Degradation of the Dinitrotoluene Isomers 2,4- and 2,6-DNT: Appraising the Role of Microorganisms

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Abstract The dinitrotoluene (DNT) isomers 2,4- and 2,6-DNT are highly toxic compounds, and their occurrence in groundwater and soils is a widespread problem mainly due to the explosive manufacturing industry and from the commercial production of polyurethane foam. Moreover, these compounds have mutagenic and carcinogenic properties making them a great hazard to the public health. Thus, their removal from the environment is paramount, and bioremediation strategies can be applied for the environmental cleanup. Pure cultures of microorganisms able to degrade at least 2,4-DNT have been recently isolated as well as a consortium and different plant species. The pure cultures are an *Arthrobacter* strain isolated from crude oil-contaminated soil, a *Rhodococcus pyridinovorans* NT2, and *Shewanella marisflavi* EP1, while the consortium named UHasselt Sofie 3 (UHS3) was formed by *Burkholderia* HC114, *Variovorax paradoxus* VM685, *Bacillus, Pseudomonas mandelii* HC88, and *Ralstonia* HC90. These microorganisms perform the degradation of the dinitrotoluenes in aerobic conditions except the marine strain *Shewanella marisflavi*. The plant species able to grow in the presence of 2,4-DNT and proposed for phytoremediation are hemp, flax, sunflower, and mustard. The intermediates reported in the majority of successful biodegradation studies are 4-methyl-5-

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nitrocatechol (4M5NC) and 2-hydroxy-5-methylquinone (2H5MQ) under aerobic conditions, while 2,4-diaminotoluene is obtained as an end product via the formation of 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) under anaerobic conditions. These microorganisms add to the growing number of isolates with the ability to degrade these types of compounds since strain DNT was isolated. This mini-review focuses on the microorganisms involved in the degradation of the dinitrotoluene isomers 2,4- and 2,6-DNT in recent years. Information related to the degradation of these contaminants is critical to understanding and predicting their fate in the environment.

Keywords Environmental pollution • Dinitrotoluene isomers • Degradation • Microorganisms

Introduction

2,4-Dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene are major groundwater and soil contaminants derived from the production of polyurethane (foam), but most importantly they are major by-products in the production of the explosive 2,4,6-trinitrotoluene (TNT). DNT-contaminated sites are a wide problem due to the explosive manufacturing and army activities all over the world (Xu and Jing 2012). Both DNT isomers are highly toxic; they have mutagenic and carcinogenic properties and are damaging the reproductive system. They can enter the body by inhalation, skin contact, or consumption of contaminated water.

Because of their abundance, toxicity, mutagenicity, and carcinogenicity, US EPA has classified the DNT isomers as priority pollutants (Dodard et al. 1999; Ellis III et al. 1979; Gong et al. 2003; Rickert et al. 1984). Thus, the removal of these contaminants is imperative in order to prevent public health problems. The bioremediation of these contaminants is a viable alternative since both DNT isomers can be degraded in aerobic (Hudcova et al. 2011; Johnson et al. 2000; Küce et al. 2015; Kundu et al. 2015; Nishino et al. 2000) and anaerobic conditions (Cheng et al. 1996, 1998; Huang et al. 2015; Hughes et al. 1999; Wang et al. 2011). Some of the first reported microorganisms capable of the dinitrotoluene degradation were the *Burkholderia* sp. strain DNT (Suen and Spain 1993), *Burkholderia cepacia* R34 (Johnson and Spain 2003), the fungal strain *Phanerochaete chrysosporium* (Valli et al. 1992), and two consortia containing the key members *Variovorax paradoxus* VM685 and *Pseudomonas* sp. VM908, respectively (Snellinx et al. 2003). Subsequent studies tested several genetically modified strains in order to enhance the degradation of the dinitrotoluene with an emphasis on the 2,4-DNT isomer (Dutta et al. 2003; Fish et al. 2000; Lin et al. 2003; Nasr et al. 2001; Patel et al. 2000). While the degradation genes from strain DNT were transferred to *Sinorhizobium meliloti,* the genes of the bacterial hemoglobin *Vitreoscilla* were added to a few strains (Fish et al. 2000; Lin et al. 2003; Nasr et al. 2001; Patel et al.

