Microbial Degradation of 2,4,6-Trinitrotoluene *In Vitro* and in Natural Environments

Harald Claus

1 Introduction

2,4,6-Trinitrotoluene (TNT) is a nitroaromatic explosive that was released into soil and water ecosystems mainly due to its massive use during the two world wars. As a result, many sites used for TNT production have become seriously contaminated with the explosive and related compounds (Fuller et al. 2004; Lewis et al. 2004). Typical contaminated sites may contain up to 10 g/kg TNT in soils and up to 100 mg/l in water. TNT and its metabolites exhibit a high toxic and mutagenic potential on both prokaryotes and eukaryotes (Spanggord et al. 1995; Honeycutt et al. 1996; Lachance et al. 1999; Maeda et al. 2006). Consequently, there is an urgent need to clean up contaminated sites to ensure environmental quality and safety. It has been estimated that nearly 3,200 sites in Germany require environmental restoration (Preuß and Eitelberg 1999). Various physical/chemical procedures for remediation of TNT contaminated soils have been established, but all are very cost-intensive. Carbon adsorption has often been used for removal of nitroaromatics from contaminated ground- and surface-waters (Wujcik et al. 1992). Unfortunately, the matrix is expensive and the spent carbon still constitutes a problematic waste (Schmidt et al. 1998).

Biological based remediation is an economical and ecological compatible approach to detoxify areas contaminated with xenobiotics (Alvarez and Illman 2005; Crawford et al. 2005; Singh and Tripathi 2006). However, TNT is resistant to oxidative microbial degradation and only low mineralization rates have been sporadically reported with bacterial consortia and several white-rot fungi. The reason is the presence of three electron withdrawing nitro-groups in TNT which introduce steric constraints and confer a high electron deficiency to the aromatic ring (Heiss and Knackmus 2002). Instead of oxidation, many bacteria catalyse the

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reduction of one or two nitro-groups of TNT to monoaminodinitrotoluenes (ADNT) and diaminonitrotoluenes (DANT). Another pathway is mediated by addition of one or two hydride ions to the aromatic ring, resulting in the formation of Meisenheimer-complexes (adducts of aromatic nitro-compounds with a nucleophile) often accompanied by release of nitrite. The electron transfer is catalyzed by different types of cytoplasmatic nitroreductases (Pak et al. 2000; Kim and Song 2005). Reactive nitroso- and hydroxylamino-intermediates can further react to condensated azoxy-dimers and acetyl-derivates of TNT. Under strictly anaerobic conditions, ADNT is further reduced to 2,4,6-triaminotoluene (TAT) which is highly reactive and can polymerize or irreversibly bind to the organic soil matrix (Thiele et al. 2002).

The reductive reactions are the basis of several treatment processes for the bioremediation of TNT-contaminated soils (Breitung 1996; Lenke et al. 1998, 2000; Fuller et al. 2004; Lewis et al. 2004). However, there is a lack of biological strategies to clean up contaminated water ecosystems. Some promising microbiological approaches for detoxification of aquatic environments are addressed in this chapter.

2 2,4,6-Trinitrotoluene

2.1 Toxicity

Due to its high blasting power and relative security of handling, TNT is still one of the most used military explosives. It has been estimated that around 1.000,000 kg TNT is produced per year (Singh et al. 2012). Five hundred thousand US gallons of water, contaminated with TNT and other nitroaromatic compounds, may be released into the environment by one TNT-manufacturing plant in one day. In USA, 15 million acres at over 2000 sites are suspected or known to be contaminated with military munitions (Montgomery et al. 2011). At some munitions manufacturing and processing sites, the contamination can be as high as 200 g TNT per 1 kg of soil (Symons and Bruce 2006).

TNT forms yellow crystals and has a water solubility of 130 mg/l. At contaminated sites, it exists as a fine dust, flakes or crystallized chunks. Its heterogeneous distribution in soil restricts mobility, microbial degradation and also its analysis.

In several studies, it has been demonstrated that TNT and most of its degradation products are toxic to fish (Osmon and Klausmeier 1972), rats and mice (Ashby et al. 1985) as well as to algae and aquatic plants (Sunahara et al. 1999). For microorganisms, such as yeasts, actinomycetes and Gram-positive bacteria, TNT is toxic at concentrations of ca. 50 mg/l (Klausmeier et al. 1973). Also precursors and metabolites of TNT are classified as very toxic, carcinogenic and mutagenic (Schneider et al. 2000; Haarck et al. 2001). Ribeiro et al. (2012)

reported that the toxic potential of effluents generated by TNT production (yellow and red waters), produced from a plant located in Brazil was extremely high to all test organisms (*Daphnia similis, Danio rerio, Escherichia coli, Pseudomonas putida* and *Pseudokircheneriella subcaptata*).

Numerous symptoms of poisoning in humans following inhalation or dermal absorption of mononitrotoluene, dinitrotoluene, and TNT are observed a few days after exposure: headache, loss of appetite, dizziness, nausea, insomnia, numbness of various parts of the skin and diarrhea. Strong changes in the hemogram are the result of exposure. A particularly striking symptom is cyanosis, a bluish-red discoloration of lips, fingernails and skin due to oxygen deficiency. That is caused by reduced metabolites of TNT which are blamed for increased methemoglobin formation and hemolysis. The metabolites of TNT cause liver damaging effects (Koss et al. 1989).

2.2 Microbial Degradation of TNT

The degradation of TNT by microorganisms has been extensively studied for many years and the results have been compiled in numerous reviews (Fritsche et al. 2000; Hawari et al. 2000; Lenke et al. 2000; Spain et al. 2000; Esteve-Núñez et al. 2001; Rodgers and Bunce 2001; Rosser et al. 2001; van Aken and Agathos 2001; Heiss and Knackmus 2002; Fuller et al. 2004; Lewis et al. 2004; Schrader and Hess 2004; Zhao et al. 2004; Stenuit and Agathos 2010; Rylott et al. 2011). Some basic reactions are listed in Table 1.

