2.

Chapter 2

Promising strategies for the mineralisation of 2,4,6-trinitrotoluene

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.

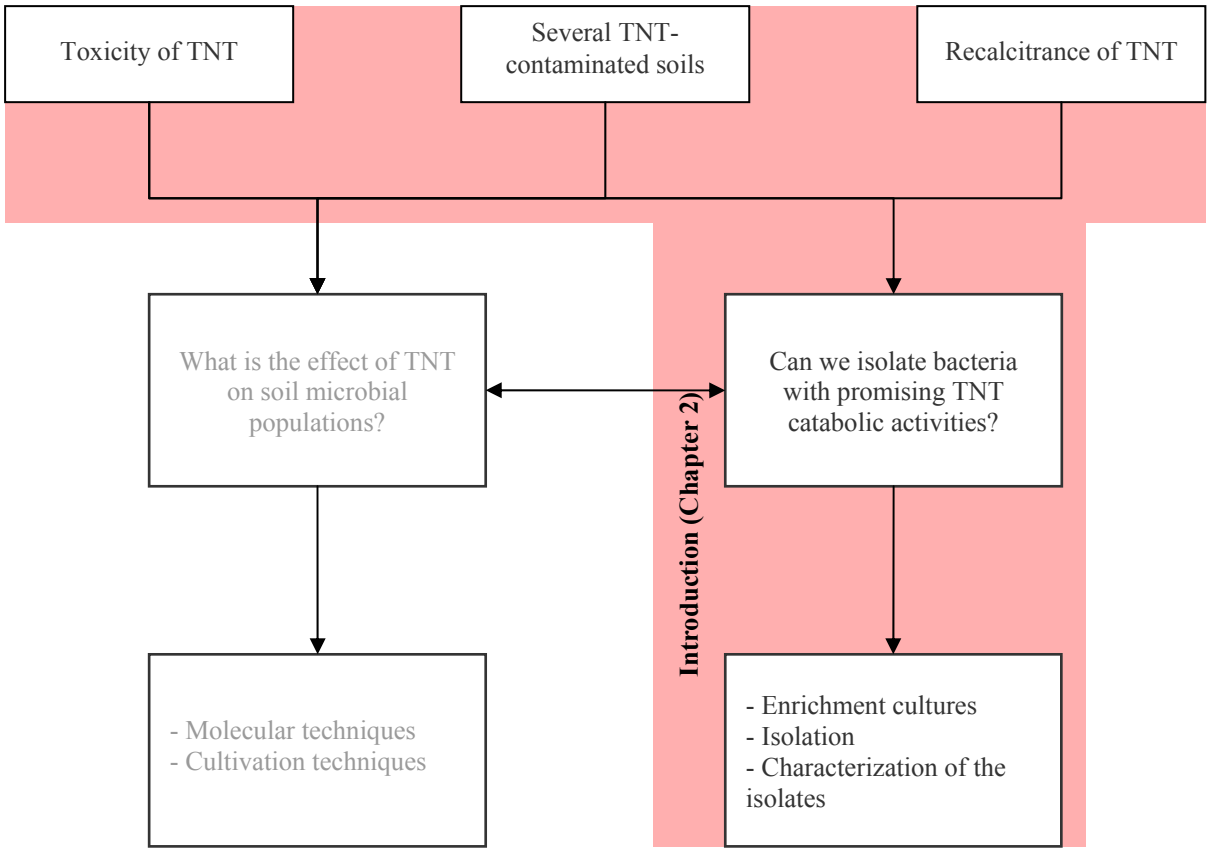
Chapter 4 compares hybridizations of in vitro transcribed 16S rRNA derived from an uncontaminated and a TNT-contaminated soil sample to a phylogenetic microarray with thermal dissociation analysis.

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?
Introduction (Chapter 2)
- Molecular techniques
- Cultivation techniques
- Enrichment cultures
- Isolation
- Characterization of the isolates

Promising strategies for the mineralisation of 2,4,6-trinitrotoluene

Ben Stenuit, Laurent Eyers[1], Saïd El Fantroussi & Spiros N. Agathos*

Unit of Bioengineering, Catholic University of Louvain, Place Croix du Sud 2/19, 1348 Louvain-la-Neuve, Belgium

*Author for correspondence.
Phone: +32-10-47-36-44
Fax: +32-10-47-30-62

Reviews in Environmental Science and Bio/Technology (2005) 4: 39-60

[1] L. Eyers participated in the writing of this paper and reviewed it.

2.1. Abstract

2,4,6-trinitrotoluene (TNT) is known to be one of the most common military explosives. In spite of its established toxicity and mutagenicity for many organisms, soils and groundwater are still being frequently contaminated at manufacturing, disposal and TNT destruction sites. The inability of natural aquatic and soil biota to use TNT as growth substrate has been recognized as the primary limitation in the application of bioremediation processes to contaminated environments. However, promising degradation pathways have been recently discovered which may lead to the mineralisation of TNT. Significant advances have been made in studying the mechanism of TNT denitration, which can be considered as the major reaction and the driving force towards beneficial biodegradation. The possibilities to favour TNT denitration are discussed based on current knowledge of the enzymology and genetics of denitration in nitroaromatic degrading organisms. The literature survey demonstrates that the only enzymes characterized so far for their denitrase activity towards TNT belong to the class I flavin-dependent β/α barrel oxidoreductases, also known as the "Old Yellow Enzyme" family. In addition, this review provides an overview of strategies and future directions towards a rational search for new catabolic activities, including metagenomic library screening, plus new possibilities to improve the activity of known catabolic enzymes acting on TNT, such as DNA shuffling.

2.2. Introduction

2.2.1. Xenobiotic compounds: a challenge for microorganisms

The term xenobiotic comes from the Greek "xenos", foreign, and "bios", life, and refers to chemically synthesized compounds containing structural elements or substituents that do not (or seldom) occur in nature. Therefore, their structure is not easily recognized by existing degradative enzymes and, as a consequence, they accumulate in the environment (Peres & Agathos 2000). This means that the natural metabolic diversity of microbes is insufficient to protect the biosphere from anthropogenic pollution. At first sight, this observation contrasts with the fact that microorganisms excel at using organic substances, natural or synthetic, as sources of nutrients and/or energy. Accordingly, microorganisms are the best candidates among all living organisms to funnel xenobiotic compounds into natural biogeochemical cycles. However, microorganisms have only recently been exposed to xenobiotic compounds and in many cases have not developed the necessary enzymes to use these compounds as sole source of nutrients and/or energy. In contrast to existing catabolic pathways that are the outcome of a long evolutionary process, one consequence of the appearance of new synthetic organic chemicals in the environment is the remarkably rapid evolution of microorganisms (Timmis & Pieper 1999; Rieger et al. 2002). In other words, the resulting gigantic "library" of microbial enzymes serves as raw material for further evolution whenever a new xenobiotic chemical becomes available (Dua et al. 2002). For example, it has been suggested that the atrazine degradative pathway has been assembled recently, over a period of decades (Martinez et al. 2001; Seffernick & Wackett 2001). In addition, repeated atrazine application in soil can lead to a rapid emergence of degradative microorganisms (Goux et al. 2003). Thus, due to their metabolic versatility and evolutionary potential, microorganisms are very promising at degrading an amazing spectrum of noxious xenobiotics such as nitroaromatic chemicals.

2.2.2. Nitroaromatic compounds

Only few aromatic compounds, bearing one nitro group on the aromatic ring, are produced by microorganisms. Natural nitroaromatic compounds described to date are chloramphenicol (Vats et al. 1987), aureothin (He & Hertweck 2003), phidolopin (Ayer et al. 1984),

nitropyoluteorin (Ohmori et al. 1978), and oxypyrrolnitrin (Hashimoto & Hattori 1966). Nitroaromatic compounds can also be formed abiotically, for example by photochemical reactions in the atmosphere (Crawford 1995). The majority of nitroarenes present in the biosphere comes from man-made sources. These compounds are used worldwide as explosives, pesticides and raw materials for the synthesis of many products, such as polyurethane foams, dyes, polymers and pharmaceuticals. Due to the massive industrial use of these chemicals in the last 75 years, together with the intense military activity of the two World Wars, nitroaromatic compounds are widely distributed in the environment. These nitroaromatic compounds differ greatly in their recalcitrance to biodegradation. The mineralization of several mono-and dinitroaromatic compounds is possible by an oxygenase attack mechanism (Ye et al. 2004 and references therein). In contrast, trinitroaromatic compounds, such as 2,4,6-trinitrotoluene, exhibit a great recalcitrance and often persist for decades in soils, sediments and groundwater.

2.2.3. TNT: a toxic chemical compound widespread in many countries

2,4,6-trinitrotoluene (TNT) is a trinitroaromatic compound (Figure 1).

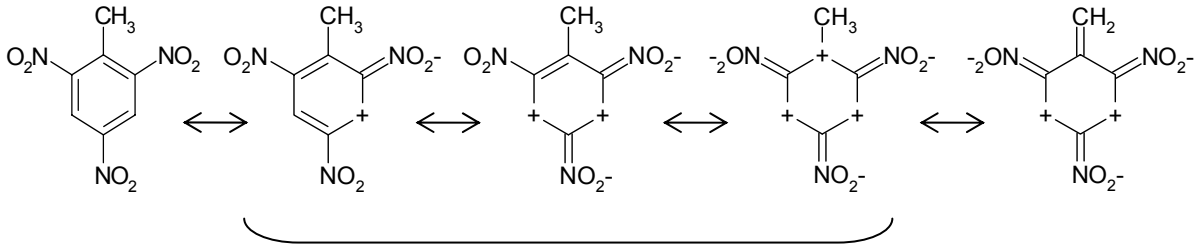


Figure 1. Canonical forms describing the resonance hybrids of TNT, adapted from Rieger and Knackmuss (1995). (a) One form of the Kekulé structure, (b) some of the potential ionic contributions, (c) one form describing the hyperconjugative effect of the methyl group.

This xenobiotic was first synthesized by Willbrand in 1863 by stepwise nitration of toluene (Davis 1943). In 1891, Haussermann with the "Chemische Fabrik Griesheim" undertook the production of TNT on an industrial scale. After 1901, its use as military explosive rapidly became generalized among the great powers, because of its low melting point, stability, low sensitivity to impact, friction and high temperature, and relatively safe methods of

manufacture (Urbanski 1964). TNT is generally considered to be satisfactory for all military purposes and has been frequently used as a solid secondary high explosive in armaments, either by itself or in mixtures such as Composition B, Octol, Pentolite, Cyclotol, Baratol, H-6, and Amatol (Kury et al. 1999). During World War II, Germany produced approximately 800 000 tons of TNT (Daun et al. 1998) and the highest amount of TNT was produced at the end of World War II, when the world production was estimated to be 150 000 tons per month (Snellinx et al. 2002). Contamination of soils and waters with TNT resulted from various military activities (manufacturing, handling, disposal (open burning/open detonation), demilitarisation, testing and training of explosives and propellants). At the present time, the US Army, which now uses only rarely TNT, has still millions of kilograms of explosives that have reached the end of their shelf-life, but that are difficult to dispose of, because of environmental concerns (Gilles 2004). The US Army alone has estimated that over 1.2 million tons of soil have been contaminated with explosives, and the impact of such contamination in other countries is of similar magnitude (Lewis et al. 2004). The concentration of TNT in "hot spots" of contaminated soil can reach 50 g/kg soil (Conder et al. 2004), with the highest levels of TNT contamination directly on or near the soil surface. In Canada, there were in 2000 an estimated 103 training sites (air and land force bases) and three open burning/open detonation sites associated with activities involving TNT (Hawari et al. 2000). In Australia, some site characterization has been done and, in the rest of the world, the scope of the problem is significant (Spain 2000). For instance, countries that have suffered serious armed conflicts (e.g., recent wars in Africa, in the Middle East and in Eastern Europe) may be faced with similar problems, even though data on the extent of explosives contamination and the need to decontaminate polluted environments are not available.

In addition to its worldwide distribution, the toxicity of TNT and its degradation products is well documented. TNT is toxic to many living organisms, from bacteria to humans (Lachance et al. 2004). For instance, the concentration of TNT causing 50 % lethality (LC_{50}) in *Daphnia magna* is around 5 mg/l (Johnson et al. 1994) and $\geq$ 143 mg/kg dry soil for the earthworm (*Eisenia andrei)* (Lachance et al. 2004; Robidoux et al. 2004). In addition, the concentration of TNT required for 50% inhibition of growth (EC_{50}) for the green alga *Selenastrum capricornutum* is around 0.9 mg/l (Sunahara et al. 1998) and ranges from 2 to 10 mg/l for the fungus *Suillus variegates* (Meharg et al. 1997). Fuller and Manning (1997; 2004) observed that the growth of pure cultures of aerobic Gram-positive bacteria was severely inhibited by concentrations of TNT as low as 10 mg/l, whereas most Gram-negative organisms were

unaffected by concentrations close to the water solubility of TNT (± 100 mg/l). Last but not least, TNT is mutagenic in the Ames test (Kaplan & Kaplan 1982). The toxic action of nitroaromatic compounds is frequently caused by enzymatic single-electron reduction of the nitro groups (Purohit & Basu 2000 and references therein), inducing an oxidative stress by the formation of reactive oxygen species (Peres & Agathos 2000; Kumagai et al. 2004). This single-electron reduction is mediated by bacterial oxygen-sensitive nitroreductases (type II) (Sarlauskas et al. 2004), that are also present in mammalian tissues (Ask et al. 2004). Redox cycling yields a nitrobenzene anion radical that can react with oxygen to form a superoxide radical and the original nitro group (Peterson et al. 1979). The phenomenon is also called a "futile cycle" because reduced pyridine nucleotides are oxidized without net reduction of the nitro group (Schenzle et al. 1999). In addition, reduction products can bind covalently to proteins and DNA (Sarlauskas et al. 2004). Finally, oxidation of the haemoglobin iron (Peres & Agathos 2000) is another mechanism of toxicity, as well as uncoupling of oxidative and photosynthetic phosphorylations (Donlon et al. 1995). Therefore, in response to growing concern about the health effects and the ecological threats posed by TNT, numerous recent studies have been focused on its toxicity (Dodard et al. 2004; Patel et al. 2004; Schaefer 2004), environmental fate (Lynch et al. 2003; Kim et al. 2004; Weiss et al. 2004b; Weiss et al. 2004c), detection methods (Altamirano et al. 2004; Borch & Gerlach 2004; Buryakov 2004; Charles et al. 2004; Jung et al. 2004; Weiss et al. 2004a), size and mass distributions of TNT particles scattered by detonation (Taylor et al. 2004) and, last but not least, (bio)degradation.

