8.

Chapter 8

Conclusions and perspectives

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.

8.1. Major results obtained from the initial objectives of this thesis

The initial objectives of this thesis were (i) to decipher the microbial populations of soils contaminated with TNT and (ii) to isolate and characterize bacteria with promising TNT-degradation activities. The results obtained from those objectives are summarized in Figure 1.

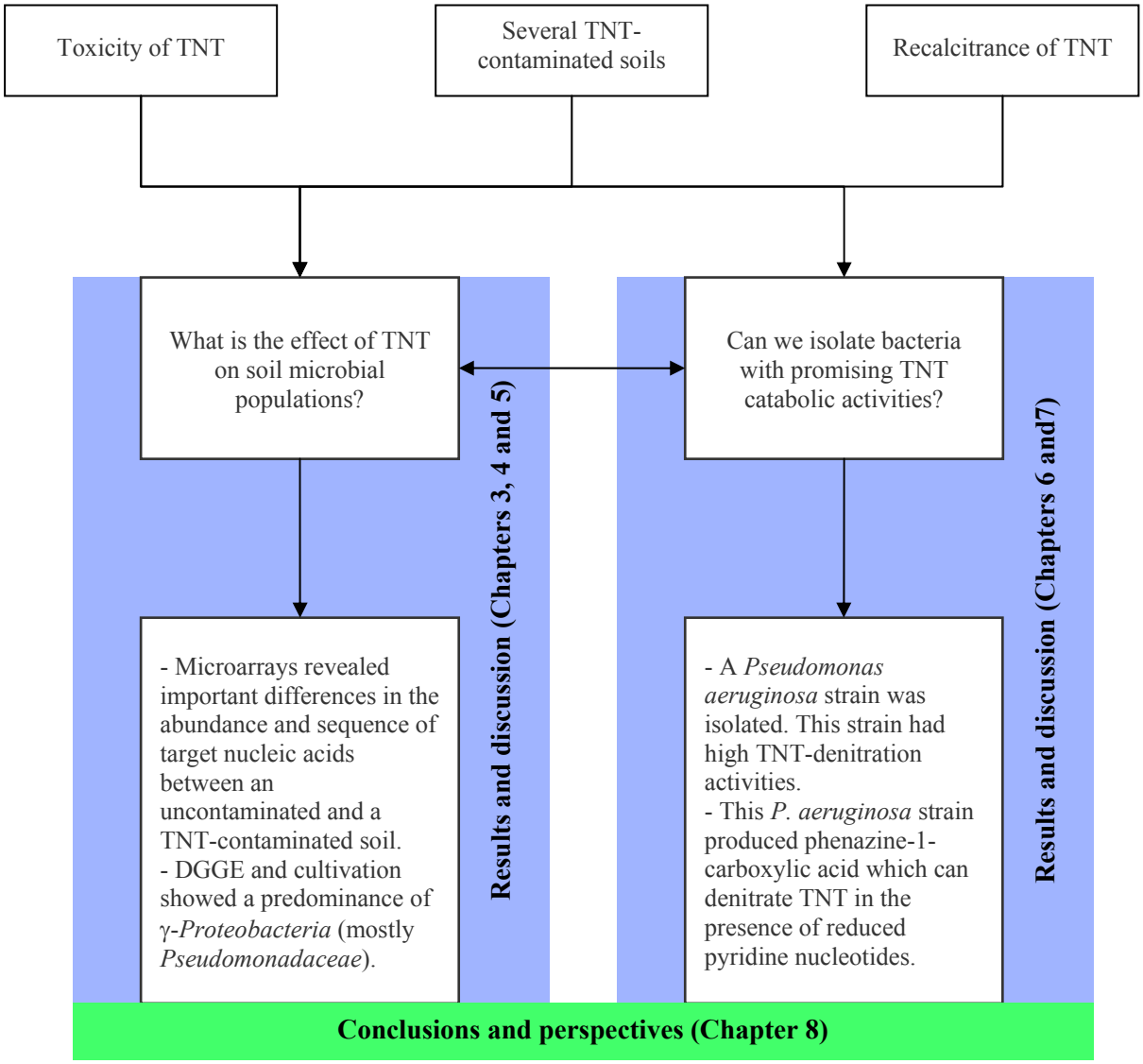

Figure 1. Major results obtained from the initial objectives of this thesis.