2.2.1 TNT Degradation by Bacteria

There is one major problem with microbial TNT degradation: the three symmetrically arranged nitro-groups induce a high electron deficiency at the aromatic ring. An oxidative degradation and the use of TNT as a source of carbon and energy is extremely unlikely. Thus, the term degradation in this context means transformation or destruction of TNT, but not mineralization, i.e., use as the sole growth substrate for a microorganism.

The initial metabolites in the biotransformation of TNT are hydroxylaminodinitrotoluenes (HADNTs) aminodinitrotoluenes (ADNTs), diaminomononitrotoluenes (DANTs) and tetranitroazoxytoluenes (AZTs) (Hawari et al. 2000; Oh et al. 2000). Because of the electron deficiency of the ring π system, the initial degradation of TNT by microorganisms is characterized by reductive reactions (Vorbeck et al. 1998). The nitro-moieties of TNT ($-NO_2$) can be successively reduced to nitroso ($-NO$), hydroxylamino ($-NHOH$) and finally amino ($-NH_2$) groups (Fig. 1). By strictly anaerobic bacteria, such as *Clostridium* sp., *Desulfovibrio* sp. and archaebacteria as *Methanococcus* sp., TNT is completely reduced

Table 1 Degradation of TNT by microorganisms

Mechanism	Products*
Primary enzymatic reactions	
Stepwise reduction of nitro-groups of the aromatic ring by nitroreductases	Nitrosodinitrotoluene Hydroxyldinitrotoluene Aminodinitrotoluene Diaminodinitrotoluene
Hydride addition to the aromatic ring by flavoproteins of the *old yellow enzyme* family (*OYE*)	Monohydride– Dihydride- Protonated Dihydride- Meisenheimer complex
Secondary abiotic reactions	
Condensation of NODNT/ HADNT	Tetranitroazoxytoluene Secondary Diarylamines
Condensation of HADNT/$2H^-$ TNT.H^+	
Covalent binding to cell compounds	Protein adducts
Secondary enzymatic reactions	
Oxidation of the methyl-group	
Acetylation of an amino-group	
Oxidation of reduced metabolites (ADNT, DANT) by fungal exoenzymes and coupling to organic soil components	Polymers Humic acids adducts

*Different isoforms are formed depending on the microorganism and enzyme specificity

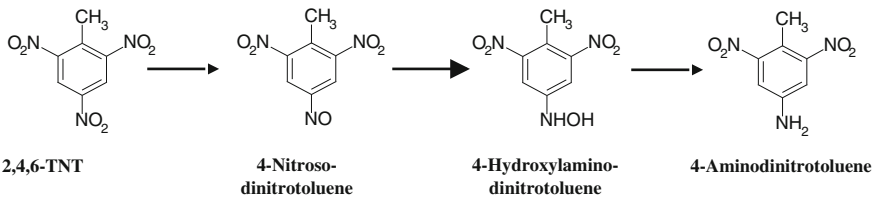

Fig. 1 Microbial transformation of TNT by reduction of nitro-groups

to 2,4,6-triaminotoluene (Boopathy and Kulpa 1994; Regan and Crawford 1994; Crawford 1995; Ederer et al. 1997).

These reduced TNT compounds present the primary products of the microbial metabolism. However, depending upon the reaction conditions (e.g., pH), they can be further converted by biotic and abiotic mechanisms to azo-, azoxy-, hydrazone-, and phenol-acetyl derivatives (Hawari et al. 2000). The intermolecular condensation of partially reduced derivatives leads to formation of azoxytetranitrotoluenes (Haidour and Ramos 1996).

The second important pathway is the direct reduction of the aromatic ring by addition of hydride-ions with the formation of monohydride-, and dihydride-Meisenheimer complexes (Fig. 2). As both pathways (nitro-reduction and aromatic

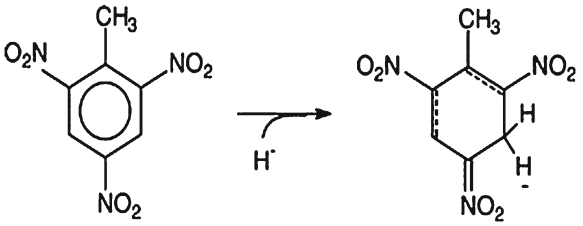

Fig. 2 Microbial transformation of TNT by hydride addition to the aromatic ring

2,4,6-TNT **Monohydride-Meisenheimer complex**

ring-reduction) may co-exist in the same cell, condensation reactions between dihydride-Meisenheimer complexes and hydroxylaminodinitrotoluene can lead to the formation of diarylamines with concomitant liberation of nitrite (Wittich et al. 2009).

TNT enters the bacterial cell most probably by passive diffusion across the cell barriers. In contrast, multi-drug efflux pumps are induced in *Pseudomonas putida* KT2440 in the presence of TNT, suggesting the importance of active extrusion systems in maintaining low intracellular TNT concentration to overcome toxicity (as reviewed by Stenuit and Agathos 2010). As a result of TNT degradation, different amounts of monomeric transformation products (ADNT, DANT) have been found extracellular (Claus et al. 2007a, b). Conclusively, active efflux systems may also exist for these compounds.

2.2.2 TNT Degradation by Fungi

The enzymatic conversions mentioned above do not imply ring opening (Hawari et al. 2000). This is the reason why TNT is transformed by bacteria, but usually not mineralized. However, white-rot fungi and the litter degrading fungus *Phanerochaete chrysosporium* as well as *Stropharia* species have been shown to mineralize TNT, at least in part, under aerobic conditions (Bumpus and Tatarko 1994; Fritsche 1998; Esteve-Núñez et al. 2001).