2.2.4. TNT recalcitrance and non-beneficial biodegradation pathways

The recalcitrance of TNT is well understood. The observation of TNT contaminated soils, 50 years after World War II, indicates that the intrinsic bioremediation capacity is very limited. The recalcitrant character of TNT is due to a high electron deficiency on the entire π-electron system (electophilic nucleus), generated by the strong electron-withdrawing properties of the nitro substituents (Rieger & Knackmuss 1995). In addition, steric effects are important due to the symmetric position of the four functional groups. Consequently, the π-electrons of the aromatic ring of TNT are shielded from any attack by classical oxidative and electrophilic mechanisms (Heiss & Knackmuss 2002), whereas oxygenase reactions do take place with mono- and dinitrotoluene compounds (Peres & Agathos 2000; Johnson et al. 2002). The

electronic nature of TNT is primarily responsible for the resistance to oxygenase attacks rather than the steric hindrance (Johnson et al. 2001). Instead of an effective and beneficial attack of the aromatic ring by microorganisms, almost all TNT biodegradation studies have shown that enzymatic and abiotic reactions on the molecule preferably take place at the external functional groups, leaving the stable aromatic system intact (Hawari et al. 2000). Although oxidation of the methyl group was reported in a rare study by Vanderberg et al. (1995), almost all TNT biodegradation studies show that the nitro groups, due to their electrophilic character, are subject to an initial reductive transformation (Figure 2).

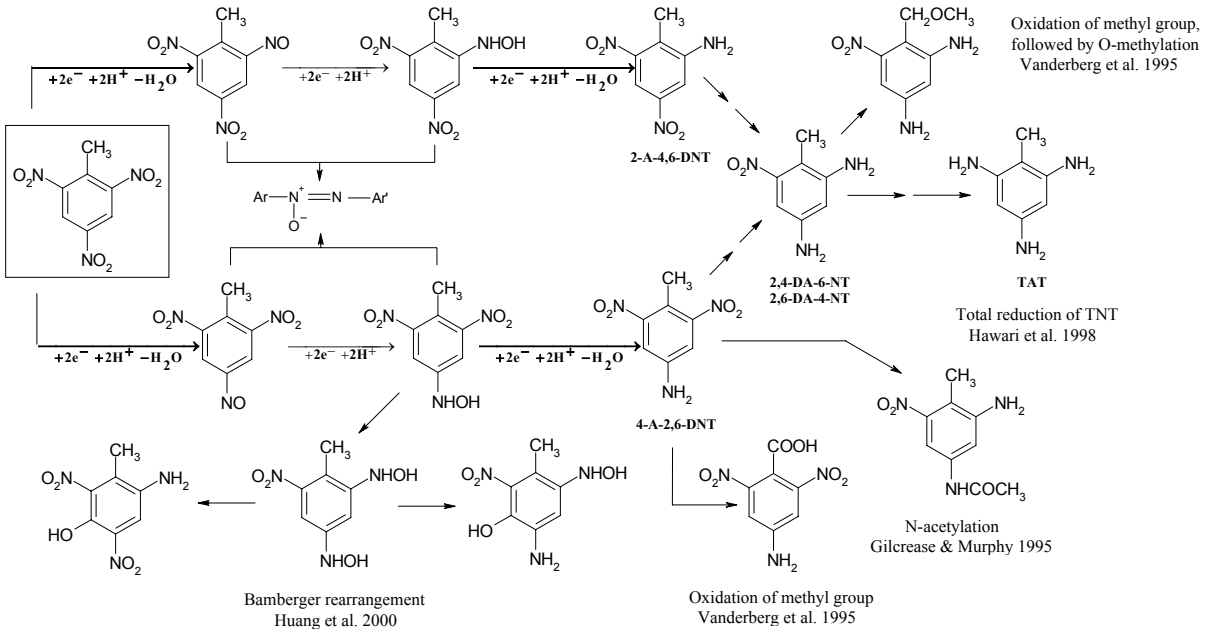


Figure 2. Non-beneficial and misrouted biotransformation pathways for 2,4,6-trinitrotoluene.

Most of these bacterial TNT-transformations belong to the class of fortuitous or gratuitous metabolism (cometabolism). This is therefore a non-beneficial process for the cell, in contrast to beneficial degradation, by which the microorganism can derive carbon, nitrogen and/or energy from the substrate. The reduction of TNT can be catalysed by a whole range of organisms, from bacteria to mammals (Peres & Agathos 2000). Most nitroreductases purified from bacteria are type I soluble flavoproteins, oxygen-insensitive, that use NAD(P)H as electron donors (Riefler & Smets 2000). The bacterial reduction of an aromatic nitro group to an amino group occurs as a series of two-electron transfers to yield successively nitroso, hydroxylamino, and amino derivatives of the parent compound (Esteve-Núñez et al. 2001). Amino derivatives include aminodinitrotoluene (4-amino-2,6-dinitrotoluene (4-A-2,6-DNT) and 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT)) and diaminonitrotoluene chemicals (2,4-

diamino-6-nitrotoluene (2,4-DA-6-NT) and 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT)). These partially reduced intermediates are recalcitrant and are of great concern due to their higher solubility, toxicity and genotoxicity compared to the parent compound (Snellinx et al. 2002). Under strictly anaerobic conditions ($E_H \leq$ -200 mV), these amino derivatives can be further transformed to an unstable and electron-rich intermediate, 2,4,6-triaminotoluene (TAT). All of these TNT derivatives can undergo further transformation to chemicals that remain recalcitrant. For instance, under aerobic conditions, nitroso- and hydroxylamino-dinitrotoluene (respectively NODNTs and HADNTs) intermediates can react with each other to produce tetranitroazoxytoluene compounds (e.g. 4,4',6,6'-tetranitro-2,2'-azoxytoluene and 2',4,6,6'-tetranitro-2,4'-azoxytoluene). Also, a fraction of HADNTs was found to rearrange to their corresponding phenolamines through a Bamberger rearrangement, the latter compounds accumulating in the medium (Hughes et al. 1998; Huang et al. 2000). The amino derivatives can be further transformed to acetylated (Gilcrease & Murphy 1995) and oxidized (Vanderberg et al. 1995) products. In addition, amino derivatives can interact with the soil matrix. In the case of TAT, its reactivity towards electrophiles and oxidizing agents can produce azo and phenolic compounds (Hawari et al. 1998), but no effective mineralisation is observed (Heiss & Knackmuss 2002).

All cometabolic processes cited above contribute to the formation of "dead-end" secondary products that misroute the TNT mineralisation process (Figure 2). This frequently observed chemical misrouting leads to the accumulation of metabolites identified as major recalcitrant co-contaminants of TNT. Such metabolites may have partition coefficients widely different from those of the original compound. For example, metabolites derived from a range of pesticide types have generally an organic carbon adsorption (K_{oc}) of a least one order of magnitude lower than that the corresponding parent compound (Boxall et al. 2004). Thus, these substances may be more likely to be transported from soils to surface and groundwaters. However, in the case of TNT-reduced metabolites, whereas an electron donor-acceptor (EDA) mechanism is assumed to account for the strong affinity of TNT on negatively charged 2:1 clay mineral surfaces saturated by low hydrated cations (Li et al. 2004), the reduced TNT metabolites are subject to covalent binding with soil organic matter (Eriksson et al. 2004). The covalent binding of the reduced amino groups of ^{15}N-TNT on ^{13}C-enriched organic matter has been demonstrated (Knicker 2003), thanks to recent advances in the application of NMR to environmental microbiology (Grivet et al. 2003). This binding is the basis of bioremediation processes such as windrow composting and bioslurries, where cometabolic

TNT transformations and subsequent incorporation of resulting reduced metabolites into the soil matrix is exploited (Knicker et al. 2001; Thorn et al. 2002). Although these processes are simple and cost-effective, the lack of information about the long-term fate of the bound metabolites is a real problem. It is a particularly critical issue since significant transport of potentially toxic TNT derivatives into surface- and groundwaters can take place by leaching of dissolved soil organic matter (DOM) in contaminated soils (Eriksson & Skyllberg 2001; Fauser & Thomsen 2002; Eriksson et al. 2004). In addition, another study has shown that a natural surfactant, i.e. cyclodextrin, is able to desorb and solubilize TNT and its metabolites from contaminated soils (Sheremata & Hawari 2000).

The widespread capacity of microbes to transform TNT into reduced metabolites has deviated investigations towards microbial immobilization technologies, such as bioslurry and composting processes. However, as mentioned above, mineralisation of TNT is preferable. To date, with the exception of white-rot fungi secreting powerful ligninolytic peroxidases (Van Aken & Agathos 2001), no significant mineralisation has been detected in biological systems (Van Aken et al. 2004). However, there are a number of recent reports describing a possible bacterial mineralisation of TNT *via* denitration activities.

In conclusion, there is a need to develop new strategies to search for cost-effective remediation technologies leading to the mineralisation of TNT. To carry out bioremediation *in situ*, it is necessary to increase the rate and extent of ongoing natural processes or to stimulate and sustain the metabolic activity of bacteria within the contaminated media.

2.3. Beneficial biodegradation pathways of nitroaromatic compounds

Before presenting promising pathways for the mineralisation of TNT, it is necessary to give a short overview of the biodegradation of mono- and dinitro-aromatic chemicals to understand the potential of TNT-metabolising activities leading to the production of the latter chemicals. There are multiple pathways allowing nitro- and dinitroaromatic substances to serve as carbon, energy, and nitrogen sources, such as the bacterial pathway for the degradation of 2,4-dinitrotoluene (Johnson & Spain 2003). In this context, a beneficial biodegradation of TNT produces metabolites, such as dinitrotoluene isomers, which can be mineralised *via* the catabolic activity of the same microorganism or *via* a syntrophic catabolism.

2.3.1. Biodegradation of nitroaromatic compounds: classical routes for mono- and dinitroaromatic compounds

Nitroaromatic compounds are catabolised by diverse pathways, and the removal of the nitro groups can occur by both oxidative and reductive reactions (Marvin-Sikkema & de Bont 1994; Crawford 1995; Nishino et al. 2000b; Peres & Agathos 2000). Catabolic enzymes and coding genes from a variety of bacteria are well characterised (Johnson et al. 2002; Johnson & Spain 2003; Ye et al. 2004).

2.3.1.1. Oxygenolytic denitration of nitroaromatic compounds

Mono- and dinitro-aromatic compounds can be degraded by an initial attack under aerobic conditions through oxygenases. This class of enzymes has the property of catalysing the incorporation of molecular oxygen into the aromatic moiety, with NAD(P)H as a cofactor. Monooxygenases generally catalyse the addition of a single hydroxyl group to compounds that already contain such a group, like 4-nitrophenol (Jain et al. 1994) and 2-nitrophenol (Zeyer et al. 1986). This initial monooxygenase attack on the aromatic ring is followed by the subsequent release of the nitro group as nitrite. In contrast, aromatic ring dioxygenases initiate the degradation of a whole range of nitroaromatic compounds through addition of two hydroxyl groups, as it is the case for nitrobenzene (Lessner et al. 2002), 2-nitrotoluene

(Haigler et al. 1994), 3-nitrobenzoate (Nadeau & Spain 1995), 2,4-dinitrotoluene (Spanggord et al. 1991; Suen & Spain 1993; Haigler et al. 1996; Suen et al. 1996) and 2,6-dinitrotoluene (Nishino et al. 2000a). The addition of two hydroxyl groups results in a hypothetical partially reduced and unstable diol intermediate, that rearranges to a catechol compound (1,2-dihydroxybenzene) with the release of nitrite. Oxidative ring cleavages are catalysed by a dioxygenase in the presence of NADPH through either *ortho*-ring cleavage between two hydroxyl groups or *meta*-ring cleavage at the bond proximal to one of two hydroxyl groups (Ye et al. 2004).