2000) achieving higher degradation rates. However, recent studies have unveiled yet more consortia and individual strains capable of the DNT isomers. Some of these strains are the marine isolate *Shewanella marisflavi, Rhodococcus pyridinovorans*, and *Arthrobacter* sp. It is important to mention that the *Rhodococcus* strain was able to degrade both isomers concomitantly and could tolerate an initial concentration of 100 ppm in only 2 days. Also consortia comprised of *Bacillus cereus* NT4, *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, and *Achromobacter* sp. NDT3 (Hudcova et al. 2011) have been reported to degrade 2,4-DNT, the most common of the isomers. The two pairs of intermediates 4-methyl-5-nitrocatechol (4M5NC) and 2-hydroxy-5-methylquinone (2H5MQ) under aerobic conditions and 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) under anaerobic conditions have been reported in most of the biodegradation studies suggesting two main degradation pathways.

Excellent reviews on the degradation of nitroaromatics have been published previously (Anuradha 2015; Gorontzy et al. 1994; Nishino and Spain 2001, 2004; Spain and Hughes 2000). However, this chapter focuses on the microorganisms that have been identified in dinitrotoluene biodegradation studies in the recent years.

Recent Studies on the Degradation of 2,4- and 2,6-DNT

The degradation of 2,4-dinitrotoluene has recently been observed by an *Arthrobacter* strain isolated from crude oil-contaminated soil in Turkey and is plasmid mediated; the plasmid name is pArK1 with an estimated size of 8.1 kb (Küce et al. 2015). The rates of degradation depended mainly on pH and temperature and the optimum conditions were 7 and 30 °C, respectively. Although the degradation of 2,4,6-TNT had been reported previously by an *Arthrobacter* strain, the study by the Turkish group seems to be the first to report the degradation of 2,4-DNT by an *Arthrobacter* strain. Another recent study reported that the strain *Rhodococcus pyridinovorans* NT2 could degrade up to 100 mg L^{-1} of both 2,4- and 2,6-DNT with a complete degradation of the contaminants in 48 h (Kundu et al. 2015). The *Rhodococcus* strain was isolated from soils and effluents in a pesticide industry in Gujarat, India. The growth yield was 0.68 and 0.65 g of cells per $gram^{-1}$, while their degradation rates were 1.38 and 2.08 mg l^{-1} h^{-1} for 2,4 and 2,6-DNT, respectively. Thus, the authors suggest that this *Rhodococcus* strain could be used as the main bioremediation agent of DNT-contaminated sites since it can use both DNT isomers as carbon, nitrogen, and energy source (Kundu et al. 2015).

Phytoremediation has also been proposed as an alternative for the bioremediation of DNT-contaminated sites (Podlipná et al. 2015; Su and Zhu 2007; Susarla et al. 2002). Several plant species such as hemp, flax, sunflower, and mustard were able to grow on up to 1.0 ppm 2,4-DNT-contaminated soil. Moreover germination was not affected when the plants were grown under 200 ppm of 2,4-DNT in the laboratory, and a low concentration of the contaminant (0.252 g kg^{-1}) even had a

stimulatory effect. Furthermore in vitro cultures of *Saponaria officinalis* (soapwort), *Senecio jacobaea* (ragwort), and *Phragmites australis* (reed) demonstrated the 2,4-DNT metabolization with 2-amino-4-nitro compounds and 4-amino-2-nitro compounds as the main intermediates. Soapwort metabolized the contaminant faster than reed, and the authors suggested the presence of specific enzymes in the former plant (Podlipná et al. 2015).