In a screening program, 91 fungal strains belonging to 32 genera of different ecological and taxonomic groups (wood and litter decaying basidiomycetes, saprophytic micromycetes) were tested for their ability to metabolize and mineralize TNT (Scheibner et al. 1997b). All these strains metabolized TNT rapidly by forming monoaminodinitrotoluenes (ADNT). Micromycetes produced higher amounts of ADNT than did wood and litter decaying basidiomycetes. A significant mineralization of (C^{14}) TNT was only observed for certain wood and litter decaying basidiomycetes. The most active strains, *Clitocybula dusenii* TMb12 and *Stropharia rugosa-annulata* DSM11372 mineralized 42 and 36 %, respectively of the initial added (C^{14}) TNT to (C^{14}) CO_2 within 64 days. However, micromycetes (deuteromycetes, ascomycetes, zygomycetes) were unable to mineralize (C^{14}) TNT significantly.

Responsible for this degradation is the ligninolytic enzyme system of white-rot fungi which consists of a number of extracellular enzymes, especially peroxidases (lignin peroxidase, Mn-dependent peroxidase) and oxido-reductases (laccases) which catalyze the degradation of the wood constituent lignin (van Aken and Agathos 2001). The metabolism starts again with reduction of the TNT nitro-groups yielding 4-ADNT and 2-ADNT. Thereafter, various acylation reactions to formylated and acetylated products may occur (Hawari et al. 1999). One of these formylated products, 2-amino-4-formamido-6-nitrotoluene has been identified as a substrate for lignin-peroxidase. The other products are broken down under ligninolytic conditions as well (Hawari et al. 1999). The manganese-dependent peroxidase is able to mineralize 4-ADNT directly, as shown for the lignin peroxidase negative fungus *Nematoloma frowardii* (Scheibner et al. 1997a). In addition, an intracellular, cytochrome P-450 dependent enzyme system has been identified which is involved in the mineralization of TNT in *Bjerkandera adusta* (Eilers et al. 1999).

2.2.3 TNT as a Source of Nitrogen or Electron Acceptor

The vast majority of studies demonstrate that TNT can be exclusively transformed in a co-metabolic manner (i.e., in the presence of a reduction equivalent donating substrate), but not mineralized by an individual bacterial strain. Nevertheless, there are some reports of at least partial mineralization of TNT by natural bacterial consortia (Robertson and Jjemba 2005; Montgomery et al. 2011). Recently, an amazing new strain VT11 of *Acinetobacter* sp. has been described which utilizes TNT as sole growth substrate (Solyanikova et al. 2012).

In contrast, it is also established that various microorganisms can use TNT as a source of nitrogen (Duque et al. 1993; Boopathy and Kulpa 1994) or as external electron acceptor (Table 2). In the case of *Pseudomonas* sp. JLR 11, nitrite is released from the aromatic ring and then further reduced to ammonium. Almost 85 % of the nitrogen of TNT can be incorporated into the cells as organic nitrogen (Esteve-Núñez and Ramos 1998; Esteve-Núñez et al. 2000). As an intermediate of nitrogen release, Meisenheimer-complexes have been identified (French et al. 1998; Heiss and Knackmus 2002). It has been proposed that the dihydride-complex slowly rearomatizes with the concomitant release of nitrite. Another mechanism of N release from TNT involves its partial reduction to hydroxylamino derivates and subsequent release of ammonium from the aromatic ring, probably through an acid-catalyzed Bamberger-like rearrangement (Stenuit and Agathos 2010).

The yeast strain *Geotrichum candidum* AN-Z4 isolated from a polluted site is able to transform TNT via the formation of unstable intermediate hydride-Meisenheimer-complexes with their subsequent destruction and accumulation of nitrite and nitrate (Ziganshin et al. 2010). Aeration of the medium promoted more profound destruction of this xenobiotic by the strain *G. candidum* AN-Z4 than static conditions.

Table 2 Microbial utilization of TNT as N-source or electron acceptor

Mode of utilization	Mechanisms	Conditions
Nitro-groups of TNT as N-source	*Pathway A*	
	Reduction of TNT to HADNTs and subsequent release of ammonium from the aromatic ring through acid-catalyzed Bamberger-like reaction	1 NAD(P) H^+ required, pH < 4.2
	Pathway B	
	Condensation of HADNTs with $[2H^-]$–TNT.H^+ to form secondary diarylamines with concomitant release of nitrite	7 NAD(P) H^+ required, 3 of them for further conversion of NO^{2-} to NH_3 by nitrite reductase
Nitro-groups of TNT as external electron acceptors	*Pathway A*	Anaerobic/respirative
	Energy generation by utilization of an electrochemical gradient	
	Pathway B	Anaerobic/fermentative
	Reoxidation of reduced electron carriers to maintain energy via substrate level phosphorylation	

Two possible pathways of TNT biodegradation were confirmed experimentally: (1) via the destruction of the TNT-monohydride complex [(3-H^-)-TNT] and (2) via the destruction of one protonated TNT-dihydride complex [(3,5-$2H^-$)-TNT.H^+].

The best proven mechanism of N release from TNT involves the abiotic condensation of hydroxylamino-dinitroluene- (HADNT) isomers and protonated dihydride-Meisenheimer-complexes to form secondary diarylamines with the concomitant release of nitrite (Wittich et al. 2009). The nitrite is probably further converted to ammonium by a nitrite reductase and finally assimilated via the glutamine synthetase-glutamate synthase reaction (Caballero et al. 2005b). The complete process requires seven reducing equivalents.

Some *Pseudomonas* strains use TNT as an alternative electron acceptor in the respiratory chain forming ATP (Esteve-Núñez et al. 2000). Anaerobic *Clostridiae* exploit TNT for reoxidation of reduced electron carriers to maintain the fermentative metabolism (Stenuit and Agathos 2010).

2.2.4 Enzymes Involved in TNT Transformation

Initial TNT transformations are catalysed by nitroreductases which are related to the old yellow enzyme (OYE) of yeast (French et al. 1998; Klausmeier et al. 2001; Williams et al. 2004; Kim and Song 2005). They catalyse the pyridine nucleotide dependent reduction of nitro-aromatic compounds. Nitroreductases are ubiquitous in bacteria and higher organisms (fungi, plants, animals). They have recently generated great interest from different points of view. Possible application areas

are not only restricted to bioremediation (Hannink et al. 2001), but also to specific biocatalysis (Kadiyala et al. 2003) and cancer therapy (Denny 2002; Knox et al. 2003). Through their activity, they decisively determine the toxicity of nitroaromatic compounds (Homma-Takeda et al. 2002; Padda et al. 2003), although their physiological relevance is still largely unknown. Nitroreductases may be classified into two types.