2.3.1.2. Partial reduction of nitroaromatic compounds, followed by beneficial biodegradation

Instead of a direct attack by oxygenases, another pathway consists in the reduction of the nitro to a hydroxylamino group, which is further released as ammonia. The hydroxylamino rearrangement and the release of ammonia are catalysed either by a lyase or a mutase to provide respectively a dihydroxy or an aminophenol compound. Such reactions are taking place in the catabolism of 4-nitrobenzoate (Groenewegen et al. 1992; Yabannavar & Zylstra 1995) where the hydroxylamino compound is converted to protocatechuate in a reaction catalysed by a hydroxylaminolyase. This pathway contrasts with the gratuitous and unspecific reductions of the nitro groups of 2,4,6-trinitrotoluene to amino groups mediated by bacteria. In this nitrobenzoate pathway, the reduction of the nitro group is beneficial and mediated by inducible and specific reductases that chemoselectively stop at the level of the hydroxylamino group (Schenzle et al. 1999). A similar pathway leads to the catabolism of 4-nitrotoluene (Haigler & Spain 1993; Rhys-Williams et al. 1993). However, with this compound, sequential oxidation of the methyl group to 4-nitrobenzoate occurs at first. Then, the resulting compound is assimilated by the aforementioned reactions (Groenewegen et al. 1992). Alternatively, the hydroxylamino group can undergo a mutase-mediated rearrangement to *ortho*-aminophenols, by a reaction similar to the nonenzymatic acid-catalysed Bamberger rearrangement (Schenzle et al. 1997; Schenzle et al. 1999; Davis et al. 2000). The aminophenols serve as ring fission substrates with the production of aminomuconic semialdehyde in a dioxygenase-mediated *meta*-ring cleavage and subsequent release of ammonia (He & Spain 1997; Spiess et al. 1998). Such an initial reduction of the nitro group to hydroxylamino by chemospecific reductases together with mutase-catalysed rearrangements has been described respectively in

Burkholderia cepacia and *Ralstonia paucula* strains capable of mineralising both 4-nitro- and 4-aminobenzoate (Peres et al. 2001). Remarkably, the main 4-nitrobenzoate degradation pathway did not have 4-aminobenzoate as an intermediate and the degradation of the two compounds involved distinct pathways converging into protocatechuate (Peres et al. 2001).

2.3.1.3. Complete reduction of nitroaromatic compounds as a mineralisation strategy

Alternatively, several microorganisms reduce the nitro group to an amino group, via a nitroso and hydroxylamino group. The aminoaromatic product can be further degraded to catechol by an aniline oxygenase. The catechol produced is further mineralised by ring-cleavage reactions and liberation of ammonia (Marvin-Sikkema & de Bont 1994). For instance, five bacterial strains have been found to metabolise both aniline and 3-chloroaniline by oxidative deamination (Boon et al. 2001). However, this catabolic pathway requires at least one more reducing equivalent than pathways with hydroxylamino as key metabolites and is less efficient for bacterial growth and little reported in the literature (Schenzle et al. 1999). Thus, the beneficial degradation of an aminoaromatic compound through the complete reduction of a nitro into an amino group has never been convincingly proven in the same bacterium. As stated above, the only cases described in the literature where both 4-nitrobenzoate and its corresponding amino compound, 4-aminobenzoate, are mineralised by the same bacteria, involved two distinct pathways rather than passage of nitro- through aminobenzoate (Peres et al. 2001).

2.3.2. Attempts to favour mineralisation of TNT

Structurally, TNT is similar to 2,4-dinitrotoluene and 2,6-dinitrotoluene, suggesting that dioxygenases could also hydroxylate TNT. However, when the number of nitro groups increases, the likelihood of a dioxygenase-catalysed reaction decreases (Peres & Agathos 2000). Because of the electron-withdrawing character of the nitro groups, each additional nitro group generates a high redox potential and increases the electron deficiency of the ring. This deficiency hinders the electrophilic attack by oxygenases. Instead, the presence of multiple nitro groups thermodynamically favours their reduction into amino groups (Rieger & Knackmuss 1995). Therefore, there are no reports describing oxidative attack of TNT.

2.3.2.1. Oxidative transformation of reduced metabolites of TNT

One strategy to favour the mineralisation of TNT is to take advantage of the ubiquitous reduction of nitro groups to amino groups. The formation of reduced TNT metabolites increases the electron density of the ring, and thus its reactivity towards further oxidative metabolism (Lenke et al. 2000). For instance, the mineralisation of TNT by wood rotting fungi takes place when TNT reduced metabolites are produced (Van Aken & Agathos 2001). In addition, Johnson and co-workers (2001) observed that oxygenolytic transformation of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene can occur with purified multicomponent nitroarene dioxygenases. The reaction produced a dihydroxylated aromatic compound with concomitant release of nitrites (Johnson et al. 2001). Subsequent degradation of the aminodinitrotoluene oxidation products was not examined, but this discovery reveals the potential of extensive bacterial transformation of TNT under aerobic conditions.

2.3.2.2. TNT denitration

Another possibility to favour mineralisation of TNT consists in a direct denitration of the molecule, without passing by a TNT-reduced intermediate. In this case, denitration activities leading to the production of mono- and dinitroaromatic compounds are very promising for the complete mineralisation of the molecule. Indeed, as described in the previous section, the removal of a nitro group will produce mono- and dinitroaromatic compounds that can be further mineralised. This route might be really profitable to microorganisms since TNT could be used as nitrogen source. Therefore, one strategy consists in obtaining enrichment cultures with TNT as sole nitrogen source to select for bacteria with TNT denitration abilities.

a. TNT denitration through Meisenheimer-complex formation

Few reports describe a possible denitration of TNT and concomitant formation of mono- and/or dinitroaromatic compounds. Table 1 summarizes studies reporting TNT denitration.

Table 1. TNT denitration reported in the literature, experimental conditions and biochemical mechanism

Conditions / Strain / Purified enzyme	**Denitration mechanism**	**Reference**
Aerobic enrichments / Mixed microbial culture	Unknown mechanism	(Tront & Hughes 2005)
Aerobic enrichments / Mixed microbial culture	Unknown mechanism	(Popesku et al. 2004)
Aerobic / Members of the Old Yellow Enzyme family	Formation of H-TNT[(1)] and 2H-TNT[(2)] complexes and selective protonation. The protonated forms might then be either hydroxylated or oxidized to yield nitrite	(Williams et al. 2004)
Anoxic enrichments / Presence of ferric oxyhydroxide	Unknown mechanism	(Eyers et al. 2004b)
Aerobic / *B. subtilis* SK1	Unknown mechanism	(Kurinenko et al. 2003)
Aerobic / *Klebsiella* sp. C1	Unknown mechanism	(Kim et al. 2002)
Aerobic / XenB reductase from *P. fluorescens* I-C	Dimerization reaction of the HADNT[(3)] isomers and 2H-TNT·H^{+}[(4)] complexes, subsequent formation of amino-dimethyltetranitrobiphenyl and nitrite release	(Pak et al. 2000)
Anoxic / *P. putida* JLR11	Unknown mechanism, key intermediates in the beneficial removal of nitro groups identified, such as 2,4,6-trinitrobenzaldheyde and 2-nitro-4-hydroxybenzoic acid	(Esteve-Núñez & Ramos 1998)
Aerobic / PETN reductase from *E. cloacae*	Formation of H-TNT and 2H-TNT complexes and suggested rearomatization with nitrite release	(French et al. 1998)
Aerobic / *Bacillus* sp.	Unknown mechanism	(Kalafut et al. 1998)
Aerobic / *P. savastanoi*	Unknown mechanism	(Martin et al. 1997)
Aerobic / *P. pseudoalcaligenes* JS52	Unknown mechanism, DHANT[(5)] as key intermediate	(Fiorella & Spain 1997)
Aerobic / *Mycobacterium* sp.	Formation of H-TNT complex and suggested rearomatization with nitrite release	(Vorbeck et al. 1994)

[1] H-TNT : hydride-Meisenheimer complex

[2] 2H-TNT : dihydride-Meisenheimer complex

[3] HADNT : hydroxylaminodinitrotoluene isomers

[4] 2H-TNT·H^{+} : protonated tautomers of dihydride-Meisenheimer complex

[5] DHANT : dihydroxylaminonitrotoluene isomers

Different mechanisms have been reported for this transformation and are summarized in Figure 3.

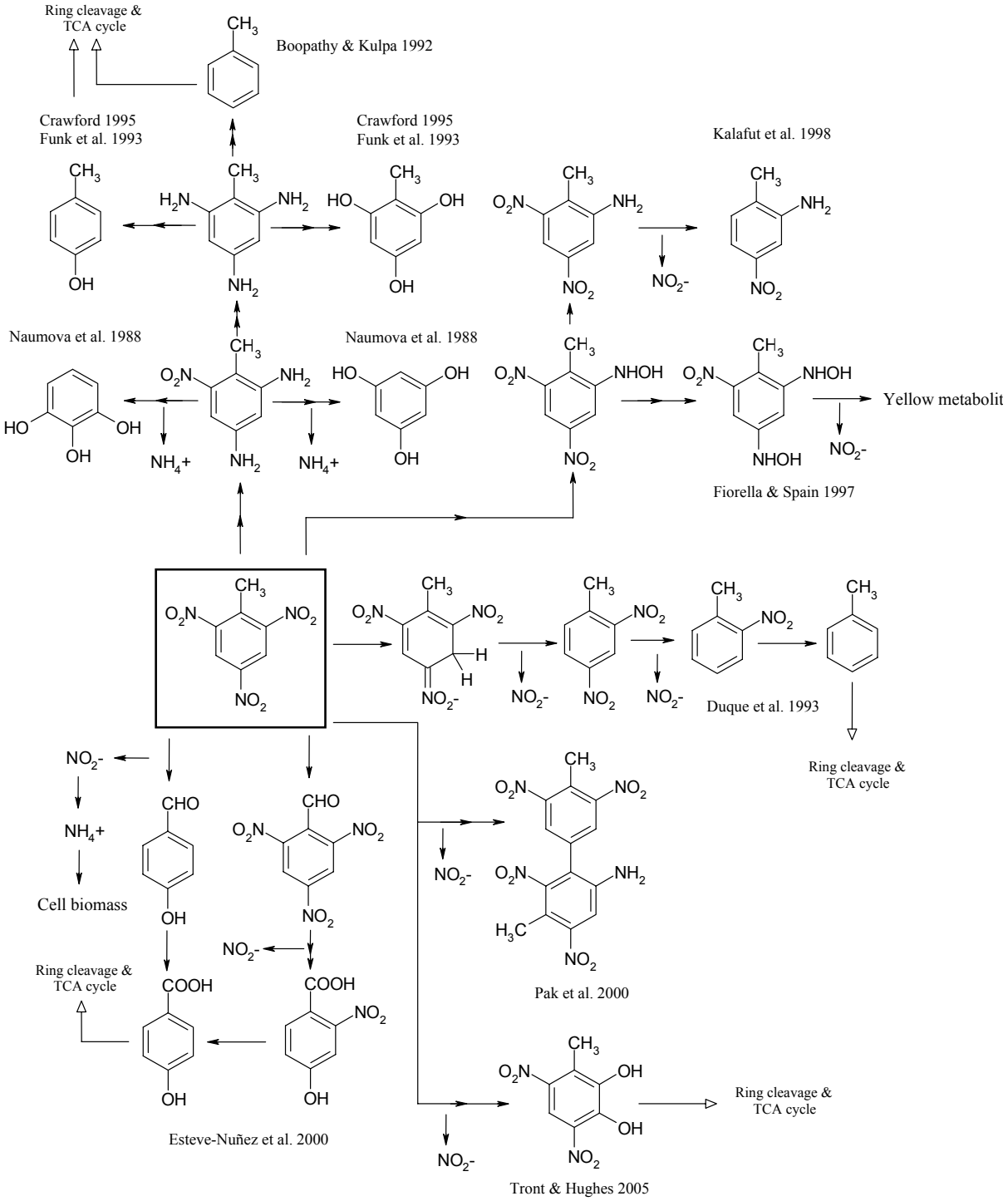


Figure 3. Different beneficial biodegradation pathways for 2,4,6-trinitrotoluene reported in the literature. The arrows with blank arrowhead represent putative ring cleavage and subsequent incorporation in the TCA cycle, which can be mediated by the same microorganism or by a syntrophic process.

The best characterized mechanism proposed for TNT denitration is a direct $2e^-$ reduction of the aromatic ring catalysed by bacteria under aerobic conditions. A similar mechanism is also observed with another trinitroaromatic compound, i.e. picric acid (2,4,6-trinitrophenol) (Lenke & Knackmuss 1992; Lenke et al. 1992; Behrend & Heesche-Wagner 1999; Ebert et al. 1999; Rieger et al. 1999; Heiss et al. 2002; Heiss et al. 2003; Hofmann et al. 2004). The initial attack consists in the addition of hydride (negatively charged nucleophile form of atomic hydrogen H^-) to polynitroarenes to form Meisenheimer complexes (Lenke & Knackmuss 1992). For example, Duque et al. (1993) isolated a *Pseudomonas* sp. clone A using TNT as sole nitrogen source with successive removal of nitro groups to yield respectively 2,4-dinitrotoluene and 2,6-dinitrotoluene, 2-nitrotoluene and toluene. Haïdour & Ramos (1996) also studied this bacterium to analyze more precisely its TNT metabolism. These authors suggested a nucleophilic attack by hydride ions to the aromatic system followed by rearomatization to hydride-Meisenheimer complexes (H-TNT). Subsequently, nitrite release and production of 2,4-dinitrotoluene was proposed (Haïdour & Ramos 1996). However, reductive denitration with this strain could not be confirmed by Vorbeck et al. (1998). These latter authors showed that products of direct hydride transfer included also a dihydride-Meisenheimer complex (2H-TNT) and several protonated tautomers of 2H-TNT (2H-TNT·H^+) (Figure 4).

