6.

Chapter 6

Denitration of 2,4,6-trinitrotoluene by Pseudomonas aeruginosa ESA-5 in the presence of ferrihydrite

The organization of the chapters is as follows:

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

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

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

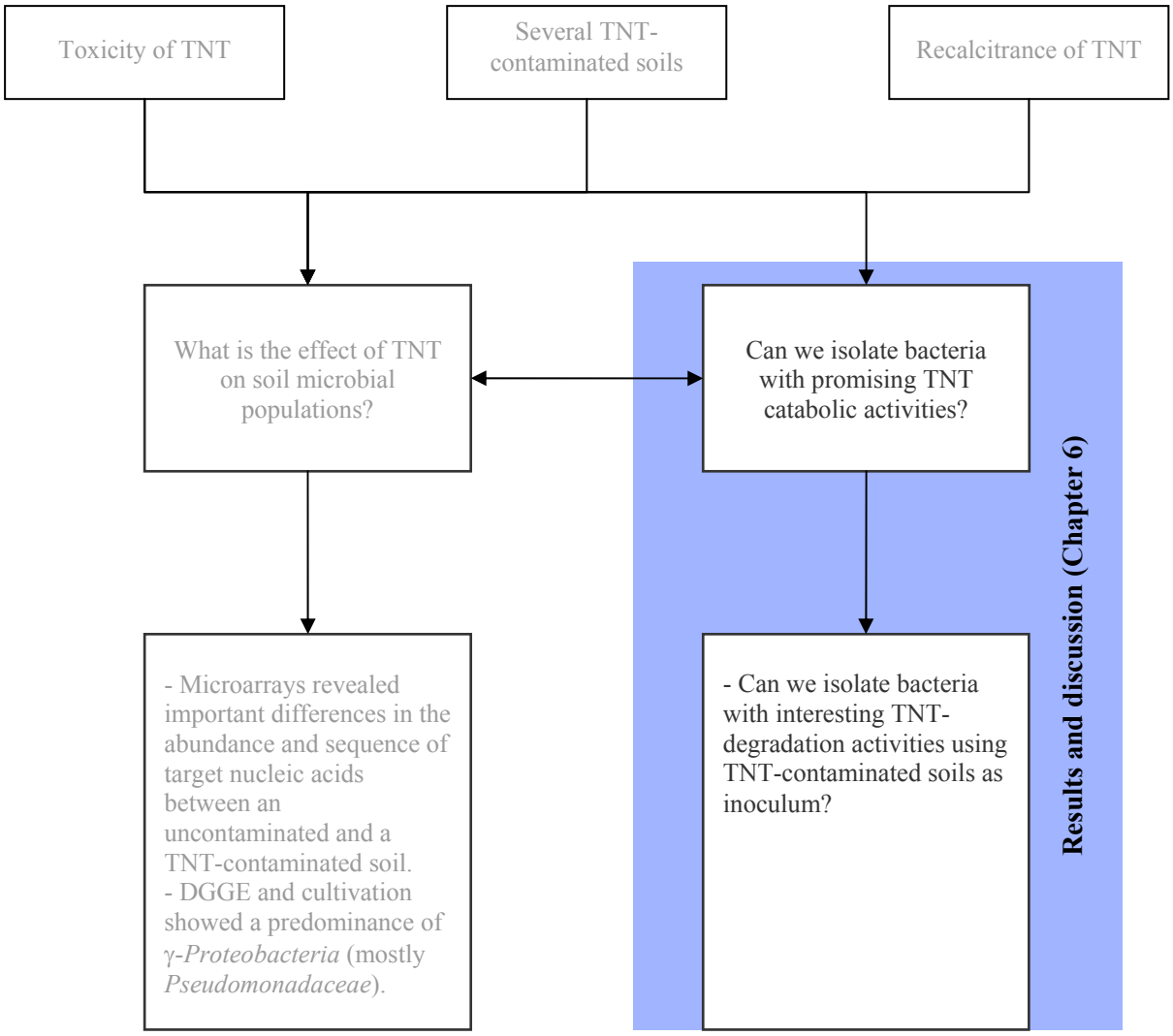
Chapter 4 compares hybridizations of in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to a phylogenetic microarray with thermal dissociation analysis.

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

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

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

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


Toxicity of TNT
Several TNT-contaminated soils
Recalcitrance of TNT
What is the effect of TNT on soil microbial populations?
Can we isolate bacteria with promising TNT catabolic activities?
- Microarrays revealed important differences in the abundance and sequence of target nucleic acids between an uncontaminated and a TNT-contaminated soil.
- DGGE and cultivation showed a predominance of γ-*Proteobacteria* (mostly *Pseudomonadaceae*).
- Can we isolate bacteria with interesting TNT-degradation activities using TNT-contaminated soils as inoculum?
Results and discussion (Chapter 6)

Denitration of 2,4,6-trinitrotoluene by Pseudomonas aeruginosa ESA-5 in the presence of ferrihydrite

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6.1. General context

We have shown with 16S rRNA microarrays (chapter 4), 16S PCR-DGGE and culturing methods (chapter 5) that soil microbial populations are significantly different between uncontaminated and TNT-contaminated soils, and that TNT induced a selection of *γ-Proteobacteria*, mostly of *Pseudomonadaceae*. Although these molecular phylogenetic approaches cannot directly establish that the detected microorganisms are capable of pollutant degradation, they might have the ability to transform and/or biodegrade TNT.