8.2. Microbial characterization of TNT-contaminated soils

Until this thesis was undertaken, the microbial composition of TNT-contaminated soils was poorly understood because only traditional methods providing non-discriminating information had been used. As mentioned in chapter 5, the methods used to analyze microorganisms present in TNT-contaminated soils had consisted in phospholipid fatty acid composition (11, 30), soil respirometric activities (12), and denitrification activities (26). Given the poor phylogenetic resolution capacities of these techniques, the bacterial composition at the group and species level was only partially resolved. The only evidence was a differential sensitivity between Gram-positive and Gram-negative bacteria in the presence of TNT (10). The development of molecular techniques based on the 16S rRNA has allowed the study of both cultivable and uncultivable fractions of microbial communities in fine detail (i.e., at the group and species level). These techniques were described in chapter 1. Among these methodologies, phylogenetic microarrays and DGGE were chosen to analyze the microbial communities in TNT-contaminated soils because they are complementary approaches.

The analysis of environmental samples with microarrays containing probes targeting the 16S rRNA is challenging because mismatch (MM) target nucleic acids are likely to hybridize to probes targeting perfect-match (PM) nucleic acids. Hacia et al. (13) showed that quantification of non-specific hybridization can be determined by subtracting signals of MM probes from PM probes. However, this approach may not be suitable with samples from complex environmental communities since the abundance of related species that have MM sequences is unknown. To solve this issue, dissociation curves resulting from thermal dissociation analysis of probe-target duplexes can be used. The exact shape of the curve depends on the sequence of the duplex. Thus, PM duplexes may produce curves that are distinguishable from MM duplexes. Although dissociation curves are critical to analyze environmental samples at the level of the 16S rRNA, the groups of Dr. D. Stahl (University of Washington, Seattle) and Dr. A. Mirzabekov (Argonne National Laboratory, Illinois) were, at the time the research of this thesis was undertaken, amongst the few teams using such a technique because it requires equipment that is not commercially available: the gel-pad microarrays contain a regular set of polyacrylamide gel pads (described in (25) - an image of a hybridized gel-pad microarray is shown in Figure S-1 of chapter 4), increase of the temperature is realized with a temperature controller and a Peltier thermotable on which the

hybridized chip is deposited (Figure 2A), collection of images is carried out with a fluorescent microscope and a CCD camera (Figure 2B), and a computer equipped with a custom data acquisition software.

A. B.

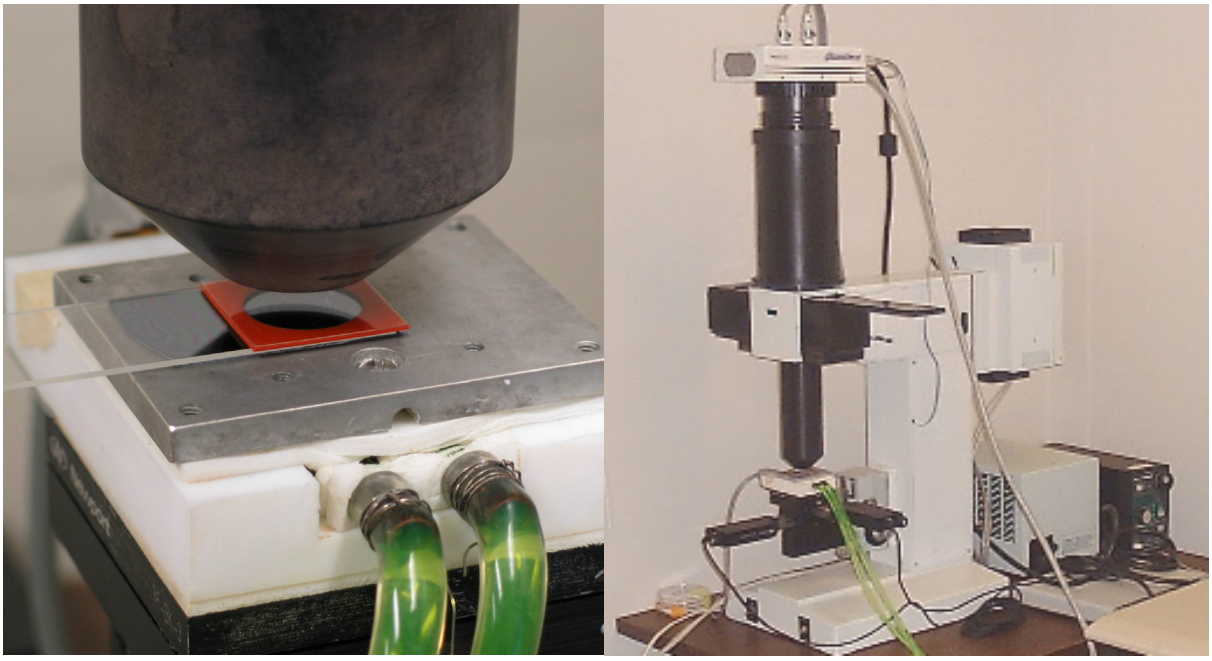

Figure 2. Detail of the Peltier system on which a hybridized microarray is deposited (A). Fluorescent microscope and CCD camera used for image collection (B).