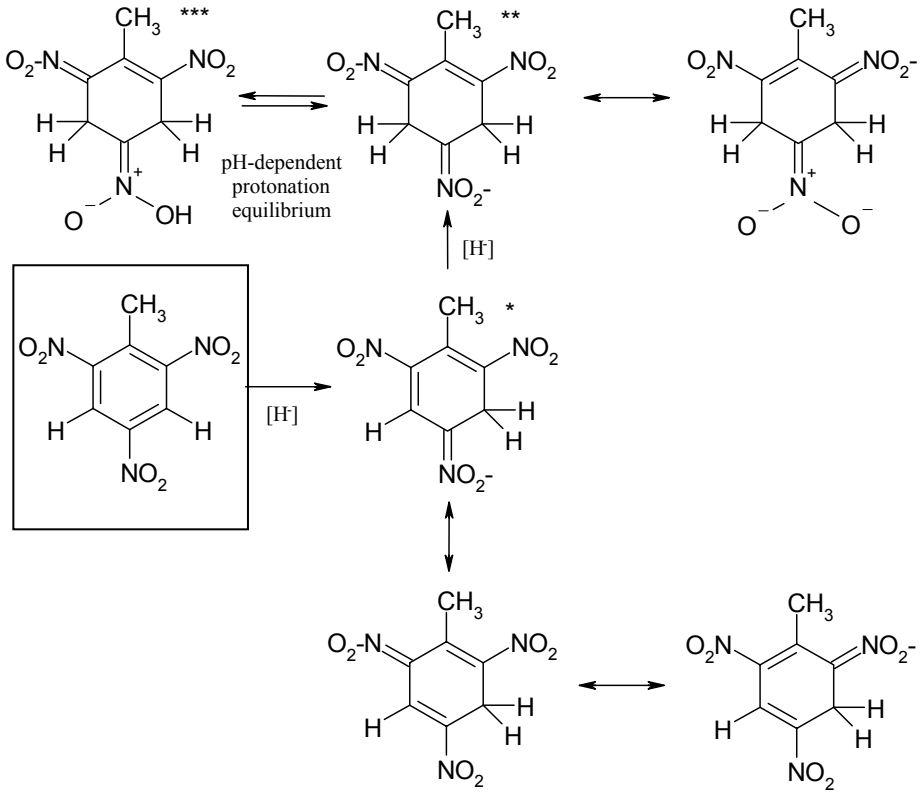


Figure 4. Attack of the aromatic ring of TNT by a hydride anion at C3 and formation of the Meisenheimer complex (H-TNT) (*), C3-C5 2H-TNT dihydride Meisenheimer complex (**), protonated 2H-TNT (***) and some resonance forms (adapted from Vorbeck et al. (1998) and Lenke et al. (2000)).

They also postulated that this route of dihydride complex formation is non-beneficial for the catabolism of TNT. In another report of Vorbeck et al. (1994), TNT biodegradation was studied with a *Mycobacterium* sp. strain HL 4NT-1 that was isolated after enrichment with 4-nitrotoluene as sole nitrogen source under aerobic conditions. In resting cell experiments, the authors detected the formation of a hydride-Meisenheimer complex and subsequent liberation of nitrite. However, this *Mycobacterium* strain did not use TNT as sole N-source (Vorbeck et al. 1994). On the other hand, French and coworkers (1998) investigated TNT biodegradation with an *Enterobacter cloacae* PB2 strain originally isolated from an explosive-contaminated site on the basis of its ability to grow with nitrate esters as sole nitrogen source. They identified a NADPH-dependent flavoprotein reductase, i.e. PETN reductase (Binks et al. 1996; Khan et al. 2002). This enzyme, like many other flavoenzymes, is able to reduce the nitro group of TNT and form hydroxylamino- and aminodinitrotoluene compounds thanks to gratuitous nitro group reduction. However, PETN reductase can also catalyse the addition of hydride to the aromatic ring of TNT, yielding hydride and dihydride Meisenheimer

complexes. In contrast to the *Mycobacterium* sp. of Vorbeck et al. (1994), *E. cloacae* PB2 was found to be able to grow with TNT as sole nitrogen source under aerobic conditions (French et al. 1998). Isomers of hydroxylaminodinitrotoluene, aminodinitrotoluene or diaminonitrotoluene were identified. However, 2,4-dinitrotoluene, 2,6-dinitrotoluene and 2-nitrotoluene were not detected with this strain (French et al. 1998). The absence of dinitro- and mononitrotoluene compounds was also observed with purified PETN reductase and recombinant *E. coli* expressing PETN reductase. This lack of detection of denitrated TNT metabolites may be explained by the study of Pak and colleagues (2000) who showed that nucleophilic attack by a hydride transfer to the aromatic ring can lead to the liberation of nitrite with the concomitant formation of dimerized compounds (Figure 3). These authors observed enzymatic transformation of TNT by a purified enzyme XenB, a NADPH-dependent flavoprotein oxidoreductase which catalysed nitro group reduction and direct reduction of the aromatic ring (Pak et al. 2000). This enzyme was purified from a *P. fluorescens* I-C strain able to use nitroglycerin as sole nitrogen source (Blehert et al. 1997). XenB catalysed the reduction of TNT either by hydride addition to the aromatic ring or by nitro group reduction, with the accumulation of various tautomers of the protonated dihydride-Meisenheimer complex of TNT and hydroxylaminodinitrotoluene compounds (Pak et al. 2000). Nitrite release was observed together with the production of various amino-dimethyl-tetranitrobiphenyl isomers (Pak et al. 2000). The authors suggested that these isomers are produced by dimerization of protonated dihydride-Meisenheimer complexes with aryl cations formed from hydroxylaminodinitrotoluene compounds (Pak et al. 2000). However, it is not known if the accumulating amino-dimethyl-tetranitrobiphenyl isomers are toxic and/or recalcitrant.

b. Other beneficial pathways

A recent report revealed a novel oxidative microbial degradation of TNT, used as the sole carbon, nitrogen and energy source (Tront & Hughes 2005). These authors showed that an aerobic microbial community was able to oxidatively denitrate TNT with the formation of 3-methyl-4,6-dinitrocatechol. This is the first documented analytical evidence of the formation from TNT of a substituted catechol, a precursor of oxygenolytic ring cleavage. However, this study brings no information on the mechanism of TNT denitration (Tront & Hughes 2005). In another set of studies related to potential TNT denitration, Esteve-Nuñez and Ramos (1998) using *Pseudomonas* sp. JLR11 observed nitrite release under anoxic conditions (no electron acceptor other than TNT) with 85% of the total N-TNT content incorporated as cell biomass.

The proposed pathway involved the formation of 2-nitro-4-hydroxybenzoic acid and 4-hydroxybenzaldehyde in a mechanism that probably does not involve the formation of Meisenheimer complexes. However, elucidation of the putative denitration mechanisms is still needed. In addition, whereas biological TNT reduction is in general a cometabolic reaction, Esteve-Nuñez et al. (2000) also demonstrated with the same strain that TNT can act as a final electron acceptor in respiratory chains under anoxic conditions, just like other xenobiotic compounds such as chloroaromatic chemicals in the halorespiration process (Sanford et al. 1996; El Fantroussi et al. 1998; Fetzner 1998; Löffler et al. 1999) (Figure 5). Recent work on mutants of strain JLR11 suggests that TNT denitration allows incorporation of N via the glutamine synthetase-glutamate synthase (GS-GOGAT) system (Caballero et al. 2005).

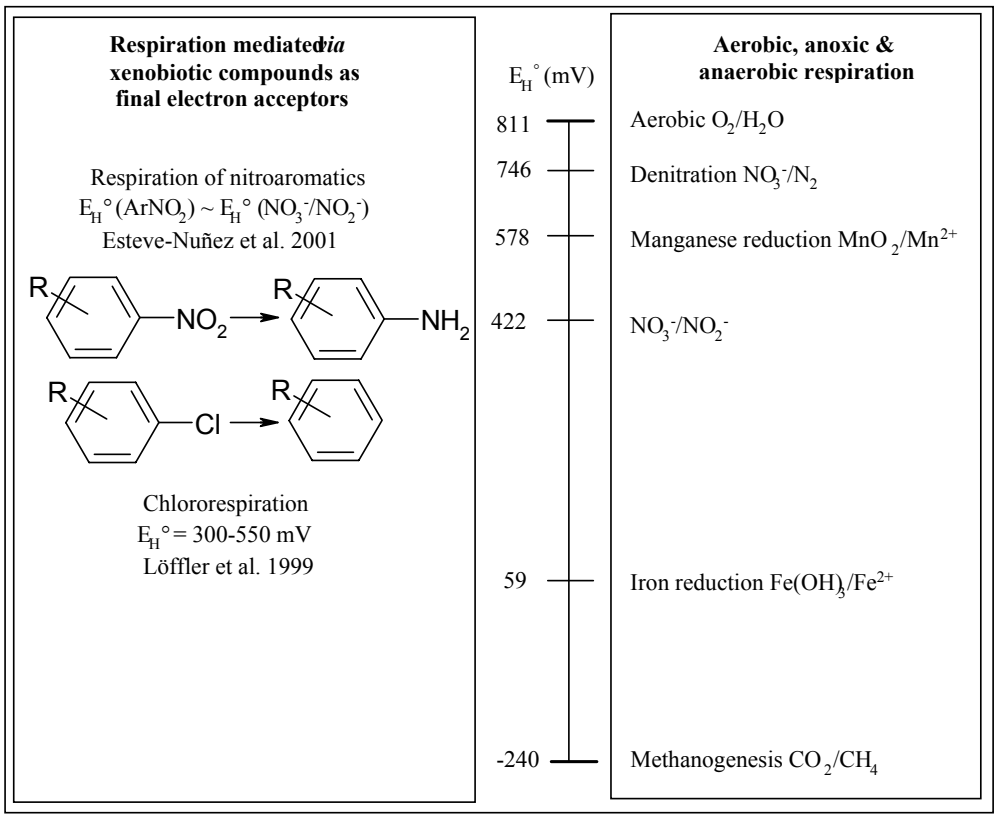


Figure 5. Redox potential for the reduction of xenobiotics in relation with some important redox couples present in nature (reduction potentials at pH 7) (adapted from Klausen et al. (1995)).

Denitration activities were also reported by Fiorella and Spain (1997) and Kalafut et al. (1998) but their mechanism needs further elucidation. In the study of Fiorella and Spain (1997), the authors detected the formation of dihydroxylaminonitrotoluene and proposed a

subsequent release of nitrite from that compound whereas Kalafut and coworkers (1998) detected the formation of 2-amino-4-nitrotoluene. Another type of denitration activity was reported by Martin and coworkers (1997) with a *P. savastanoi* strain able to denitrate TNT under aerobic conditions when it was provided as sole C-source. They identified dinitrotoluene compounds as metabolites resulting from the denitration of TNT. In another study involving a *Klebsiella* sp. strain C1 isolated from activated sludge, Kim and colleagues (2002) also observed TNT denitration with subsequent production of dinitrotoluene metabolites.

Recently, high nitrite release from TNT has been observed with TNT contaminated soil enrichment cultures under anoxic conditions, in the presence of ferric oxyhydroxide (Eyers et al. 2004b). The mechanism of denitration remains to be determined. However, the process of that study involved both high initial TNT concentrations and high nitrite release and therefore offers an exciting opportunity for the treatment of sites highly contaminated with TNT. In addition, Robertson & Jjemba (2005) investigated the mineralisation of TNT by a bacterial consortium isolated from a TNT-contaminated sediment. Nearly half of the ^{14}C-TNT was mineralised and one member of the consortium, an *Enterobacter* sp., was found to significantly contribute to the TNT mineralisation. In another recent study, Popesku et al. (2004) observed that a mixed culture was able to rapidly transform TNT and to denitrate it. This mixed culture was obtained by subculture in a basal salts medium supplemented with crude oil and other hydrocarbon sources for several years in cyclone fermentors (Ward et al. 2003). Because toluene is a component of petroleum, it is postulated that the petroleum-degrading culture of Popesku et al. (2003) could contain the TOL plasmid, which is capable of breaking the benzene ring to provide carbon for bacterial growth. This investigation to test a petroleum-degrading mixed bacterial culture for their ability to degrade TNT was inspired from the original study of Duque et al. (1993), cited previously, in which TNT was transformed into toluene, itself further mineralisable *via* the "metabolic funnel". For instance, the TOL plasmid of *P. putida* contains genes coding for the enzymes involved in funneling aromatic compounds, such as toluene and xylenes, into central metabolism. These genes coding for upper and lower pathways are clustered in two operons, independently but coordinately regulated (Abril et al. 1989). To promote complete mineralisation of TNT, Duque et al. (1993) had transferred the TOL pWWO-Km plasmid to *Pseudomonas* sp. clone A. The constructed bacterium was finally able to use TNT as C- and N-source. This hybrid pathway allowed complete mineralisation of 50% of TNT metabolites, although reduction of

nitroaromatic groups to dead-end aminoaromatic groups represented a large proportion of the mass balance.

On the other hand, Naumova et al. (1988) investigated a possible TNT mineralisation with a *P. fluorescens* strain cultivated with aminodinitrotoluene compounds as the N-source. With experiments using cell extracts, TNT was metabolized to diaminonitrotoluene compounds that were further transformed to two polar compounds: phloroglucinol (1,3,5-trihydroxybenzene) and pyrogallol (1,2,3-trihydroxybenzene). Release of 30% of the nitrogen of diaminonitrotoluene compounds as ammonium was observed (Naumova et al. 1988). However, the biochemical mechanism of this particular pathway is far from clear (Figure 3).