The degradation of 2,4-DNT has also been recently reported under anaerobic conditions. The microorganism *Shewanella marisflavi* EP1was able to degrade 2,4-DNT through anaerobic respiration. Furthermore the degradation occurred at a 7.0–9.0 pH range and at a wide range of temperatures (4–40 °C) and salinity (2–8% NaCl concentration) (Huang et al. 2015). Although several strains had been reported to degrade 2,4-DNT in laboratory studies with diverse strains such as *Lactococcus lactis*, *Clostridium acetobutylicum*, and *Pseudomonas aeruginosa* (Cheng et al. 1998; Hughes et al. 1999; Noguera and Freedman 1996; Shin et al. 2005; VanderLoop et al. 1999), the study involving *Shewanella marisflavi* was conducted in situ, adding information to a previous report on marine sediments (Yang et al. 2009). Strain EP1 was able to completely reduce (100 μM) 2,4-DNT in less than 24 h with lactate as the electron donor. Moreover the authors also proved that lactate oxidation is necessary for electron release to reduce the contaminant and support the growth of EP1 under anaerobic conditions. Two main intermediates are produced during the 2,4-DNT reduction: 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) which in turn become 2,4-diaminotoluene (2,4-DAT). 4A2NT was preferentially formed over 2A4NT, and the authors attribute it to the shielding effect of the methyl group on the ortho-nitro group as explained previously and to the terminal reductases in each of the microorganisms (Huang et al. 2015). Although the complete degradation pathway was not proposed, the authors were able to conclude that dehydrogenase, menaquinone, cytochromes, and flavins play an important role in the electron transport process during the reduction. Furthermore the strain's ability to degrade the 2,4-DNT at a wide range of pH, temperature, and salinity makes it an important potential bioremediation tool in natural environments.

Another recent study reported the successful enhancement of *Arabidopsis* root length under 2,4-DNT stress by the UHasselt Sofie 3 consortium (UHS3) (Thijs et al. 2014). The consortium was formed by *Burkholderia* HC114*, Variovorax paradoxus* VM685*, Bacillus, Pseudomonas mandelii* HC88, and *Ralstonia* HC90 species, and it helped the plant to double the main root length after 9 days when exposed to 1.0 mg l^{-1} of 2,4-DNT (Thijs et al. 2014). Furthermore three consortia UHS1, UHS2, and UHS3 successfully degraded 2,4-DNT, but UHS3 reported the best degradation when compared to UHS1 and UHS2 since no intermediates accumulated, degradation started earlier, and it showed the fastest degradation rate. Thus the authors propose consortium UHS3 to be used in 2,4-DNT-contaminated environments (Thijs et al. 2014). Table 1 summarizes the studies on the degradation of the DNT isomers in the recent years with an emphasis on the microorganisms responsible of the degradation.

Table 1 Recent studies reporting the degradation of at least one of the DNT isomers

