gel and incubated overnight at 4°C in 35 µl of sterile dH_2O. Next, 1 to 8 µl of this solution (depending on the intensity of the band that was cut) was used for a subsequent PCR amplification (40 µl volume) with 20 µl of ReadyMix (Sigma) and 0.25 µM

of primers BACT63F and UNIV518R. The same amplification cycle was used as described above, except that 25 cycles were performed instead of 30. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequencing was carried out using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems). The 20-µl sequencing mixture contained 15 to 30 ng of purified PCR product and 0.5 µM of primer BACT63F or UNIV518R. This sequencing protocol gave unambiguous sequences for most DGGE bands. When ambiguous, the DGGE bands were cloned prior to sequencing using the pDrive vector (Qiagen).

Comparative sequence analysis and construction of the phylogenetic tree. Sequences were aligned to 16S rRNA sequences obtained from the National Centre for Biotechnology Information GenBank database by using the BLAST 2.2.10 search program (Altschul et al. 1997). The closest match with sequences > 1300 bp was used to assign the bands to taxonomic groups. Partial sequences corresponding to the DGGE bands were aligned with about 31000 homologous complete bacterial 16S rRNA gene sequences by using the automated aligning tool of the ARB program package (http://www.mikro.biologie.tu-muenchen.de) (Ludwig et al. 2004). Then, the partial sequences were inserted into the optimized tree derived from the complete sequence data by using the maximum-parsimony criterion and a special ARB parsimony tool that did not affect the initial tree topology. The resulting tree was pruned to save space, and the closest relatives were retained.

Nucleotide sequence accession numbers. Sequences have been deposited in the GenBank database under accession numbers EF121875 to EF121949.

5.4. Results

Physico-chemical characteristics of the soil samples. Among the samples collected at the Bourges site, 4 samples were uncontaminated and 10 were contaminated with relatively high concentrations of TNT (from 3.59 to 36.2 g TNT/kg soil, Table 1), resulting from the use of this site as a TNT-destruction field over the past 20 years. All samples had a pH above 7.6. Soil texture was mostly sandy, except for sample KF4 which was mostly silty. The percentage of carbon varied significantly between the samples, with values ranging between 0.7 to 14.5 %C. There was no correlation between these various parameters (e.g., the presence of TNT did not result in a decrease in the percentage of carbon).

On the contrary, the samples collected at the site located in El Gordo contained relatively low concentrations of TNT (from 0.01 to 1.57 g TNT/kg soil, Table 1). One sample was uncontaminated. The pH measurements also indicated that they were more acidic than the soil samples collected at Bourges. These soil samples were loamy sands (they contained on average 82% sand, 10% silt and 8% clay) and had an organic matter content of 0.8% (P. van Dillewijn, personal communication).

Table 1. Physico-chemical characteristics of the soil samples used in this study

Sample	TNT level mean ± SD [a] (g TNT/kg dry soil)	pH	% sand - silt - clay	% C mean ± SD (dry soil)
Series from Bourges				
REF	nd	8.03	60.8 - 12.7 - 26.4	5.12 ± 0.21
KF4	nd	7.97	15.5 - 81.9 - 2.6	6.56 ± 0.17
F1	nd	7.88	94.2 - 5.7 - 0.1	4.21 ± 1.17
F2	nd	8.03	78.1 - 15.5 - 6.4	1.70 ± 0.05
F3	3.59 ± 0.59	7.85	76.8 - 17.4 - 5.8	4.23 ± 0.08
KX1	26.6 ± 2.0	7.68	75.2 - 10.1 - 14.6	4.83 ± 0.16
KL1	36.2 ± 3.1	8.05	73.7 - 19.2 - 7.0	14.5 ± 0.1
KL2	23.0 ± 2.2	7.74	87.9 - 9.2 - 2.8	8.19 ± 0.56
KF1	13.3 ± 3.5	8.35	68.5 - 6.0 - 25.5	1.32 ± 0.19
KF2	4.12 ± 0.96	8.42	52.3 - 28.0 - 19.7	0.68 ± 0.06
KF5	6.40 ± 1.09	8.07	58.2 - 10.9 - 30.9	1.61 ± 0.03
KF6	19.5 ± 3.3	8.40	69.4 - 8.1 - 22.5	4.54 ± 0.10
KF6b	13.0 ± 3.2	8.33	66.3 - 14.6 - 19.0	4.06 ± 0.29
KF6c	19.2 ± 2.8	8.35	71.3 - 10.5 - 18.2	4.43 ± 0.29
Series from El Gordo				
OUT	nd	6.30	nm	nm
UEE	0.010	5.23	nm	nm
IN	1.57	6.03	nm	nm
C15	0.040	5.84	nm	nm
J15	0.036	6.31	nm	nm
C29	0.056	6.50	nm	nm
J29	0.044	6.37	nm	nm
Louvain-la-Neuve				
Pristine	nd	7.40	23.8 - 63.7 - 12.4	nm

nd: not detected (detection limit of 1 mg TNT/kg soil)

nm: not measured

[a] TNT concentrations were measured in triplicates for the soil samples from Bourges. For the soils from El Gordo, they were measured in duplicates due to the very low amount of soil samples provided to us from this site. Therefore no SD is available for the latter.

DNA extraction. The amount of soil DNA extracted was related to the level of TNT contamination in the soil samples (Figure 1). With the soil samples from the Bourges site (Figure 1A), it was much higher for the uncontaminated soils (REF, KF4, F1, and F2) than for the heavily contaminated ones (F3, KX1, KL1, KL2, KF1, KF2, KF5, KF6, KF6b, and KF6c). The same difference was observed at the El Gordo site (Figure 1B) between the uncontaminated (OUT) and the "moderately" contaminated soil samples (UEE, IN, C15, J15, C29, J29). However, the amount of DNA extracted from the "moderately" contaminated soils from El Gordo was higher than from the heavily contaminated soils from Bourges. Indeed, for the latter, the DNA extracted was hardly visible or not visible at all on the gel. These results suggested a lower bacterial biomass in the TNT-contaminated soils or problems of DNA extraction related to the presence of TNT.

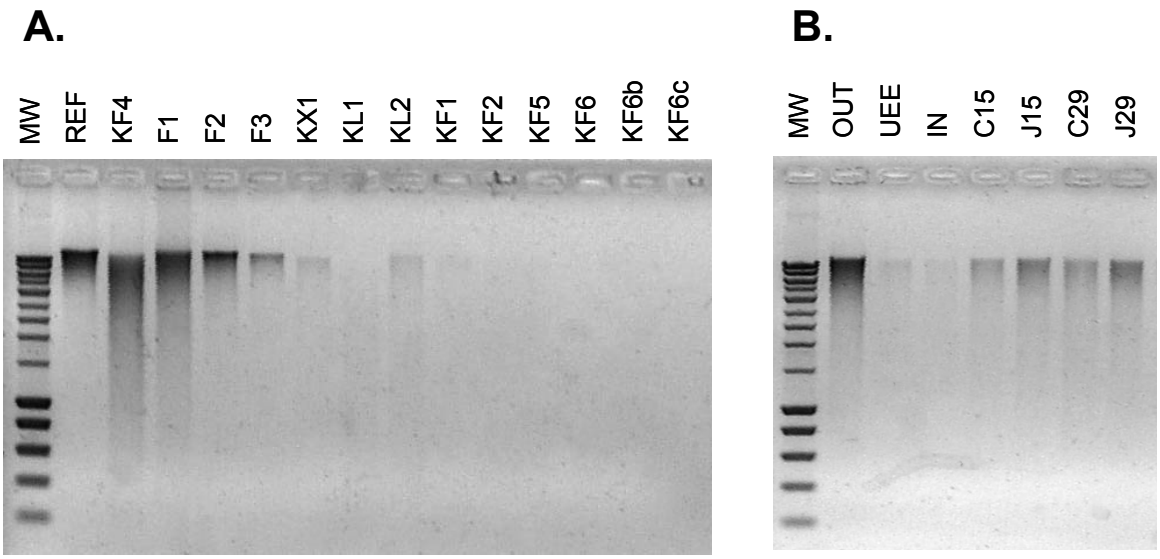


Figure 1. Extraction of DNA from different soil samples collected in Bourges, France (**A**) and in El Gordo, Spain (**B**). Five µl of molecular weight marker (MW) Smartladder (Eurogentec, Seraing, Belgium) was loaded in the first well and 5 µl of the DNA extracted was loaded in the next wells.