The next step is to compare dissociation curves, for instance dissociation curves from PM and single MM probe-target duplexes. If the dissociation curves are different, then PM probe-target duplexes can be effectively discriminated from MM probe-target duplexes. The comparison of dissociation curves was generally done by using the temperature corresponding to 50% probe-target dissociation (i.e., T_d values). T_d values of several probe-target duplexes were statistically compared using Student's *t*-tests. Alternatively, since no statistical calculator was available to compare dissociation curves in their entirety, the curves were compared visually (e.g., 6). This was a limitation to an objective comparison and interpretation of dissociation curves.

For this reason, a statistical calculator was developed (chapter 3). This calculator was based on a functional ANOVA model with P-splines to smooth data points and obtain continuous curves. This model was initially developed for the analysis of electroencephalograms and was

adapted to the analysis of dissociation curves obtained from microarray hybridizations. More specifically, the functional ANOVA calculator allowed the detection and rejection of outlier dissociation curves (which may result from experimental artefacts such as movement of the microarray during thermal dissociation analysis, formation of bubbles within the hybridization chamber at high temperatures, and so on). Then, individual dissociation curves were normalized and averaged for each probe-target duplex and an interval of confidence was calculated. Intervals of confidence were used to compare average curves; if the interval of confidence of two average curves did not overlap at a given temperature, then these two curves were significantly different. This functional ANOVA was shown to be more useful than the T_d approach because this latter method only compared curves at the temperature corresponding to 50% probe-target dissociation and could not identify differences at lower or higher temperatures. The functional ANOVA calculator was developed under Matlab as an open-source so that users can view and modify the algorithms or add and substitute modules to perform different functions. In addition to providing the first calculator with a robust statistical framework for the comparison of dissociation curves from microarray hybridizations, this calculator can also be used to analyze the curves from other dissociation experiments, such as real-time PCR experiments.

Now that dissociation curves can be effectively compared, the functional ANOVA calculator was used to analyze hybridizations from in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to a phylogenetic microarray containing probes with PM to known sequences (chapter 4). The PM probes targeted universal-, domain-, group- and species-specific probes. Two single MM probes were also spotted on the microarray. With the PM probe Eub338 targeting *Bacteria*, there were no significant differences in dissociation curves between nucleic acids derived from a reference pure strain (*P. putida*) and from uncontaminated and TNT-contaminated soil samples, demonstrating that robust and reproducible reference curves could be established for each probe. With nucleic acids derived from the two soil samples, most of the dissociation curves collected from PM probes could be differentiated from the dissociation curves of single MM probes. Therefore, a specificity of a single nucleotide change could be achieved for most of the probes. From a total of 23 probes, 16 group- and species-specific probes had initial signal intensities significantly different between the two soil samples. Also, 11 probes had significantly different dissociation curves between the two samples, even though initial signal intensities for 3 of the 11 probes did not vary. This suggests that important differences existed between

the two soil samples, in terms of both abundance and sequence of target nucleic acids. This is the first report providing microbial characterization of a TNT-contaminated soil compared to an uncontaminated soil sample using microarrays with probes targeting nucleic acids at different levels of phylogenetic affiliations. This is also the first report presenting a fully elaborated experimental (i.e., presence of two MM probes to estimate the specificity of each PM probe) and statistical approach (i.e., use of a functional ANOVA calculator) for the generation and analysis of dissociation curves. As such, this work provides the fundations for further development of the microarray technology. For instance, the design of PM probes is based on sequences found in databases. Although the number of sequences in these databases is impressive and is growing each day, these sequences cover only a minority of the sequence diversity commonly found in ecosystems. As a consequence, the design of probes might not be as specific as required for ecological characterization or biological prospection. To evaluate whether PM probes are specific enough with complex environmental samples, an experimental and statistical approach similar to the one used in this study might be used. A second line of development could consist in a database of dissociation curves obtained from hybridizations of PM probes to reference strains. This database could be used to analyze dissociation curves collected from hybridizations to environmental samples having unknown target sequences in unknown abundance. If no significant differences are found between dissociation curves of a PM probe hybridized to a reference curve and an unknown sample, then a sequence can be attributed, with a great level of confidence, to the target nucleic acid of the environmental sample.