Among the various catalytic activities displayed by bacteria, denitration of TNT is an important step for further mineralization (cfr. chapter 2). Various bacteria with TNT-denitration activities have been isolated under aerobic conditions, but a single report mentioned the denitration of TNT under anoxic conditions with a *P. putida* strain (chapter 2). It is becoming increasingly apparent that microorganisms can degrade many contaminants with alternative electron acceptors, such as nitrate, sulfate and Fe(III) oxides. The objective of this study was to enrich for microorganisms with TNT-denitration activities using a TNT-contaminated soil sample and electron acceptors different from oxygen.

6.2. Abstract

Denitration of 2,4,6-trinitrotoluene (TNT) was evaluated in oxygen-depleted enrichment cultures. These cultures were established starting with an uncontaminated or a TNT-contaminated soil inoculum and contained TNT as sole nitrogen source. Incubations were carried out in the presence or absence of ferrihydrite. A significant release of nitrite was observed in the liquid culture containing TNT, ferrihydrite and inoculum from a TNT-contaminated soil. Under these conditions, *Pseudomonas aeruginosa* was the predominant bacterium in the enrichment, leading to the isolation of *P. aeruginosa* ESA-5 as a pure strain. The isolate had TNT denitration capabilities as confirmed by nitrite release in oxygen-depleted cultures containing TNT and ferrihydrite. Concomitantly, TNT-reduced compounds were detected as well as unidentified polar metabolites. The concentration of nitrite released from TNT was proportional to the concentration of ferrihydrite in the medium. The release of nitrite was lower when the concentration of initially spiked TNT was reduced by one order of magnitude. Under these conditions, the concentration of nitrite peaked and then its concentration slowly decreased and the production of ferrous ions was detected. A decrease of nitrite concentration and production of ferrous ions were observed when TNT was omitted and nitrite and ferrihydrite were provided. These results suggest that nitrite-reducing conditions were initially achieved, followed by iron-reducing conditions.

6.3. Introduction

2,4,6-trinitrotoluene (TNT) is an environmental pollutant frequently found at sites associated with military activities. TNT is toxic to many organisms, from bacteria to humans, and has mutagenic activities (16). Therefore, intensive research has been conducted to identify microorganisms degrading this compound. Due to its xenobiotic character and structural features, TNT is very recalcitrant to microbial attack. The capacity to reduce the nitro groups of TNT is a ubiquitous reaction among many microorganisms. However, the reduced TNT metabolites (aminodinitrotoluene (ADNT) and diaminonitrotoluene (DANT) chemicals) are not degraded further and are as toxic as TNT itself. A very promising catabolic pathway consists in denitration (defined as the release of nitrite from TNT or other TNT metabolite) since related aromatic molecules with less than three nitro groups are completely mineralized by bacteria (e.g., 29).

Few reports describe the release of nitrite from TNT. Stenuit et al. (34) provided a detailed review of these reports. Briefly, nucleophilic addition of a hydride ion to the TNT molecule with the formation of a hydride-Meisenheimer complex has been observed under aerobic conditions (9). Nitrite release from the hydride-Meisenheimer complex and formation of dinitrotoluene compounds were reported using a *Pseudomonas* sp. (8, 17), although this could not be confirmed by Vorbeck et al. (38). Pak et al. (31) showed that protonated dihydride-Meisenheimer complexes and aryl cations formed from hydroxylaminodinitrotoluene compounds produced in vitro by the action of a purified oxidoreductase from a *P. fluorescens* strain reacted with each other to form a dimeric molecule with the release of nitrite. This was confirmed by Stenuit et al. (35) with whole cells and cell-free extracts of *Escherichia coli*. Another aerobic denitration pathway from a dihydroxylamino-dinitrotoluene metabolite of TNT was proposed by Fiorella and Spain (13) using a *P. pseudoalcaligenes* strain, whereas Kalafut et al. (20) reported the production of nitrite and 2-amino-4-nitrotoluene from TNT using Gram-positive and Gram-negative bacteria. Aerobic denitration of TNT and formation of dinitrotoluene compounds were reported by Kim et al. (22) and by Martin et al. (27) with a *Klebsiella* and a *P. savastanoi* strain, respectively. Recently, oxidative denitration of TNT by a bacterial consortium with 3-methyl-4,6-dinitrocatechol as a metabolite was reported (36). All of these studies have shown that denitration of TNT occurred under aerobic conditions.

A number of biodegradative reactions occurring with terminal electron acceptors different from oxygen have been described. However, in the case of TNT, a single report refers to the denitration of TNT under anoxic conditions using a *P. putida* strain[1] (10). In this work, a temporary release of nitrite was detected and particular denitrated compounds including 2-nitro-4-hydroxy-benzoic acid and 4-hydroxy-benzoic acid were identified. With terminal electron acceptors such as sulfate or carbon dioxide, TNT is rapidly reduced to 2,4,6-triaminotoluene (TAT) (9). This is due to the low redox potential associated with these acceptors. Under such redox conditions, the reduction of TNT prevents its denitration. On the other hand, the redox potential associated with iron(III) is higher than with sulfate or carbon dioxide. For instance, the redox potential of ferrihydrite (i.e., amorphous $Fe(OH)_3$) is +150 mV at neutral pH (39)). However, there have been no reports of a TNT denitration in the presence of Fe(III).

The objective of this study was to investigate the denitration of TNT in oxygen depleted enrichment cultures containing TNT as sole nitrogen source and ferrihydrite (also commonly referred to ferric oxyhydroxide). Another objective was to identify bacteria enriched under these conditions and characterize their physiology towards the denitration of TNT.