In another intriguing study, beneficial biodegradation of TNT through total reduction to 2,4,6-triaminotoluene (Figure 3) was reported with a *Desulfovibrio* sp. strain B, isolated from a continuous anaerobic digester (Boopathy & Kulpa 1992). With this strain, TNT was presumably reduced at first to triaminotoluene (TAT), the latter being subsequently deaminated reductively to toluene. However, this speculative pathway identified neither TAT nor the intermediates between TAT and toluene. In contrast, the formation of methylphloroglucinol (2,4,6-trihydroxytoluene) and *p*-cresol (4-hydroxytoluene) was observed by Funk et al. (1993) with anaerobic incubation of a consortium from a munitions-fed soil-sewage sludge bioreactor. The actual physiological mechanism(s) whereby the nitrogenous substituents were liberated from triaminotoluene remain(s) to be determined (Crawford 1995; Shen et al. 2000). In addition, Shen et al. (2000) demonstrated that *p*-cresol is not a TNT-metabolite, but an intermediate of aromatic amino acid degradation.

In conclusion, different potentially beneficial pathways of TNT degradation have been reported. Among these, TNT denitration seems to be the critical requirement for complete mineralisation, since dinitrotoluene compounds can be mineralised by oxygenolytic attacks.

2.4. Perspectives for denitration of 2,4,6-trinitrotoluene

An explosive compound such as TNT possesses an enormous potential free energy. Therefore, as pointed out by Shelley and coworkers (1996), successive denitration steps of TNT should be characterized by relatively large negative Gibbs free energy changes as compared to the glycolytic pathway and TCA cycle. These authors estimated the free energy change (ΔG°') of reactions involved in the denitration of TNT. They obtained a value of -11.7 kcal/mol for the release of one nitro group from TNT to 2,4-dinitrotoluene[2]. Instead, reduction of one nitro to an amino group (i.e. formation of 4-amino-2,6-dinitrotoluene from TNT) was more energetically favourable (-95.4 kcal/mol) (Shelley et al. 1996)[3]. Thus, as microorganisms utilize enzymatic reactions that produce the greatest possible energy return for the cell, the reduction of the nitro group will be strongly favoured. This is also exemplified by catabolic reactions of other xenobiotics, such as chlorinated benzenes: from 19 possible dechlorination reactions, only seven reactions with the highest energy release are taking place (Beurskens et al. 1994). Therefore, when less energetically favourable reactions are sought, it is important to focus on increasing their enzyme specificity or otherwise altering their regulation. For example, despite the fact that TNT denitration has been observed with the purified enzyme XenB (Pak et al. 2000), studies with *P. fluorescens* I-C harbouring this enzyme revealed that accumulation of aminodinitrotoluene compounds increased and that a decrease of nitrite liberation was observed (Pak et al. 2000). These authors suggested that other enzymes enhanced production of these TNT reduced metabolites and competed with the XenB biocatalytic activity. Whereas nitro reduction activity is apparently significant enough to compete with the energy and biomass yielding route of biotransformation, these authors brought to the fore that a large consumption of NAD(P)H equivalents was required in order to produce one equivalent of utilizable nitrogen. Thus, this substantial energy demand and the limited nitrite release may effectively prevent utilization of TNT as sole nitrogen source. Therefore, a real challenge exists to search for novel biocatalytic activities leading to TNT denitration. One possible approach is to identify new naturally occurring prokaryotes and/or engineer new microorganisms capable of mineralising TNT. However, the relatively unsuccessful isolation experiments of TNT-mineralising bacteria might not look very

[2] Such denitration pathway has been described by Duque et al. (1993) and Haidour and Ramos (1996). However, it has to be noted that other TNT-denitration pathways have been found (cfr. Figure 3).

[3] The kinetics of those reactions has not been considered in this example.

promising, considering the isolation experiments realised over the last two decades. Through recent advancements in the field of microbial diversity, it is known that classical enrichment techniques are limited, given that more than 99% of microbes living in natural environments are refractory to cultivation (Torsvik & Ovreas 2002). Therefore, an alternative approach for the search of novel biocatalytic genes is based on metagenomic libraries. Metagenomics is the culture-independent genomic analysis of entire microbial communities (Schloss & Handelsman 2003). A growing interest in this discipline is fuelled by the quest to analyse the unexplored immense wealth of physiological diversity in our microbial communities. In other words, metagenomics provides access to the pool of genomes of a given environment without the need for cultivation. This strategy might be useful in finding genes involved in TNT denitration as described by Eyers et al. (2004a).

Another strategy for the mineralisation of TNT lies in the formation of hydride-Meisenheimer complexes. For example, several bacteria of the *Actinomycetales* family (notably of the genera *Rhodococcus* and *Nocardioides*) are able to grow aerobically on 2,4,6-trinitrophenol (picric acid) and/or dinitrophenol and utilize these compounds as sole nitrogen, carbon, and energy sources, through the formation of hydride-Meisenheimer complexes (Hofmann et al. 2004). A second reductive step leads to the formation of a dihydride- Meisenheimer complex followed by a denitration reaction, which leads to total and beneficial degradation through hydrolytic ring cleavage. However, picric acid is more amenable to complete degradation than TNT. Hence, the obvious question is: why does nitrite release occur with dihydride-picric acid but not with dihydride-TNT? Despite the analogy between picric acid and TNT for the formation of hydride-Meisenheimer complexes, the inherent difference between a methyl and a hydroxyl substitution on the ring might explain these differences in nitrite release. Heiss and Knackmuss (2002) ventured the hypothesis that the amount of dihydride-TNT generated may be too low for subsequent efficient nitrite release, due to less efficient enzymes. In addition, and as stated above for TNT, nitro group reduction and formation of hydride-Meisenheimer complexes can occur simultaneously by two different pathways. On the contrary, no nitro group reduction has been observed during the formation of hydride-Meisenheimer complexes of picric acid (Lenke et al. 2000; Hofmann et al. 2004). Thus, although ring hydrogenation of TNT might provide the opportunity for developing a catabolic pathway for the mineralisation of TNT and its use as a nitrogen source, this beneficial pathway is apparently not efficient enough to denitrate TNT and avoid the formation of dead-end metabolites. Therefore, a future

challenge consists in improving the pathway towards hydride-Meisenheimer complexes without reduction of nitro groups.

2.5. Enzymology of beneficial TNT transformation: The "Old Yellow Enzyme" family

Understanding TNT biodegradation by known enzymes and finding novel biocatalytic activities such as TNT denitration will open new perspectives for bioremediation. For instance, screening for TNT biodegradation activities in polluted sites will be very useful to define bioremediation strategies. The presence and the level of abundance of theses catabolic activities will determine whether an intrinsic bioremediation is possible compared to a bioaugmentation process involving natural or engineered TNT degrading strains. In this context, a particular class of enzymes was recently described, with catabolic activities potentially leading to ring fission and possible mineralisation of TNT. The structure of these enzymes was investigated as well as their kinetic properties (Khan et al. 2002; Basran et al. 2003; Khan et al. 2004). These investigations have provided atomic level insight into reductive TNT-catabolic activities.

Among all enzymes involved in the biotransformation of TNT, the previously mentioned oxygen-insensitive type I nitroreductases represent the best characterized enzymatic group. This group of enzymes catalyses the ubiquitous reduction of the nitro groups to amino groups, through two-electron increments. The type I nitroreductases are either monomeric or homodimeric flavin mononucleotide (FMN)-containing flavoproteins with a subunit size of approximately 25 kDa and use NAD(P)H as an electron donor (Blehert et al. 1999). Another class of bacterial flavoproteins was recently shown as able to catalyse the degradation of TNT. These enzymes are members of the old yellow enzyme family of flavoproteins, which is responsible for diverse transformations (Williams & Bruce 2002). Over the last few years, an increasing number of proteins have been isolated from bacterial, yeast and plant sources, sharing significant sequence homology with old yellow enzyme (OYE, EC 1.6.99.1). OYE was originally isolated from brewer's bottom yeast, and contains a bound flavin (FMN) as prosthetic group (Kataoka et al. 2004). The enzyme is a mixture of homodimers and heterodimers, with each monomer of approximately 45 kDa binding a molecule of flavin mononucleotide (Meah et al. 2001). Flavin, either FMN or FAD, is typically found to participate in biological redox reactions where enzyme-bound oxidized flavin serves as a temporary sink of electrons, which are then passed on to another electron-accepting protein or substrate (Fitzpatrick et al. 2004). The overall catalytic reaction can be separated into a

reductive and an oxidative half-reaction. The physiological reductant of OYE is probably NAD(P)H, whereas substrates capable of reoxidizing OYE include methylene blue, Fe^{3+}, quinones, cytochrome *c* and ferricyanide (Williams & Bruce 2002). Although many fundamental biochemical and spectroscopic characterizations have been performed, the physiological role of the OYE family remains obscure. It has been assumed that OYE may serve as a detoxification enzyme in antioxidant defense systems (Kohli & Massey 1998). Interestingly, some members of the OYE family are also able to reduce certain xenobiotic compounds containing nitro functional groups, such as TNT or nitrate ester explosives. These FMN-containing flavoproteins catalyse the NAD(P)H-dependent reductive cleavage of nitrate esters to give alcohol and nitrite. Among these enzymes, the aforementioned pentaerythritol tetranitrate (PETN) reductase was purified and characterized from *Enterobacter cloacae* on the basis of its ability to use nitrate ester explosives (Binks et al. 1996). Enzymes sharing homologies with PETN reductase, i.e. glycerol tetranitrate (GTN) reductase from *Agrobacterium radiobacter* (Snape et al. 1997) and xenobiotic reductase from *P. fluorescens* and *P. putida* (Blehert et al. 1997), also show reactivity against explosive substrates. These reductases are monomeric flavoproteins with a range of subunit size from 40 to 50 kDa. Their structure is similar to that of *N*-ethylmaleimide reductase from *Escherichia coli* (Miura et al. 1997), morphinone reductase from *P. putida* (Barna et al. 2002) and old yellow enzyme OYE3 from *S. cerevisiae* (Niino et al. 1995). The discovery that PETN reductase and xenobiotic reductase B are able to denitrate TNT has triggered investigations towards related orthologous enzymes with potentially the same catalytic activities. Indeed, contrary to many flavoenzymes (Sarlauskas et al. 2004), PETN reductase and xenobiotic reductase B are able to reduce TNT through two reductive reactions in two competing pathways within the oxidative half-reaction of the enzyme (Pak et al. 2000, Figure 6). The recent work of Bruce's team has provided new biochemical insights into TNT biodegradation (Williams et al. 2004). In addition, they demonstrated that other members of the OYE family are able to transform TNT, i.e. NEM reductase, morphinone reductase and OYE from *Saccharomyces carlsbergensis* (Williams et al. 2004). However, only NEM reductase is able to liberate significant levels of nitrites, while other investigated enzymes catalyse sequential reduction of one or more nitro groups to amino derivatives (Figure 6). Williams and coworkers (2004) observed that a structural difference might explain the two distinct pathways, following characterization of an active site mutant of PETN reductase. Whereas a histidine residue at position 184 is present in PETN and NEM reductase, OYE and morphinone reductase contain an asparagine residue. Hydride addition to the ring of TNT was inhibited in a H184N mutant

of PETN reductase. In contrast, the hydride-Meisenheimer complex (H-TNT) was still an effective substrate for the modified enzyme, suggesting that this His residue at position 184 is critical for the initial reduction of TNT, and therefore the eventual partition between ring-reduced and nitro-reduced products (Williams et al. 2004). In addition, the same authors reported the disappearance of dihydride-Meisenheimer complex isomers (2H-TNT), but did not identify resulting metabolites. However, the formation of hydrophobic dimeric compounds, as proposed by Pak et al. (2000), did not occur. These results are very promising, compared to previous studies showing that 2H-TNT isomers accumulated (Vorbeck et al. 1998).