Because a database of dissociation curves from reference strains was not available, the sequence of target nucleic acids in the TNT-contaminated soil sample could not be determined. Also, as pointed out by one of the anonymous scientists who reviewed chapter 4 for publication, the differences in initial signal intensities and dissociation curves between the uncontaminated and TNT-contaminated soil samples might be due to the presence or absence of TNT, but also to spatial heterogeneity (e.g., pH, soil texture and carbon content). Additional studies were therefore needed to better assess bacterial communities of TNT-contaminated soils. This was done by collecting several TNT-contaminated soil samples from two sites. One site had been used as TNT-destruction field for 20 years and another one was artificially contaminated with TNT for 7 months. In the TNT-contaminated soil samples, the quantity of DNA extracted was lower than in the uncontaminated soil samples. These soil samples were also analyzed by DGGE with primers targeting the 16S rRNA. In the

uncontaminated soil sample, several faint bands were present, with none of them dominating the fingerprint. This suggests the presence of diverse bacteria in these soils, with none of them prevailing within the population. On the other hand, the fingerprints of the TNT-contaminated soil samples were very different from the ones of the uncontaminated soil samples since fewer but brighter bands were observed. These bands corresponded to γ-*Proteobacteria*, and more specifically to *Pseudomonadaceae* and *Xanthomonadaceae*. There was no correlation between the amount of DNA extracted and the DGGE fingerprints with parameters such as pH, soil texture or carbon content. The presence or absence of TNT was the main factor explaining the differences in the amount of DNA extracted and the DGGE fingerprints between the uncontaminated and TNT-contaminated soil samples. This is the first report unambiguously showing that TNT had an important impact on soil microbial populations by selecting for few dominant bacteria. Without contest, such an effect is remarkable. It also shows how anthropogenic activities can profoundly modify the ecosystem.

Although cultivation is a method often criticized by scientists because a major part of the total microflora of most environmental samples is not cultivable, the results obtained by plating on agar dishes an inoculum consisting in an uncontaminated or a TNT-contaminated soil pointed towards the same conclusions as the DNA extraction and DGGE results of the total microflora. In other words, the cultivation technique indicated that a lower biomass was present in the TNT-contaminated than the uncontaminated soil samples. In addition, most of the CFUs isolated on agar plates corresponded to *Pseudomonadaceae*.

Since the presence of TNT clearly impacted microbial populations, the effect of its concentration and time exposure to microbial communities was studied in more detail. At relatively high concentrations of TNT (1.4 and to 28.5 g TNT/kg soil), its impact on soil microbial populations was rapid since a shift in microbial communities was already observed by DGGE after 7 days of incubation. This shift resulted in a predominance of *Pseudomonadaceae*. At a relatively low concentration of TNT (140 mg TNT/kg soil), there was almost no shift in the DGGE fingerprints, even after 121 days of incubation. The amount of DNA extracted was affected in the soils having a relatively high concentration of TNT, but this phenomenon was only detected after 4 months of incubation. In other words, the observation of the influence of TNT contamination on the amount of DNA extracted was delayed compared to the rapid shift observed in DGGE fingerprints. This is probably the result of the relatively high stability of DNA in soil matrices. Extraction of RNA would have

been more appropriate but it is more challenging that the extraction of DNA. Nonetheless, these results showed that TNT rapidly induced shifts in bacterial communities at relatively high concentration of this xenobiotic. At low concentration of TNT, this effect was moderate. This is the first experimental report on the influence of TNT concentration and time exposure upon soil microbial populations.

In addition, these results extend beyond the pure 'eco-toxicological' assessment. These results provide further insights for the enrichment and isolation of 'superbugs' capable of degrading TNT. Although the biodegrading potential of the predominant bacteria in the TNT-contaminated soil samples was not tested in chapter 5, these samples are ideal candidates for the isolation of such 'superbugs'. Evidence for this is the fact that most of the predominant bacteria were *Pseudomonadaceae* and that TNT-degrading bacteria of the genus *Pseudomonas* have been already isolated from various contaminated environments (reviewed in chapter 2).

8.3. Isolation of bacteria with promising TNT-degrading activities

Enrichment cultures are a method of choice for isolating bacteria that express specific phenotypes. It is based on the idea that certain microorganisms are better suited for certain catabolic tasks and environments than others. Basically, enrichment culturing uses a sample of a naturally occurring microbial community as an inoculum for laboratory-prepared growth medium that is designed to select a small subset of the initial community. For instance, if one is interested in finding aerobic microorganisms that can grow on benzene, then the enrichment medium would contain benzene as the sole carbon and energy source, and oxygen as the electron acceptor. If a soil sample is used as inoculum, one would expect that a small proportion of the species present in the soil sample would grow on benzene. After a given incubation time, benzene degraders would become dominant. Then, individual colonies of benzene degraders would be isolated by plating small volumes of the enrichment culture on selective solid medium. The isolated colonies would be further characterized using appropriate physiological, biochemical, and/or genetic procedures.