DGGE fingerprints of soil samples. Among the samples from the Bourges site, the uncontaminated soil samples (REF, KF4, F1, and F2) showed a complex DGGE fingerprint with no dominant bands emerging from the profiles, except two bands for soil F1 (Figure 2A). The latter corresponded to *Pseudomonas* sp. and an uncultured bacterium clone (Table 2, Figure 6). In the contaminated soils (F3, KX1, KL1, KL2, KF1, KF2, KF5, KF6, KF6b, and KF6c), the profiles were dominated by a low number of bright bands (Figure 2A). The DGGE

fingerprints of these contaminated soils were similar, especially for samples KF1, KF2, KF5, KF6, KF6b, KF6c (note that for the two latter samples, the profile was faint because of a low PCR signal). These dominant bands (bands no. 1 to 14) were identified as *Pseudomonas* spp. (Table 2, Figure 6). Soils KL1 and KL2 had additional dominant bands identified as *Acidovorax* sp., *Parvibaculum* sp. and uncultured γ-*Proteobacteria* (Table 2, Figure 6). One additional dominant band was identified in soils KF6b and KF6c as *Thermomonas* sp. Also, a band corresponding to *Brevundimonas* sp. (band no. 18) was present in most contaminated soils.

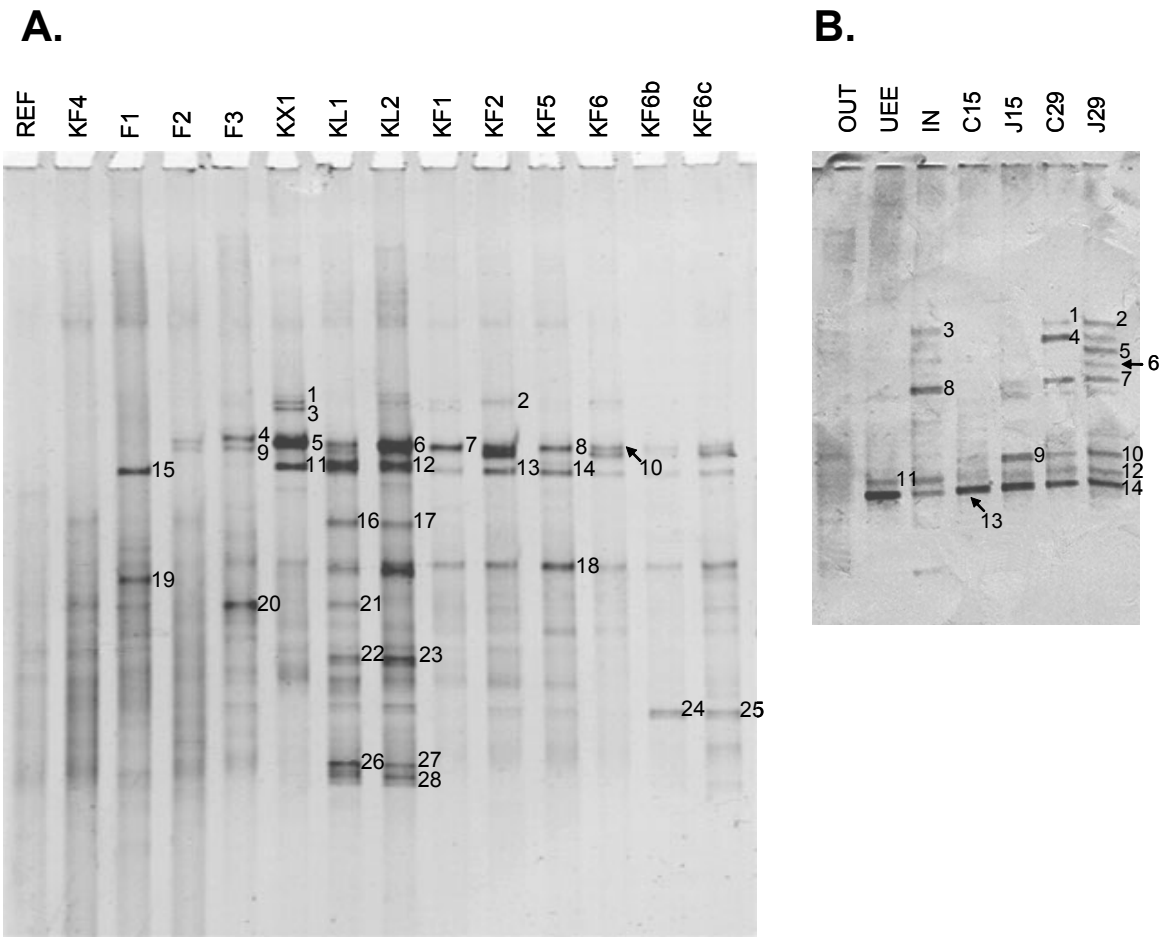


Figure 2. DGGE fingerprints of 16S rRNA gene fragments amplified from DNA extracted from uncontaminated and TNT-contaminated soil samples collected in Bourges, France (**A**) and in El Gordo, Spain (**B**). Numbers correspond to bands that were excised and sequenced. Their assignment to taxonomic groups is listed in Table 2 and 3.

Table 2. Sequence similarity of DNA amplicons excised from the DGGE of Figure 2A

Band[a]	Soil sample	Identity[b]	Taxonomic group	% similarity[c]
1	KX1	*Pseudomonas* sp. (e.g., AY785733)	*γ-Proteobacteria - Pseudomonadaceae*	100 (436)
2	KF2	*Pseudomonas* sp. (e.g., AY785733)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (446)
3	KX1	*Pseudomonas* sp. (e.g., AY785733)	*γ-Proteobacteria - Pseudomonadaceae*	100 (449)
4	F3	*Pseudomonas* sp. (e.g., AF074383)	*γ-Proteobacteria - Pseudomonadaceae*	100 (402)
5	KX1	*Pseudomonas* sp. (e.g., Z76654)	*γ-Proteobacteria - Pseudomonadaceae*	100 (371)
6	KL2	*Pseudomonas* sp. (e.g., AJ551161)	*γ-Proteobacteria - Pseudomonadaceae*	100 (448)
7	KF1	*Pseudomonas* sp. (e.g., AY456704)	*γ-Proteobacteria - Pseudomonadaceae*	99.7 (390)
8	KF5	*Pseudomonas* sp. (e.g., AJ551161)	*γ-Proteobacteria - Pseudomonadaceae*	100 (420)
9	F3	*Pseudomonas syringae* (AE016853) and many other *Pseudomonas* spp. (e.g., AJ492827)	*γ-Proteobacteria - Pseudomonadaceae*	100 (434)
10	KF6	*Pseudomonas syringae* (AE016853) and many other *Pseudomonas* spp. (e.g., AY179326)	*γ-Proteobacteria - Pseudomonadaceae*	100 (451)
11	KX1	*Pseudomonas* sp. (e.g., AM114526)	*γ-Proteobacteria - Pseudomonadaceae*	100 (322)
12	KL2	*Pseudomonas* sp. (e.g., AY456702)	*γ-Proteobacteria - Pseudomonadaceae*	99.7 (362)
13	KF2	*Pseudomonas* sp. (e.g., AY250110)	*γ-Proteobacteria - Pseudomonadaceae*	100 (433)
14	KF5	*Pseudomonas* sp. (e.g., AM114526)	*γ-Proteobacteria - Pseudomonadaceae*	100 (438)
15	F1	*Pseudomonas putida* (AY491973)	*γ-Proteobacteria - Pseudomonadaceae*	99.5 (433)
16	KL1	*Acidovorax* sp. (e.g., AJ012070)	*β-Proteobacteria - Comamonadaceae*	100 (428)
17	KL2	*Acidovorax* sp. (e.g., AJ012070)	*β-Proteobacteria - Comamonadaceae*	100 (429)
18	KF5	*Brevundimonas* sp. (AJ244710)	*α-Proteobacteria - Caulobacteraceae*	98.6 (364)
19	F1	Uncultured bacterium clone (AY532574)		99.8 (420)
20	F3	Uncultured bacterium clone (AY439192)		99.2 (374)
21	KL1	Uncultured γ-proteobacterium (e.g., AY921806)	*γ-Proteobacteria*	96.3 (436)
22	KL1	*Parvibaculum lavamentivorans* (AY387398)	*α-Proteobacteria - Rhizobiaceae*	99.0 (390)
23	KL2	*Parvibaculum lavamentivorans* (AY387398)	*α-Proteobacteria - Rhizobiaceae*	100 (384)
24	KF6b	*Thermomonas haemolytica* (e.g., AF508106)	*γ-Proteobacteria - Xanthomonadaceae*	98.3 (436)
25	KF6c	*Thermomonas haemolytica* (e.g., AF508106)	*γ-Proteobacteria - Xanthomonadaceae*	98.4 (451)
26	KL1	Uncultured γ-proteobacterium (AJ581594)	*γ-Proteobacteria*	98.1 (432)
27	KL2	Uncultured γ-proteobacterium (AJ581594)	*γ-Proteobacteria*	97.7 (432)
28	KL2	Uncultured γ-proteobacterium (AJ581598)	*γ-Proteobacteria*	98.8 (431)