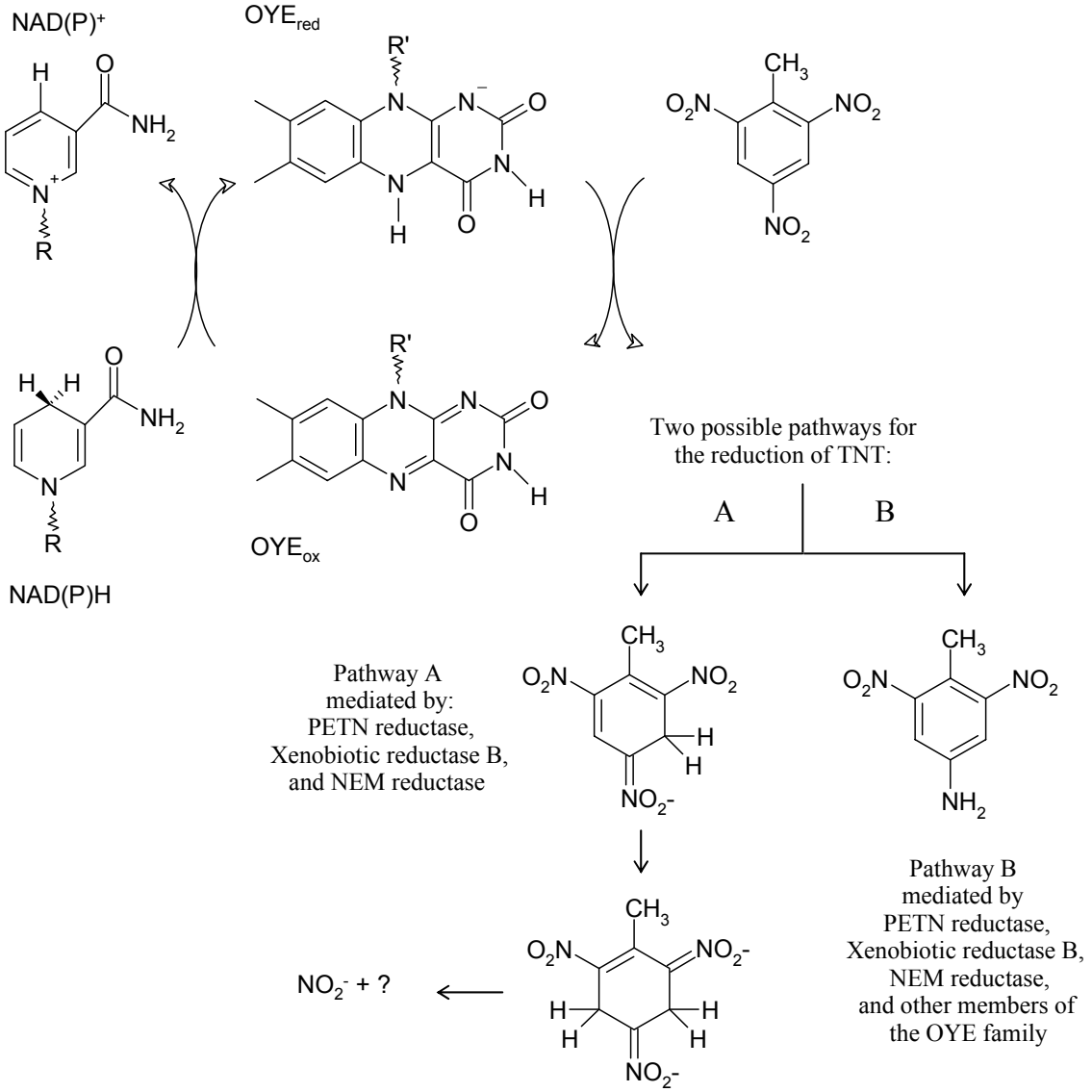


Figure 6. Catalytic cycle of members of the "Old Yellow Enzyme" (OYE) family, in which TNT can act as substrate for the turnover reaction with NAD(P)H. A: Reduction of TNT by hydride attack, formation of hydride-Meisenheimer complexes and subsequent nitrite release; B: Classic reduction of nitro group with formation of amino group (adpted from Kohli & Massey (1998)).

So far, proteins of the OYE family are the best candidates for TNT biodegradation, through ring reduction, Meisenheimer complex formation and subsequent nitrite release. The ever-increasing information on their structure and function offers further possibilities for their optimization. In this context, the performance of an enzyme can be increased through direct manipulation of the enzymatic structure. Indeed, the effect of substituting different amino acids at the active site of an enzyme can have a dramatic effect on the activity (Cirino & Arnold 2002). Consequently, sequence alignments can help in identifying critical residues for enzyme activity. As demonstrated by Williams and colleagues (2004), even small differences

in the amino acid sequences lead to major differences in the enzymatic properties. Thus, because some bacterial flavoproteins share high homologies in structure and exhibit very relaxed substrate specificities and a potentially highly efficient TNT biodegradation, protein engineering of OYE family flavoenzymes may generate improved enzymes, that could reduce preferentially the ring rather than nitro groups.

Another approach to improve the biocatalytic activity of enzymes consists in DNA shuffling, a method that involves random recombination of selected genes by random fragmentation and PCR reassembly (Crameri et al. 1998; Zhang et al. 2002). The subsequent modified genes can be introduced into the parent hosts and replace the original genes. DNA shuffling might be the ideal choice to direct the evolution of enzymes or pathways with highly specialized traits (Dua et al. 2002). The main advantage of this "irrational" approach is that no prior information on the enzymatic structure and homologies is needed. Nonetheless, this random technique can be laborious since it generally requires the screening of numerous clones. However, the recent work of Dai & Copley (2004) has greatly boosted the efficiency of *Sphingobium chlorophenolicum* for the degradation of high concentrations of pentachlorophenol (PCP), through just three rounds of genomic shuffling. In a related method, i.e. family shuffling, homologous genes are recombined *in vitro* or *in vivo* using one of several recombination methods (Joern et al. 2002). Family shuffling utilizes naturally occurring nucleotide substitutions among genes of the same family as the driving force of evolution (Kikuchi et al. 2000; Hsu et al. 2004). This method assumes that chimeric analogs derived from various homologous proteins can gain favourable properties (Pieper & Reineke 2000). For instance, Hsu et al. (2004) have obtained the most active penicillin G-specific expandase known to date by using family shuffling, whereas Suen et al. (2004) used DNA family shuffling to improve the activity of lipase B from *C. antarctica* towards the hydrolysis of diethyl dichlorophenylglutarate (DDG). Likewise, family shuffling of OYE family flavoenzyme genes might be a very attractive approach to enhance TNT denitration activity.

NOTE ADDED IN PROOF: As this review was going to press, a review was published by Ramos et al. (2005) analyzing recent advances in the removal of polynitrated aromatics, such as picric acid and TNT, by microbes, plants and plant-microbe associations.

Acknowledgements

The first author gratefully acknowledges FRIA (Fund for the Promotion of Research in Industry and Agriculture, Belgium) for a doctoral fellowship. Additional material support comes from grant QLK-3-CT-2001-00345 (MADOX) funded by the EU under the 5th Framework Programme (FP5).

2.6. References

Abril M-A, Michan C, Timmis KN & Ramos JL (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. Appl. Environ. Microbiol. 171: 6782-6790

Altamirano M, Garcia-Villada L, Agrelo M, Sanchez-Martin L, Martin-Otero L, Flores-Moya A, Rico M, Lopez-Rodas V & Costas E (2004) A novel approach to improve specificity of algal biosensors using wild-type and resistant mutants: An application to detect TNT. Biosens. Bioelec. 19: 1319-1323

Ask K, Decologne N, Asare N, Holme JA, Artur Y, Pelczar H & Camus P (2004) Distribution of nitroreductive activity toward nilutamide in rat. Toxicol. Appl. Pharmacol. 201: 1-9

Ayer S, Andersen RJ, Cun-Heng H & Clardy J (1984) Phidolopin, a new purine derivative from the bryozoan *Phidolopora pacifica*. J. Org. Chem. 49: 3869-3870

Barna T, Messiha HL, Petosa C, Bruce NC, Scrutton NS & Moody PCE (2002) Crystal structure of bacterial morphinone reductase and properties of the C191A mutant enzyme. J. Biol. Chem. 277: 30976-30983

Basran A, Harris RJ, Sutcliffe MJ & Scrutton NS (2003) H-tunneling in the multiple H-transfers of the catalytic cycle of morphinone reductase and in the reductive half-reduction of the homologous pentaerythritol tetranitrate reductase. J. Biol. Chem. 278: 43973-43982

Behrend C & Heesche-Wagner K (1999) Formation of hydride-Meisenheimer complexes of picric acid (2,4,6-trinitrophenol) and 2,4-dinitrophenol during mineralization of picric acid by *Nocardioides* sp. strain CB 22-2. Appl. Environ. Microbiol. 65: 1372-1377

Beurskens JEM, Dekker CGC, van den Heuvel H, Swart M & de Wolf J (1994) Dechlorination of chlorinated benzenes by an anaerobic microbial consortium that selectively mediates the thermodynamic most favorable reactions. Environ. Sci. Technol. 28: 701-706

Binks PR, French CE, Nicklin S & Bruce NC (1996) Degradation of pentaerythritol tetranitrate by *Enterobacter cloacae* PB2. Appl. Environ. Microbiol. 62: 1214-1219

Blehert DS, Fox BG & Chambliss GH (1999) Cloning and sequence analysis of two *Pseudomonas* flavoprotein xenobiotic reductases. J. Bacteriol. 181: 6254-6263

Blehert DS, Knoke KL, Fox BG & Chambliss GH (1997) Regioselectivity of nitroglycerin denitration by flavoprotein nitroester reductases purified from two *Pseudomonas* species. J. Bacteriol. 179: 6912-6920

Boon N, Goris J, De Vos P, Verstraete W & Top EM (2001) Genetic diversity among 3-chloroaniline- and aniline-degrading strains of the *Comamonadaceae*. Appl. Environ. Microbiol. 67: 1107-1115

Boopathy R & Kulpa CF (1992) Trinitrotoluene (TNT) as a sole nitrogen source for a sulfate-reducing bacterium *Desulfovibrio* sp. (B strain) isolated from an anaerobic digester. Curr. Microbiol. 25: 235-241

Borch T & Gerlach R (2004) Use of reversed-phase high-performance liquid chromatography-diode array detection for complete separation of 2,4,6-trinitrotoluene metabolites and EPA Method 8330 explosives: influence of temperature and an ion-pair reagent. J. Chrom. A 1022: 83-94

Boxall ABA, Sinclair CJ, Fenner K, Kolpin D & Maund SJ (2004) When synthetic chemicals degrade in the environment. Environ. Sci. Technol. 38: 368A-375A

Buryakov IA (2004) Express analysis of explosives, chemical warfare agents and drugs with multicapillary column gas chromatography and ion mobility increment spectrometry. J. Chrom. B 800: 75-82

Caballero A, Esteve-Núñez A, Zylstra GJ & Ramos JL (2005) Assimilation of nitrogen from nitrite and trinitrotoluene in *Pseudomonas putida* JLR11. J. Bacteriol. 187: 396-399

Charles PT, Shriver-Lake LC, Francesconi SC, Churilla AM, Rangasammy JG, Patterson J, C.H., Deschamps JR & Kusterbeck AW (2004) Characterization and performance evaluation of *in vivo* and *in vitro* produced monoclonal anti-TNT antibodies for the detection of TNT. J. Immunol. Methods 284: 15-26

Cirino PC & Arnold FH (2002) Protein engineering of oxygenases for biocatalysis. Curr. Op. Chem. Biol. 6: 130-135

Conder JM, La Point TW & Bowen AT (2004) Preliminary kinetics and metabolism of 2,4,6-trinitrotoluene and its reduced metabolites in an aquatic oligochaete. Aquatic Toxicol. 69: 199-213

Crameri A, Raillard S-A, Bermudez E & Stemmer WPC (1998) DNA shuffling of a family of genes from diverse species accelerates directed evolution. Nature 391: 288-291

Crawford RL (1995) The microbiology and treatment of nitroaromatic compounds. Curr. Op. Biotechnol. 6: 329-336

Dai M & Copley SD (2004) Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. Appl. Environ. Microbiol. 70: 2391-2397

Daun G, Lenke H, Reuss M & Knackmuss H-J (1998) Biological treatment of TNT-contaminated soil. 1. Anaerobic cometabolic reduction and interaction of TNT and metabolites with soil components. Environ. Sci. Technol. 32: 1956-1963

Davis JK, Paoli GC, He Z, Nadeau LJ, Somerville CC & Spain JC (2000) Sequence analysis and initial characterization of two isozymes of hydroxylaminobenzene mutase from *Pseudomonas pseudoalcaligenes* JS45. Appl. Environ. Microbiol. 66: 2965-2971

Davis TL (1943) The Chemistry of Powder and Explosives. John Wiley & Sons, Inc., New York

Dodard SG, Powlowski J & Sunahara GI (2004) Biotransformation of 2,4,6-trinitrotoluene (TNT) by enchytraeids (*Enchytraeus albidus*) *in vivo* and *in vitro*. Environ. Poll. 131: 263-273

Donlon B, Razo-Flores E, Field JA & Lettinga G (1995) Toxicity of *N*-substitued aromatics to acetoclastic methanogenic activity in granular sludge. Appl. Environ. Microbiol. 61: 3889-3893

Dua M, Singh A, Sethunathan N & Johri AK (2002) Biotechnology and bioremediation: successes and limitations. Appl. Microbiol. Biotechnol. 59: 143-152

Duque E, Haïdour A, Godoy F & Ramos JL (1993) Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J. Bacteriol. 175: 2278-2283

Ebert S, Rieger P-G & Knackmuss H-J (1999) Function of coenzyme F420 in aerobic catabolism of 2,4,6-trinitrophenol and 2,4-dinitrophenol by *Nocardioides simplex* FJ2-1A. J. Bacteriol. 181: 2669-2674

El Fantroussi S, Naveau H & Agathos SN (1998) Anaerobic dechlorinating bacteria. Biotechnol. Progr. 14: 167-188

Eriksson J, Krankki S, Shchukarev A & Skyllberg U (2004) Binding of 2,4,6-trinitrotoluene, aniline, and nitrobenzene to dissolved and particulate soil organic matter. Environ. Sci. Technol. 38: 3074-3080

Eriksson J & Skyllberg U (2001) Binding of 2,4,6-trinitrotoluene and its degradation products in a soil organic matter two-phase system. J. Environ. Qual. 30: 2053-2061

Esteve-Núñez A, Caballero A & Ramos JL (2001) Biological degradation of 2,4,6-trinitrotoluene. Microbiol. Mol. Biol. Rev. 65: 335-352

Esteve-Núñez A, Lucchesi G, Philipp B, Schink B & Ramos JL (2000) Respiration of 2,4,6-trinitrotoluene by *Pseudomonas* sp. strain JLR11. J. Bacteriol. 182: 1352-1355

Esteve-Núñez A & Ramos JL (1998) Metabolism of 2,4,6-trinitrotoluene by *Pseudomonas* sp. JLR11. Environ. Sci. Technol. 32: 3802-3808

Eyers L, George I, Schuler L, Stenuit B, Agathos SN & El Fantroussi S (2004a) Environmental genomics: exploring the unmined richness of microbes to degrade xenobiotics. Appl. Microbiol. Biotechnol. 66: 123-130

Eyers L, Stenuit B, El Fantroussi S & Agathos SN (2004b) Microbial characterization of TNT-contaminated soils and anaerobic TNT degradation: high and unusual denitration activity, Verstraete W (Ed), Proceedings of the European Symposium on Environmental Biotechnology, ESEB 2004 (pp 51-54), Taylor and Francis Group plc, London, U.K.

