7.

Chapter 7

Denitration of 2,4,6-trinitrotoluene by a phenazine molecule produced by Pseudomonas aeruginosa ESA-5

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.

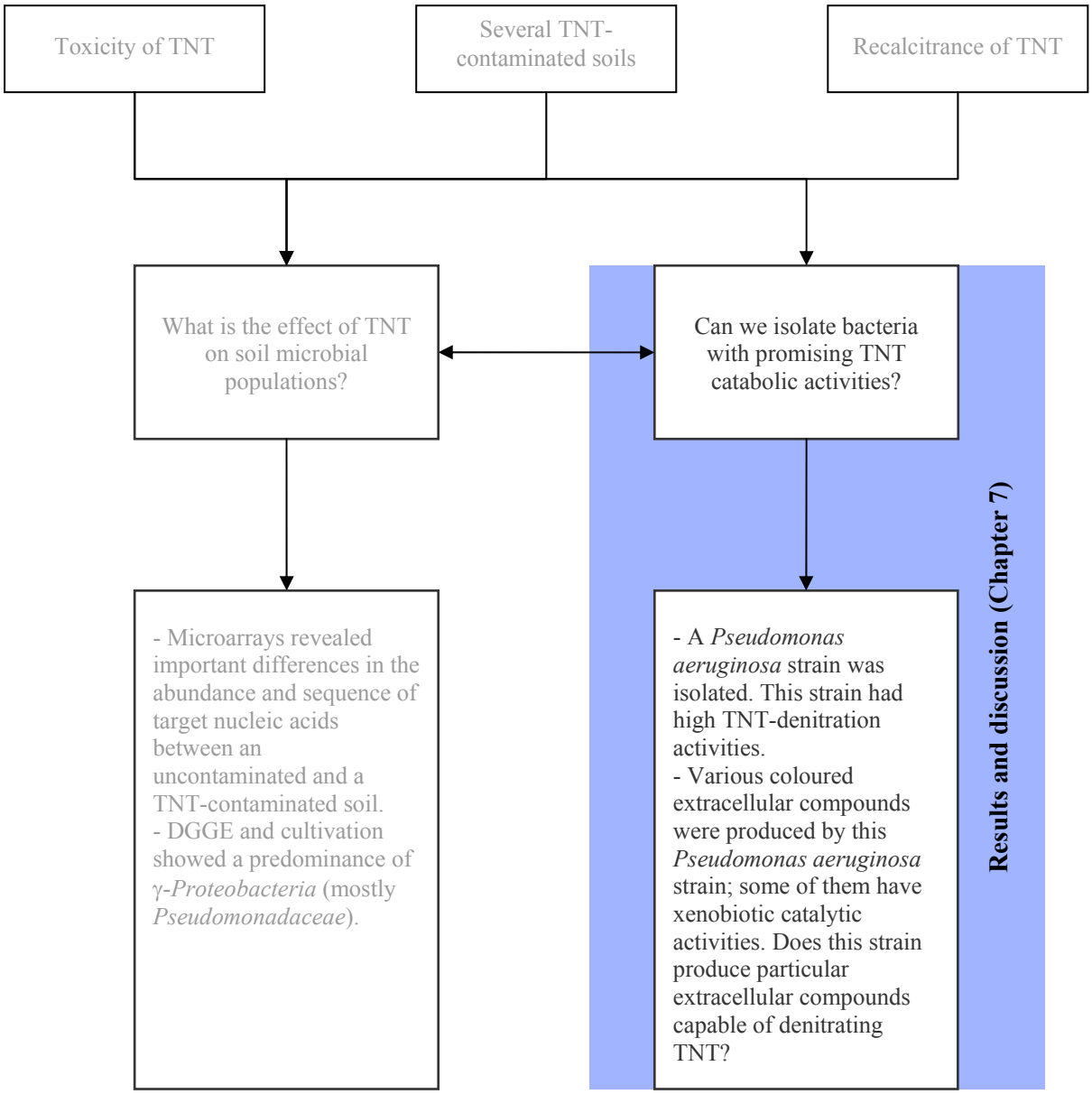
Chapter 4 compares hybridizations of in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to a phylogenetic microarray with thermal dissociation analysis.

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*).
- A *Pseudomonas aeruginosa* strain was isolated. This strain had high TNT-denitration activities.
- Various coloured extracellular compounds were produced by this *Pseudomonas aeruginosa* strain; some of them have xenobiotic catalytic activities. Does this strain produce particular extracellular compounds capable of denitrating TNT?
Results and discussion (Chapter 7)

Denitration of 2,4,6-trinitrotoluene by a phenazine molecule produced by Pseudomonas aeruginosa ESA-5

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7.1. Abstract

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

7.2. Introduction

TNT is a xenobiotic and recalcitrant pollutant. The strong electron-withdrawing properties of the three nitro groups confer a high electron deficiency on the entire π-electron system of the aromatic ring (51). In addition, the presence of four functional groups on the ring generates steric hindrance. Johnson et al. (27) indicated that the electronic nature of TNT was primarily responsible for the absence of electrophilic attacks by oxygenases rather than steric hindrance. On the other hand, TNT is easily transformed reductively by diverse bacteria, with successive reduction of the nitro groups to nitroso-, hydroxylamino- and amino groups. These TNT-reduced metabolites accumulate because they are not transformed further. In addition, they are as toxic as TNT itself (49).

Abiotically, TNT is mineralized by highly reactive hydroxyl radicals generated through Fenton reactions. Biotically, white-rot fungi have the ability to partially mineralize TNT through initial reduction to hydroxylamino- and amino-dinitrotoluene compounds followed by oxidative attack by powerful peroxidases (56). Among bacteria, a single report has demonstrated the productive attack of aminodinitrotoluene compounds by recombinant dioxygenases in *Escherichia coli* with release of nitrite and production of 3-amino-4-methyl-5-nitrocatechol (27). Similarly to white-rot fungi, hydroxylation of the aromatic ring does not take place if TNT is not initially reduced to 4-amino-dinitrotoluene (27). Another report indicates the release of nitrite from TNT and formation of a 3-methyl-4,6-dinitrocatechol intermediate by an aerobic bacterial consortium (55). The authors did not provide evidence of the potential involvement of oxygenases, but they found that oxidative attack occurred without prior reduction to amino-dinitrotoluene compounds.

Another bacterial attack consists in the addition of hydride ions (H^-) to the TNT molecule to form hydride-Meisenheimer complexes. For instance, release of nitrite from hydride-Meisenheimer complex and production of dinitrotoluene compounds has been reported with a *Pseudomonas* sp. strain (11, 19). However, no evidence of denitration (defined as the release of nitrite from TNT or any TNT metabolite) with the same strain could be found by Vorbeck et al. (58). Although nitrite liberation through hydride addition with this *Pseudomonas* strain seems controversial, effective removal of the nitro group from TNT-Meisenheimer complex has been established with other bacterial strains. For instance, another study of Vorbeck et al.

(57) indicated that a *Mycobacterium* strain released nitrite from TNT Meisenheimer-complex. Additionally, two unidentified metabolites were produced but were not dinitrotoluene compounds (57). With a *P. fluorescens* strain, Pak et al. (43) found that the strain produced hydride-Meisenheimer complex and hydroxylaminodinitrotoluene compounds that further reacted with each other to form a dimeric molecule with the release of nitrite. This pattern was confirmed by Stenuit et al. (52) with whole cells and cell extracts of *E. coli*. Denitration of TNT and production of amino-nitrotoluene (28) or dinitrotoluene compounds (30, 33) were reported with other bacterial strains, but the mechanism of denitration was not determined. In summary, these reports indicating the production of catechol or dinitrotoluene metabolites are promising for further mineralization of TNT because the catechol metabolites are precursors of oxygenolytic ring cleavage and dinitrotoluene compounds are attacked by oxygenases (e.g., 40).

We recently isolated a *P. aeruginosa* strain ESA-5 that released nitrite from TNT (Eyers et al., manuscript in preparation; thesis chapter 6), but the mechanism of denitration was not determined. *P. aeruginosa* is known to naturally produce several extracellular coloured metabolites. Among them, pyoverdines are fluorescent yellow-green peptides involved in the uptake of iron (siderophores) (35, 46). These peptides may have catalytic activities to degrade xenobiotic compounds. For instance, pyoverdines have been found to cleave triphenyltin to diphenyltin (25, 26). The mechanism of this cleavage was not investigated, but Xiao and Kisaalita (63) showed that pyoverdines oxidized ferrous ion, suggesting the involvement of redox reactions in the degradation of triphenyltin. *P. aeruginosa* also produces several phenazine molecules that have distinct colour and spectral properties (34). These phenazine compounds have antibiotic activity against a wide variety of microorganisms (22, 54) and possess redox properties. Depending on the redox potential, phenazine molecules oxidize or reduce several chemicals. For instance, phenazine compounds operate as bacterial electron shuttles to reduce Fe(III) (24) or oxidize NAD(P)H or 2',7'-dichlorodihydrofluorescein (41, 42). In addition, under aerobic conditions, phenazine molecules undergo non-enzymatic reduction by NAD(P)H and reduced phenazine molecules rapidly react with oxygen to produce the reactive oxygen species superoxide ($O_2^{-\cdot}$) and, by dismutation, hydrogen peroxide (H_2O_2) (8). Pyochelin is another extracellular coloured metabolite synthesized by *P. aeruginosa*. It is a siderophore involved in the uptake of iron (6). Britigan et al. (2) showed that Fe-bound pyochelin catalyzed the formation of $OH^{\cdot}$ radicals via the Haber-Weiss (18) reaction between $O_2^{-\cdot}$ and H_2O_2 produced by a phenazine molecule known as pyocyanin (5-

methyl-1-hydroxyphenazine). Abiotically, such $OH^{\cdot}$ radicals mineralize TNT (32). Moreover, pyochelin has been reported to cleave triphenyltin to diphenyltin (53). In summary, these studies showed that various pigments of *P. aeruginosa* may have properties enabling them to degrade xenobiotic compounds such as TNT. In the case of *P. aeruginosa* strain ESA-5, we noticed the production of a greenish extracellular compound when the strain was grown in a chemically defined medium. The objective of this study was to characterize the green pigment produced by *P. aeruginosa* ESA-5 and investigate its activity towards the denitration of TNT.