The use of enrichment cultures is valuable for several reasons. The characterization of microorganisms performing xenobiotic catabolic reactions allows us to gain insights into the various and distinct catabolic pathways operated by microorganisms. Also, it helps us to understand the physiology of these microorganisms. The type of catalytic reactions carried out and the physiology of the bacteria present in contaminated environments are essential to determine the natural biodegradation potential of these environments (i.e., commonly referred to “bioattenuation”), but also to make an efficient use of electron acceptors/donors, water, and nutrients to increase the natural potential of biodegradation (i.e., “biostimulation”). Last but not least, the isolation and use in situ of microorganisms with specific xenobiotic catabolic properties is helpful when effective pollutant-degrading populations are missing at a polluted site and external inoculation is contemplated (i.e., “bioaugmentation”).

Enrichment culturing was used in the framework of this thesis in search for promising TNT biodegrader species. It is important to note the rationale used in carrying out such enrichments. First of all, a soil sample historically contaminated by TNT was the inoculum source. The microbial ecology of this soil sample was already extensively studied by various

molecular (i.e., phylogenetic microarrays and DGGE targeting 16S rRNA genes) and culturing (i.e., study of colonies growing on agar plates with this soil sample as inoculum) techniques. Its microbial composition was very different from uncontaminated soil samples. For this reason, but also to make a useful comparison, an uncontaminated soil sample was also used as enrichment inoculum. A second refinement in this enrichment culturing consisted in focusing specifically on the denitration of TNT since this is probably the most promising catabolic reaction to achieve a complete mineralization of TNT (reviewed in chapter 2). To enrich for bacteria able to denitrate TNT and eventually use the released nitrogen, TNT was the only nitrogen source provided. Although several bacteria have already been described to denitrate TNT, the denitration activities have been reported under fully aerobic conditions. These are likely not the conditions prevailing at contaminated environments (i.e., soils, sediments and groundwater). Given the scarcity of oxygen in many contaminated subsurface environments, oxygen is sometimes provided using various engineered design. However, supplying oxygen is technically challenging because of its low solubility and because it reacts rapidly with reduced soil components such as ferrous iron. In oxygen-depleted environments, bacteria can use several electron acceptors such as nitrate, iron(III) or sulfate to oxidize a variety of organic compounds. Iron (III) is one of the most abundant electron acceptors in subsurface environments (18). In view of this fact, enrichment cultures were degassed with argon and ferrihydrite was provided.

Interestingly, a high nitrite release was observed in the enrichment culture inoculated with a soil sample chronically contaminated with TNT. With an uncontaminated soil sample, the release of nitrite was relatively low. This finding suggested that during the 20 years of chronic contamination, the bacterial community of the TNT-contaminated soil had evolved to a population that could degrade TNT. This is also supported by the fact that the bacterium isolated in the enrichment culture with the TNT-contaminated inoculum was a member of the *Pseudomonadaceae* whereas the bacteria found with the uncontaminated inoculum belonged to *Enterobacteriaceae*.

The isolated *Pseudomonas aeruginosa* strain ESA-5 had exceptional TNT-denitration activities. The yields of nitrite release reported in the literature commonly range between 0.04 and 0.40 mol nitrite released/mol of TNT (Table 1). A single report mentioned a yield of 1.02 mol nitrite/mol TNT under anoxic conditions with a *P. putida* JLR 11 (8). However, although this strain was extensively tested in the framework of this thesis, no significant release of

nitrite from TNT nor growth when TNT was provided as the sole nitrogen source were ever observed. For this reason, the results described by Esteve-Nunez et al. (7, 8) are questionable. In this study with *P. aeruginosa* strain ESA-5, the yield of nitrite released amounted up to 0.82 mol nitrite release/mol of TNT. Initially, the redox conditions were probably anoxic (i.e., use of the released nitrite as electron acceptor), and when the concentration of nitrite was sufficiently low, the redox potential was likely associated with iron reduction (iron (II) was only detected when most of the nitrite had been transformed).

Table 1. Yield values of nitrite released from TNT as reported in the literature.