[a] Number as indicated in Figure 2A.

[b] Closest match to band sequence obtained by comparison with GenBank database. When several matches with the same percentage of similarity were obtained, a single example was provided and indicated by 'e.g.'. Only sequences with more than 1300 bp in the GenBank database were considered. Numbers in parentheses indicate the GenBank accession number.

[c] Numbers in parentheses indicate the number of alignment positions used.

The bacterial diversity of the soil samples from El Gordo was investigated using the same DGGE approach. Although those soils had been exposed to lower amounts of TNT and for a shorter period of time, the same qualitative shift in the DGGE fingerprints was observed (Figure 2B): the DGGE fingerprint of the uncontaminated soil (OUT) consisted in a faint smear of bands, whereas a few prominent bands were visible in the TNT-contaminated soils (UEE and IN). These bands were identified as *Pseudomonas* spp. (bands no. 3 and 8, Table 3; Figure 6) and *Rhodanobacter* spp. (band no. 11, Table 3; Figure 6). The same two genera were found in the soil samples from plots planted with corn (C15, J15, C29 and J29). Of note, an evolution of the DGGE fingerprints was observed between samples collected 15 and 29 days after planting.

Table 3. Sequence similarity of DNA amplicons excised from the DGGE of Figure 2B

Band[a]	Soil sample	Identity[b]	Taxonomic group	% similarity[c]
1	C29	*Pseudomonas* sp. (AJ002813)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (440)
2	J29	*Pseudomonas* sp. (e.g., DQ490324)	*γ-Proteobacteria - Pseudomonadaceae*	100 (440)
3	IN	*Pseudomonas* sp. (e.g., AM403728)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (440)
4	C29	*Pseudomonas* sp. (e.g., AY450557)	*γ-Proteobacteria - Pseudomonadaceae*	98.9 (454)
5	J29	*Pseudomonas* sp. (e.g., AM184301)	*γ-Proteobacteria - Pseudomonadaceae*	100 (455)
6	J29	*Pseudomonas* sp. (e.g., AJ551157)	*γ-Proteobacteria - Pseudomonadaceae*	99.6 (456)
7	J29	*Pseudomonas* sp. (e.g., DQ178233)	*γ-Proteobacteria - Pseudomonadaceae*	100 (440)
8	IN	*Pseudomonas* sp. (e.g., DQ178233)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (440)
9	J15	*Rhodanobacter fulvus* (AB100608)	*γ-Proteobacteria - Xanthomonadaceae*	98.4 (444)
10	J29	*Rhodanobacter fulvus* (AB100608)	*γ-Proteobacteria - Xanthomonadaceae*	98.4 (444)
11	UEE	Uncultured bacterium clone (e.g., DQ125804)		98.8 (411)
		Rhodanobacter sp. (e.g., AB100608)	*γ-Proteobacteria - Xanthomonadaceae*	95.6 (411)
12	J29	Uncultured bacterium clone (e.g., DQ125804)		97.5 (399)
		Rhodanobacter sp. (e.g., AM087226)	*γ-Proteobacteria - Xanthomonadaceae*	96.7 (399)
13	C15	Uncultured bacterium clone (e.g., DQ125804)		100 (411)
		Rhodanobacter lindaniclasticus (e.g., DQ370020)	*γ-Proteobacteria - Xanthomonadaceae*	99.7 (362)
14	J29	Uncultured bacterium clone (e.g., DQ125804)		100 (411)
		Rhodanobacter lindaniclasticus (e.g., DQ370020)	*γ-Proteobacteria - Xanthomonadaceae*	99.7 (362)

[a] Number as indicated in Figure 2B.

[b] Closest match to band sequence obtained by comparison with GenBank database. When several matches with the same percentage of similarity were obtained, a single example was provided and indicated by 'e.g.'. Only sequences with more than 1300 bp in the GenBank database were considered. Numbers in parentheses indicate the GenBank accession number.

[c] Numbers in parentheses indicate the number of alignment positions used.

Cultivation experiments. Cultivation experiments were carried out with an uncontaminated (REF) and a TNT-contaminated soil sample (KX1) in order to determine whether TNT contamination had patterned the cultivable community of these soils as well. These two soils had relatively similar pH, soil texture and carbon content values (Table 1).

After spreading on agar plates containing 200-fold dilute nutrient broth ("diluted NB"), a clear difference in the number of CFU was observed between the uncontaminated and the TNT-contaminated soil (Figure 3A). This experiment was repeated and gave very similar results (data not shown). After 91 days of incubation, the number of CFU reached 2.28 x 10^7

CFU/g for soil REF versus 1.15 x 10^7 CFU/g for soil KX1. The number of CFU was stable after 21 days for soil KX1 whereas it had not yet reached a plateau after 91 days of incubation for soil REF.