7.3. Materials and methods

Chemicals. TNT was obtained from Nobel Explosives (Chatelet, Belgium). [U-^{15}N]-labeled TNT was synthesized by Dr. M. Göbel (Faculty of Biology, University of Konstanz) and labeling of the three nitro groups was confirmed by NMR. 2-Amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene were synthesized as previously described (56). 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-Trinitrobenzyl alcohol, 2,4,6-trinitrobenzaldehyde, and 2,4,6-trinitrobenzoic acid were kindly supplied by M. Régis (GIAT Industries, France). Other chemicals were purchased from Sigma (St Louis, MO).

Strains. *P. aeruginosa* strains used in this study consisted of strain ESA-5 (Eyers et al., manuscript in preparation) and PAO1 (P. Cornelis, Laboratory of Microbial Interactions, Vrije Universiteit Brussel).

Culture conditions. CAA medium for pyochelin production contained 0.25% casamino acids (Difco) and 0.2 mM $MgCl_2$ at pH 7.5 (6). Production of pyochelin was tested after extraction of the culture supernatant with ethyl acetate, separation by thin-layer chromatography, and spraying with an iron and a phenolate reagent (6). A peptone-glycerol medium (9) was used to produce pyocyanin. It was extracted from the liquid culture with chloroform as previously described (4). For production of the green *P. aeruginosa* ESA-5 pigment, M8 minimal medium was used (11), except that ferric citrate was replaced by ferric chloride. A mixture of the four carbon sources glucose, acetate, succinate and pyruvate (6 mM each) was added. The nitrogen source was ammonium (5 mM). All cultures were conducted aerobically in the dark at 35°C on an orbital shaker set at 170 rpm.

Extraction of the *P. aeruginosa* ESA-5 green pigment and its use in nitroaromatic-degradation studies. When the OD_{600} of the culture reached 0.6, cells were removed by centrifugation. The supernatant was filtered through a 0.22 µm filter. The pigment was absorbed on a cartridge containing 2 g of C18 sorbent (Alltech, Deerfield, IL). One cartridge was typically sufficient to absorb the pigment of 500 ml of supernatant. The cartridge was washed with 10 ml of water and the pigment was desorbed with 5 ml of 50/50 (v/v) methanol/water. Methanol was subsequently removed by continuous evaporation (rotavapor

set at 35°C) and the working volume was reduced to approximately 500 µl. The concentration of the green pigment was calculated using the extinction coefficient of phenazine-1-carboxylic acid at 368 nm (10).

Nitroaromatic compounds were dissolved in acetone (50 mM) and provided in the reaction mix at a final concentration of 275 µM. The green pigment was added to a final concentration of 132.5 µM, except when mentioned otherwise. NAD(P)H was provided at concentrations stated in the Results section. To study the effect of various inhibitors, these were provided at a final concentration of 10 mM. The reaction (final volume of 5 ml) was carried out in 15 ml tubes agitated at 35°C in the dark. Experiments were done in triplicates.

TNT degradation studies with *P. aeruginosa* ESA-5 liquid cultures. M8 minimal medium containing glucose, acetate, succinate and pyruvate (6 mM each) was used. Three different conditions were evaluated: TNT as sole nitrogen source (4.4 mM) and addition of the green pigment (26.5 µM), addition of TNT and no green pigment, and addition of green pigment and no TNT. Experiments were done in triplicates.

Analytical methods. Preparation of samples, analysis of TNT and its derivatives with by HPLC, and spectrophotometric determination of nitrite were carried out as previously described (12). Nitrate and ammonium concentration were spectrophotometrically determined with a nitrate and ammonium Spectroquant® kit (Merck, Darmstadt, Germany), respectively. TNT interfered with ammonium determination and was removed by passing the samples through a Sep-Pak C18 cartridge (Waters, Milford, MA).

Liquid chromatography - mass spectrometry analysis was carried out with an HPLC system of Thermo Separation Products (San Jose, CA) equipped with a P1000 XR pump and an AS 3000 autosampler. This system was coupled with a UV 6000-LP detector (Thermo Separation Products) followed by a Quantum triple-quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA). All mass spectra were acquired by atmospheric pressure chemical ionization (APCI) in the negative ion mode. Since the mass spectrometer was connected after the UV detector, a difference of retention time of 0.07 min between the two detectors was found. The operating conditions of the mass spectrometer were as follows: vaporizer temperature 400°C; capillary temperature 220°C and collision energy 25 (arbitrary unit). The initial mobile phase consisted of 100% water at a flow rate of 0.7 ml/min. At 0.2 min after sample injection, the

concentration of acetonitrile was increased linearly to 100% over 55 min. At 60 min, a linear gradient was set to return to the initial mobile phase composition. The column was equilibrated for 15 min before the next run.

7.4. Results

Identification of the green pigment produced by *P. aeruginosa* ESA-5. In peptone-glycerol medium, *P. aeruginosa* ESA-5 produced a blue pigment that was extracted. The pigment went red under acidic conditions. This is a typical feature of pyocyanin (4, 29). In CAA medium, *P. aeruginosa* ESA-5 produced a yellow pigment which, after extraction and separation by thin-layer chromatography, appeared as a yellow-green fluorescent band when viewed under UV light, and turned red and deep blue with the iron and phenolate spray reagent, respectively. Those are typical features of pyochelin (6). When *P. aeruginosa* ESA-5 was grown in a chemically defined medium (four carbon sources, ammonium as the nitrogen source), the production of a green pigment was detected. This pigment was not detected in peptone-glycerol or CAA medium. In the same chemically defined medium, no green pigment production was observed with *P. aeruginosa* PAO1. When iron was omitted from the chemically defined medium inoculated with *P. aeruginosa* ESA-5, the green pigment was still produced. The pigment was adsorbed on a C18 HPLC column and presented a particular UV spectrum (Figure 1A). Its big absorption peak at 251 nm (Figure 1A) was similar to that of phenazine-1-carboxylic acid (PCA) as recorded by Mavrodi et al. (34). The UV spectrum was identical whether iron was provided in the culture or not, and the spectrum of the extracted green pigment was not modified by the addition of Fe^{3+}. These results indicated that the pigment was not a siderophore. The mass spectrum of the green pigment presented a deprotonated molecular mass ion $[M-H]^-$ at *m/z* 223 (MW, 224, Figure 1B (note that the *m/z* at 224 may result from incomplete deprotonation of the molecule since optimal mass detection was realized for nitroaromatic compounds but not for the green pigment)). Taken together, the evidence from the UV and mass spectra indicated that the green pigment was PCA (Figure 1C).