Oxygen-insensitive Type I Nitroreductases

These are localized in the cytoplasm where they are constitutively expressed. They are present as either monomeric or homodimeric flavin-mononucleotide (FMN) containing proteins with a subunit size of approximately 25 kDa and use NAD(P)H as an electron donor (Stenuit and Agathos 2010). They catalyze the ubiquitous reduction of aromatic nitro-groups to amino-groups through two-electron increments. The arising nitroso- and hydroxylamine-intermediates readily undergo condensations reactions with themselves or other organic molecules (proteins, humic acids) yielding polymeric products (Sarlauskas et al. 2004). Some of these enzymes attack directly the aromatic ring by hydride-ion addition to TNT and other nitroaromatics (Ramos et al. 2005). Type I nitroreductases have been described in many Gram-negative bacteria, such as *Escherichia coli* (Whiteway et al. 1998), *Salmonella enterica* (Nokhbeh et al. 2002), *Enterobacter cloacae* (Haynes et al. 2002), *Helicobacter pylori* (Goodwin et al. 1998), *Vibrio harveyi* (Lei et al. 1994), *Vibrio fisherii* (Riefler and Smets 2002), *Rhodobacter capsulatus* (Blasco and Castillo 1993), *Thermus thermophilus* (Park et al. 1992), *Pseudomonas pseudoalcaligenes* (Sommerville et al. 1995), *Pseudomonas putida* (Caballero et al. 2005a, b), *Pseudomonas fluorescens* (Pak et al. 2000), *Selenomonas ruminatium* (Anderson et al. 2002) and *Klebsiella* sp. (Shin and Song 2009).

Type II Hydride Transferases

They belong to the $(\beta/\alpha)_8$ barrel *OYE* family of flavoproteins (Stenuit and Agathos 2010). The enzymatic catalysis is characterized by a ping-pong reaction comprising two half-reactions. In the reductive part, the enzyme is reduced by NAD(P)H to yield the enzyme-bound $FMNH_2$. In the oxidative half-reaction, $FMNH_2$ is reoxidized by TNT in two competing pathways: (a) the ubiquitous nitro-reduction of TNT and (b) the specific nucleophilic addition of hydride-ions to TNT, leading to formation of mono- and dihydride-Meisenheimer-complexes.

Because of different redox-potentials, the reduction of the first nitro-group of TNT occurs faster than that of the remaining groups. The reaction mostly starts at the *para*-nitro group (Pak et al. 2000; Riefler and Smets 2002; Kim and Song 2005). However, some bacterial strains produce mainly the *ortho*-derivate (2-ADNT) which may be due to differences in substrate specificities of the degrading enzymes (Oh et al. 2003; Maeda et al. 2006). In addition to nitro-group reduction, nitroreductases of *Enterobacter cloacae*, *E. coli, Pseudomonas fluorescens* and *Pseudomonas putida* catalyze reduction of TNT by hydride-additions to the aromatic ring, yielding orange coloured hydride- and dihydride-Meisenheimer-complexes under the release of nitrite (French et al. 1998; Khan et al. 2004; Williams et al. 2004; Caballero et al. 2005b).

Besides the two major enzyme classes, an only iron hydrogenase from *Clostridium acetobutylicum* has been found capable of reducing TNT to its dihydroxylamino-derivate in a hydrogen depending manner (Symons and Bruce 2006).

Recent experiments indicated that the enzymes from *Raoultella terrigena* HB prefer the nitro-group reduction pathway (Claus et al. 2007a, b) similar to its close relative *Klebsiella* sp. (Kim and Song 2005). Apart from TNT, cells of *R. terrigena* HB transformed dinitrotoluene and nitrobenzenes. The comparison of microbial transformation of whole cells as opposed to a cell-free extract suggests that nitrophenolic compounds are substrates for the reducing enzymes, but they presumably do not pass the bacterial cell membrane and/or act as metabolic inhibitors. In contrast, nitrobenzenes were as good substrates for whole cells and cell extracts.

Secondary transformations of TNT metabolites can be catalyzed by enzymes, generally known as laccases (EC 1.10.3.2, para-benzenediol:dioxygen oxidoreductases). These are multi-copper proteins that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism. The enzyme has been found in eukaryotes (fungi, higher plants, insects) and more recently in many bacteria (Claus and Strong 2010). Numerous articles have touted its diverse potential application in various biotechnological processes. This is attributed to the enzyme's broad-substrate spectrum, the use of readily available oxygen as the final electron acceptor and apart from copper, no requirement for co-factors or peroxide (Claus and Strong 2010).

TNT itself is not a substrate for these oxidative enzymes. However, after conversion by nitroreductases, the reduced metabolites, such as aminodinitrotoluenes (ADNT), azoxy-compounds and diaminonitrotoluenes, can be efficiently oxidized by laccase to polymeric products (Strong and Claus 2011). One approach for the bioremediation of contaminated sites presents the immobilization of TNT and its metabolites into the complex soil organic matter during composting or during anaerobic and aerobic slurry treatment. The potential of laccases from different white-rot fungi for immobilizing TNT degradation metabolites into the humic matrix has been demonstrated by several research groups (Dawel et al. 1997; Thiele et al. 2002; Wang et al. 2002).