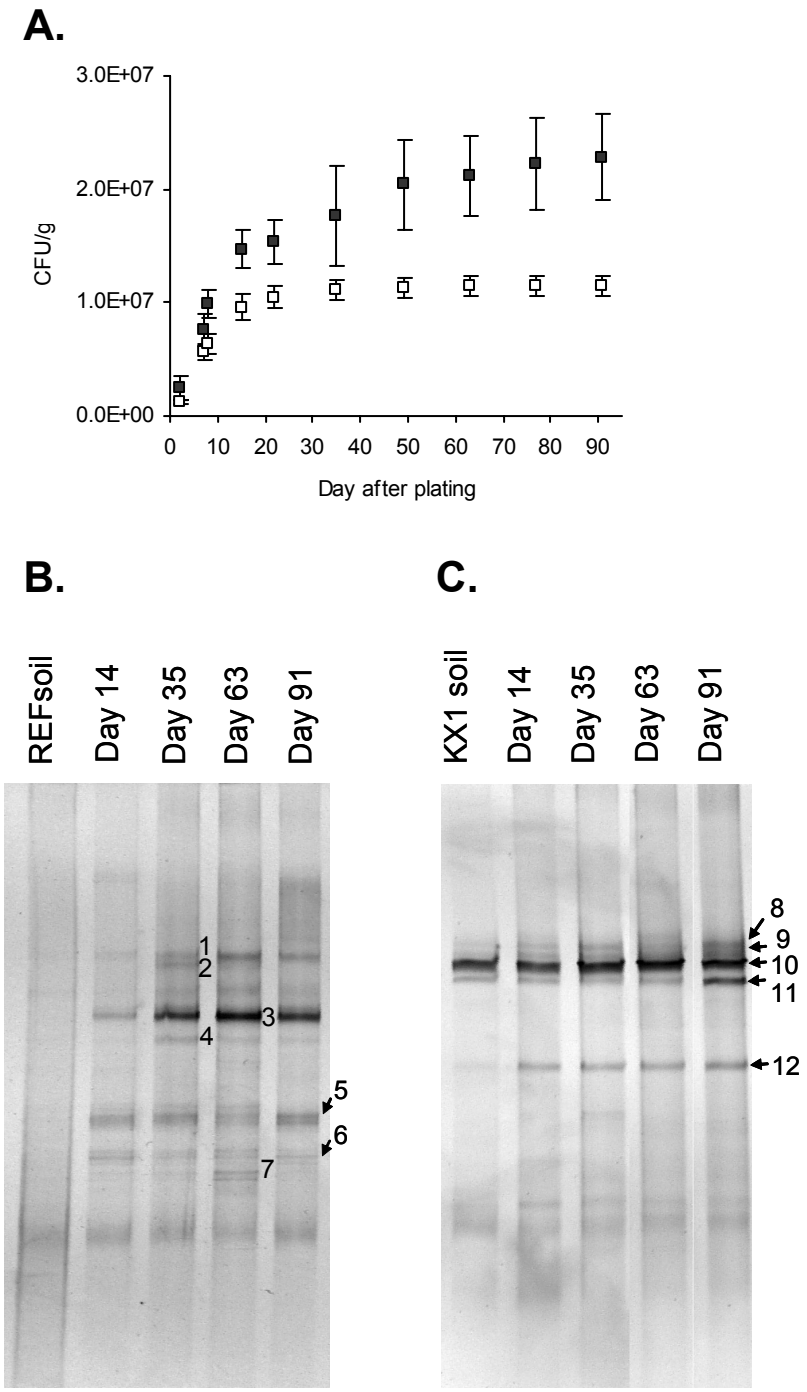


Figure 3. Evolution of the number of CFU per g of dry soil on agar plates supplemented with dilute nutrient broth as a function of incubation time (**A**). Closed and open squares are CFUs from soil REF (uncontaminated) and KX1 (TNT-contaminated), respectively. Error bars represent the standard deviation of triplicates to quintuplicates of the same dilution. DGGE fingerprints of 16S rRNA gene fragments amplified from DNA extracted from the cultivable bacterial microflora of soil REF (**B**) and soil KX1 (**C**) after 14, 35, 63 and 91 days of incubation. The first lane shows the DGGE fingerprint of 16S rRNA genes directly amplified from the two soils (as in Figure 2A). Numbers correspond to bands that were excised and sequenced. Their assignment to taxonomic groups is listed in Table 4.

The bacterial diversity of CFUs collected by plate washing was analyzed by DGGE (Figure 3B and 3C). These profiles corresponded to plates with the same dilution (1,000-fold dilution). The DGGE fingerprints of the CFUs from soil REF showed a moderate change from day 14 to day 91 (Figure 3B). The few dominant bands in these fingerprints corresponded to *Pseudomonas* sp., *Acinetobacter* sp., *Variovorax* spp., *Rhizobium* sp., *Paracoccus* sp., and *Achromobacter/Alcaligenes* sp. (Table 4, Figure 6). The DGGE fingerprints of the CFUs from soil KX1 were similar to the pattern of soil KX1 (Figure 3C). No change in that DGGE fingerprint was observed between days 14 and 91. Except for a single band corresponding to *Brevundimonas* sp., all dominant bands were identified as *Pseudomonas* spp. (Table 4, Figure 6).

Table 4. Sequence similarity of DNA amplicons excised from the DGGE of Figure 3B and Figure 3C

Band[a]	Soil inoculum	Identity[b]	Taxonomic group	% similarity[c]
1	REF	*Pseudomonas* sp. (e.g., DQ453822)	*γ-Proteobacteria - Pseudomonadaceae*	100 (486)
2	REF	*Acinetobacter* sp. (e.g., DQ536511)	*γ-Proteobacteria - Moraxellaceae*	99.8 (487)
3	REF	*Variovorax* sp. (e.g., AY599733)	*β-Proteobacteria - Comamonadaceae*	99.8 (483)
4	REF	*Variovorax* sp. (e.g., AM269508)	*β-Proteobacteria - Comamonadaceae*	99.8 (483)
5	REF	*Rhizobium* sp. (e.g., AY500260)	*α-Proteobacteria - Rhizobiaceae*	100 (408)
6	REF	*Paracoccus* sp. (AJ244715)	*α-Proteobacteria - Rhodobacteraceae*	99.8 (435)
7	REF	*Achromobacter* / *Alcaligenes* sp. (e.g., DQ530072)	*β-Proteobacteria - Alcaligenaceae*	99.8 (483)
8	KX1	*Pseudomonas* sp. (e.g., AY785733)	*γ-Proteobacteria - Pseudomonadaceae*	100 (409)
9	KX1	*Pseudomonas* sp. (e.g., AY785733)	*γ-Proteobacteria - Pseudomonadaceae*	100 (395)
10	KX1	*Pseudomonas* sp. (e.g., AY512608)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (447)
11	KX1	*Pseudomonas* sp. (e.g., AM114526)	*γ-Proteobacteria - Pseudomonadaceae*	100 (409)
12	KX1	*Brevundimonas* sp. (AJ244710)	*α-Proteobacteria - Caulobacteraceae*	98.4 (435)

[a] Number as indicated in Figure 3B and 3C.

[b] Closest match to band sequence obtained by comparison with GenBank database. When several matches with the same percentage of similarity were obtained, a single example was provided and indicated by 'e.g.'. Only sequences with more than 1300 bp in the GenBank database were considered. Numbers in parentheses indicate the GenBank accession number.

[c] Numbers in parentheses indicate the number of alignment positions used.

Artificial contamination of a pristine soil. In order to evaluate the effect of TNT on a pristine soil, a soil sample from Louvain-la-Neuve was artificially contaminated with different concentrations of TNT and changes in the amount of DNA extracted and in the DGGE fingerprints were monitored for 4 months. The amount of DNA in the soil contaminated with different concentrations of TNT was similar at t=0 (Figure 4) and remained relatively stable over a 61-day period (data not shown). After 4 months of incubation, a significant reduction in the amount of DNA extracted was observed in the samples containing high concentrations of TNT (Figure 4). The DNA extracted from the soil samples was quantified by agarose gel analysis: in the control sample (no TNT added) and in the sample containing 140 mg TNT/kg soil, a reduction of 11% and 12% of the initial DNA content was measured after 4 months, respectively. In the samples containing 1.4 and 28.5 g TNT/kg soil, the reduction amounted to 79% of the initial DNA content.

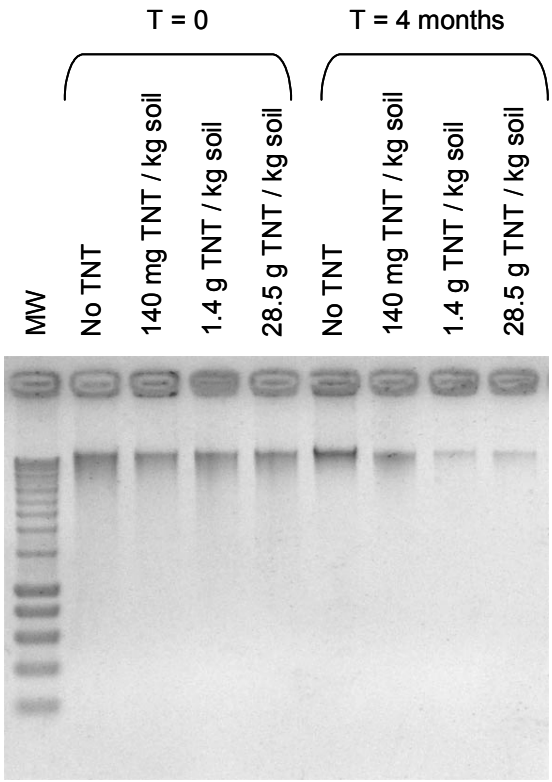


Figure 4. Extraction of DNA from a pristine soil at t=0 and 121 days after artificial contamination with different concentrations of TNT. Five µl of molecular weight marker (MW) Smartladder was loaded in the first well and 5 µl of the DNA extracted was loaded in the next wells.