[1] This strain was provided by Drs. A. Esteve-Nunez and J.L. Ramos (CSIC, Granada, Spain). L. Eyers extensively tested the denitration activities of this strain in growing cell experiments, but growth and nitrite release were not significant when TNT was provided as sole nitrogen source. Also, the various metabolites reported by Drs. A. Esteve-Nunez and J.L. Ramos were not detected. In this connection, it is interesting to note that, with another strain isolated from the same research institute, a similar problem was reported by Vorbeck et al. (38); these authors could not confirm the reductive denitration reported by Duque et al. (8) with a *Pseudomonas* clone A.

6.4. Materials and methods

Chemicals. TNT was obtained from Nobel Explosives (Chatelet, Belgium). 2-Amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) were synthesized as previously described (37). Formylamido-dinitrotoluene compounds were obtained by reacting formic acid with aminodinitrotoluene compounds (18). 2,4-Diamino-6-nitrotoluene (2,4-DANT), 2,2'-azoxy-4,4',6,6'-tetranitrotoluene and 4,4'-azoxy-2,2',6,6'-tetranitrotoluene were graciously donated by Dr. D. Bruns-Nagel (University of Marburg, Germany). 2,4,6-Trinitrobenzaldehyde (TNBA) and 2,4,6-trinitrobenzoic acid were kindly supplied by M. Régis (GIAT Industries, France). Other chemicals were purchased from Sigma (St Louis, MO).

Soil inocula. Samples were collected from TNT-contaminated soil (29.1 ± 2.8 g TNT/kg dry soil) and uncontaminated soil (TNT was not detected by HPLC with a detection limit of 1 mg TNT/kg soil) at a site in Bourges, France that had been used for TNT destruction over the past 20 years (12). The uncontaminated soil, which was sampled a few meters from the contaminated soil, was not polluted due to the presence of a protecting wall at the destruction field. Both sets of samples were collected from the top 5-cm layer of soil and homogenized. Other soil characteristics (pH, granulometry, water content and chemically oxidable carbon) are described elsewhere (George et al., manuscript in preparation). Two g of soil per 150 ml of medium were used as inoculum.

Media. As a basal medium, M8 minimal medium was used for both enrichment and pure cultures (17). Except where mentioned otherwise, TNT was the sole nitrogen source provided. TNT was added at a concentration of 440 or 4400 μM (440 μM corresponds to its limit of solubility in water). Oxygen was removed in all liquid cultures. To remove oxygen, standard anaerobic techniques were used. In brief, argon was passed through heated reduced copper filings to remove traces of oxygen and boiling M8 medium was flushed with this oxygen-free argon for 2 hours. Except where mentioned otherwise, the carbon source was a mixture of glucose, acetate, succinate and pyruvate (6 mM each). Amorphous ferrihydrite was freshly prepared as described by Lovley and Phillips (26). For the cultures containing ferrihydrite, a concentration of 400 mM was used, except where stated otherwise. The prepared ferrihydrite was indeed amorphous and its chemical composition was $Fe(OH)_3$ (M. Régis, personal

communication). Abiotic controls consisted of cultures in which $HgCl_2$ (200 ppm) was added and the carbon sources were omitted. The pH of each liquid culture was adjusted to 6.8. Cultures were sealed with a rubber stopper and an aluminium crimp. They were incubated in the dark at 35°C in an orbital shaker set at 170 rpm. Experiments were carried out in triplicate.

Seed culture. The medium used to obtain a sufficient inoculum of *P. aeruginosa* ESA-5 consisted of 150 ml of M8 minimal medium containing glucose, acetate, succinate and pyruvate (6 mM each) and ammonium (5 mM). It was inoculated with *P. aeruginosa* ESA-5 and incubated aerobically in the dark at 35°C in an orbital shaker at 170 rpm. When the OD_{600} was close to 0.6, cells were harvested by centrifugation (6000 x g, 15 min, and 4°C) and the pellet was washed three times with M8 minimal medium. The washed pellet was used to inoculate cultures to an OD_{600} of 0.225.

DNA extraction, PCR amplification and denaturing gradient gel electrophoresis. For denaturing gradient gel electrophoresis (DGGE), two ml of the enrichment cultures were centrifuged and the pellet was used for DNA extraction. DNA was extracted with the UltraClean Soil DNA isolation kit from Mo Bio (Carlsbad, CA) following the manufacturer's instructions. PCR amplification of a portion of the 16S rRNA gene was carried out with primers P63f containing a 40 bp GC-clamp attached to its 5'-end and P518r (11) in a thermal cycler (Perkin-Elmer, Norwalk, CT). The PCR mix (100 µl) contained 50 µl of Red'y'Star mix (Eurogentec, Seraing, Belgium), 0.25 µM of each primer, and 100 ng of template DNA. An initial denaturation step of 10 min at 94°C was followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A final extension at 72°C for 10 min was done. DGGE was performed with a D-Code System (Bio-Rad, Hercules, CA). PCR products (~600 ng) were loaded on a 6% polyacrylamide gel with a denaturing gradient of 35 to 55%. The electrophoresis was carried out at 150V for 7 hr in 0.5 X TAE buffer maintained at 60°C. Gels were stained with ethidium bromide and photographed on a transillumination table.

Bands of the DGGE to be sequenced were excised from the gel with a scalpel, placed in 100 µl of sterile water, and incubated overnight at 4°C. A 5 µl volume of the DNA diffused in water served as a template for PCR amplification. The PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Valencia, CA) before being sent for sequencing with primer P518r.

For sequencing of the 16S rRNA gene of the isolated *P. aeruginosa* strain ESA-5, its DNA was extracted with the UltraClean Soil DNA isolation kit from Mo Bio. PCR amplification was done with primers Bact11F (21) and S-D-Bact-1512-a-A-16 (15) under conditions described above. Amplicons were purified with a Qiaquick PCR purification kit and sent for sequencing with primers Bact11F, Univ907r (2) and S-D-Bact-1512-a-A-16.