Fauser P & Thomsen M (2002) Sensitivity analysis of calculated exposure concentrations and dissipation of DEHP in a topsoil compartment: the influence of the third phase effect and dissolved organic matter (DOM). Sci. Total Environ. 296: 89-103

Fetzner S (1998) Bacterial dehalogenation. Appl. Microbiol. Biotechnol. 50: 633-657

Fiorella PD & Spain JC (1997) Transformation of 2,4,6-trinitrotoluene by *Pseudomonas pseudoalcaligenes* JS52. Appl. Environ. Microbiol. 63: 2007-2015

Fitzpatrick TB, Auweter S, Kitzing K, Clausen T, Amrhein N & Macheroux P (2004) Structural and functional impairment of an Old Yellow Enzyme homologue upon affinity tag incorporation. Prot. Exp. Purif. 36: 280-291

French CE, Nicklin S & Bruce NC (1998) Aerobic degradation of 2,4,6-trinitrotoluene by *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase. Appl. Environ. Microbiol. 64: 2864-2868

Fuller ME & Manning JF (1997) Aerobic Gram-positive and Gram-negative bacteria exhibit differential sensitivity to and transformation of 2,4,6-trinitrotoluene (TNT). Curr. Microbiol. 35: 77-83

Fuller ME & Manning JF (2004) Microbiological changes during bioremediation of explosives-contaminated soils in laboratory and pilot-scale bioslurry reactors. Bioresource Technol. 91: 123-133

Funk SB, Roberts DJ, Crawford DL & Crawford RL (1993) Initial-phase optimization for bioremediation of munition compound-contaminated soils. Appl. Environ. Microbiol. 59: 2171-2177

Gilcrease PC & Murphy VG (1995) Bioconversion of 2,4-diamino-6-nitrotoluene to a novel metabolite under anoxic and aerobic conditions. Appl. Environ. Microbiol. 61: 4209-4214

Gilles N (2004) Green explosives: collateral damage. Nature 427: 580-581

Goux S, Shapir N, El Fantroussi S, Lelong S, Agathos SN & Pussemier L (2003) Long-term maintenance of rapid atrazine degradation in soils inoculated with atrazine degraders. Water, Air Soil Pollut. Focus 3: 131-142

Grivet J-P, Delort A-M & Portais J-C (2003) NMR and microbiology: from physiology to metabolomics. Biochimie 85: 823-840

Groenewegen PE, Breeuwer P, van Helvoort JM, Langenhoff AA, de Vries FP & de Bont JA (1992) Novel degradative pathway of 4-nitrobenzoate in *Comamonas acidovorans* NBA-10. J. Gen. Microbiol. 138: 1599-1605

Haïdour A & Ramos JL (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

Haigler BE & Spain JC (1993) Biodegradation of 4-nitrotoluene by *Pseudomonas* sp. strain 4NT. Appl. Environ. Microbiol. 59: 2239-2243

Haigler BE, Suen WC & Spain JC (1996) Purification and sequende analysis of 4-methyl-5-nitrocatechol oxygenase from *Burkholderia* sp. strain DNT. J. Bacteriol. 178: 6019-6024

Haigler BE, Wallace WH & Spain JC (1994) Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42. Appl. Environ. Microbiol. 60: 3466-3469

Hashimoto M & Hattori K (1966) Oxypyrrolnitrin: a metabolite of *Pseudomonas*. Chem. Pharm. Bull. 14: 1314-1316

Hawari J, Beaudet S, Halasz A, Thiboutot S & Ampleman G (2000) Microbial degradation of explosives: biotransformation versus mineralization. Appl. Microbiol. Biotechnol. 54: 605-618

Hawari J, Halasz A, Paquet L, Spencer B, Ampleman G & Thiboutot S (1998) Characterization of metabolites in the biotransformation of 2,4,6-trinitrotoluene with anaerobic sludge : Role of triaminotoluene. Appl. Environ. Microbiol. 64: 2200-2206

He J & Hertweck C (2003) Iteration as programmed event during polyketide assembly; molecular analysis of the aureothin biosynthesis gene cluster. Chem. Biol. 10: 1225-1232

He Z & Spain JC (1997) Studies of the catabolic pathway of degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* JS45: removal of the amino group from 2-aminomuconic semialdehyde. Appl. Environ. Microbiol. 63: 4839-4843

Heiss G, Hofmann KW, Trachtmann N, Walters DM, Rouvière P & Knackmuss H-J (2002) *npd* gene functions of *Rhodococcus* (*opacus*) *erythropolis* HL PM-1 in the initial steps of 2,4,6-trinitrophenol degradation. Microbiology 148: 799-806

Heiss G & Knackmuss H-J (2002) Bioelimination of nitroaromatic compounds: immobilization versus mineralization. Curr. Op. Biotechnol. 5: 282-287

Heiss G, Trachtmann N, Abe Y, Takeo M & Knackmuss H-J (2003) Homologous *npdGI* genes in 2,4-dinitrophenol- and 4-nitrophenol-degrading *Rhodococcus* spp. Appl. Environ. Microbiol. 69: 2748-2754

Hofmann KW, Knackmuss H-J & Heiss G (2004) Nitrite elimination and hydrolytic ring cleavage in 2,4,6-trinitrophenol (picric acid) degradation. Appl. Environ. Microbiol. 70: 2854-2860

Hsu J-S, Yang Y-B, Deng C-H, Wei C-L, Liaw S-H & Tsai Y-C (2004) Family shuffling of expandase genes to enhance substrate specificity for penicillin G. Appl. Environ. Microbiol. 70: 6257-6263

Huang S, Lindahl PA, Wang C, Bennett GN, Rudolph FB & Hughes JB (2000) 2,4,6-trinitrotoluene reduction by carbon monoxide dehydrogenase from *Clostridium thermoaceticum*. Appl. Environ. Microbiol. 66: 1474-1478

Hughes JB, Wang C, Yesland K, Richardson A, Bhadra R, Bennett G & Rudolph F (1998) Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. Environ. Sci. Technol. 32: 494-500

Jain RK, Dreisbach JH & Spain JC (1994) Biodegradation of *p*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. Appl. Environ. Microbiol. 60: 3030-3032

Joern JM, Meinhold P & Arnold FH (2002) Analysis of shuffled gene libraries. J. Mol. Biol. 316: 643-656

Johnson GR, Jain RK & Spain JC (2002) Origins of the 2,4-dinitrotoluene pathway. J. Bacteriol. 184: 4219-4232

Johnson GR, Smets BF & Spain JC (2001) Oxidative transformation of aminodinitrotoluene isomers by multicomponent dioxygenases. Appl. Environ. Microbiol. 67: 5460-5466

Johnson GR & Spain JC (2003) Evolution of catabolic pathways for synthetic compounds: bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. Appl. Microbiol. Biotechnol. 62: 110-123

Johnson LR, Davenport R, Balbach H & Schaeffer DJ (1994) Phototoxicology 3. Comparative toxicity of trinitrotoluene and aminodinitrotoluenes to *Daphnia magna*, *Dugesia dorotocephala*, and sheep erythrocytes. Ecotoxicol. Environ. Saf. 27: 34-49

Jung CM, Newcombe DA, Crawford DL & Crawford RL (2004) Detection and decontamination of residual energetics from ordnance and explosive scrap. Biodegradation 15: 41-48

Kalafut T, Wales ME, Rastogi VK, Naumova RP, Zaripova SK & Wild JR (1998) Biotransformation patterns of 2,4,6-trinitrotoluene by aerobic bacteria. Curr. Microbiol. 36: 45-54

Kaplan DL & Kaplan AM (1982) Mutagenicity of 2,4,6-trinitrotoluene-surfactant complexes. Bull. Environ. Contam. Toxicol. 28: 33-38

Kataoka M, Kotaka A, Thiwthong R, Wada M, Nakamori S & Shimizu S (2004) Cloning and overexpression of the old yellow enzyme gene of *Candida macedoniensis*, and its application to the production of a chiral compound. J. Biotechnol. 114: 1-9

Khan H, Barna T, Harris RJ, Bruce NC, Barsukov I, Munro AW, Moody PCE & Scrutton NS (2004) Atomic resolution structures and solution behaviour of enzyme-substrate complexes of *Enterobacter cloacae* PB2 pentaerythritol tetranitrate reductase. J. Biol. Chem. 279: 30563-30572

Khan H, Harris RJ, Barna T, Craig DH, Bruce NC, Munro AW, Moody PCE & Scrutton NS (2002) Kinetic and structural basis of reactivity of pentaerythritol tetranitrate reductase with NADPH, 2-cyclohexenone, nitroesters, and nitroaromatic explosives. J. Biol. Chem. 277: 21906-21912

Kikuchi M, Ohnishi K & Harayama S (2000) An effective family shuffling method using single-stranded DNA. Gene 243: 133-137

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

Kim J, Sung K, Corapcioglu MY & Drew MC (2004) Solute transport and extraction by a single root in unsaturated soils: model development and experiment. Environ. Poll. 131: 61-70

Klausen J, Tröber SP, Haderlein SB & Schwarzenbach RP (1995) Reduction of substituted nitrobenzenes by Fe(II) in aqueous mineral suspensions. Environ. Sci. Technol. 29: 2396-2404

Knicker H (2003) Incorporation of ^{15}N-TNT transformation products into humifying plant organic matter as revealed by one- and two-dimensional solid state NMR spectroscopy. Sci. Tot. Environ. 308: 211-220

Knicker H, Achtnich C & Lenke H (2001) Solid-state nitrogen-15 nuclear magnetic resonance analysis of biologically reduced 2,4,6-trinitrotoluene in a soil slurry remediation. J. Environ. Qual. 30: 403-410

Kohli RM & Massey V (1998) The oxidative half-reaction of Old Yellow Enzyme. The role of tyrosine 196. J. Biol. Chem. 273: 32763-32770

Kumagai Y, Kikushima M, Nakai Y, Shimojo N & Kunimoto M (2004) Neuronal nitric oxide synthase (NNOS) catalyzes one-electron reduction of 2,4,6-trinitrotoluene, resulting in decreased nitric oxide production and increased *nNOS* gene expression: implication for oxidative stress. Free Radical Biol. Med. 37: 350-357

Kurinenko BM, Yakovleva GY, Denivarova NA & Abreimova YV (2003) Specific toxic effects of 2,4,6-trinitrotoluene on *Bacillus subtilis* SK1. Appl. Biochem. Microbiol. (Russia) 39: 275-278

Kury JW, Breithaupt RD & Tarver CM (1999) Detonation waves in trinitrotoluene. Shock Waves 9: 227-237

Lachance B, Renoux AY, Sarrazin M, Hawari J & Sunahara GI (2004) Toxicity and bioaccumulation of reduced TNT metabolites in the earthworm *Eisenia andrei* exposed to amended forest soil. Chemosphere 55: 1339-1348

Lenke H, Achtnich C & Knackmuss H-J (2000) Perspectives of bioelimination of polynitroaromatic compounds In: Spain JC, Hughes JB & Knackmuss H-J (Eds) Biodegradation of Nitroaromatic Compounds and Explosives (pp 91-126). Lewis Publishers, New York

Lenke H & Knackmuss H-J (1992) Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. Appl. Environ. Microbiol. 58: 2933-2937

Lenke H, Pieper DH, Bruhn C & Knackmuss H-J (1992) Degradation of 2,4-dinitrophenol by two *Rhodococcus erythropolis* strains, HL 24-1 and HL 24-2. Appl. Environ. Microbiol. 58: 2928-2932

Lessner DJ, Johnson GR, Parales RE, Spain JC & Gibson DT (2002) Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. Strain JS765. Appl. Environ. Microbiol. 68: 634-641

Lewis TA, Newcombe DA & Crawford RL (2004) Bioremediation of soils contaminated with explosives. J. Environ. Manag. 70: 291-307

Li H, Teppen BJ, Johnston CT & Boyd SA (2004) Thermodynamics of nitroaromatic compound adsorption from water by smectite clay. Environ. Sci. Technol. 38: 5433-5442

Löffler FE, Tiedje JM & Sanford RA (1999) Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. Appl. Environ. Microbiol. 65: 4049-4056

Lynch JC, Brannon JM, Hatfield K & Delfino JJ (2003) An exploratory approach to modeling explosive compound persistence and flux using dissolution kinetics. J. Cont. Hydrol. 66: 147-159

Martin JL, Comfort SD, Shea PJ, Kokjohn TA & Drijber RA (1997) Denitration of 2,4,6-trinitrotoluene (TNT) by *Pseudomonas savastanoi*. Can. J. Microbiol. 43: 447-455

Martinez B, Tomkins J, Wackett LP, Wing R & Sadowsky MJ (2001) Complete nucleotide sequence and organization of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* sp. strain ADP. J. Bacteriol. 183: 5684-5697

Marvin-Sikkema FD & de Bont JAM (1994) Degradation of nitroaromatic compounds by microorganisms. Appl. Microbiol. Biotechnol. 42: 499-507