The DGGE fingerprints of the pristine soil and the soil containing 140 mg TNT/kg soil showed little change during the 121-d incubation period (Figure 5). Some of the bands of these fingerprints were successfully sequenced and identified as *Methylosinus* sp., *Methylocystis* sp., *Lysobacter* sp. and *Ralstonia* sp. (although the match was not very good for the latter) (Table 5, Figure 6). In the soil containing 140 mg TNT/kg soil, one new band appeared after 121 days of incubation, which was identified as *Pseudomonas* sp. With the soil containing 1.4 g TNT/kg soil, bright bands already appeared after 7 and 14 days of incubation. They corresponded to *Pseudomonas* sp., *Variovorax* sp., and *Lysobacter* sp. After 61 days, several even brighter bands were observed. In particular, the band corresponding to the *Pseudomonas* sp. became brighter, and two additional bands corresponding to another *Pseudomonas* sp. and *Brevundimonas* sp. were detected. Finally, with the soil containing 28.5 g TNT/kg soil, several bright bands were present after 7 days of incubation and the DGGE fingerprint remained similar during further incubation. They all corresponded to *Pseudomonas* spp., except two bands identified as *Delftia* sp. (Table 5, Figure 6).

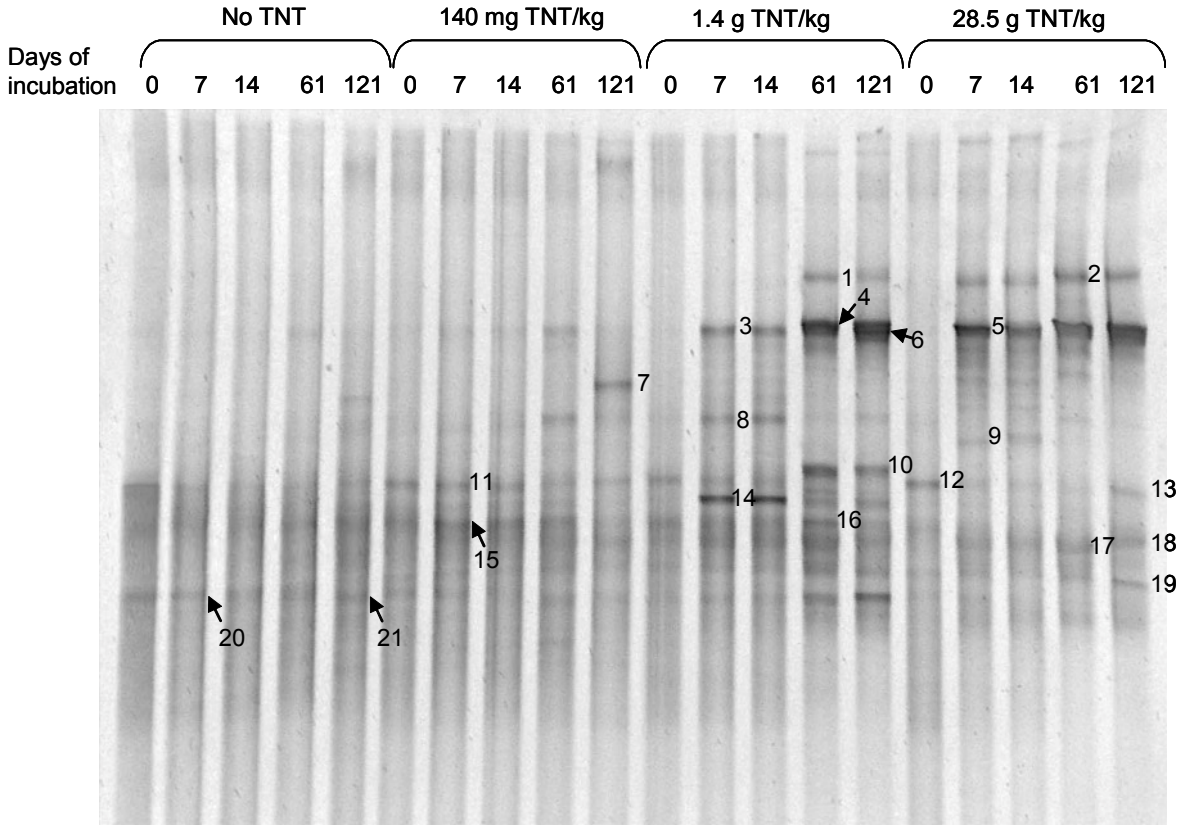


Figure 5. DGGE fingerprints of 16S rRNA gene fragments amplified from DNA extracted from a pristine soil at t=0 and 7, 14, 61 and 121 days after artificial contamination with different concentrations of TNT. Numbers correspond to bands that were excised and sequenced. Their assignment to taxonomic groups is listed in Table 5.

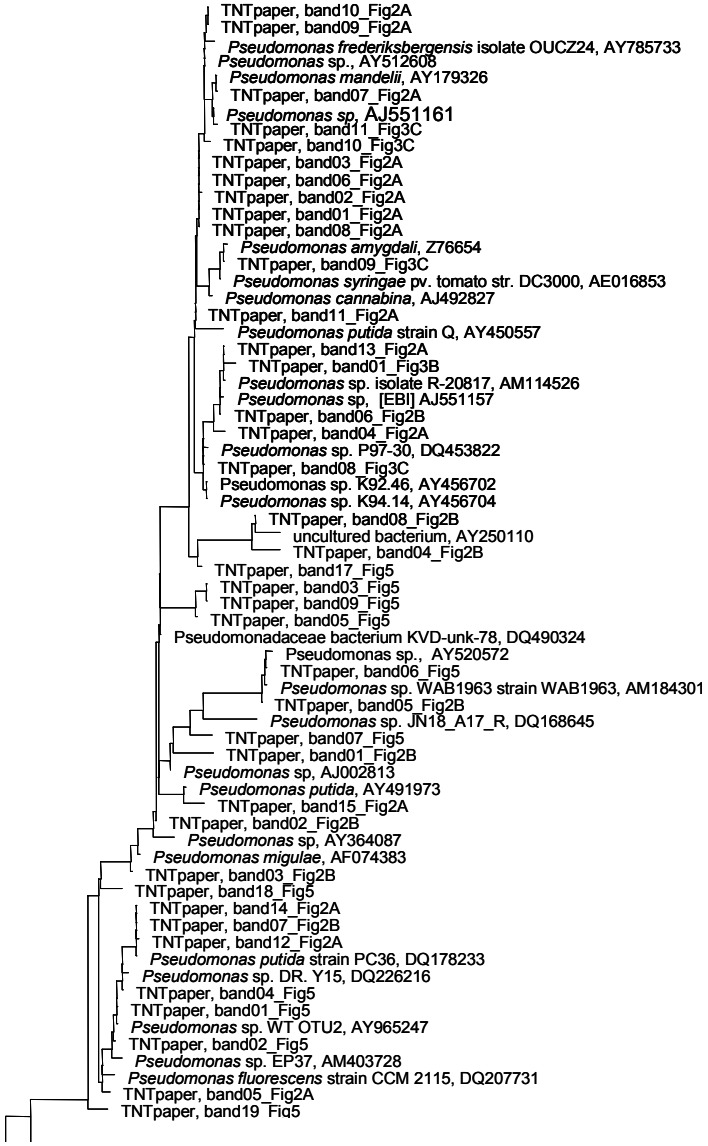
Table 5. Sequence similarity of DNA amplicons excised from the DGGE of Figure 5