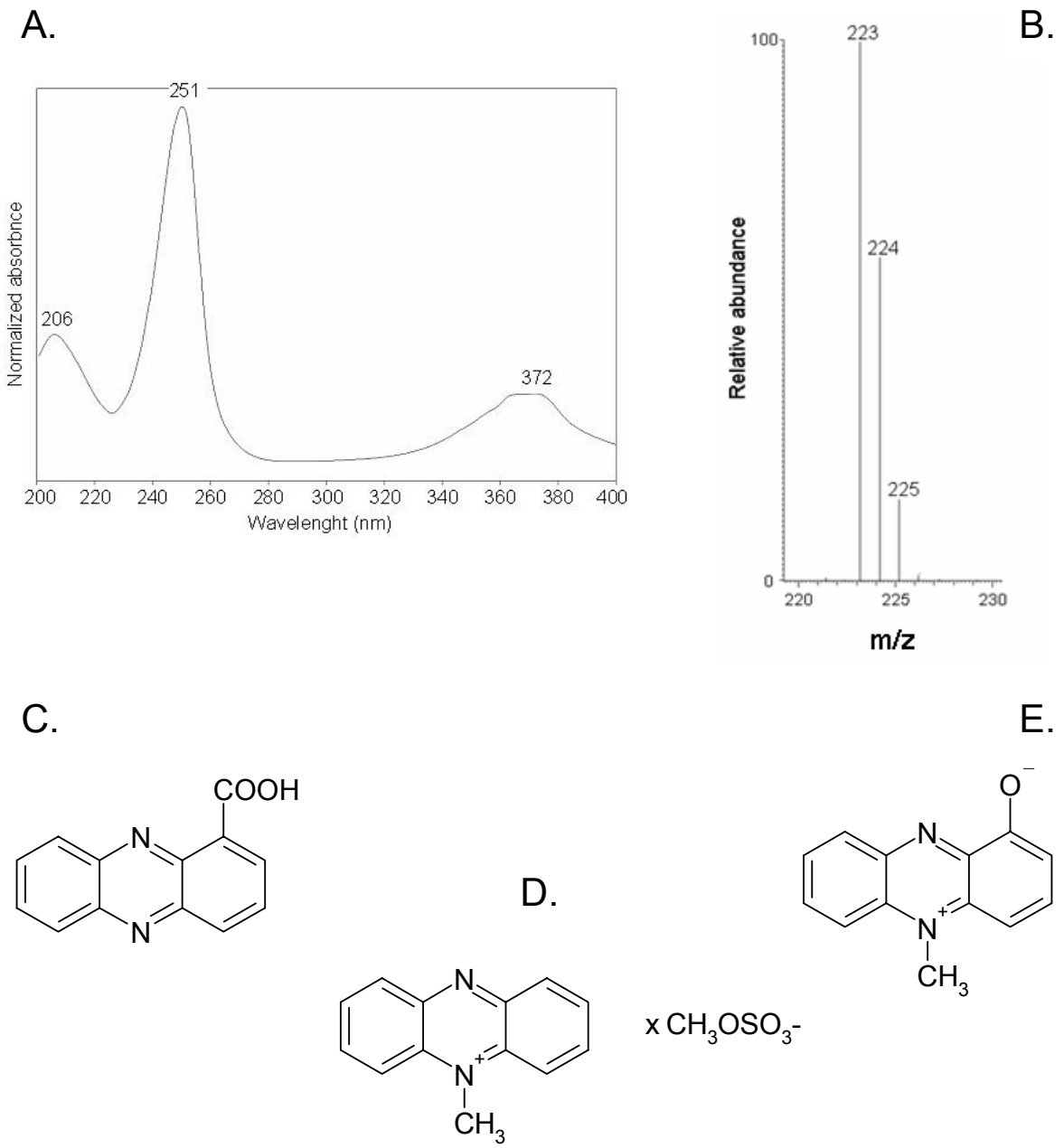

Figure 1. UV spectrum (A), mass spectrum (B) and chemical structure of PCA (C). Chemical structures of phenazine methosulfate (D) and pyocyanin (E). The solvent used for recording the UV spectrum of PCA was distilled water.

Denitration of TNT by PCA. The concentrations of nitrite detected when PCA, TNT (275 µM) and NADH (2000 µM) were incubated singly or in mixtures of two of the three compounds were not significant (measured concentrations were comprised between 0.0 to 4.0 µM, Figure 2). When PCA, TNT and NADH were incubated together, a significant release of nitrite occurred. Most of the nitrite was released within the first 19 h (124.4 µM, Figure 2). Then, nitrite concentration remained stable (130.6 µM after 91 h). Neither nitrate nor

ammonium was detected in the medium. These results showed that the presence of all three compounds together in the reaction mix (PCA, TNT and NADH) was required to obtain significant nitrite release.

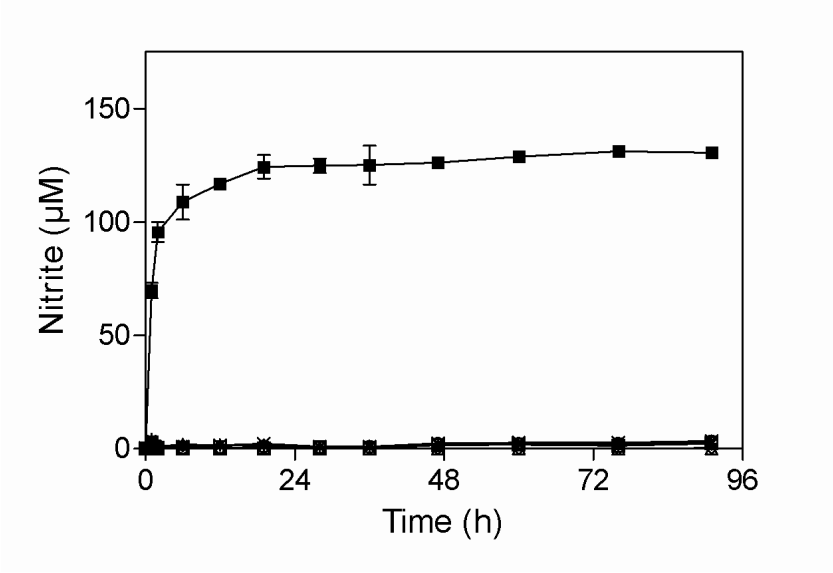

Figure 2. Nitrite release measured under different conditions with PCA, TNT, NADH incubated singly or in mixtures of two of the three compounds. PCA (open squares), NADH (open triangles), TNT (open lozenges). PCA and NADH (open circles), NADH and TNT (x crosses), and PCA and TNT (* crosses). PCA, NADH and TNT (closed squares). PCA was added to a final concentration of 132.5 µM. TNT and NADH were provided at a concentration of 275 and 2000 µM, respectively. Error bars represent the standard deviation of triplicates.

When the concentration of PCA was diminished by a factor 10 (i.e., 13.3 µM) or 100 (i.e., 1.3 µM), the release of nitrite dropped accordingly, with 51.8 µM and 22.6 µM detected after 19 h, respectively (Figure 3A). Most of the nitrite was released after 19 h with the 10-fold diluted PCA, whereas nitrite release continued over a 48-h incubation with the 100-fold diluted PCA. Concomitantly with nitrite release, a reddish color developed and the concentration of TNT decreased (Figure 3B). When PCA was not diluted, most of the TNT was eliminated after 2 h of incubation, from an initial TNT concentration of 274.0 µM down to 32.1 µM. With 10-fold or 100-fold diluted PCA, TNT concentration was reduced to a lower extent and at a slower pace (from 268.3 µM to 209.8 µM after 19 h, and from 275.5 µM to 250.0 µM after 91 h, respectively). The relationship between the kinetics of nitrite release and TNT elimination, and the absence of nitrite release when TNT was omitted from the reaction mix suggested that

TNT was at the origin of the nitrite. In addition, these results indicated that the concentration of PCA affected directly the extent of nitrite release and TNT elimination[1, 2].

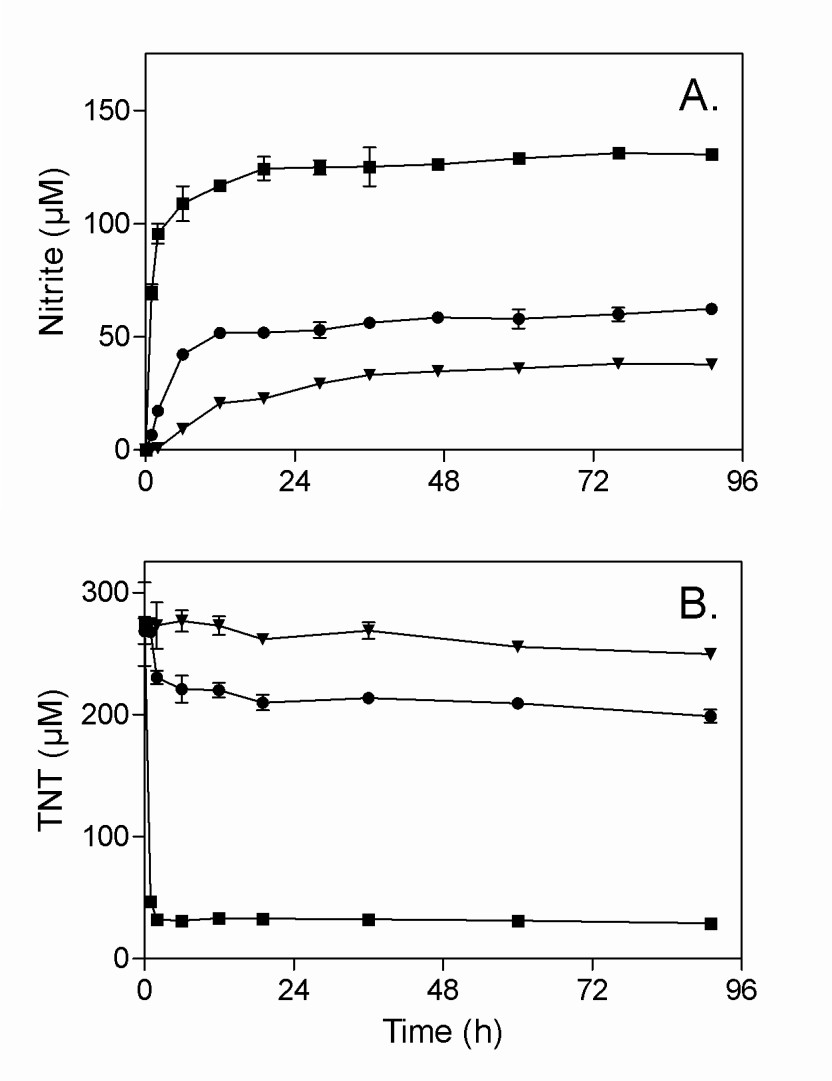

Figure 3. Nitrite release (A) and TNT concentration (B) in the presence of TNT (275 μM), NADH (2000 μM) and different concentrations of PCA. PCA added to a final concentration of 132.5 (closed squares), 13.3 (closed circles) and 1.3 μM (closed triangles). Error bars represent the standard deviation of triplicates.

[1] The maximum rate of nitrite release was calculated with undiluted, 10-fold diluted and 100-fold diluted PCA from the data of Figure 3A. These rate values were plotted against the concentration of PCA. From linear regression, the maximum yield rate of nitrite release amounted to 0.53 μM nitrite released / (μM PCA * hr) (under conditions of initial concentrations of TNT and NADH of 275 and 2000 μM, respectively).