Strain	Conditions	mol nitrite released/ mol of TNT	Reference
P. aeruginosa	Aerobic	0.04	(23)
Bacillus sp.	Aerobic	0.13	(15)
E. coli EPI300™	Aerobic	0.17	(27)
B. subtilis SK-1	Aerobic	0.22	(17)
P. savastanoi	Aerobic	0.26	(21)
Klebsiella sp. strain C1	Aerobic	0.29	(16)
P. fluorescens I-C	Aerobic	0.37	(24)
P. pseudoalcaligenes JS52	Aerobic	0.40	(9)
P. aeruginosa strain ESA-5	Degassed cultures/presence of ferrihydrite	0.82	This thesis
P. putida JLR11	Anoxic	1.02	(8)

This is the first report showing denitration in the presence of ferrihydrite. When the concentration of ferrihydrite was reduced from 400 mM to 200 mM, the concentration of nitrite was diminished by a factor of 2. When no ferrihydrite was provided, no nitrite was detected. Clearly, the release of nitrite was dependent on the presence of ferrihydrite. However, the exact role of ferrihydrite remains to be determined. A likely explanation comes from the studies of Cooper et al. (3) and Coby and Picardal (2). With *Shewanella putrefaciens*, but also with nitrate-contaminated sediments, Cooper et al. (3) observed a significant reduction in the rate of nitrite reduction in the presence of a crystalline form of ferrihydrite. Cooper et al. (3) and Coby and Picardal (2) showed that Fe^{2+} was microbially produced and reacted with nitrite to form N_2O. In the course of this reaction, Fe^{2+} was oxidized to Fe(III) that formed a dense coating on the cell surface, thus physically blocking the transport of nitrite into the cell (Figure 3). If such a dense coating was formed during the TNT-denitration operated by *P. aeruginosa* strain ESA-5 in the presence of ferrihydrite, then

nitrite would likely accumulate in a higher concentration than in the absence of ferrihydrite. This suggests that TNT-denitration was also taking place in the absence of ferrihydrite, but that nitrite was transformed so rapidly that it was not detectable. Indeed, *P. aeruginosa* is a known denitrifying bacterium. Also, experiments without TNT have shown that *P. aeruginosa* strain ESA-5 transforms nitrite much more rapidly in the absence of ferrihydrite than in its presence.

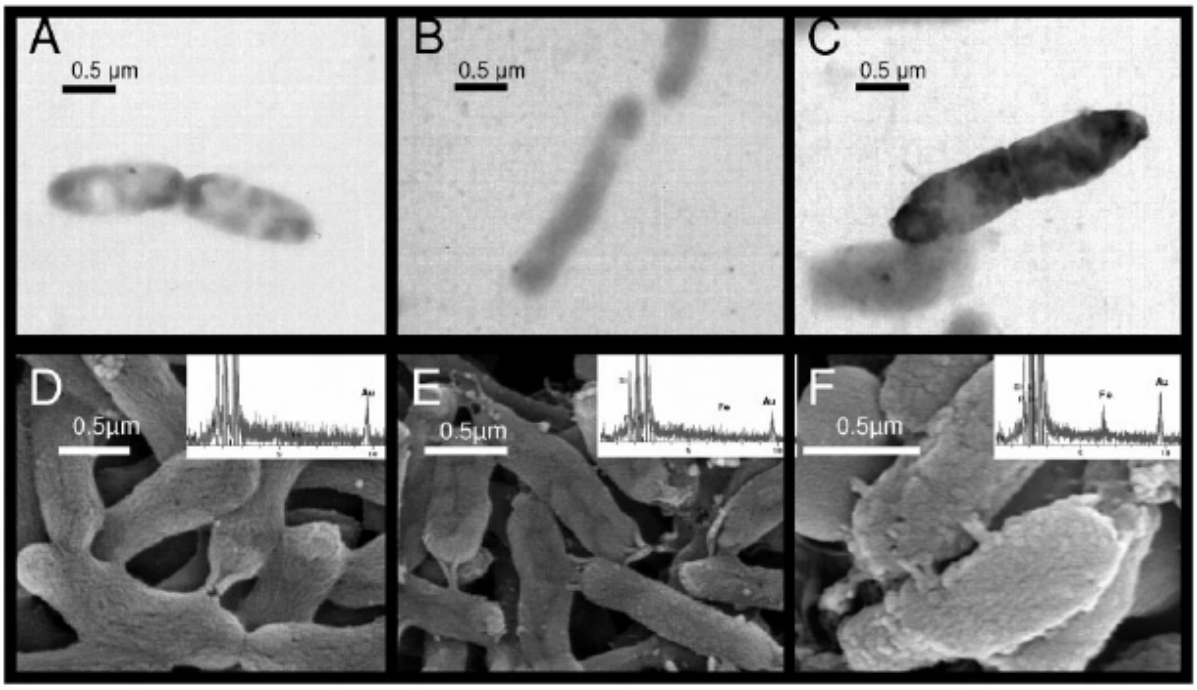

Figure 3. Transmission and scanning electron micrographs (SEM) of *Shewanella putrefaciens* under three treatments. (A and D) Cells treated with NO_2^-; (B and E) cells treated with Fe^{2+}; (C and F) cells treated with both Fe^{2+} and NO_2^-. The electron-dense coating on the cells in panel C and the rough surface on the cells in panel F represent an Fe (oxy)hydroxide coating formed from the reaction between surface-bound Fe^{2+} and NO_2^-. The inset on each SEM image is an electron dense surface point scan that corresponds to the cells in the image. Adapted from Coby and Picardal (2).