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 (1). 16S rRNA gene sequences of DGGE bands 1 to 4 (Figure 2) and of *P. aeruginosa* ESA-5 are available in the GenBank database under accession numbers DQ641676 to DQ641680, respectively.

Analytical methods. TNT and its metabolites were extracted from liquid culture samples by mixing 1 ml of the sample with 1 ml of acetonitrile. After centrifugation (18,000 x g, 20 min), the supernatant was analyzed using a HPLC system (multisolvent delivery system, controller and autosampler (Waters, Milford, MA)). The column was a 4 µm C18 Nova-Pak, 3.9 x 300 mm (Waters). The mobile phase consisted of 30 % acetonitrile and 70 % phosphate buffer (10 mM, pH 3.2) and the flow rate was maintained at 1 ml/min. After 2 min, a linear gradient was applied to reach a composition of 90 % acetonitrile and 10 % phosphate buffer in 18 min. Then, a linear gradient was set for 5 min to reach the initial mobile phase composition and this composition was maintained for 15 min. Compounds were monitored by a photodiode array detector (Waters). TNT metabolites were identified and quantified by comparing their UV spectra and peak absorbance with authentic standards.

The concentrations of nitrite, nitrate and ammonium were quantified in the supernatants obtained after centrifugation of the samples (18,000 x g, 20 min). Nitrite concentration was colorimetrically determined using a nitrite Spectroquant® kit from Merck (Darmstadt, Germany). To avoid the interference of colored samples, a supernatant without the addition of reactants for the determination of nitrite was used as reference. Nitrite was also quantified by HPLC with an IC-PAK Anion HC column (Waters). The mobile phase was a borate/gluconate eluent (pH 8.5) with the following constituents (in ml per liter of bi-distilled water): 40 ml of n-butanol, 240 ml of acetonitrile and 40 ml of borate/gluconate concentrate. One liter of the borate/gluconate concentrate contained 16 g of sodium gluconate, 18 g of boric acid, 25 g of

sodium tetraborate decahydrate and 250 ml of glycerol. Nitrite was detected with a UV detector (Pharmacia, Uppsala, Sweden) set at 210 nm. Nitrate and ammonium concentrations were spectrophotometrically determined with a nitrate and ammonium Spectroquant® kit (Merck), respectively. TNT interfered with ammonium determination and was removed by passing the samples through a Sep-Pak C18 cartridge (Waters).

The concentration of Fe^{2+} was colorimetrically determined with an iron Spectroquant® kit (Merck) on samples containing ferrihydrite. Samples were diluted to a total iron final concentration of approximately 20 mM in 2 M HCl and the iron precipitate was dissolved for 45 min. Then, 50 µl were diluted in 950 µl dH_2O, and 67.5 µl of solution 'Fe-2' (solution provided with the iron Spectroquant kit) was added. Fe^{2+} concentration was determined by measuring the absorbance at 510 nm after a 10 min reaction.

6.5. Results

Enrichment cultures. Denitration was investigated in enrichment cultures degassed with argon and with TNT (4400 μM) as sole nitrogen source. When ferrihydrite was omitted, a relatively low concentration of nitrite was detected with the uncontaminated soil (initial nitrite concentration was 1.0 μM and reached 50.5 μM after 56 days of incubation). With the TNT-contaminated soil, the concentration of nitrite was not significant (0.8 to 2.0 μM over the same timescale). When ferrihydrite was present, a significant release of nitrite was observed. With the uncontaminated soil inoculum, a concentration of nitrite of 236.0 μM after 56 days was measured (Figure 1). Nitrite concentration reached 475.0 μM after 112 days. Even more remarkable was the evolution of nitrite concentration with the TNT-contaminated soil inoculum. Nitrite concentration peaked to 3923.8 μM after 39 days and decreased thereafter (2825.7 μM after 112 days, Figure 1). The accuracy of this measure was confirmed by anionic chromatography (results not shown). Neither nitrate nor ammonium was detected in these samples. When TNT was omitted, the concentration of nitrite measured (between 0.8 to 2.9 μM during the entire time course of the experiment) was not significant with either inoculum, indicating that TNT was at the origin of the release of nitrite. Two abiotic controls (i.e., presence of the uncontaminated or TNT-contaminated inoculum, presence of ferrihydrite and $HgCl_2$, and absence of C-sources) were also run. The concentrations of nitrite detected in these controls were much lower than those attained biotically (Figure 1). Taken together, these results indicated that (i) the marked release of nitrite observed in the presence of ferrihydrite resulted from a biotic denitration of TNT, and (ii) this denitration was more significant with the TNT-contaminated soil inoculum.