[2] The maximum rate of TNT transformed was calculated with undiluted, 10-fold diluted and 100-fold diluted PCA from the data of Figure 3B. These rate values were plotted against the concentration of PCA. From linear regression, the maximum yield rate of TNT transformed amounted to 1.72 μM TNT transformed / (μM PCA * hr) (under conditions of initial concentrations of TNT and NADH of 275 and 2000 μM, respectively).

The concentration of NADH also influenced the extent of nitrite release and TNT elimination (Figure 4). After 19 h of incubation in the presence of PCA, TNT and various concentrations of NADH, a higher release of nitrite was detected with 800, 2000, 4000 and 10000 μM (63.2, 112.7, 144.3 and 147.1 μM of nitrite, respectively) than with 0, 100, 200 and 400 μM of NADH (2.7, 4.3, 5.7 and 21.7 μM of nitrite, respectively)[3]. When PCA was omitted, the levels of nitrite measured at different concentrations of NADH were negligible (between 0.0 and 2.8 μM). When NADPH was provided together with PCA and TNT, release of nitrite occurred at a similar extent as seen with the same concentrations of NADH, PCA and TNT (results not shown). Therefore, the presence of reduced pyridine nucleotides was necessary for TNT denitration.

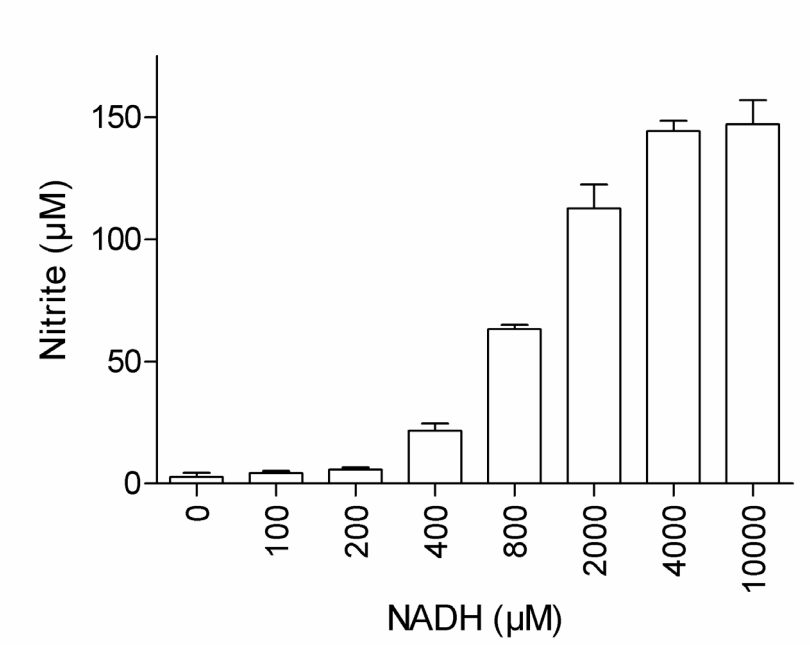

Figure 4. Nitrite release in the presence of TNT (275 μM), PCA (132.5 μM) and different concentrations of NADH after a 19 h of incubation. Error bars represent the standard deviation of triplicates.

[3] The maximum rate of nitrite release occurred with 800 μM of NADH. Under these conditions (PCA and TNT at initial concentrations of 132.5 and 275 μM, respectively), the maximum yield rate of nitrite release amounted to 0.0042 μM nitrite / (μM NADH * hr). Note that this experiment was carried out with an incubation time of 19 hr and that a shorter incubation time might likely result in higher maximum yield rates.

We also tested different phenazine compounds. With 5 μM of phenazine methosulfate (structure given in Figure 1D), TNT (275 μM) and NADH (2000 μM), nitrite was released, reaching 40.6 μM at 22 h, levelling off at 47.7 μM by the end of incubation at 94 h. In a reaction mix containing the extracted pyocyanin (structure given in Figure 1E) obtained from cells of *P. aeruginosa* strain ESA-5 grown in peptone-glycerol medium, TNT (275 μM) and NADH (500 μM), 84.3 μM of nitrite was detected after 120 h of incubation. On the contrary, when the extracted pyochelin obtained from *P. aeruginosa* strain ESA-5 grown in CAA medium was used in the place of PCA or pyocyanin in the same mix of TNT (275 μM) and NADH (500 μM), there was no measurable nitrite release. Finally, the addition of pyochelin to the mixture of pyocyanin, TNT and NADH did not increase nitrite release.

Denitration of other nitroaromatic compounds by PCA. Different TNT-related molecules (275 μM) were tested with PCA and NADH (2000 μM). No denitration was observed with 3,5-dinitroaniline, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,4,6-trinitrophenol (picric acid), and trinitrobenzoic acid. On the contrary, a reddish colour developed immediately upon addition of NADH in a medium containing trinitrobenzaldehyde and PCA. Under such conditions, the release of nitrite was more rapid and more extensive than with TNT (Figure 5). Trinitrobenzaldehyde was rapidly eliminated (from an initial concentration of 275.0 μM to 8.6 μM after 1 h of incubation)[4]. Denitration of 2,4,6-trinitrobenzyl alcohol was also observed but it was slower and less significant than with TNT (Figure 5). Also, trinitrobenzyl alcohol disappeared more slowly than TNT (from an initial level of 275.7 μM to 42.5 μM after 1 h of incubation)[5]. No reddish colour was observed.

[4] Under these conditions (i.e., initial concentrations of trinitrobenzaldehyde and NADH of 275 and 2000 μM, respectively), the maximum yield rate of nitrite release and trinitrobenzaldehyde transformed amounted to 0.61 μM nitrite / (μM PCA * hr) and 2.01 μM trinitrobenzaldehyde transformed / (μM PCA * hr), respectively.

[5] Under these conditions (i.e., initial concentration of trinitrobenzyl alcohol and NADH of 275 and 2000 μM, respectively), the maximum yield rate of nitrite release and trinitrobenzyl alcohol transformed amounted to 0.18 μM nitrite / (μM PCA * hr) and 1.76 μM trinitrobenzaldehyde transformed / (μM PCA * hr), respectively.

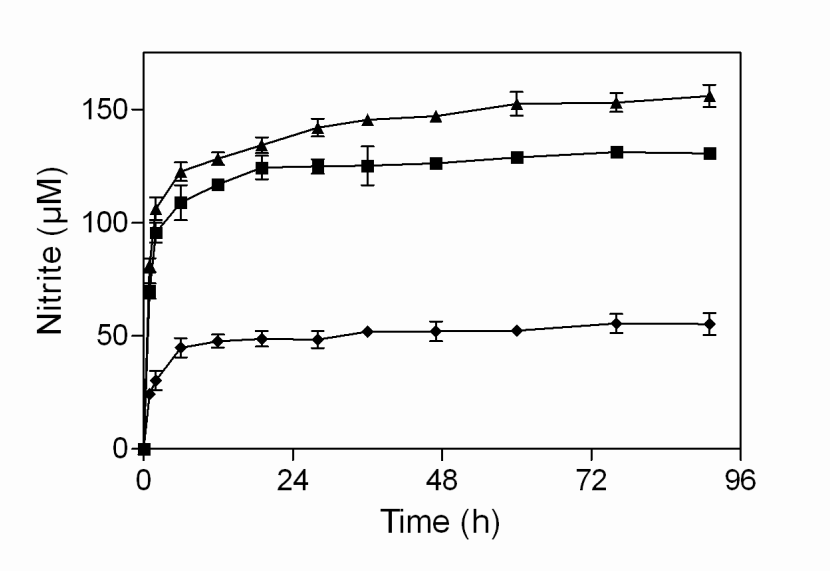

Figure 5. Nitrite release in the presence of PCA (132.5 μM), NADH (2000 μM) and various trinitroaromatic compounds (275 μM). 2,4,6-trinitrotoluene (closed squares), 2,4,6-trinitrobenzaldehyde (closed triangles) and 2,4,6-trinitrobenzyl alcohol (closed lozenges). Error bars represent the standard deviation of triplicates.

Identification of TNT metabolites produced upon PCA-mediated denitration. At low concentrations of NADH (i.e., 0, 100, 200, 400 and 800 μM), TNT-reduced metabolites were not detected in the presence of TNT and PCA. However, when the concentration of NADH was increased (i.e., 2000, 4000 and 10000 μM), 4-amino-2,6-dinitrotoluene and 4,4'-azoxy-2,2',6,6'-tetranitrotoluene were identified. These compounds were not detected when TNT was replaced by trinitrobenzaldehyde or trinitrobenzyl alcohol.