It is interesting to note that pure culture microbiology fell into disfavour among many microbial ecologists over the past two decades as an irrational exuberance about the power of environmental molecular analyses took hold. The reasons for this disfavour are the artefacts related to the enrichment procedure. The basis of such artefacts consists in the responsiveness of both individual microorganisms and entire microbial communities to environmental changes implicit in habitat disturbance (i.e., samples are usually physically disturbed by removal from the field) and enrichment culture conditions, and the fact that many processes

are not catalyzed by individual bacteria, but instead by cooperating populations (consortia). In addition, it is known that most of the microorganisms are unculturable by traditional techniques (for instance, less than 1% of soil bacteria have been cultured so far (29)) making the usefulness of cultivation steps limited. In other words, controlled model laboratory experiments allow a logical reductionistic progression to proceed from field sample, to laboratory incubation, to enrichment cultures, to the isolation of pure cultures, and to the elucidation of cellular and subcellular processes (19, 20). With each simplification step, the likelihood of the resultant information being ecologically relevant diminishes (i.e., are the selected bacteria responsible for the bioremediation process in the environment or are they just 'weeds' that grew rapidly in the laboratory?).

In this study, enrichment culturing was used and a *P. aeruginosa* strain ESA-5 was isolated. *Pseudomonadaceae* are known to be easily cultivated under laboratory conditions. Therefore, we could cautiously state that this enrichment led to the isolation of a 'weed' that is not representative of the soil bacterial population. Therefore, the use of such a 'weed' for bioremediation purposes should be of dubious value. However, the microbial characterization of TNT-contaminated soils by molecular and plate culturing techniques showed that the dominant bacteria in these contaminated soils did, indeed, belong to the *Pseudomonadaceae*. In other words, enrichment culturing selected for a bacterium that was representative of the indigenous microbial community within the TNT-contaminated soil. Also, the isolation of *P. aeruginosa* strain ESA-5 provided a certain understanding of the biodegradation reactions carried out by this bacterium, but also other aspects of its physiology that are likely to control its growth and activity in contaminated environments. In summary, given its TNT-denitration activities and its phylogenetic affiliation to the *Pseudomonadaceae*, this strain is potentially very promising for the bioremediation of TNT-contaminated environments.

When grown in chemically defined medium, *P. aeruginosa* strain ESA-5 produced an extracellular greenish compound (Figure 4). *P. aeruginosa* is known to produce several coloured metabolites that are siderophores (pyochelin, pyoverdines) or molecules with antibiotic activity (e.g., pyocyanin). Since pyochelin and pyoverdines are able to degrade several xenobiotic compounds (e.g., 14, 28) and phenazine compounds such as pyocyanin produce several reactive species (4) that might have properties to degrade TNT, the isolation and characterization of this greenish metabolite was carried out. This compound turned out to be phenazine-1-carboxylic acid (PCA). In the presence of reduced pyridine nucleotides such

as NADH, a significant release of nitrite from the TNT molecule was observed. This release was also taking place with two TNT-related compounds (i.e., 2,4,6-trinitrobenzaldehyde and 2,4,6-trinitrobenzyl alcohol). Other phenazine compounds were also found to denitrate TNT in the presence of NADH. The release of nitrite was coupled with the formation of TNT metabolites having a single nitro group. Since phenazine compounds are known to produce several reactive oxygen species, inhibitors of reactive oxygen species were tested to determine whether those species were involved in the denitration of TNT. This experiment showed that no denitration was observed with inhibitors of singlet oxygen and superoxide. Additional recent studies with specific inhibitors clearly indicated that superoxide was at the origin of TNT denitration (B. Stenuit, personal communication). This is the first report showing that a phenazine compound can denitrate TNT and that this denitration leads to metabolites bearing a single nitro group.

Figure 4. Production of a greenish compound (phenazine-1-carboxylic acid) by *P. aeruginosa* strain ESA-5 in chemically defined medium (the composition of this medium can be found in (5)).

When PCA was added to a medium inoculated with *P. aeruginosa* strain ESA-5 containing a mixture of C-sources and TNT as sole nitrogen source, growth was observed and was more significant than in the absence of TNT. A plausible hypothesis is that PCA was intracellularly reduced by NADH, then exported to the extracellular medium where it reduced oxygen to superoxide, the latter leading to TNT-denitration and use of nitrite by *P. aeruginosa* strain ESA-5 as nitrogen source. In the absence of exogenously added PCA, growth was also observed with TNT as sole nitrogen source but it was less significant than with exogenously added PCA. Under these conditions, the production of PCA was not detected by HPLC.