2.3 TNT Contamination in Germany

The total production of TNT, the main explosive of the last World Wars amounted to about 800,000 tons in Germany. As a result of manufacture, accidents and improper disassembling, TNT, precursors and by-products were heavily discharged into soils and groundwater (Preuß 1996; Preuß and Eitelberg 1999). A study in 1996 listed 3,240 suspected locations, of which at least 750 might be contaminated with explosives (Preuß 1996). It has been estimated that the contaminated area extends to over 10,000 km^2 (Preuß 1996). After the cessation of production and removal of the relevant installations, most sites of the former weapon factories were converted to residential or commercial areas (Schneider 1989). A notable example is the

terrain of a former TNT factory near Hamburg, where a nuclear power plant has been installed. Due to high water consumption for the production process, explosives plants were located in water-rich areas, i.e., important sources for drinking water. After rainfalls, the nitroaromatics are still continuously leached out and hence require expensive activated carbon filter systems to protect groundwater. An ancient bomb factory from World War I ("Espagit" at Hallschlag, Rhineland-Palatinate) was not fully restored, but only secured in the core zone by a soil cover. The leakage of water is collected by an underground ring pipeline and purified through activated charcoal (Preuß and Eitelberg 1999).

2.4 Treatment of Contaminated Soils

TNT and some of its degradation products have a high persistence, toxicity and mutagenicity (Spanggord et al. 1995; Honeycutt et al. 1996; Lachance et al. 1999). For this reason, the fate of these compounds is of interest and remediation of the contaminated sites is inevitable. Traditional remediation methods for TNT-contaminated sites have been primarily conducted by physical–chemical methods, including incineration, landfilling, thermal desorption and soil washing. A study published in 1999 on the economics of various methods of soil remediation suggested that biological soil remediation procedures in Germany are more favorable for technical reasons vis-à-vis soil incineration or soil washing (Jansky and Neumann 1999). Furthermore, incineration of soil to get rid of explosives can result in the exposure of workers to high levels of toxins (Symons and Bruce 2006).

In the past, various bioremediation technologies have been developed for soil environments (Held et al. 1997; Daun et al. 1998; Drzyzga et al. 1999; Lenke et al. 2000; Fuller et al. 2004; Kröger et al. 2004; Lewis et al. 2004). Biological *ex situ* methods rely upon the microbial community to treat contaminated media which include soil slurry reactors, land farming and soil composting. Soil slurry is created by transferring contaminated soil to a reactor, where aerated mixed with nutrients the xenobiotics are degraded by indigenous microflora. Land farming involves mixing of the contaminated soil with the surface layer of an uncontaminated soil (0–30 cm depth) where added nutrients (fertilizers) and moisture synergistically maximize indigenous microbial activity on nitroaromatic degradation. Soil composting (static piles or windrows) is similar to land farming, but includes addition of organic amendments, such as biosolids or green/animal manures and subsequent mixing of contaminated soil with the amendments (Makris et al. 2010).

As a serious drawback, *ex situ* treatment may not be economically feasible for a large-scale remediation of TNT-contaminated sites, as found in Europe and USA (Makris et al. 2010). If we improve the biological methods for the *in situ* remediation, it will be economical. Furthermore, the degradation of pollutants by microorganisms is a very ecofriendly method, as the structure and biological function of soils are not disturbed (Fritsche 1998). Various *in situ* bioremediation methods have been developed and tested in the lab/field with relative success, such

as natural attenuation, biostimulation, bioaugmentation and phytoremediation. Natural attenuation is the most simple and inexpensive bioremediation method that relies upon the activity of the indigenous microbial community on the xenobiotics which often takes long time (decades). Biostimulation of indigenous microorganisms with the chemical amendments (N and P fertilizers) is another method that relies on the adjustment of soil properties, such as nutrient content, pH, and redox potential, resulting in enhanced microbial activity on the contaminant. Bioaugmentation is defined as the addition of specific microorganisms (wild-type, or genetically engineered) to the contaminated soil which have been previously tested in the lab for their degradation ability. Phytoremediation is a low-cost and environment-friendly bioremediation method which has shown a high promise for use in TNT-contaminated soils (Makris et al. 2010; Rylott et al. 2011).

In experiments with radio-labeled TNT, it was repeatedly found that a major part of TNT and its metabolites, independently of the individual process, quickly and irreversibly bind to the soil matrix (Drzyga et al. 1998, 1999; Spain et al. 2000; Weiss et al. 2004a). This could be demonstrated in an aerobic–aerobic soil slurry process (>99 % bound residues), various composting variants (80 % bound residues), and by the use of fungi (86 % bound residues). The radioactivity was determined in each case in the aqueous and methanolic-extracts as well as in the organic fractions, i.e., fulvic acids, humic acids and humin.

Weiss et al. (2004b) studied the fate of (N^{15})-TNT in the course of an aerobic bioremediation reactor process with *Stropharia rugosoannulata*. About 2 % of the (N^{15}) label was found as NO_3^- and NH_4^+, indicating simultaneous processes of direct TNT denitration and reduction with cleavage of the amino groups. The enrichment of NO_2^-/NO_3^- [up to 7.5 atom % of (N^{15})] suggested the formation of Meisenheimer-complexes and denitration. The enrichment of N_2O [38 % of the (N^{15}) label] demonstrated that both N atoms were generated from the labeled TNT and indicated a novel formation process. The authors propose the generation of N_2O by cleavage from condensed azoxy-metabolites. In addition, 1.7 % of the (N^{15}) label was detected as biogenic amino-acids in the wheat straw containing the fungus. Overall, 60 to 85 % of the applied (N^{15})-TNT was degraded and 52–64 % was found as nonextractable residues in the soil matrix. Three percent was detected as 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene.

Park et al. (2012) evaluated the toxicity of TNT contaminated soils after passing through composting and slurry-phase bioreactor processes using the *Salmonella* mutagenicity assay. For composting, the percentage of mutagenicity reductions of final composts in strain TA98 and TA100 with S9 metabolic activation were 90.3–93.7 % and 96.7–97.5 %, respectively. For slurry-phase bioreactor processes, the percentage reductions of final residuals in strain TA98 and TA100 with S9 metabolic activation were 95.0 and 99.1 % for anaerobic, 96.2 and 99.2 % for anaerobic/aerobic and 96.6 and 97.4 % for anaerobic treatment. It was implied that slurry-phase treatment was a more effective process than composting in reducing toxicity.