At the beginning of the above experiment with TNT (275 µM), PCA and NADH (2000 µM), 4-amino-2,6-dinitrotoluene and 4,4'-azoxy-2,2',6,6'-tetranitrotoluene were below detection limits. Then, low levels of 4-amino-2,6-dinitrotoluene (RT 29.16 min under HPLC conditions for MS detection) and of 4,4'-azoxy-2,2',6,6'-tetranitrotoluene (RT 42.21 min) were detected (results not shown). The concentration of 4-amino-2,6-dinitrotoluene increased during the experiment and reached 7.3 µM after 91 h. On the contrary, 4,4'-azoxy-2,2',6,6'-tetranitrotoluene accumulated rapidly at a higher concentration (18.3 µM after 1 h) which remained thereafter fairly constant (16.8 µM after 91 h)[6]. An unidentified peak (RT 39.74 min) was present after 1 h of incubation and its peak area remained constant throughout the rest of the experiment. This metabolite showed a mass spectrum with a deprotonated molecular mass ion $[M-H]^-$ at 391. Interestingly, Hawari et al. (23) detected two TNT metabolites with similar retention times and mass spectra as this unidentified peak, and tentatively identified them as reduced forms of azo TNT-derivatives. Our unidentified peak was further characterized. When the HPLC fraction of this compound was collected and dried, it reacted with acetyl chloride and gave rise to 2 new HPLC peaks. This indicated the presence of two acetyl chloride reactive groups. When ion at *m/z* 391 was fragmented, ions with *m/z* of 345 and 196 were found. The first fragment resulted from the loss of a nitro group. The ion at *m/z* of 196 had half the mass of the parent molecule (MW, 392) and the same mass as the deprotonated molecule of amino-dinitrotoluene. These results are consistent with the hypothesis of a reduced form of an azo TNT-derivative, with each monomer consisting in an amino-dinitrotoluene molecule. This azo TNT-derivative probably contained monomers of 4-amino-2,6-dinitrotoluene given that 2-amino-4,6-dinitrotoluene was not detected and that 4,4'-azoxy-2,2',6,6'-tetranitrotoluene was the sole azoxy compound produced.

[6] After 91 h of incubation, the residual concentration of TNT was equal to 29.2 µM. Taking into account the concentration of TNT-reduced metabolites (i.e., 4-amino-dinitrotoluene and 4,4'-azoxy-2,2',6,6'-tetranitrotoluene) produced as molar equivalent of initial TNT (i.e., 1 mole of 4-amino-dinitrotoluene and of 4,4'-azoxy-2,2',6,6'-tetranitrotoluene correspond to 1 and 2 mole(s) of TNT, respectively), 203.8 µM of TNT was transformed into unidentified TNT metabolites (bearing an unknown number of nitrogen atoms). Also, 130.6 µM of nitrite was released after 91 h of incubation. This corresponds to a ratio 'concentration of nitrite released / concentration of TNT transformed into unidentified compounds' of 0.64. When TNT was replaced by trinitrobenzaldehyde and trinitrobenzyl alcohol, this ratio was (after 91 h of incubation) 0.58 and 0.22, respectively.

Two polar metabolites appeared when TNT, trinitrobenzaldehyde or trinitrobenzyl alcohol was incubated with PCA and NADH. They were poorly retained by the C18 HPLC column as they appeared at the very beginning of the chromatogram (RT of 2.26 and 2.67 min). When mass detection was set up in order to select for metabolites bearing one or several nitro groups (i.e., detection of a neutral loss of 46), two metabolites corresponding to the above polar compounds were observed (Figure 6A). Their mass spectra presented a deprotonated molecular mass ion $[M-H]^-$ at *m/z* 180 Da (MW, 181) (Figure 6B). When this ion was fragmented, a major ion at *m/z* of 134 appeared (Figure 6C), resulting from the loss of a nitro group. Given the mass of the parent molecule, these metabolites had an odd number of nitro groups (nitrogen rule). To determine the exact number of nitro groups, [U-^{15}N]-labeled TNT was used in a similar incubation experiment together with PCA and NADH. Again, two polar metabolites appeared. They showed a deprotonated molecular mass ion $[M-H]^-$ at *m/z* 181 (MW, 182) (Figure 6D), indicating that they were produced from the loss of 2 nitro groups from TNT. A mass calculator was used to assign an empirical formula for the two polar metabolites. Inputs of molecular weight and error range were 181 and 0.8 amu, respectively. One nitrogen and a minimum of two oxygen atoms (i.e., one nitro group) were allowed. Additional boundaries involved the index of hydrogen deficiency (IHD; for instance, the IHD of one nitro group, one ring or one aromatic ring are 1, 1 and 4, respectively). Given the results of mass fragmentation indicating the presence of a single nitro group, a minimum IHD value of 1 was set. Seven empirical formulas were found: $C_3H_3O_8N_1$ (IHD of 3), $C_4H_7O_7N_1$ (IHD of 2), $C_5H_{11}O_6N_1$ (IHD of 1), $C_7H_3O_5N_1$ (IHD of 7), $C_8H_7O_4N_1$ (IHD of 6), $C_9H_{11}O_3N_1$ (IHD of 5), $C_{10}H_{15}O_2N_1$ (IHD of 4). The addition of one or more carbon atom(s) on the TNT structure appeared unlikely (see Discussion). Among the four remaining formulas, three of them corresponded to highly oxygenated compounds that had lost two or more carbons and the aromatic ring. The $C_7H_3O_5N_1$ formula was the only formula consistent with the hypothesis of no carbon loss and presence of an aromatic ring. The high IHD value associated with this formula restricted the number of possible structures. The only combination was a benzoquinone bearing one nitro and one aldehyde group (Figure 6C).

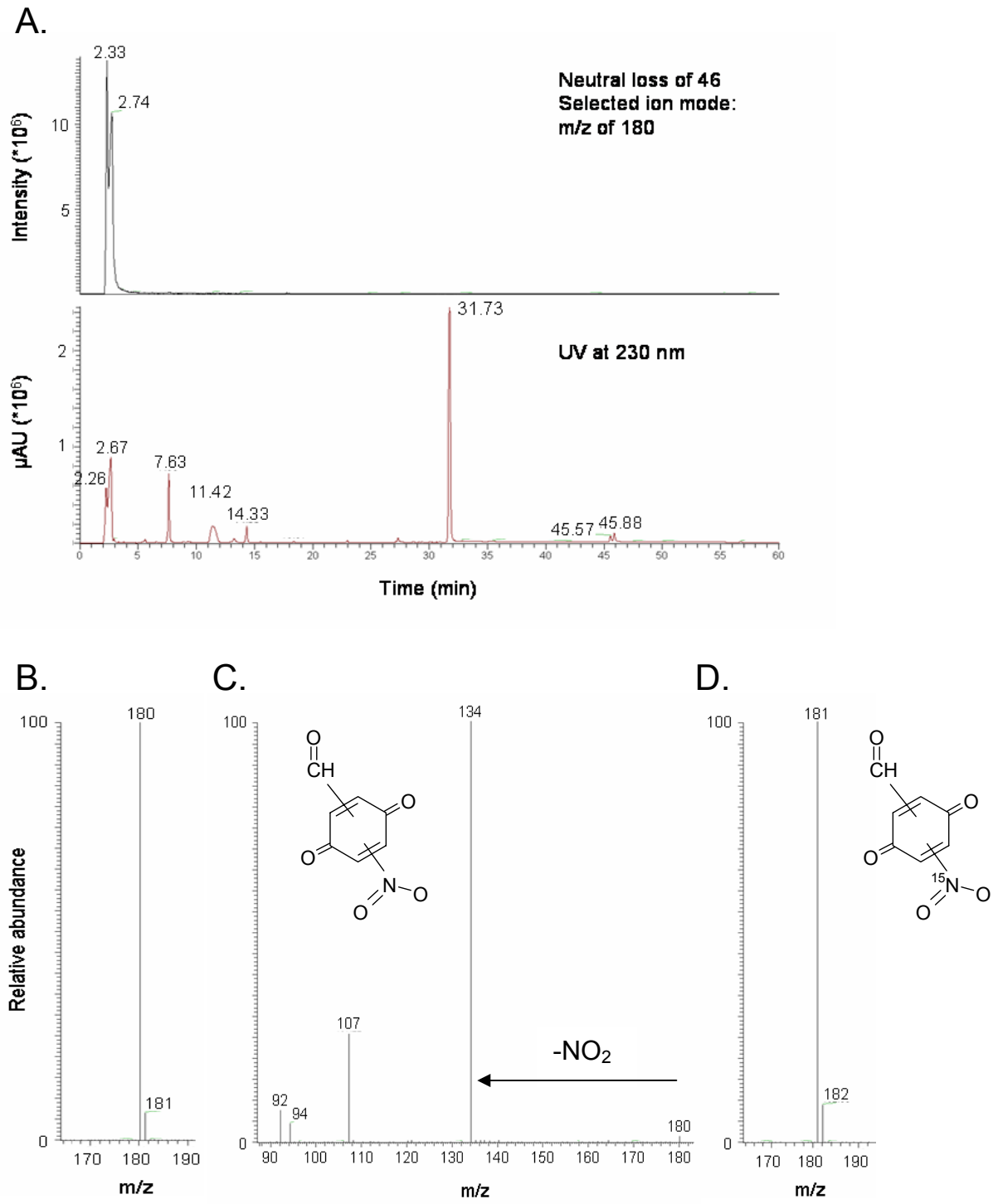

Figure 6. Metabolites identified in incubation of mixture of PCA (132.5 µM), TNT (275 µM) and NADH (400 µM). Chromatograms obtained with mass and UV detection. For mass detection, neutral loss of 46 amu with selected ion mode at an *m/z* of 180 was used. For UV detection, a 230 nm wavelength was selected (A). TNT (RT 31.73 min), PCA (RT 11.42 min), and two polar metabolites (RT 2.26 and 2.67). Mass spectrum of the two polar metabolites (B). MS-MS spectrum of the two polar metabolites (C). Fragmentation of the *m/z* of 180 was used. Mass spectrum of the two polar metabolites with [U-^{15}N]-labeled TNT (D).