Either PCA was produced at levels below detection limit or *P. aeruginosa* strain ESA-5 had another catabolic pathway to use TNT as sole nitrogen source. On way to test this hypothesis would be by constructing mutants of *P. aeruginosa* strain ESA-5.

Given that the denitration of TNT by PCA is likely mediated by superoxide and thus requires the presence of oxygen, the release of nitrite by *P. aeruginosa* strain ESA-5 observed in oxygen degassed cultures in the presence of ferrihydrite was probably not resulting from the action of PCA. This suggests that *P. aeruginosa* strain ESA-5 has different catabolic pathways to use TNT as sole nitrogen source. Of note, *P. aeruginosa* PAO1 has been reported as harbouring a xenobiotic reductase belonging to the "Old Yellow Enzyme" family. This family of flavoproteins has been described in chapter 2 as a family of enzymes with TNT-denitration activities. The xenobiotic reductase of *P. aeruginosa* PAO1 (accession no. AAG07744) shows identity with "Old Yellow Enzymes" having TNT-denitration activities such as the PETN reductase of *E. cloacae* (accession no. AAB38683), the NEM reductase of *E. coli* (accession no. P77258), and the xenobiotic reductase B of *P. fluorescens* (accession no. AF154062) (1) with identities of 45, 47, and 81%, respectively. It will be interesting to probe the corresponding gene and investigate the expression of this protein in *P. aeruginosa* strain ESA-5 and its potential involvement in the denitration of TNT. Although the TNT-denitration pathways have not yet been elucidated, *P. aeruginosa* strain ESA-5 is capable of *beneficial* TNT degradation under various conditions. For this reason, this strain is a versatile bacterium and is therefore a strong candidate for the bioremediation of TNT-contaminated soils.

8.4. Perspectives

The objectives of this thesis were (i) to determine the bacterial composition of TNT-contaminated soil samples compared to uncontaminated soil samples, and (ii) to isolate and characterize a consortium or a bacterium with promising TNT-degradation activities from one of these TNT-contaminated soil samples. This thesis revealed that (i) a selection for γ-*Proteobacteria* (mostly *Pseudomonadaceae*) occurred in TNT-contaminated soil samples and that (ii) an isolated *P. aeruginosa* strain ESA-5 denitrated TNT under different conditions. This strain can be considered as robust bacterium ('superbug') of potential usefulness to bioremediate sites contaminated with TNT. To determine its effective use in situ, several fundamental questions need to be answered:

(i) The role of ferrihydrite in the biotic denitration of TNT should be investigated. It was hypothesized that ferrihydrite formed a dense coating on cells which physically blocked the transport of nitrite. If such a hypothesis is correct, *P. aeruginosa* strain ESA-5 should also be able to denitrate TNT in the absence of ferrihydrite. If this hypothesis is incorrect, then ferrihydrite is necessary for effective TNT denitration. Whatever the mechanism, the role of ferrihydrite should be determined as it will provide both basic understanding as well as application insights for designing effective TNT-bioremediation strategies.

(ii) The possible involvement of superoxide during the denitration of TNT by PCA should be elucidated. Furthermore, the generality of this mechanism should be studied in view of developing a robust biomimetic system for the degradation of persistent molecules.

(ii) The metabolic pathway(s) leading to the denitration of TNT by *P. aeruginosa* strain ESA-5 should be determined. The identification of genes that are crucial for the denitration of TNT will also allow the assessment of the performance of *P. aeruginosa* strain ESA-5 in situ. For instance, monitoring key genes involved in the production of PCA will be informative to evaluate the performance of *P. aeruginosa* strain ESA-5 to denitrate TNT in various contaminated ecosystems. Recently developed high-throughput molecular techniques are very promising to detect expression of hundreds of phylogenetic and catabolic genes, and production of proteins. An overview of these techniques is provided in Appendices 1, 2 and 3.

(iii) Ideally, the TNT molecule should be completely mineralized. If TNT-metabolites accumulate, their toxicity should be assessed. If toxic, they should be degraded further or transformed into innocuous compounds (e.g., by the use of a consortium of strains).

(iv) Strains of *P. aeruginosa* are pathogenic (22). The pathogenicity of *P. aeruginosa* strain ESA-5 should be estimated. If pathogenic, its use in situ should be considered on a case-by-case basis (e.g., can we use this strain in uninhabited environments?).

Responses to these questions will determine the real potential of this strain to bioremediate TNT-contaminated ecosystems.

8.5 References

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