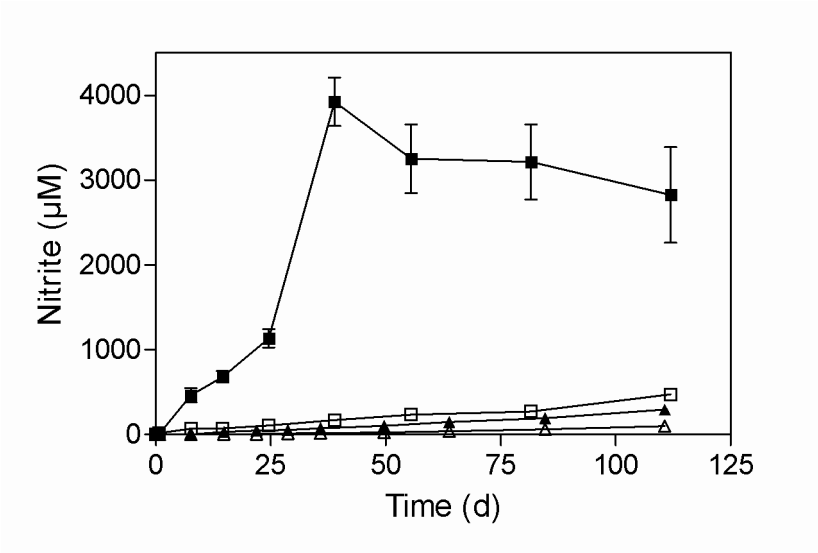

Figure 1. Nitrite release in the presence of ferrihydrite and TNT (4400 µM). TNT-contaminated soil inoculum (closed squares), uncontaminated soil inoculum (open squares). Abiotic controls (200 ppm $HgCl_2$ and omission of C-sources) with inoculum from TNT-contaminated soil (closed triangles) and uncontaminated soil (open triangles). Error bars represent the standard deviation of triplicates.

DGGE analysis. DGGE fingerprints of the enrichment cultures with TNT as sole nitrogen-source and supplemented with ferrihydrite are depicted in Figure 2. At the starting time point, two faint bands were initially observed with the uncontaminated soil inoculum. Subsequently, three brighter bands appeared after 34 days. At day 55, the DGGE fingerprint was similar to the one after 34 days of incubation. Two of these three bands were sequenced (bands 1 and 2, Figure 2) and were found to correspond to *Enterobacter cloacae* (100% identity on a 442 bp fragment) and to *E. ludwigii* (100% identity on 442 bp). With the TNT-contaminated soil inoculum, five bands were observed at the starting point. Among them, a brighter band (band 3) corresponded to *P. amygdali* (99.8% identity on 409 bp). At day 34, this band disappeared. Instead, another bright band (band 4) was detected and corresponded to *P. aeruginosa* (99.8% identity on 428 bp). This dominant band was still present after 55 days of incubation.

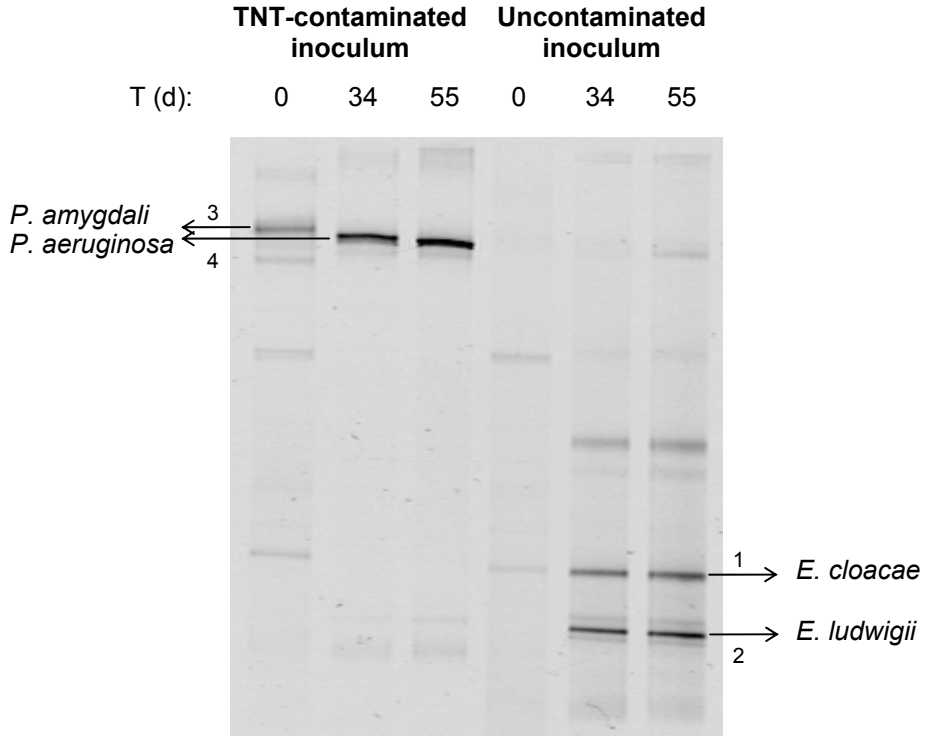

Figure 2. DGGE fingerprints of 16S rRNA gene fragments amplified from DNA extracted from enrichment cultures. Samples were collected from enrichment cultures with TNT, ferrihydrite, and TNT-contaminated or uncontaminated soil inoculum. Arrows indicate bands that were extracted and sequenced. A negative of the original DGGE image is shown.

Isolation and characterization of *P. aeruginosa* ESA-5. At day 55, a sample of the enrichment culture with the TNT-contaminated soil inoculum, TNT and ferrihydrite was taken and used to inoculate *Pseudomonas* isolation agar plates (Difco, Detroit, MI). All the colonies on the plates appeared very similar. Several of them were picked up and analysed by DGGE. For each colony, a single band was detected and migrated at the same position as the *P. aeruginosa* band of the enrichment culture with the TNT-contaminated soil inoculum. Subsequently, one of these analysed colonies was chosen for additional characterization. When the strain was grown in peptone-glycerol medium (7), a blue pigment was extracted. Under acidic conditions, this pigment turned red. This behaviour is typical of pyocyanin (6), a pigment associated with *P. aeruginosa*.

Sequencing of the 16S rRNA gene of this isolate confirmed that it was a *P. aeruginosa* strain (99.9% identity on 1422 bp[2]). This strain produced pyoverdine group I (P. Cornelis and S. Matthijs, personal communication), a feature of *P. aeruginosa* PAO1 (28). Our isolate was named *P. aeruginosa* strain ESA-5.