Effect of specific inhibitors. Various inhibitors of reactive radical oxygen species were added at a concentration of 10 mM to vials containing PCA, TNT (275 µM) and NADH (2000 µM). In the presence of KI, mannitol or formate, the release of nitrite was similar to the control containing PCA, TNT and NADH (Figure 7). With ascorbate, the concentration of nitrite was significantly lower than the control (14.5 versus 130.6 µM). With reduced glutathione, no nitrite release was detected (Figure 7).

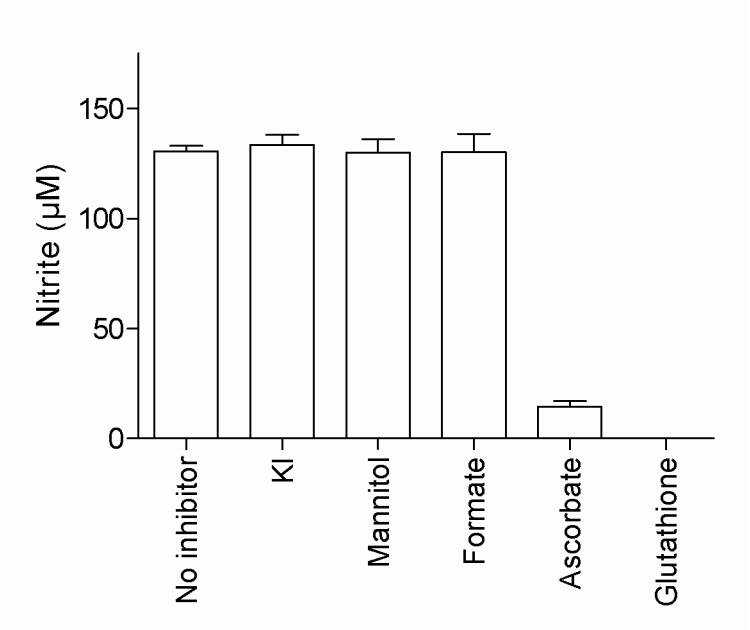

Figure 7. Nitrite release in the presence of PCA (132.5 µM), TNT (275 µM), NADH (2000 µM) and various inhibitors (10 mM). Error bars represent the standard deviation of triplicates.

Addition of PCA to a *P. aeruginosa* ESA-5 culture with TNT as sole nitrogen source. PCA was added to a culture inoculated with *P. aeruginosa* ESA-5. The composition of the culture medium was identical to the one used to produce and extract PCA, except that ammonium was replaced by TNT (4.4 mM). Under such conditions, PCA promoted growth of *P. aeruginosa* ESA-5 (Figure 8). When PCA was omitted, growth was characterized by a longer lag phase and it was less extensive than with PCA. No growth was observed when PCA was present and TNT was omitted (Figure 8), and when PCA and TNT were omitted (results not shown). These results supported the utilization by *P. aeruginosa* ESA-5 of TNT as a nitrogen source. However, nitrite was not detected in the medium. The HPLC area of the PCA peak remained stable in the experiments involving supplementation of the culture with PCA and medium with or without TNT. In the cultures with TNT and without exogenously added PCA, we did not detect the production of PCA. In addition, the two polar metabolites

found in vitro with mixtures of TNT, NADH and PCA were also detected in the inoculated liquid culture containing PCA and TNT. These two metabolites were not observed in the inoculated culture containing TNT but not PCA.

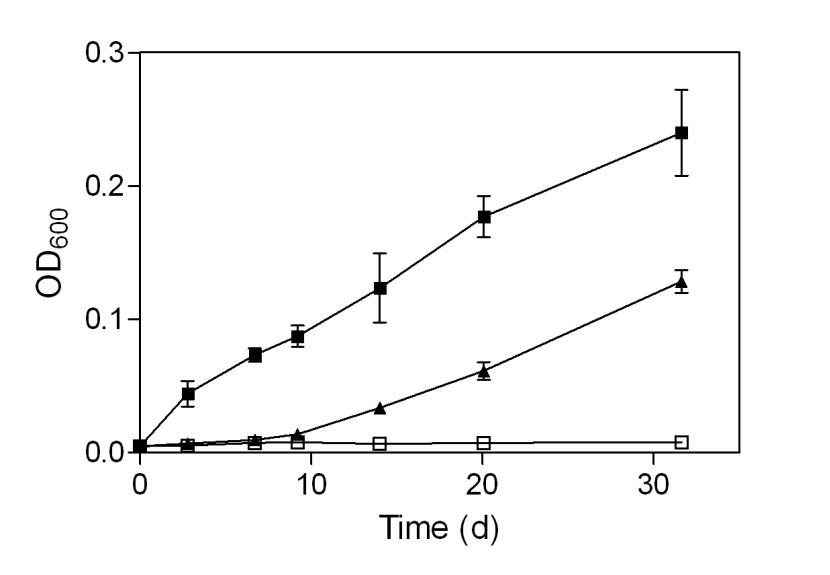

Figure 8. Evolution of the OD_{600} of *P. aeruginosa* ESA-5 liquid cultures. PCA (open squares), TNT (closed triangles), and PCA and TNT (closed squares). PCA and TNT were added at a final concentration of 26.5 µM and 4.4 mM, respectively. Error bars represent the standard deviation of triplicates.

7.5. Discussion

Several results demonstrated that the green pigment isolated from *P. aeruginosa* ESA-5 grown in a chemically defined medium was PCA. This compound has a green colour (54) that is different from the yellowish green colour of pyoverdine (5), the yellow colour of pyochelin (7) or the blue colour of pyocyanin (29). These colour differences were confirmed in the present study with the isolated PCA, pyochelin and pyocyanin of *P. aeruginosa* ESA-5. The UV spectrum of the green pigment was identical to the previously reported spectrum of PCA (34) and different than the spectrum of an isolated pyoverdine (5) and pyochelin (6) of *P. aeruginosa* PAO1. In addition, the UV spectrum of the pigment was not modified by the addition of Fe^{3+}, providing evidence that it was not a siderophore. Mass spectrometry indicated the presence of a compound of 224 amu. These results are consistent with PCA. Also, two related phenazine compounds (i.e., phenazine methosulfate and pyocyanin) had similar TNT denitration activities as PCA in the presence of NADH.

The denitration of TNT by PCA in the presence of NADH produced two polar metabolites. It was previously reported that some Old Yellow Enzymes used the reducing power of NAD(P)H to transfer a hydride to the TNT molecule with the formation of a hydride Meisenheimer complex (43, 60). Subsequently, nitrite from this TNT-Meisenheimer complex was released with the production of dinitrotoluene (11, 19) or amino-dimethyl-tetranitrobiphenyl (43) compounds. In our study with PCA, we chemically synthesized hydride-Meinsenheimer complexes (13) but we did not find evidence of formation of such complexes in mixtures of TNT, PCA and NADH. This indicated that denitration did not occur through hydride addition. Moreover, we did not detect the formation of dinitrotoluene or dimethyl-tetranitrobiphenyl chemicals by comparison with chemically-synthesized standards of dinitrotoluene and biologically-synthesized standards of amino-dimethyl-tetranitrobiphenyl (52) compounds, respectively.

On the other hand, phenazine compounds are known to produce several reactive oxygen species (ROS) (8, 21)[7]. Among these, hydroxyl radicals ($OH^{\cdot}$) are powerful oxidants that mineralize TNT (32). Another ROS is superoxide ($O_2^{\cdot -}$) which is a moderately reactive compound (20). However, as pointed out by Fridovich (14), $O_2^{\cdot -}$ can diffuse a considerable distance before it encounters a suitable, and possibly critical target. Superoxide oxidizes a variety of compounds such as nitrated polycyclic aromatic hydrocarbons (15), polyphenols, thiols, catecholamines and sulfite (14), and inactivates several bacterial enzymes (37). A third ROS is hydrogen peroxide (H_2O_2), a reactive oxidant damaging cell constituents such as membranes, enzymes and DNA (59). Last but not least, singlet oxygen ($^1O_2$) is yet another ROS. It is produced chemically, enzymatically and photochemically (31). Singlet oxygen reacts with electron-rich compounds containing double bonds (38) such as aromatic hydrocarbons (1) or amino acids (36).

Mannitol and formate react with hydroxyl radicals with rate constants of $1.8*10^9$ (17) and $2.8*10^9$ M^{-1} s^{-1} (3), respectively. They are therefore frequently used to scavenge hydroxyl radicals. In our study, we noticed that mannitol and formate did not inhibit the denitration of TNT in the presence of PCA and NADH (Figure 8). Also, KI is a scavenger of $OH^{\cdot}$ (rate constant of $1.1*10^{10}$ M^{-1} s^{-1} (16)) and it decomposes H_2O_2. KI did not influence the denitration of TNT, providing evidence that denitration did not involve $OH^{\cdot}$ and H_2O_2. On the contrary, reduced glutathione and ascorbate inhibited denitration of TNT. Reduced glutathione and ascorbate are scavengers of $^1O_2$ (rate constants of $2.4*10^6$ and $8.3*10^6$ M^{-1} s^{-1} (44), respectively) and of $O_2^{\cdot -}$ (rate constants of $1*10^4$ (61) and $5.4*10^6$ M^{-1} s^{-1} (50), respectively). These results did not allow to determine conclusively which of the two reactive oxygen species was involved in the observed denitration of TNT.

[7] Under the experimental conditions used, O_2 was likely not at a limiting concentration given the reaction volume (reactions were carried out in 15 mL test tubes, with 5 mL of reaction volume (at 35°C, 100 kPa, and 10 mL gas volume left, there are 90 µmole of O_2)) and number of samplings (each time a sampling was realized, the cap of the test tube was opened for 5 min, allowing for gas exchanges.