In order to biostimulate the capacity of bacteria to degrade TNT, Muter et al. (2012) amended varying concentrations of nutrients consisting of inorganic salts,

plant extracts, and molasses to soil and liquid media. For the inoculum, they used a consortium of bacteria which was isolated from explosives contaminated soils and exhibited the ability to degrade TNT. Phylogenetically, the clones clustered into seven different genera: *Klebsiella, Raoultella, Serratia*, *Stenotrophomonas, Pseudoxanthomonas, Achromobacter* and *Pseudomonas*. The addition of the consortium to a liquid environment along with 100 % nutrient amendment decreased the amount of TNT (and its degradation products) by up to 90 % after 14 days incubation. When the total amount of TNT was less than 100 mg/l, the concentration of TNT did not influence the amount of sugar consumed by the bacterial consortium. In soil media, the TNT degradation process was dependent on the concentration of nutrients added. At higher initial concentrations of TNT (500 mg/kg), bioaugmentation (i.e., addition of a bacterial inoculum) had a significant effect, especially when also nutrients were added to the soil.

Remedial measures are of increasing interest with plants (phytoremediation). These methods are economical, but limited by the relatively low tolerance of plants to TNT. In future, detoxification capacities might be enhanced using genetic modifications (Makris et al. 2010; Rylott et al. 2011). In accordance with these developments, Zhu et al. (2012) presented a system for TNT phytoremediation by overexpressing the old yellow enzyme (OYE3) gene from *Saccharomyces cerevisiae*. The resulting transgenic *Arabidopsis* plants demonstrated significantly enhanced TNT tolerance and a strikingly higher capacity to remove TNT from the media.

Various *on-site* biological methods of soil treatment were tested in Germany as a part of feasibility studies. For example in Hallschlag, a two-stage reactor process (soil suspension) and an anaerobic/aerobic composting process were tested, each with 50 tons of soil (Schmitz 1995). Worldwide, there have been or will be also *off-site* systems and highly developed *in situ* biological methods to clean up TNT-contaminated soils. Time will show whether they pass the proof of sustainability (Reinhard and Feldmann 1998; Thomas et al. 2001). For aqueous phases (without solid matrix) such methods are unsuitable, even though the spread of contaminants through groundwater and leachate is a particular hazard.

2.5 Treatment of Contaminated Waters

2.5.1 Microbial Entrapment

For contaminated soils, TNT biotransformation and immobilization to the organic matrix represents an effective and low-cost procedure, if not to eliminate, but at least to detoxify TNT and its metabolites. For aquifers, these procedures are not applicable as long as TNT and its potential toxic transformation products remain in the solution. So far, they have to be removed by expensive physico-chemical adsorption till alternative microbiological procedures are not available.

In the following section the isolation and characterization of a TNT transforming bacterial strain will be described which may be useful for this purpose (Claus et al. 2007a, b). The microbiological and analytical methods presented might concomitantly serve as a short practical outline for the study of microbial TNT degradation.

Water and soil samples were collected from abandoned TNT production sites in Germany (Hallschlag/Rhineland-Palatinate, Moschwig/Saxony-Anhalt). Microorganisms were enriched in nutrient broth supplemented with TNT (10 mg/l). From these cultures, single colonies were obtained on nutrient agar containing TNT (10 mg/l) and further characterized. Bacterial identification was done by sequencing of the 16S rDNA genes amplified by polymerase chain reaction (PCR). By this procedure about 20 isolates of mainly Gram-negative bacteria were obtained which were frequently isolated from contaminated soil in other studies (Muter et al. 2012). About half of the isolates contained one or more plasmids which is an indication of adaption to a stressful environment. The most efficient isolate, *R. terrigena* strain HB, grew in the presence of 100 mg TNT/l, a concentration which is toxic to many microorganisms (Fuller and Manning 1997). It grows well at temperatures between 4 and 40 °C with an optimum at 30 °C. Growth at low temperatures is a hallmark of the genus *Raoultella* which is facultative anaerobe, having both a respiratory and a fermentative type of metabolism (Drancourt et al. 2001).

For TNT degradation studies, the mineral-salt medium of Kalafut et al. (1998) was used. Nitroaromatic compounds were added at concentrations between 40 and 400 μM to the mineral-salt medium before autoclaving. For (^{14}C) studies, the medium was spiked with uniformly ring labeled (^{14}C)-TNT (33.3 kBq/ml). Glucose concentrations were set to 0.3 and 3.0 % (w/v), respectively. The pH of the culture medium was adjusted between 5.0 and 8.0 using a 200 mM Na phosphate buffer. *R. terrigena* was precultured in Standard I nutrient-broth for 16 h at 30 °C on a shaker before inoculation into mineral-salt media. These cultures were incubated for 7 days under aerobic conditions on a rotary shaker at temperatures between 10 °C and 37 °C. At regular intervals, aliquots were taken to determine transformation products. At the end, cells and insoluble material were separated by centrifugation at 40,000 × *g* for 30 min. The bacterial cell mass was washed twice with phosphate-buffered saline solution (pH 7.4), extracted with acetonitrile for 16 h at 30 °C and centrifuged as above. The resulting fractions (supernatant, washings, acetonitrile extract) were analysed by HPLC and Radio-HPLC.

On minimal-salt agar supplemented with TNT (100 mg/l), *R. terrigena* strain HB produced brownish pigments within and around the growth zone. Growth in liquid media was determined by colony counts and optical density. After a lag time of 24 h, the colony forming units (cfu) in the minimal-salt medium with TNT increased rapidly and reached the same level as in the medium without TNT. The significant increase of the optical density of cells grown in the presence of TNT was thus not a result of higher cell densities, but obviously attributed to altered spectroscopic properties of the cells and the culture media by accumulation of coloured TNT metabolites, similar to those observed on solid agar-media.

Determination of the optical density is thus not an appropriate parameter for estimating bacterial growth on dependence of TNT.