Denitration of TNT by *P. aeruginosa* ESA-5. *P. aeruginosa* ESA-5 was used to inoculate liquid cultures containing TNT (4400 μM) as sole nitrogen source. When ferrihydrite was present at a concentration of 200 or 400 mM, a significant release of nitrite was detected and its concentration increased over the time course of the experiment (Figure 3). At day 9, 142.9 and 281.5 μM of nitrite were measured with 200 and 400 mM of ferrihydrite, respectively. Neither nitrate nor ammonium was detected. When ferrihydrite or TNT was not provided, nitrite concentration values were negligible (i.e., between 0.7 to 1.8 μM over the time course of the experiment). Abiotically (inoculated cultures, absence of C-sources, presence of ferrihydrite (400 mM) and $HgCl_2$), nitrite concentration values were also not significant (i.e., between 0.8 and 2.8 μM). These results indicated, once again, that denitration of TNT was a biotic process and that nitrite release was only detected in the presence of ferrihydrite. The concentration of Fe^{2+} was close to the detection limit (0.4 mM) and did not evolve during the 9 days of the culture.

[2] The sequence differed from the sequence of *P. aeruginosa* PAO1 by a single nucleotide (A in the sequence of strain ESA-5 vs. T in the sequence of *P. aeruginosa* PAO1). By looking at the sequence chromatogram of strain ESA-5, the peak corresponding to this A nucleotide was confounded by a peak corresponding to a T. Therefore, it was not possible to determine of this single nucleotide difference was due to an experimental artefact or not.

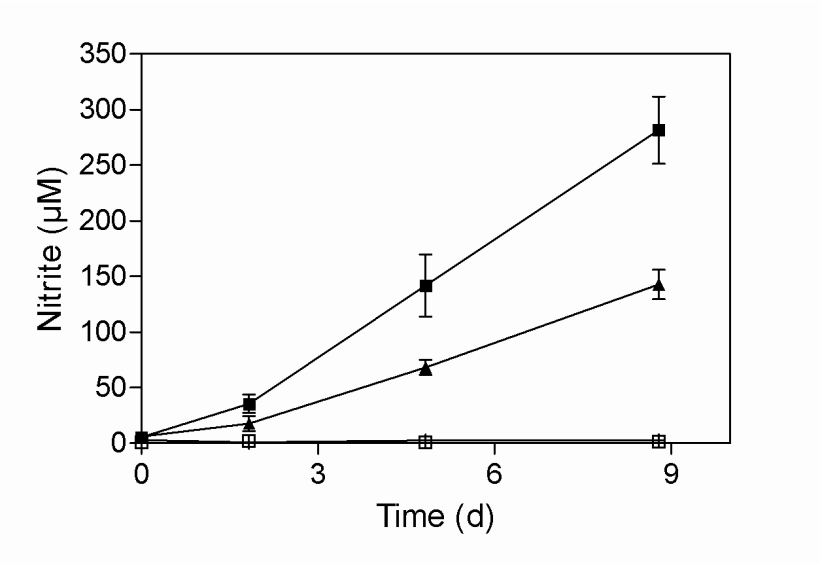

Figure 3. Nitrite release by *P. aeruginosa* ESA-5 in the presence of various concentrations of ferrihydrite and TNT (4400 µM). Concentrations of ferrihydrite used: 400 mM (closed squares), 200 mM (closed triangles), no ferrihydrite (open squares). Abiotic control (200 ppm $HgCl_2$ and omission of C-sources, represented by X crosses (superimposed with the open squares)). Error bars represent the standard deviation of triplicates.

To test the influence of the carbon source in the denitration of TNT by *P. aeruginosa* ESA-5 with 400 mM of ferrihydrite, each carbon source was added individually (final concentration of 25 mM) instead of a mixture of 4 carbon sources. With acetate, succinate and pyruvate, no denitration occurred. With glucose, a significant release of nitrite was observed (200.8 µM after 7 days).

Different metabolites resulting from the reduction of the nitro group(s) were detected in the presence or absence of ferrihydrite. Among them, 2-ADNT, 4-ADNT, 2,4-DANT and 2,6-DANT were identified. The dimeric metabolites 2,2'-azoxy-4,4',6,6'-tetranitrotoluene and 4,4'-azoxy-2,2',6,6'-tetranitrotoluene were also found. In the presence of ferrihydrite, additional TNT-reduced metabolites included the formylated derivatives of ADNT, i.e., 2-formylamido-4,6-dinitrotoluene and 4-formylamido-2,6-dinitrotoluene. Several other metabolites were also detected but were not identified. These metabolites were more polar than TNT, suggesting that they may bear less than three nitro groups.

When the concentration of initially spiked TNT was reduced by one order of magnitude to 440 µM, the release of nitrite in the presence of ferrihydrite (400 mM) was lower (Figure 4). Nitrite concentration peaked at 53.5 µM after 3 days and then its concentration slowly

decreased reaching 4.2 µM by day 15. Again, nitrite concentration was not significant when ferrihydrite was omitted (0.1 to 1.7 µM, Figure 4). Interestingly, the concentration of Fe^{2+} was low until day 3 (0.4 to 0.6 mM, Figure 4). After that point, a significant increase in the concentration of Fe^{2+} was observed, concomitantly with a decrease in the concentration of nitrite. The concentration of Fe^{2+} was maximal (3.8 mM) after 15 days.