Band[a]	TNT concentration - days of incubation	Identity[b]	Taxonomic group	% similarity[c]
1	1.4 g/kg - 61	*Pseudomonas* sp. (e.g., AY965247)	*γ-Proteobacteria - Pseudomonadaceae*	100 (450)
2	28.5 g/kg - 61	*Pseudomonas* sp. (e.g., AY965247)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (461)
3	1.4 g/kg - 7	*Pseudomonas* sp. (e.g., AY364087)	*γ-Proteobacteria - Pseudomonadaceae*	99.3 (462)
4	1.4 g/kg - 61	*Pseudomonas* sp. (e.g., DQ226216)	*γ-Proteobacteria - Pseudomonadaceae*	100 (461)
5	28.5 g/kg – 7	*Pseudomonas* sp. (e.g., AY364087)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (449)
6	1.4 g/kg - 121	*Pseudomonas* sp. (e.g., AY520572)	*γ-Proteobacteria - Pseudomonadaceae*	99.8 (459)
7	140 mg/kg - 121	*Pseudomonas* sp. (DQ168645)	*γ-Proteobacteria - Pseudomonadaceae*	98.9 (450)
8	1.4 g/kg - 7	*Variovorax* sp. (DQ256488)	*β–Proteobacteria - Comamonadaceae*	97.5 (447)
9	28.5 g/kg – 7	*Pseudomonas* sp. (e.g., AY364087)	*γ-Proteobacteria - Pseudomonadaceae*	99.3 (459)
10	1.4 g/kg - 121	*Brevundimonas* sp. (AJ227785)	*α–Proteobacteria – Caulobacteraceae*	99.0 (410)
11	140 mg/kg – 7	*Ralstonia* sp. (e.g., DQ847128)	*β–Proteobacteria - Burkholderiaceae*	94.0 (353)
12	28.5 g/kg – 0	*Delftia* sp. (e.g., AM180725)	*β–Proteobacteria - Comamonadaceae*	99.4 (454)
13	28.5 g/kg – 121	*Delftia* sp. (e.g., AM180725)	*β–Proteobacteria - Comamonadaceae*	97.8 (412)
14	1.4 g/kg – 7	*Lysobacter* sp. (DQ462462)	*γ–Proteobacteria - Xanthomonadaceae*	98.9 (467)
15	140 mg/kg – 7	*Lysobacter* sp. (DQ249997)	*γ–Proteobacteria - Xanthomonadaceae*	98.5 (406)
16	1.4 g/kg - 61	*Lysobacter* sp. (AB161361)	*γ–Proteobacteria - Xanthomonadaceae*	96.4 (467)
17	28.5 g/kg - 61	*Pseudomonas* sp. (e.g., DQ207731)	*γ-Proteobacteria - Pseudomonadaceae*	99.6 (460)
18	28.5 g/kg – 121	*Pseudomonas* sp. (e.g., AY965247)	*γ-Proteobacteria - Pseudomonadaceae*	99.6 (460)
19	28.5 g/kg – 121	*Pseudomonas* sp. (e.g., DQ226216)	*γ-Proteobacteria - Pseudomonadaceae*	97.3 (330)
20	No TNT – 7	*Methylosinus* sp. (AJ868424)	*α–Proteobacteria – Methylocystaceae*	96.2 (346)
21	No TNT – 121	*Methylocystis* sp. (AJ458497)	*a-Proteobacteria – Methylocystaceae*	98.8 (341)

[a] Number as indicated in Figure 5.

[b] Closest match to band sequence obtained by comparison with GenBank database. When several matches with the same percentage of similarity were obtained, a single example was provided and indicated by 'e.g.'. Only sequences with more than 1300 bp in the GenBank database were considered. Numbers in parentheses indicate the GenBank accession number.

[c] Numbers in parentheses indicate the number of alignment positions used.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TNTpaper, band09_Fig2A
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Pseudomonas sp., AY512608
Pseudomonas mandelii, AY179326
TNTpaper, band07_Fig2A
Pseudomonas sp. AJ551161
TNTpaper, band11_Fig3C
TNTpaper, band10_Fig3C
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TNTpaper, band06_Fig2A
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TNTpaper, band01_Fig2A
TNTpaper, band08_Fig2A
Pseudomonas amygdali, Z76654
TNTpaper, band09_Fig3C
Pseudomonas syringae pv. tomato str. DC3000, AE016853
Pseudomonas cannabina, AJ492827
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Pseudomonas sp, [EBI] AJ551157
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TNTpaper, band04_Fig2A
Pseudomonas sp. P97-30, DQ453822
TNTpaper, band08_Fig3C
Pseudomonas sp. K92.46, AY456702
Pseudomonas sp. K94.14, AY456704
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uncultured bacterium, AY250110
TNTpaper, band04_Fig2B
TNTpaper, band17_Fig5
TNTpaper, band03_Fig5
TNTpaper, band09_Fig5
TNTpaper, band05_Fig5
Pseudomonadaceae bacterium KVD-unk-78, DQ490324
Pseudomonas sp., AY520572
TNTpaper, band06_Fig5
Pseudomonas sp. WAB1963 strain WAB1963, AM184301
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TNTpaper, band01_Fig2B
Pseudomonas sp, AJ002813
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Pseudomonas sp, AY364087
Pseudomonas migulae, AF074383
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TNTpaper, band18_Fig5
TNTpaper, band14_Fig2A
TNTpaper, band07_Fig2B
TNTpaper, band12_Fig2A
Pseudomonas putida strain PC36, DQ178233
Pseudomonas sp. DR. Y15, DQ226216
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Pseudomonas sp. WT OTU2, AY965247
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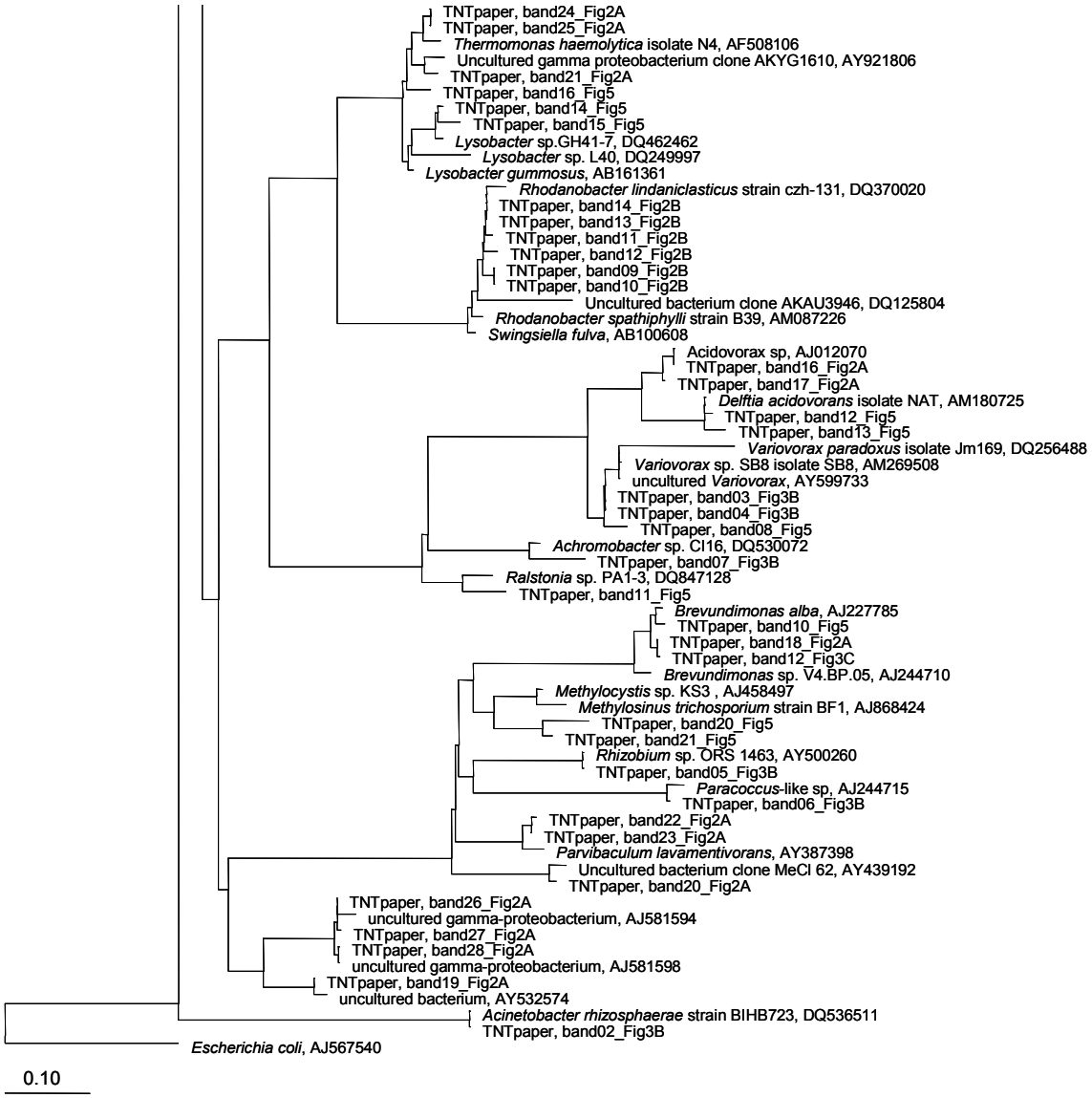