Meah Y, Brown BJ, Chakraborty S & Massey V (2001) Old yellow enzyme: reduction of nitrate esters, glycerin trinitrate, and propylene 1,2-dinitrate. Proc. Natl. Acad. Sci. USA 98: 8560-8565

Meharg AA, Dennis GR & Caimey JWG (1997) Biotransformation of 2,4,6-trinitrotoluene (TNT) by ectomycorrhizal basidiomycetes. Chemosphere 35: 513-521

Miura K, Tomioka Y, Suzuki H, Yonoezawa M, Hishinuma T & Mizugaki M (1997) Molecular cloning of the *nemA* gene encoding *N*-ethylmaleimide reductase from *Escherichia coli*. Biol. Pharm. Bull. 20: 110-112

Nadeau L & Spain J (1995) Bacterial degradation of *m*-nitrobenzoic acid. Appl. Environ. Microbiol. 61: 840-843

Naumova RP, Selivanovskaya SLU & Mingatina FA (1988) Possibilities for the deep bacterial destruction of 2,4,6-trinitrotoluene. Mikrobiologiya 57: 218-222

Niino YS, Chakraborty S, Brown BJ & Massey V (1995) A new old yellow enzyme of *Saccharomyces cerevisiae*. J. Biol. Chem. 270: 1983-1991

Nishino SF, Paoli GC & Spain JC (2000a) Aerobic degradation of dinitrotoluenes and pathway for bacterial degradation of 2,6-dinitrotoluene. Appl. Environ. Microbiol. 66: 2139-2147

Nishino SF, Spain JC & He Z (2000b) Strategies for aerobic degradation of nitroaromatic compounds by bacteria: process discovery to field application In: Spain JC, Hughes JB & Knackmuss H-J (Eds) Biodegradation of Nitroaromatic Compounds and Explosives (pp 7-62). Lewis Publishers, New York

Ohmori T, Hagiwara S, Ueda A, Minoda Y & Yamada K (1978) Production of pyoluteorin and its derivatives from n-paraffin by *Pseudomonas aeruginosa* S10B2. Agric. Biol. Chem. 42: 2031-2036

Pak JW, Knoke KL, Noguera DR, Fox BG & Chambliss GH (2000) Transformation of 2,4,6-trinitrotoluene by purified xenobiotic reductase B from *Pseudomonas fluorescens* I-C. Appl. Environ. Microbiol. 66: 4742-4750

Patel N, Cardoza V, Christensen E, Rekapalli B, Ayalew M & Stewart J, C.N. (2004) Differential gene expression of *Chlamydomonas reinhardtii* in response to 2,4,6-trinitrotoluene (TNT) using microarray analysis. Plant Science 167: 1109-1122

Peres CM & Agathos SN (2000) Biodegradation of nitroaromatic pollutants : from pathways to remediation. Biotechnol. Ann. Rev. 6: 197-220

Peres CM, Russ R, Lenke H & Agathos SN (2001) Biodegradation of 4-nitrobenzoate, 4-aminobenzoate and their mixtures: new strains, unusual metabolites and insights into pathway regulation. FEMS Microbiol. Ecol. 37: 151-159

Peterson FJ, Mason RP, Hovsepian J & Holtzman JL (1979) Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes. J. Biol. Chem. 254: 4009-4014

Pieper DH & Reineke W (2000) Engineering bacteria for bioremediation. Curr. Op. Biotechnol. 11: 262-270

Popesku JT, Singh A, Zhao J-S, Hawari J & Ward OP (2003) High TNT-transforming activity by a mixed culture acclimated and maintained on crude-oil-containing media. Can. J. Microbiol. 49: 362-366

Popesku JT, Singh A, Zhao J-S, Hawari J & Ward OP (2004) Metabolite production during transformation of 2,4,6-trinitrotoluene (TNT) by a mixed culture acclimated and maintained on crude oil-containing media. Appl. Microbiol. Biotechnol. 65: 739-746

Purohit V & Basu AK (2000) Mutagenicity of nitroaromatic compounds. Chem. Res. Toxicol. 13: 673-692

Ramos JL, González-Pérez MM, Caballero A & van Dillewijn P (2005) Bioremediation of polynitrated aromatic compounds: plants and microbes put up a fight. Curr. Op. Biotechnol. 16: 275-281

Rhys-Williams W, Taylor SC & Williams PA (1993) A novel pathway for the catabolism of 4-nitrotoluene by *Pseudomonas*. J. Gen. Microbiol. 139: 1967-1972

Riefler RG & Smets BF (2000) Enzymatic reduction of 2,4,6-trinitrotoluene and related nitroarenes: kinetics linked to one-electron redox potentials. Environ. Sci. Technol. 34: 3900-3906

Rieger P-G & Knackmuss H-J (1995) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil In: Spain JC (Ed) Biodegradation of nitroaromatic compounds (pp 1-18). Plenum Press, New York

Rieger P-G, Meier H, Gerle M, Vogt U, Groth T & Knackmuss H-J (2002) Xenobiotics in the environment: present and future strategies to obviate the problem of biological persistence. J. Biotechnol. 94: 101-123

Rieger P-G, Sinnwell V, Preub A, Francke W & Knackmuss H-J (1999) Hydride-Meisenheimer complex formation and protonation as key reactions of 2,4,6-trinitrophenol biodegradation by *Rhodococcus erythropolis*. J. Bacteriol. 181: 1189-1195

Robertson BK & Jjemba PK (2005) Enhanced bioavailability of sorbed 2,4,6-trinitrotoluene (TNT) by a bacterial consortium. Chemosphere 58: 263-270

Robidoux PY, Gong P, Sarrazin M, Bardai G, Paquet L, Hawari J, Dubois C & Sunahara GI (2004) Toxicity assessment of contaminated soils from an antitank firing range. Ecotoxicol. Environ. Saf. 58: 300-313

Sanford RA, Cole JR, Loffler FE & Tiedje JM (1996) Characterization of *Desulfitobacterium chlororespirans* sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate. Appl. Environ. Microbiol. 62: 3800-3808

Sarlauskas J, Nemeikaite-Ceniene A, Anusevicius Z, Miseviciene L, Julvez MM, Medina M, Gomez-Moreno C & Cenas N (2004) Flavoenzyme-catalyzed redox cycling of hydroxylamino- and amino

metabolites of 2,4,6-trinitrotoluene: implications for their cytotoxicity. Arch. Biochem. Biophys. 425: 184-192

Schaefer M (2004) Assessing 2,4,6-trinitrotoluene (TNT)-contaminated soil using three different earthworm test methods. Ecotoxicol. Environ. Saf. 57: 74-80

Schenzle A, Lenke H, Fischer P, Williams PA & Knackmuss H-J (1997) Catabolism of 3-nitrophenol by *Ralstonia eutropha* JMP 134. Appl. Environ. Microbiol. 63: 1421-1427

Schenzle A, Lenke H, Spain JC & Knackmuss H-J (1999) Chemoselective nitro group reduction and reductive dechlorination initiate degradation of 2-chloro-5-nitrophenol by *Ralstonia eutropha* JMP134. Appl. Environ. Microbiol. 65: 2317-2323

Schloss PD & Handelsman J (2003) Biotechnological prospects from metagenomics. Curr. Op. Biotechnol. 14: 303-310

Seffernick JL & Wackett LP (2001) Rapid evolution of bacterial catabolic enzymes: a case study with atrazine chlorohydrolase. Biochemistry 40: 12747-12753

Shelley MD, Autenrieth RL, Wild JR & Dale BE (1996) Thermodynamic analysis of trinitrotoluene biodegradation and mineralization pathways. Biotechnol. Bioeng. 50: 198-205

Shen CF, Hawari JA, Ampleman G, Thiboutot S & Guiot SR (2000) Origin of p-cresol in the anaerobic degradation of trinitrotoluene. Can. J. Microbiol. 46: 119-124

Sheremata TW & Hawari J (2000) Cyclodextrins for desorption and solubilization of 2,4,6-trinitrotoluene and its metabolites from soils. Environ. Sci. Technol. 34: 3462-3468

Snape JR, Walkley NA, Morby AP, Nicklin S & White GF (1997) Purification, properties, and sequence of glycerol trinitrate reductase from *Agrobacterium radiobacter*. J. Bacteriol. 179: 7796-7802

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

Spain JC (2000) Introduction In: Spain JC, Hughes JB & Knackmuss H (Eds) Biodegradation of Nitroaromatic Compounds and Explosives (pp 1-6). Lewis Publishers, New York

Spanggord RJ, Spain JC, Nishino SF & Mortelmans KE (1991) Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. Appl. Environ. Microbiol. 57: 3200-3205

Spiess T, Desiere F, Fischer P, Spain JC, Knackmuss H-J & Lenke H (1998) A new 4-nitrotoluene degradation pathway in a *Mycobacterium* strain. Appl. Environ. Microbiol. 64: 446-452

Suen WC, Haigler BE & Spain JC (1996) 2,4-dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J. Bacteriol. 178: 4926-4934

Suen WC & Spain JC (1993) Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation. J. Bacteriol. 175: 1831-1837

Suen W-C, Zhang N, Xiao L, Madison V & Zaks A (2004) Improved activity and thermostability of *Candida antarctica* lipase B by DNA family shuffling. Prot. Eng. Design Selection 17: 133-140

Sunahara GI, Dodard S, Sarrazin M, Paquet L, Ampleman G, Thiboutot S, Hawari J & Renoux AY (1998) Development of a soil extraction procedure for ecotoxicity characterization of energetic compounds. Ecotoxicol. Environ. Saf. 39: 185-194

Taylor S, Hewitt A, Lever J, Hayes C, Perovich L, Thorne P & Daghlian C (2004) TNT particle size distributions from detonated 155-mm howitzer rounds. Chemosphere 55: 357-367

Thorn KA, Pennington JC & Hayes C (2002) ^{15}NMR investigation of the reduction and binding of TNT in an aerobic bench scale reactor simulating windrow composting. Environ. Sci. Technol. 36: 3797-3805

Timmis KN & Pieper DH (1999) Bacteria designed for bioremediation. Trends Biotechnol. 17: 201-204

Torsvik V & Ovreas L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr. Op. Microbiol. 5: 240-245

Tront JM & Hughes JB (2005) Oxidative microbial degradation of 2,4,6-trinitrotoluene via 3-methyl-4,6-dinitrocatechol. Environ. Sci. Technol. 39: 4540-4549

Urbanski T (1964) Chemistry and Technology of Explosives. Pergamon Press, Oxford, UK, Oxford, UK

Van Aken B & Agathos SN (2001) Biodegradation of nitro-substituted explosives by white-rot fungi: a mechanistic approach. Adv. Appl. Microbiol. 48: 1-77

Van Aken B, Yoon JM & Schnoor JL (2004) Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* x *nigra* DN34). Appl. Environ. Microbiol. 70: 508-517

Vanderberg LA, Perry JJ & Unkefer PJ (1995) Catabolism of 2,4,6-trinitrotoluene by *Mycobacterium vaccae*. Appl. Microbiol. Biotechnol. 43: 937-945

Vats S, Stuttard C & Vining LC (1987) Transductional analysis of chloramphenicol biosynthesis genes in *Streptomyces venezuelae*. J. Bacteriol. 169: 3809-3813

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

Vorbeck C, Lenke H, Fischer P, Spain JC & Knackmuss H-J (1998) Initial reductive reaction in aerobic microbial metabolism of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. 64: 246-252

Ward OP, Singh A & Van Hamme JD (2003) Accelerated biodegradation of petroleum waste. J. Ind. Microbiol. Biotechnol. 30: 260-270

Weiss JM, Mckay AJ, Derito C, Watanabe C, Thorn KA & Madsen EL (2004a) Development and application of pyrolysis gas chromatography/mass spectrometry for the analysis of bound trinitrotoluene residues in soil. Environ. Sci. Technol. 38: 2167-2174

Weiss M, Geyer R, Gunther T & Kaestner M (2004b) Fate and stability of ^{14}C-labeled 2,4,6-trinitrotoluene in contaminated soil following microbial bioremediation processes. Environ. Toxicol. Chem. 23: 2049-2060

Weiss M, Geyer R, Russow R, Richnow HH & Kastner M (2004c) Fate and metabolism of [^{15}N]2,4,6-trinitrotoluene in soil. Environ. Toxicol. Chem. 23: 1852-1860

Williams RE & Bruce NC (2002) 'New uses for an Old Enzyme' - the Old Yellow Enzyme family of flavoenzymes. Microbiology 148: 1607-1614

Williams RE, Rathbone DA, Scrutton NS & Bruce NC (2004) Biotransformation of explosives by the old yellow enzyme family of flavoproteins. Appl. Environ. Microbiol. 70: 3566-3574

Yabannavar AV & Zylstra G (1995) Cloning and characterization of the genes for *p*-nitrobenzoate degradation from *Pseudomonas pickettii* YH105. Appl. Environ. Microbiol. 61: 4284-4290

Ye J, Singh A & Ward OP (2004) Biodegradation of nitroaromatics and other nitrogen-containing xenobiotics. World J. Microbiol. Biotechnol. 20: 117-135

Zeyer J, Kocher HP & Timmis KN (1986) Influence of *para*-substituents on the oxidative metabolism of *o*-nitrophenols by *Pseudomonas putida* B2. Appl. Environ. Microbiol. 52: 334-339

Zhang Y-X, Perry K, Vinci VA, Powell K, Stemmer WPC & del Cardayré SB (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415: 644-646