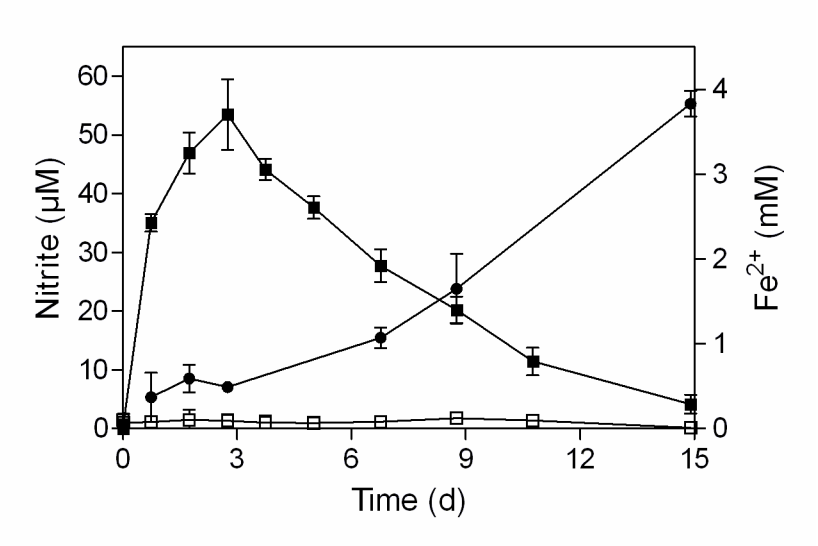

Figure 4. Nitrite release and Fe^{2+} production by *P. aeruginosa* ESA-5 in the presence of TNT (440 µM). Nitrite release (closed squares) and Fe^{2+} production (closed circles) with 400 mM ferrihydrite. Nitrite concentration (open squares) without ferrihydrite.

Elimination of nitrite by *P. aeruginosa* ESA-5. To better understand the relationship between the decrease of nitrite concentration and production of Fe^{2+}, *P. aeruginosa* ESA-5 was inoculated in a liquid medium containing nitrite and ferrihydrite (400 mM) in the absence of TNT. From a nitrite concentration of 1012.5 µM at time zero, the concentration of nitrite decreased to 27.5 µM in 15 days (Figure 5). The initial concentration of Fe^{2+} was 0.2 mM and peaked to 3.3 mM at day 15.

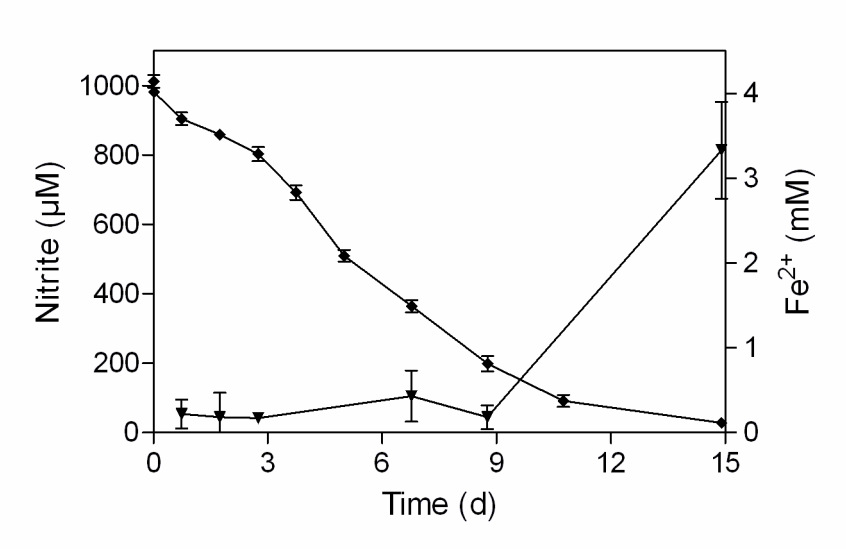

Figure 5. Nitrite elimination by *P. aeruginosa* ESA-5. Nitrite elimination (closed lozenges) and Fe^{2+} production (closed triangles) in oxygen-depleted medium with 400 mM ferrihydrite and absence of TNT.

6.6. Discussion

Under abiotic conditions in oxygen depleted medium containing TNT as sole nitrogen source in the presence of ferrihydrite, we detected some nitrite release with the uncontaminated and TNT-contaminated soil inoculum (Figure 1). This suggested that some nitrite release occurred abiotically under these conditions. However, this release of nitrite was much lower than what was observed with a live culture.

Biotically, a release of 475.0 µM nitrite was detected at day 112 in the enrichment culture inoculated with the uncontaminated soil sample. Two enterobacteria were the predominant species. This suggested that these two enterobacteria likely had TNT-denitration activities. However, we did not investigate this further by isolating these bacteria. With nitramine explosive-contaminated soil, Kitts et al. (23) isolated three enterobacteria (identified as *Morganella morganii*, *Providencia rettgeri* and *Citrobacter freundii*) that reduced hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under oxygen-depleted liquid cultivation. *M. morganii* and another isolated enterobacterium *E. cloacae* strain 96-3 were shown to reduce both RDX and TNT (24). Under aerobic conditions, French et al. (14) isolated an *E. cloacae* strain PB2 that was capable of growth with TNT as the sole nitrogen source. Release of nitrite was not reported by these authors with growing cells, but denitration of TNT through the formation of hydride-Meisenheimer complexes was confirmed with a purified PETN reductase of *E. cloacae* PB2 (14).