Figure 6. Phylogenetic tree showing the affiliation of the different sequenced DGGE bands of the total and culturable microflora of uncontaminated and TNT-contaminated soil samples to their closest relatives. The bar indicates 10% sequence variation.

5.5. Discussion

Amount of DNA extracted from the soil samples. A preliminary step prior to DGGE analysis consisted in isolating DNA from the soil samples. The amount of DNA extracted was related to the concentration of TNT in these soil samples: uncontaminated soils yielded a higher amount of DNA than the TNT-contaminated soils (Figure 1). This suggested that soils contaminated with TNT contained less DNA than uncontaminated ones, or that TNT had an inhibitory effect on the extraction of DNA with the Mo Bio kit.

When a pristine soil was artificially contaminated with TNT, no significant difference in the amount of DNA extracted was observed at t=0 and during the first two months of incubation. This indicated that TNT had no inhibitory effect on the extraction method but rather affected the real content of DNA in the soil samples.

Among the contaminated soils, the amount of DNA extracted was higher in the samples from El Gordo than the ones from Bourges. This probably resulted from differences in pH, texture, carbon content, history of contamination (7 months versus 20 years) and concentrations of TNT (mg versus g TNT/kg soil). The experiment carried out with an artificially contaminated pristine soil clearly indicated that the history of contamination and the concentration of TNT were indeed two key factors affecting the amount of DNA extracted, as a significant decrease in the amount of DNA extracted was already observed after 4 months of incubation and at TNT concentrations equal to or above 1.4 g TNT/kg soil.

DGGE fingerprints of the soils from Bourges and El Gordo. The DGGE fingerprints of the uncontaminated soils consisted in a smear of many bands, with none of them dominating the fingerprint (except for soil F1, Figure 2A). On the other hand, the DGGE fingerprints of the TNT-contaminated soil samples revealed the presence of a few dominant bands (Figure 2A and 2B). Despite different histories of TNT contamination and different physico-chemical characteristics in addition to variable concentrations of TNT, all contaminated soil samples displayed a bacterial diversity strongly impacted by the presence of TNT. Using phospholipid fatty acid analyses, Fuller and Manning (1998) found a predominance of Gram-negative over Gram-positive bacteria in TNT-contaminated soils. However, the identity of the species was not determined. In our study, the sequencing of the DGGE bands allowed the identification of

the major species prevailing under conditions of chronic contamination. We did not retrieve any sequences corresponding to Gram-positive bacteria in the contaminated soil samples, and we found a clear predominance of *Proteobacteria*, and more specifically of *Pseudomonas* spp., *Brevundimonas* sp. and *Rhodanobacter* spp. In most cases, the phylogenetic affiliation of the DGGE bands could not be resolved at the species level because their sequence equally matched several Genbank sequences corresponding to different species within the same genus. This may be due to the size of 16S rRNA gene analyzed in this study, as the set of primers BACT63F and UNIV518R amplified approximately 450 bp of the 16S rRNA gene. Moreover, the high degree of conservation of the 16S rRNA gene may hamper a detailed phylogenetic study at the species level for some groups like *Pseudomonas*. This is the reason why several other genes have been proposed for phylogenetic analyses (Fox et al. 1992, De Vos et al. 1998, Ludwig et al. 1998, Dahllöf et al. 2000, Yamamoto et al. 2000, Tayeb et al. 2005).

Cultivation experiments. A dilute nutrient broth solidified with agar and long incubation times (> 10 weeks) were chosen because these approaches have made possible the cultivation of a larger proportion of soil microbes, including bacteria that were formerly known as unculturable, such as important members within the divisions *Actinobacteria*, and *Acidobacteria* (Janssen et al. 2002). The viable count profile obtained with sample REF (Figure 3A) was similar to the viable count profile observed by Janssen et al. (2002), i.e., the viable counts gradually increased over a 91-day period of incubation. However, by day 91, the number of CFUs was approximately two-fold lower with sample KX1 than sample REF. This suggests a direct correlation between the presence of TNT, the amount of DNA extracted and the number of CFUs. Likewise, in metal contaminated soils, Ellis et al. (2003) observed a lower number of cultivable bacteria in the soils with the greatest metal content.

The assessment of culturable bacterial diversity on dilute nutrient broth solidified with agar was carried out by Janssen et al. (2002) by picking individual colonies. In our study, we washed the plates and performed a DGGE targeting 16S rRNA genes with general primers. With the uncontaminated soil inoculum, dominant bands were identified as *Pseudomonas* sp., *Acinetobacter* sp., *Variovorax* spp., *Rhizobium* sp., *Paracoccus* sp., and *Achromobacter / Alcaligenes* sp., whereas a predominance of *Pseudomonas* spp. was already clearly in evidence after 14 days of incubation with the TNT-contaminated soil sample. These data are consistent with the DGGE fingerprints of the total microflora in the TNT-contaminated soil

samples indicating a predominance of *Pseudomonas* spp. Although the dominant bands in the DGGE fingerprints from plates incubated with the two soil samples corresponded to bacterial genera with many known culturable representatives (Table 4), we found a greater diversity of species in the uncontaminated than in the TNT-contaminated soil inoculum. These results do not mean that fastidious microorganisms did not grow on the diluted medium, but rather that they did not show up among the dominant species on the DGGE fingerprint. DGGE patterns are indeed limited to the analysis of major constituents of the community analyzed. Therefore, the detection of rare community members is limited by the sensitivity of this method (Muyzer 1999). This bias can be improved by the application of group-specific PCR primers or the identification of community members by hybridization of blotted DGGE gels with group-specific probes (Muyzer 1999, Heuer et al. 1999). More recently, the use of fluorophore-labeled primers has been shown to improve the sensitivity of the technique as well (Neufeld & Mohn 2005). For example, we have detected among the CFUs from soil REF various members of the unculturable phylum *Acidobacteria* by using *Acidobacteria*-specific PCR primers, whereas none was detected among the CFUs from soil KX1 (George et al., manuscript in preparation). With rich media solidified with agar, Fuller and Manning (1998) also observed a significantly lower number of CFUs in a highly TNT-contaminated soil (9.30 g TNT/kg soil) than in a soil with a low concentration of TNT (0.02 g TNT/kg soil). The authors found that 93% of the plating isolates from the highly TNT-contaminated soil were Gram-negative bacteria, with 75% of them identified as *Pseudomonas* species. Several studies dealing with pure cultures have shown that Gram-negative bacteria were indeed less sensitive than Gram-positive bacteria to the presence of TNT (Fuller & Manning 1997, Zaripov et al. 2004). For example, Fuller & Manning (1997) determined that the concentration of TNT in liquid cultures causing 50% reduction in growth was 7.5 ppm for Gram-positive bacteria, whereas such a reduction in growth was not reached for Gram-negative bacteria at the limit of TNT solubility (100 ppm). However, the mechanisms underlying the differential sensitivity of Gram-positive and Gram-negative bacteria to TNT were not fully deciphered.