However, several pieces of evidence point toward the implication of $O_2^{\cdot-}$ and not of $^1O_2$. First of all, the production of $O_2^{\cdot-}$ by reduction of phenazine compounds with NADH has been previously described (8, 21)[8], but the generation of $^1O_2$ has only been observed under very specific conditions not achieved in our study (i.e., visible light illumination and presence of rose bengal or riboflavin (47)). Secondly, NADH reacts much more rapidly with $^1O_2$ (rate constant of $4.3*10^7$ M^{-1} s^{-1} (45)) than with $O_2^{\cdot-}$ (rate constant lower than 27 M^{-1} s^{-1} (16)). Therefore, if $^1O_2$ was produced by PCA, it would rapidly react with NADH making this latter unavailable for further reaction with PCA. Thirdly, $^1O_2$ has an electrophilic nature (1) whereas $O_2^{\cdot-}$ is a nucleophile (15). The grafting of electron-releasing groups on the site of $^1O_2$ addition increases the rate constants in the order $H < C_6H_5 < CH_3 \leq OCH_3$ (1). For instance, 1-methylnaphthalene reacts slowly with $^1O_2$ whereas naphthalene is completely unreactive (1). With $O_2^{\cdot-}$, Fukuhara and Miyata (15) found that the presence of nitro substituents on aromatic molecules was a prerequisite for oxidation of the substrate. Since the presence of three nitro groups on the TNT aromatic ring increases its electrophilic nature, it would be an ideal candidate for oxidation by $O_2^{\cdot-}$ [9] but not by $^1O_2$. Also, it is interesting to note that TNT derivatives containing an electron-donating group (e.g., aminodinitrotoluene compounds and picric acid) were not denitrated by PCA. In addition, the results obtained with TNT, trinitrobenzaldehyde and trinitrobenzyl alcohol indicated that denitration was more rapid when the aromatic ring contained an aldehyde group than a methyl or a methyl alcohol group. These results support a nucleophilic attack by $O_2^{\cdot-}$. With trinitrobenzoic acid, no denitration was detected although the carboxyl group has a strong electron-withdrawing character.

[8] The transfer of electrons from NADH to PCA and finally to O_2 to generate $O_2^{\cdot-}$ is thermodynamically favourable (Table 1).

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

Half reaction of reductive transformation		$\Delta G°'$ (kJ/e$^-$)	Reference
$O_2 + 1\ e^-$	$\leftrightarrow O_2^{\cdot-}$	+ 0.03	(62)
$PCA_{ox} + 2\ H^+ + 2e^-$	$\leftrightarrow PCA_{red}$	+ 8.5	(24)
$NAD^+ + H^+ + 2\ e^-$	$\leftrightarrow$ NADH	+ 15.4	(24)

[9] From a thermodynamic point of view, this reaction is possible (Table 2).

Table 2. Gibbs free energy changes at 25°C and pH of 7.0 of selected redox couples

Half reaction of reductive transformation		$\Delta G°'$ (kJ/e$^-$)	Reference
$TNT + 1\ H^+ + 2\ e^-$	$\leftrightarrow$ dinitrotoluene + NO_2^-	- 24.5	(48)
$O_2 + 1\ e^-$	$\leftrightarrow O_2^{\cdot-}$	+ 0.03	(62)

However, the carboxyl group of the molecule was deprotonated under our conditions since no buffer was used (2,4,6-trinitrobenzoic acid has a pK_a of 0.65). Therefore, the deprotonated carboxyl group was not a strong electron-withdrawing group.

Mass spectrometric analysis restricted the possible chemical formula of the two polar metabolites to 7 possibilities. Three of them involved the addition of one or more carbon atom(s). If denitration of TNT occurred through $O_2^{\cdot-}$ attack as suggested above, the addition of carbon atoms was unlikely. The four other possible formulas indicated highly oxidized molecules which are consistent with an attack by $O_2^{\cdot-}$. If no carbon loss occurred and the aromatic ring was preserved, the only possible structure was a benzoquinone containing an aldehyde and a nitro group (Figure 6C).

When PCA was added to a *P. aeruginosa* ESA-5 culture with TNT as sole nitrogen source, growth was higher than without PCA. Using endothelial cells, Muller (39) reported the intracellular reduction of pyocyanin by NADPH. The reduced pyocyanin then exported reducing equivalents to the extracellular space were O_2 was reduced to $O_2^{\cdot-}$. A similar mechanism was probably taking place at the level of *P. aeruginosa* ESA-5 cells, with $O_2^{\cdot-}$ leading to denitration of TNT and further consumption of the released nitrite by *P. aeruginosa*. The reason for the absence of nitrite detected in the culture may be explained by the fast consumption/transformation of nitrite by *P. aeruginosa* ESA-5: additional studies indicated that *P. aeruginosa* ESA-5 consumed/transformed 1000 µM of nitrite in less than 5.8 h (results not shown). On the other hand, the production of PCA was not detected when it was not provided in the culture although growth was observed. This suggested that, in addition to the PCA-mediated denitration of TNT, *P. aeruginosa* ESA-5 likely had other TNT-denitration catabolic pathway(s).

Although further studies are required to determine the mechanism of action of PCA and the chemical structure of the polar metabolites, this study clearly demonstrated a denitration of TNT by a phenazine compound of *P. aeruginosa*. This is the first report showing that phenazine compounds have catabolic activities towards the degradation of a recalcitrant xenobiotic such as TNT. Noteworthy, it is also the first report providing evidence of the production of mono-nitrated metabolites of TNT with a compound of biotic origin. Also, this study shows an interaction between PCA and *P. aeruginosa* ESA-5 to use TNT as sole nitrogen source.

7.6. References

1. **Aubry, J.M., C. Pierlot, J. Rigaudy, and R. Schmidt.** 2003. Reversible binding of oxygen to aromatic compounds. Acc. Chem. Res. **36:**668-675.
2. **Britigan, B.E., T.L. Roeder, G.T. Rasmussen, D.M. Shasby, M.L. McCormick, and C.D. Cox.** 1992. Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radical and causes synergistic damage to endothelial cells. J. Clin. Invest. **90:**2187-2196.
3. **Burch, P.E.** 1989. DNA damage and cell lethality by photodynamically produced oxygen radicals. Thesis, Houston, TX.
4. **Cox, C.D.** 1986. Role of pyocyanin in the acquisition of iron from transferrin. Infect. Immun. **52:**263-270.
5. **Cox, C.D., and P. Adams.** 1985. Siderophore activity of pyoverdin for *Pseudomonas aeruginosa*. Infect. Immun. **48:**130-138.
6. **Cox, C.D., and R. Graham.** 1979. Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. J. Bacteriol. **137:**357-364.
7. **Cox, C.D., K.L. Rinehart, M.L. Moore, and J.C. Cook.** 1981. Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **78:**4256-4260.
8. **Davis, G., and P.J. Thornalley.** 1983. Free radical production from the aerobic oxidation of reduced pyridine nucleotides catalysed by phenazine derivatives. Biochim. Biophys. Acta **724:**456-464.
9. **Degiorgi, C.F., R.O. Fernandez, and R.A. Pizarro.** 1996. Ultraviolet-B lethal damage on *Pseudomonas aeruginosa*. Curr. Microbiol. **33:**141-146.
10. **Dietrich, L.E.P., A. Price-Whelan, A. Petersen, M. Whiteley, and D.K. Newman.** 2006. The phenazine pyocyanin is a terminal signalling factor in the quorum sensing of *Pseudomonas aeruginosa*. Mol. Microbiol. **61:**1308-1321.
11. **Duque, E., A. Haidour, F. Godoy, and J.L. Ramos.** 1993. Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J. Bacteriol. **175:**2278-83.
12. **Eyers, L., B. Stenuit, S. El Fantroussi, and S.N. Agathos** 2004. Microbial characterization of TNT-contaminated soils and anaerobic TNT degradation: high and unusual denitration activity, p. 51-54. *In* W. Verstraete (ed.), Proceedings of the European Symposium on Environmental Biotechnology, ESEB 2004. Taylor and Francis Group plc, London, UK
13. **French, C.E., S. Nicklin, and N.C. Bruce.** 1998. Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. Appl. Environ. Microbiol. **64:**2864-2868.
14. **Fridovich, I.** 1998. Oxygen toxicity: a radical explanation. J. Exp. Biol. **201:**1203-1209.

15. **Fukuhara, K., and N. Miyata.** 1995. Chemical oxidation of nitrated polycyclic aromatic hydrocarbons: hydroxylation with superoxide anion radical. Chem. Res. Toxicol. **8:**27-33.

16. **Goldstein, S., and G. Czapski.** 2000. Reactivity of peroxynitrite versus simultaneous generation of $^{\cdot}NO$ and $O_2^{\cdot-}$ toward NADH. Chem. Res. Toxicol. **13:**736-741.

17. **Golstein, S., and G. Czapski.** 1984. Mannitol as an $OH^{\cdot}$ scavenger in aqueous solutions and in biological systems. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. **46:**725-729.

18. **Haber, F., and J. Weiss.** 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Lond. A. Math. Phys. Soc. **147:**332-351.

19. **Haïdour, A., and J.L. Ramos.** 1996. Identification of products resulting from the biological reduction of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and 2,6-dinitrotoluene by *Pseudomonas* sp. Environ. Sci. Technol. **30:**2365-2370.