A higher release of nitrite was observed biotically with the TNT-contaminated soil than the uncontaminated soil inoculum. A *P. amygdali* was initially a predominant bacterium. However, *P. amygdali* did not remain in the enrichment culture during the incubation time. Instead, our culture conditions selected for a *P. aeruginosa*. This latter strain was responsible for the TNT-denitration (Figures 2 to 4). Several authors previously reported a TNT-denitration activity with *P. aeruginosa* under aerobic conditions. For instance, Oh et al. (30) detected small amounts of 2,4-dinitrotoluene in an aerobic medium containing TNT (260 µM) and yeast extract (1 g/l) that was inoculated with a *P. aeruginosa* strain MX. Concentrations of 18 and 9 µM nitrite were detected in inoculated and uninoculated medium, respectively. Kalafut et al. (20) detected a transient production of 2-amino-4-nitrotoluene and of 13 µM of nitrite with another *P. aeruginosa* strain inoculated into an aerobic medium with TNT (440

μM) and another nitrogen source. In both of these studies, TNT was not the sole nitrogen source and the concentration of nitrite release was relatively low. Therefore, the origin of nitrite release was unclear. Our own results clearly show that the isolated *P. aeruginosa* strain ESA-5 had significant TNT-denitration activities, given that TNT was the sole nitrogen source and that the concentration of nitrite released was significantly higher than those reported by others.

P. aeruginosa is capable of utilizing O_2. It is also a denitrifying[3] bacterium knows to use nitrate (3) and nitrite (40) as terminal electron acceptors for carbon metabolism. *P. aeruginosa* can also reduce Fe(III) (25). When ferrihydrite was added in oxygen-depleted medium, a production of Fe^{2+} was only observed when most of the nitrite was eliminated and its concentration was low, i.e., approximately 30 μM (Figure 4 and 5). In other words, nitrite at higher concentrations inhibited ferrihydrite reduction. This probably resulted from differences in the rate of electron transport between nitrite and ferrihydrite that have evolved to allow preferential use of the most favourable oxidant (5). In addition, nitrite reduction by microorganisms typically precedes ferrihydrite reduction because nitrite tends to be more available to the cells than the highly insoluble forms of Fe(III) (4). All together, this suggested that the redox state of the medium was initially dominated by the potential associated with nitrite reduction. Subsequently, the reduction of iron took place. However, the role of ferrihydrite in the denitration of TNT was not determined[4].

[3] Denitrification involves the successive reduction of nitrate to nitrite, nitric oxide, nitrous oxide and nitrogen gas (NO_3^- -> NO_2^- -> NO -> N_2O -> N_2).

[4] From a thermodynamic point of view, a TNT-denitration with Fe^{2+} (that might be produced biotically from $Fe(OH)_3$) is possible (Table 1). However, under such conditions, Hofstetter et al. (19) have previously shown that TNT is preferentially reduced to amino derivatives.

Table 1. Gibbs free energy changes at 25°C and pH of 7.0 of selected redox couples

Half reaction of reductive transformation		ΔG°' (kJ/e$^-$)	Reference
$N_2O + 2H^+ + 2\ e^-$	$\leftrightarrow N_2 + H_2O$	- 65.1	(41)
$2NO + 2\ H^+ + 2e^-$	$\leftrightarrow N_2O + H_2O$	- 56.9	(41)
trinitrotoluene + $6\ H^+ + 6\ e^-$	$\leftrightarrow$ aminodinitrotoluene + $3\ H_2O$	- 66.5	(32)
$NO_2^- + 2\ H^+ + 1\ e^-$	$\leftrightarrow NO + H_2O$	- 35.7	(41)
trinitrotoluene + $1\ H^+ + 2\ e^-$	$\leftrightarrow$ dinitrotoluene + NO_2^-	- 24.5	(32)
$Fe(OH)_3 + 3\ H^+ + 1\ e^-$	$\leftrightarrow Fe^{2+} + 3\ H_2O$	- 14.5	(39)

In a study by Cooper et al. (5), the presence of goethite (a crystalline form of ferric oxyhydroxide) resulted in a 10-fold decrease in the rate of nitrite reduction by *Shewanella putrefaciens* 200. With enrichment cultures, these authors showed that inhibition of nitrite reduction was not limited to *S. putrefaciens*. When nitrite and insoluble Fe(III) were both present, Cooper et al. (5) and Coby and Picardal (4) demonstrated that Fe^{2+} was microbially produced from Fe(III) and that Fe^{2+} abiotically reacted with nitrite to form N_2O. During this reaction, Fe^{2+} was oxidized back to insoluble Fe(III) that formed a dense coating on the surface of the cell. Formation of such a coating was demonstrated with *S. putrefaciens* and *Paracoccus denitrificans* (4). This coating inhibited reduction of nitrite by physically blocking transport into the cell (4). In addition, Coby and Picardal (4) observed that inhibition of nitrite reduction by *S. putrefaciens* 200 was lower when hematite (specific surface area: ~9 m^2 g^{-1}) was used instead of goethite (~72 m^2 g^{-1}). To explain these differences, Coby and Picardal (4) hypothesized a difference in the reactivity of Fe^{2+} adsorbed on Fe(III) surfaces. This hypothesis was supported by the finding that Fe^{2+} adsorbed on Fe(III) surfaces reduced nitrite more rapidly than aqueous Fe^{2+} (33). The consequence of that would be more Fe^{2+} oxidized to an insoluble cell coating of Fe(III). Under our experimental conditions, one likely hypothesis could be that TNT denitration with *P. aeruginosa* ESA-5 was also taking place in the absence of ferrihydrite, but that nitrite was not detected because it was rapidly transformed by the strain. If an inhibition of *P. aeruginosa* ESA-5 mediated nitrite reduction occurred by the action of ferrihydrite due to the formation of an insoluble coating on the cells, then the release of nitrite would be more easily detected, as was indeed the case.

Clearly, the exact role of ferrihydrite in the denitration of TNT remains to be determined. However, this study shows that an isolated *P. aeruginosa* strain can denitrate TNT and release a significant concentration of nitrite in oxygen-depleted medium in the presence of ferrihydrite, a feature that has not been reported previously.

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