The growth was coincident with the disappearance of TNT from the culture media within 4 h incubation under optimum aerobic conditions (pH 7.0, 30 °C). Already low nutrient concentrations (≥0.05 % glucose) were sufficient to promote growth and TNT removal by *R. terrigena* strain HB. The need for nutrient supplementation and lack of ($^{14}CO_2$) production from ring-labelled TNT clearly indicated a co-metabolic process. This was further confirmed by the effective TNT transformation by resting cells.

In the culture supernatants, 2-ADNT and 4-ADNT were detected along with small amounts of 2,4-DANT and tetranitroazoxy-compounds. In contrast to the culture supernatant, the main transformation products found in the cell extracts were azoxy-dimers. The radiochromatogram of the extract identified 3 peaks, two of which could be assigned to TN-2,2′-azoxy and TN-4,4′-azoxy, respectively, in a ratio of 1:10. The third peak corresponded to either TN-2,4′-azoxy or TN-2′,4-azoxy, or a mixture of these condensation products.

The (^{14}C)-balance revealed that about 15 % of the initial radioactivity remained in the culture supernatants, whereas up to 85 % was found in the cell pellet. Our finding, that the main fraction of TNT metabolites is cell-associated, is deviated from most other reports, where the main fraction of transformation products remained in the supernatant in the form of ADNTs (Kalafut et al. 1998; Kim et al. 2002; Zhao et al. 2004). Similar to our study, a strain of *Pseudomonas aeruginosa* MX accumulated 71 % of the initial (^{14}C)-TNT in the cell pellet, leaving 21 % in the supernatant. In the latter fraction, 2-ADNT was the main metabolite and TN-2,2′-azoxy accumulated in the cells (Oh et al. 2003). As *R. terrigena* strain HB grows rapidly at low temperatures and different redox conditions, it is a promising candidate for the detoxification of TNT-contaminated waters under *in situ* conditions. The metabolites associated with the cell fraction can be removed together with the biomass, e.g. by filtration or flocculation (Fig. 3).

In order to optimize this process, we investigated the effects of culture conditions on the TNT transformation in more details. Although TNT elimination was observed at all incubation temperatures tested, pH 8.0 and 37 °C may regarded as optimum with respect to the transformation velocity. Similar conditions have been found for the biodegradation of TNT by *Pseudomonas putida* (Park et al. 2003). TNT was completely eliminated at all concentrations tested, however, the amount of glucose in the mineral salt media had a significant impact on the quantitative and qualitative distribution of metabolites in the supernatants and cells. At low glucose conditions (0.3 %), mainly ADNTs were detected along with the formation of smaller amounts of tetranitroazoxytoluenes. In contrast, at a tenfold higher glucose concentration (3 % glucose), 2,4-DANT was the almost exclusively detectable metabolite in the culture medium, accompanied by only minor amounts of azoxy-dimers in the cell pellet. One explanation is that at high glucose concentrations, an excess of reduction equivalents is produced by aerobic metabolism. As six electrons, provided by NAD(P)H, are needed for the complete reduction of one nitro group in TNT as shown in Fig. 3 (Vorbeck et al. 1998; Heiss and

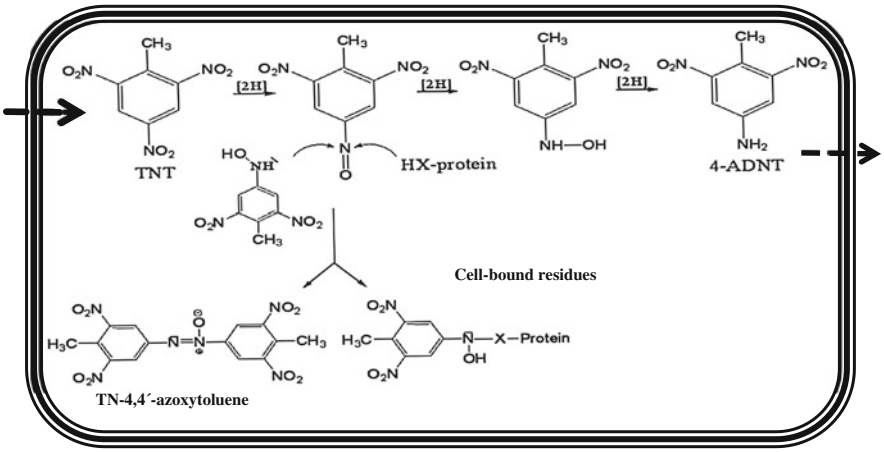

Fig. 3 Model of TNT transformation and entrapment by *R. terrigena* HB. TNT enters the bacterial cell by diffusion and is enzymatically reduced by nitroreductases. The products are about 10–20 % ADNTs which are found extracellular in the solution. Another 80–90 % of the initial TNT is converted to intra- or intermolecular coupling products which remain in the cell in form of insoluble tetranitroazoxytoluenes or bound to proteins. In the course of TNT transformation, *R. terrigena* HB forms brownish cells which can be removed from the solution by sedimentation or filtration (Claus et al. 2006, 2007a, b)

Knackmus 2002), the surplus of NAD(P)H may be used for the reduction of a further nitro-group. Farmore, high amounts of NAD(P)H will preclude the accumulation of nitroso-dinitritoluenes, thus preventing azoxy-dimer formation (Williams et al. 2004).

The efficiency of TNT removal under nearly *in situ* like conditions, was demonstrated in experiments with water and soil samples originating from contaminated sites which contained a complex mixture of nitroorganic compounds.

Conclusively, these results have shown that *R. terrigena* strain HB eliminates low and high TNT concentrations from water samples, but the efficiency of the process is regulated by controlling temperature, nutrient and pH conditions. In addition to TNT, the bacterium may be useful for the treatment of other nitroaromatic wastes as well.