20. **Halliwell, B., and J.M.C. Gutteridge.** 1989. Free radicals in biology and medicine. Clarendon Press, Oxford, UK.

21. **Hassan, H.M., and I. Fridovich.** 1980. Mechanism of the antibiotic action of pyocyanine. J. Bacteriol. **141:**156-163.

22. **Hasset, D.J., L. Charniga, D.E. Ohman, and M.S. Cohen.** 1992. Response of *Pseudomonas aeruginosa* to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. Infect. Immun. **60:**328-336.

23. **Hawari, J., A. Halasz, S. Beaudet, L. Paquet, G. Ampleman, and S. Thiboutot.** 1999. Biotransformation of 2,4,6-trinitrotoluene with *Phanerochaete chrysosporium* in agitated cultures at pH 4.5. Appl. Environ. Microbiol. **65:**2977-2986.

24. **Hernandez, M.E., A. Kappler, and D.K. Newman.** 2004. Phenazines and other redox-active antibiotics promote microbial mineral reduction. Appl. Environ. Microbiol. **70:**921-928.

25. **Inoue, H., O. Takimura, H. Fuse, K. Murakami, K. Kamimura, and Y. Yamaoka.** 2000. Degradation of triphenyltin by a fluorescent Pseudomonad. Appl. Environ. Microbiol. **66:**3492-3498.

26. **Inoue, H., O. Takimura, K. Kawagucchi, T. Nitoda, H. Fuse, K. Murakami, and Y. Yamaoka.** 2003. Tin-carbon cleavage of organotin compounds by pyoverdine from *Pseudomonas chlororaphis*. Appl. Environ. Microbiol. **69:**878-883.

27. **Johnson, G.R., B.F. Smets, and J.C. Spain.** 2001. Oxidative transformation of aminodinitrotoluene isomers by multicomponent dioxygenases. Appl. Environ. Microbiol. **67:**5460-5466.

28. **Kalafut, T., M.E. Wales, V.K. Rastogi, R.P. Naumova, S.K. Zaripova, and J.R. Wild.** 1998. Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. Curr. Microbiol. **36:**45-54.

29. **Kanner, D., N.N. Gerber, and R. Bartha.** 1978. Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. J. Bacteriol. **134:**690-692.

30. **Kim, H.Y., G. Benett, and H.G. Song.** 2002. Degradation of 2,4,6-trinitrotoluene by *Klebsiella* sp. isolated from activated sludge. Biotechnol. Lett. **24:**2023-2028.

31. **Krinsky, N.** 1977. Singlet oxygen in biological systems. Trends Biochem. Sci. **2:**35-38.

32. **Liou, M.J., M.C. Lu, and J.N. Chen.** 2003. Oxidation of explosives by Fenton and photo-Fenton processes. Wat. Res. **37:**3172-3179.

33. **Martin, J.L., S.D. Comfort, P.J. Shea, T.A. Kokjohn, and R.A. Drijber.** 1997. Denitration of 2,4,6-trinitrotoluene by *Pseudomonas savastanoi*. Can. J. Microbiol. **43:**447-455.

34. **Mavrodi, D.V., R.F. Bonsall, S.M. Delaney, M.J. Soule, G. Phillips, and L.S. Thomashow.** 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **183:**6454-6465.

35. **Meyer, J.M., A. Stinztzi, D. De Vos, P. Cornelis, R. Tappe, K. Taraz, and H. Budzikiewicz.** 1997. Use of siderophores to type Pseudomonads: the three *Pseudomonas aeruginosa* pyoverdine systems. Microbiology **143:**35-43.

36. **Michaeli, A., and J. Feitelson.** 1994. Reactivity of singlet oxygen toward amino acids and peptides. Photochem. Photobiol. **59:**284-289.

37. **Miller, R.A., and B.E. Britigan.** 1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10:**1-18.

38. **Min, D.B., and J.M. Boff.** 2002. Chemistry and reaction of singlet oxygen in foods. Comp. Rev. Food Sci. Food Saf. **1:**58-72.

39. **Muller, M.** 2002. Pyocyanin induces oxidative stress in human endothelial cells and modulates the glutathione redox cycle. Free Radic. Biol. Med. **33:**1527-1533.

40. **Nishino, S.F., G.C. Paoli, and J.C. Spain.** 2000. Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. **66:**2139-2147.

41. **O'Malley, Y., K.J. Reszka, and B.E. Britigan.** 2004. Direct oxidation of 2',7'-dichlorohydrofluorescein by pyocyanin and other redox-active compounds independent of reactive oxygen species production. Free Radic. Biol. Med. **36:**90-100.

42. **O'Malley, Y., K.J. Reszka, D.R. Spitz, G.M. Denning, and B.E. Britigan.** 2004. *Pseudomonas aeruginosa* pyocyanin directly oxidizes glutathione and decreases its level in airway epithelial cells. Am. J. Physiol. Lung Cell. Mol. Physiol. **287:**L94-L103.

43. **Pak, J.W., K.L. Knoke, D.R. Noguera, B.G. Fox, and G.H. Chambliss.** 2000. Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Appl. Environ. Microbiol. **66:**4742-4750.

44. **Peters, G., and M.A. Rodgers.** 1981. Single-electron transfer from NADH analogues to singlet oxygen. Biochim. Biophys. Acta **673:**43-52.

45. **Petrat, F., S. Pindiur, M. Kirsch, and H. de Groot.** 2003. NAD(P)H, a primary target of $^1O_2$ in mitochondria of intact cells. J. Biol. Chem. **278:**3298-3307.

46. **Ravel, J., and P. Cornelis.** 2003. Genomics of pyoverdine-mediated iron uptake in pseudomonads. Trends Microbiol. **11:**195-200.

47. **Reszka, K.J., G.M. Denning, and B.E. Britigan.** 2006. Photosensitized oxidation and inactivation of pyocyanin, a virulence factor of *Pseudomonas aeruginosa*. Photochem. Photobiol. **82:**466-473.

48. **Shelley, M.D., R.L. Autenrieth, J.R. Wild, and B.E. Dale.** 1996. Thermodynamic analysis of trinitrotoluene biodegradation and mineralization pathways. Biotech. Bioeng. **51:**198-205.

49. **Snellinx, Z., A. Nepovim, S. Taghavi, J. Vangronsveld, T. Vanek, and D. van der Lelie.** 2002. Biological remediation of explosives and related nitroaromatic compounds. Environ. Sci. Pollut. Res. Int. **9:**48-61.

50. **Som, S., C. Raha, and I.B. Chatterjee.** 1983. Ascorbic acid: a scavenger of superoxide radical. Acta Vitaminol. Enzymol. **5:**243-250.

51. **Stenuit, B., L. Eyers, S. El Fantroussi, and S.N. Agathos.** 2005. Promising strategies for the mineralisation of 2,4,6-trinitrotoluene. Rev. Environ. Science Bio/Technol. **4:**39-60.

52. **Stenuit, B., L. Eyers, R. Rozenberg, J.L. Habib-Jiwan, and S.N. Agathos.** 2006. Aerobic growth on 2,4,6-trinitrotoluene (TNT) as sole nitrogen source by *Escherichia coli* and evidence of TNT denitration with whole cells and cell-free extracts. Appl Environ Microbiol. **72:**7945-7948.

53. **Sun, G.X., W.Q. Zhou, and J.J. Zhong.** 2006. Organotin decomposition by pyochelin, secreted by *Pseudomonas aeruginosa* even in an iron-sufficient environment. Appl. Environ. Microbiol. **72:**6411-6413.

54. **Timms-Wilson, T.M., R.J. Ellis, A. Renwick, D.J. Rhodes, D.V. Mavrodi, D.M. Weller, L.S. Thomashow, and M.J. Bailey.** 2000. Chromosomal insertion of phenazine-1-carboxylic acid biosynthesis pathway enhances efficacy of damping-off disease control *Pseudomonas fluorescens*. MPMI **13:**1293-1300.

55. **Tront, J.M., and J.B. Hughes.** 2005. Oxidative microbial degradation of 2,4,6-trinitrotoluene via 3-methyl-4,6-dinitrocatechol. Environ. Sci. Technol. **39:**4540-4549.

56. **Van Aken, B., and S.N. Agathos.** 2002. Implication of manganese (III), oxalate, and oxygen in the degradation of nitroaromatic compounds by manganese peroxidase (MnP). Appl. Microbiol. Biotechnol. **58:**345-351.

57. **Vorbeck, C., H. Lenke, P. Fischer, and H. Knackmuss.** 1994. Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. J. Bacteriol. **176:**932-934.

58. **Vorbeck, C., H. Lenke, P. Fischer, J.C. Spain, and H.J. Knackmuss.** 1998. Initial reductive reactions in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. **64:**246-252.

59. **Weiss, S.J.** 1986. Oxygen, ischemia and inflammation. Acta Physiol. Scan. Suppl. **548:**9-37.

60. **Williams, R.E., D.A. Rathbone, N.S. Scrutton, and N.C. Bruce.** 2004. Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl. Environ. Microbiol. **70:**3566-3574.

61. **Winterbourn, C., and D. Metodiewa.** 1994. The reaction of superoxide with reduced glutathione. Arch. Biochem. Biophys. **314:**284-290.

62. **Wood, P.M.** 1988. The potential diagram fro oxygen at pH 7. Biochem. J. **253:**287-289.

63. **Xiao, R., and W.S. Kisaalita.** 1998. Fluorescent Pseudomonad pyoverdines bind and oxidize ferrous iron. Appl. Environ. Microbiol. **64:**1472-1476.