Ellis et al. (2003) evaluated the bacterial composition of metal-contaminated soils using culture-independent and -dependent methods. These authors found that DGGE fingerprints from the contaminated soils were similar although the level of metal contamination varied between the soil samples. On the other hand, the DGGE fingerprints from plate wash differed significantly between the samples. Although the DGGE fingerprints of the total and the cultivable microflora of these contaminated soils were not compared to uncontaminated soil

samples, the authors concluded that metal contamination did not affect soil bacterial diversity but affected the physiological status of the bacteria, so that the number of bacteria capable of responding to laboratory culture and their taxonomic distribution were altered (Ellis et al. 2003). Unlike Ellis et al. (2003), in our study, *both* culture-independent and -dependent approaches showed the selective pressure of TNT on soil bacterial diversity. On one hand, direct comparison of the DGGE fingerprints of the total microflora showed clear differences between uncontaminated and TNT-contaminated soil samples. On the other hand, DGGE analysis of the cultivable microflora indicated differences as well between uncontaminated and TNT-contaminated soil samples. Finally, plate counts gave additional insights of the number of cultivable bacteria in uncontaminated and TNT-contaminated soil samples.

Artificial contamination of a pristine soil. In a previous study, Eyers et al. (2006) analyzed the bacterial population of the uncontaminated soil sample REF and of the TNT-contaminated soil sample KX1 from Bourges by hybridization of in vitro transcribed 16S rRNA derived from these two soil samples to a phylogenetic oligonucleotide microarray. Significant differences in hybridizations were found between these two soil samples, but the origin of these differences (e.g., presence of TNT, pH, soil texture, carbon content) was not clearly determined. Our study of soils from Bourges and from El Gordo indicated that the history of contamination and the concentration of TNT had a decisive influence on their bacterial diversity.

The experiment of artificial TNT contamination of a pristine soil confirmed that the presence of the pollutant and the history of pollution were indeed two key factors affecting bacterial populations. As mentioned before, a decrease in the amount of DNA extracted was observed after 4 months of incubation in the microcosms with the greatest TNT concentration, which suggested a lower bacterial biomass in these soils and confirmed observations on the soils from Bourges and El Gordo. Moreover, clear shifts in the bacterial community structure were observed after 7 days of incubation on the DGGE fingerprints of soil samples artificially contaminated with 1.4 and 28.5 g TNT/kg of soil. These changes led to bacterial populations dominated by *Pseudomonadaceae* (*Pseudomonas* spp.), *Comamonadaceae* (*Variovorax* sp. and *Delftia* sp.), *Xanthomonadaceae* (*Lysobacter* spp.) and *Caulobacteraceae* (*Brevundimonas* sp.), i.e., the same dominant bacterial families that were found in the TNT-contaminated soils from Bourges and El Gordo.

The predominance of *Pseudomonas* spp. in the soil samples from Bourges and from El Gordo, in the plate counts, and in the artificially contaminated soil may be related to a lower sensitivity of these bacteria to the toxic effect of TNT. As previously mentioned by Fuller and Manning (1997), TNT brought about a more significant reduction of growth of Gram-positive than Gram-negative bacteria. The predominance of *Pseudomonas* spp. may also be related to their degradative properties. For instance, several *Pseudomonas* spp. have been isolated from contaminated environments and were shown to transform TNT to their amino derivatives (Esteve-Nunez et al. 2001). However, these amino derivatives tended to accumulate and were not degraded further. On the other hand, some bacteria have been reported to denitrate TNT (Esteve-Nunez et al. 1998, Stenuit et al. 2005) with the production of metabolites known to be easily mineralized (Nishino et al. 2000). Interestingly, a *Pseudomonas aeruginosa* strain was recently isolated by Eyers et al. (manuscript in preparation) from the soil sample KX1 located at the site of Bourges. This bacterium is able to denitrate TNT with the release of significant concentrations of nitrite.

Bacteria belonging to *Comamonadaceae* (*Acidovorax, Variovorax, Delftia genera*), *Xhanthomonadaceae* (*Thermomonas*, *Rhodanobacter*, *Lysobacter* genera), *Caulobacteraceae* (*Brevundimonas* genus) and *Rhizobiaceae* (*Parvibaculum* genus) were also detected in the various TNT-contaminated soil samples, which suggest that they may be at least tolerant to the presence of TNT. Most of these bacterial genera have been cited in the literature in relation to the biodegradation of different environmental pollutants. *Acidovorax* spp. were reported to degrade halobenzoate (Song et al. 2000) and polycyclic aromatic hydrocarbons (PAHs) (Eriksson et al. 2003). *Variovorax* spp. were shown to degrade trichloroethylene (Futama et al. 2005), the herbicides linuron (Dejonghe et al. 2003) and 2,4-dichlorophenoxyacetic acid (Kamagata et al. 1997), and PAHs (Eriksson et al. 2003). *Delftia* spp. (some of them formerly classified as *Comamonas* spp.) were reported to participate in the mineralization of linear alkylbenzenesulfonates (LAS) (Schleheck et al. 2004a) and to degrade phenanthrene (Vacca et al. 2005), aniline (Sheludchenko et al. 2005) and various herbicides (Müller et al. 1999, Dejonghe et al. 2003). *Thermomonas* spp. were identified in a petroleum land treatment unit (Kaplan & Kitts 2004). *Rhodanobacter lindaniclasticus* was reported to degrade the insecticide lindane (Nalin et al. 1999) and a *Rhodanobacter* sp. was identified in a benzo(a)pyrene-mineralizing consortium (Kanaly et al. 2002). *Brevundimonas spp.* (some of them formely classified as Pseudomonads) were identified in consortia or as pure cultures degrading various herbicides (Hamdi & Tewfik 1969, Smejkal et al. 2003), the

insecticide dimethoate (Deshpande et al. 2004), the solvent dichloromethane (Krausova et al. 2006) as well as isoquinoline (Lehmann et al. 1994). Finally, *Parvibaculum lamentivorans* was reported to degrade LAS (Schleheck et al. 2004b) and to grow on crude oil (Sanchez et al. 2005). However, apart from *Stenotrophomonas* sp. OK-5 (*Xhanthomonadaceae*), which is able to resist TNT-mediated stress by producing stress shock proteins (Ho et al. 2004) and to grow on TNT as sole nitrogen source (Oh & Kim 1998), no other member of the families *Comamonadaceae, Xhanthomonadaceae* and *Caulobacteraceae* has ever been reported to metabolize TNT. Bacteria known to degrade TNT aerobically and/or anaerobically belong indeed to *Pseudomonadaceae* (*Pseudomonas* spp.), *Enterobacteriaceae* (*Enterobacter* spp., *Serratia* spp.), *Mycobacteriaceae* (*Mycobacterium* spp.), *Nocardiaceae* (*Rhodococcus* spp.), *Desulfovibrionaceae* (*Desulfovibrio* spp.), *Clostridiaceae* (*Clostridium* spp.) and *Bacillaceae* (*Bacillus* spp.) (Peres & Agathos 2000, Stenuit et al. 2005, Stenuit et al. 2006). Therefore, the present study opens the field of investigation towards new TNT-degrading bacteria.

In summary, this study showed that TNT had a significant ecological impact on soil bacterial populations. To our knowledge, this is the first study providing an extensive characterization of TNT-contaminated soils sampled at different locations, and of a pristine soil artificially contaminated with TNT. Given its adverse impact, there is an important need for decontaminating sites chronically polluted with TNT.

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5.6. References

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