2.5.2 Use of Immobilised Microorganisms

Another promising strategy to eliminate TNT from aquifers may be the use of immobilized microorganisms in batch or continuously operating systems. As an example, a *Bacillus* sp. YRE1 strain was isolated from red effluent and cells immobilized on charcoal and polystyrene were checked for their ability to degrade TNT by exposing them to different temperatures (Ullah et al. 2010). It was found that both charcoal and polystyrene immobilized bacteria degraded TNT most efficiently at 37 °C. Maximum percentage reduction in case of charcoal

immobilized *Bacillus* sp. YRE1 was calculated as 73.35 % at 37 °C, whereas, polystyrene immobilized bacteria showed 70.58 % reduction. *Bacillus* sp. YRE1 immobilized on charcoal, showed maximum degradation at pH 7 with 93.81 % reduction in TNT. Similarly, pH 5 was found to be optimum for the degradation of TNT by polystyrene immobilized bacteria, with percentage reduction as high as 94 %. Charcoal immobilized cells showed increased transformation with 96 % reduction in the presence of Tween 20, whereas, polystyrene immobilized cultures caused 87.77 % reduction in TNT.

A combined process of immobilized microorganisms/biological filter to degrade TNT in an aqueous solution was studied by Wang et al. (2010). The results showed that the procedure could effectively degrade TNT to an extent that it was not detected in the effluent of the system. GC/MS analysis identified 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene and 2,4-diamino-6-nitrotoluene as the main anaerobic degradation products. Ethanol as the electron donor played a major role in the TNT biodegradation. Environment Scan Electron Microscope analysis revealed that a large number of globular microorganisms were successfully immobilized on the surface of the carrier. Further analysis by Polymerase Chain Reaction (PCR)-Denaturing Gradient Gel Electrophoresis (DGGE) demonstrated that a special bacterial community for TNT degradation could be generated during the adaptation to the explosive for 150 days. Nevertheless, one should be aware that toxic degradation products of TNT may still remain in the contaminated water after treatment with immobilized bacteria.

2.5.3 Application of Enzymes

TNT is no substrate for oxidoreductases, but small organic mediators have been shown to increase the oxidative potential of laccase and allow the enzymatic attack of molecules which are no natural laccase substrates (Claus and Strong 2010). The presence of such a co-substrate may also facilitate the removal of recalcitrant TNT metabolites from water environments. The addition of phenolic compounds (200 mM ferulic acid and guaiacol) during the reductive transformation of TNT by the fungus *Trametes modesta* prevented the accumulation of all major stable TNT metabolites by at least 92 % (Nyanhongo et al. 2006). Acute toxicity tests of individual TNT metabolites and in *T. modesta* cultures supplemented with 200 μM TNT demonstrated that the biodegradation process leads to less toxic metabolites. The presence of phenolics during the laccase reaction were very effective in immobilizing the typical TNT metabolites (ADNTs and 2,2,6,6-azoxytetranitrotoluene). When laccase from *Trametes villosa* was added to a solution containing 4-ADNT and TNT, only 30 % of the 4-ADNT and none of the TNT was transformed. When the same experiment was done in the presence of catechol, 4-ADNT was complete and up to 80 % of TNT was removed from the solution. This was attained at close to a neutral pH which is beneficial for treatment of natural environments (Wang et al. 2002).

2.6 Preventive Approaches to Minimize TNT Contamination

An innovative microbiological approach to reduce TNT contamination takes advantage of the TNT-transforming *Bacillus* sp. strain SF, whose spores were incorporated into an explosive formulation containing TNT and ammonium nitrate (Nyanhongo et al. 2009). Upon addition of water to this new explosive mixture, vegetative *Bacillus* cells grow out which immediately initiate TNT transformation even after a 5-year storage of the bioexplosive at room temperature (Nyanhongo et al. 2009). The development of these self-cleaning explosive formulations opens new perspectives for the application of specific TNT-transforming microorganisms, such as spores of *Clostridium bifermentans* KMR-1 which can be used as a relatively stable inoculant for TNT biodegradation (Sembries and Crawford 1997). The possibility to lyophilize a *Pseudomonas putida* strain in the presence of cryoprotectants was also investigated for the application of non-sporulating microorganisms into TNT-based explosive formulations (Nyanhongo et al. 2009). However, the survival of *P. putida* cells was limited in the bioexplosive formulation, underlining the need to optimize the cryoprotective media and the lyophilization conditions (Nyanhongo et al. 2009). This is a challenge since *Pseudomonadaceae*, a catabolically versatile and ecologically important group of bacteria, is also the most studied family for bacterial TNT biodegradation. Recent studies on the microbial ecology of different TNT-polluted soil samples using DGGE fingerprinting have also demonstrated the predominance of members of *Pseudomonadaceae* in both long- and short-term contaminated sites (George et al. 2008; Travis et al. 2008a, b). In addition, Gram-negative bacteria are the best candidates for the microbial incorporation in self-cleaning explosive formulations since they are more tolerant to TNT than Gram-positive bacteria (Fuller and Manning 1997). In conclusion, the development of self-cleaning explosive formulations using microbial catabolic capabilities has recently emerged as an attractive strategy to prevent further TNT contamination (Stenuit and Agathos 2010).

A new environmentally benign synthesis route to manufacture military grade TNT, which eliminates the production of red water, arising from the sulfiting process for removing unsymmetrical trinitrotoluene isomers, was introduced by Millar et al. (2011).

3 Conclusions

The metabolic capacities of microorganisms (bacteria, fungi) have been successfully exploited to clean up TNT contaminated soils by strategies generally referred as bioattenuation or bioaugmentation. Comparable microbiological methods to treat waters charged with nitroaomatics are scarce. The entrapment of TNT metabolites within bacterial cells offers an opportunity to detoxify contaminated waters after separation of the biomass, at least as long biotransformation and not biomineralization is the state of art.

Alternative or additional strategies for cleaning contaminated aquifers and waters may take advantage of immobilized microorganisms or oxidative enzymes to enhance the formation of insoluble metabolites. Some innovative microbial strategies are currently under development to minimize the danger of TNT contamination during manufacturing process itself or by the design of innovative explosive formulations.

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