Microorganism/study details	Conditions	References
Shewanella marisflavi EP1	Anaerobic	Huang et al. (2015)
Rhodococcus pyridinovorans NT2	Aerobic	Kundu et al. (2015)
Hemp, flax, sunflower, mustard	Anaerobic	Podlipná et al. (2015)
Arthrobacter sp. K1 pArK1 gene	Aerobic	Küce et al. (2015)
Bioreporters	Aerobic	Yagur-Kroll et al. (2014, 2015)
Tolerant strains: *Burkholderia, Variovorax, Bacillus, Pseudomonas, Ralstonia*	Aerobic	Thijs et al. (2014)
Agar absorption	Aerobic	O'Sullivan et al. (2010)
Bacillus cereus NT4, *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, *Achromobacter* sp. NDT3. Individually and as a mixture	Aerobic	Hudcova et al. (2011), Paca et al. (2009)
Suspended (chemostat) and attached (columns) systems. Lowest DNT concentrations to support growth	Aerobic	Han et al. (2011), Fortner et al. (2003)
Closest relatives of DGGE excised bands: Anaerobic reactor: *Pseudomonas* sp SRU_14 & *Chryseobacterium* sp. (FJ 202055) Aerobic reactor: *Riemerella* sp., *Flavobacteriaceae* sp. and *Agrobacterium* sp.	Aerobic/ Anaerobic	Wang et al. (2011)
2,4-DNT degradation with ZVI addition	Aerobic	Oh et al. (2010)
Mixed enrichment cultures in freshwater	Aerobic	Bausum et al. (1992)
Biosensor	Aerobic	Rodríguez et al. (2006)
Lactococcus lactis	Anaerobic	Shin et al. (2005)
Burkholderia strain DNT degrades 2,5 DNT	Aerobic	Leungsakul et al. (2005)
Burkholderia strain YV1 (biodegradation enhancement)	Aerobic	Lin et al. (2003), Nasr et al. (2001), Patel et al. (2000)
Consortium 1 (4 species): *Variovorax paradoxus* VM685 & *Pseudomonas* sp. VM908 Consortium 2 (6 species): *Pseudomonas marginalis* VM683, *P. aeruginosa* VM903, *Stenotrophomonas maltophilia* VM905, *P. viridiflava* VM907	Aerobic	Snellinx et al. (2003)
Sinorhizobium meliloti (GMO) PJS1 biodegradative plasmid introduced from *Burkholderia* strain DNT	Aerobic	Dutta et al. (2003)
Pseudomonas putida UO83	Aerobic	Walia et al. (2002)
Burkholderia cepacia R34	Aerobic	Johnson et al. (2000, 2002), Johnson and Spain (2003)
2,4 & 2,6-DNT *Burkholderia cepacia* JS850 and *Hydrogenophaga paleronii* JS863	Aerobic	Nishino et al. (2000), Nishino and Spain (2001)
Soil slurries	Aerobic	Zhang et al. (2000)
Continuous flow lab fermentor	Anaerobic	Cheng et al. (1996)
Denitrifying conditions bed reactor	Anaerobic	VanderLoop et al. (1999)

(continued)

Table 1 (continued)

Microorganism/study details	Conditions	References
Burkholderia sp. strain DNT	Aerobic	Ortega-Calvo et al. (1999)
Clostridium acetobutylicum	Anaerobic	Hughes et al. (1999)
Ethanol, methanol and acetic acid as substrates	Anaerobic	Cheng et al. (1996, 1998)
Pseudomonas aeruginosa	Anaerobic	Noguera and Freedman (1996)
Burkholderia sp. strain DNT	Aerobic	Haigler et al. (1994, 1996, 1999), Spanggord et al. (1991), Suen et al. (1996), Suen and Spain (1993)
Phanerochaete chrysosporium	Anaerobic	Valli et al. (1992)

Biosensors and Bioreporters for 2,4-DNT Detection

Several studies have described the construction of bioreporters for the detection of 2,4-DNT (Behzadian et al. 2011; Burlage et al. 1998; Lönneborg et al. 2012; Radhika et al. 2007; Rodríguez et al. 2006). One of the studies is centered in the detection of 4-methyl-5-nitrocathecol (4M5NC) and 2,4,5-trihidroxytoluene (2,4,5-THT). These compounds are derivatives in the 2,4-DNT biodegradation and are recognized as polyphenol oxidase (PPO). The authors devised an amperometric biosensor that involved the immobilization of the enzyme polyphenol oxidase into a composite matrix of glossy carbon microspheres and mineral oil (Rodríguez et al. 2006; Yagur-Kroll et al. 2014). The biosensor was able to detect 4M5NC even in the presence of large quantities of 2,4-DNT. The biosensor used the *dntA* (*dntA-aAbAcAd*) genes that encode the 2,4-DNT dioxygenase from *Burkholderia* sp. strain DNT from plasmid pJS37 (Suen et al. 1996). The study confirmed a biosensor lack of response when there are no quinones formed by the PPO from phenols and catechols or in the absence of the enzyme. Thus the authors suggest the biosensor as a great alternative to decentralize environmental testing of 2,4-DNT (Rodríguez et al. 2006).

A couple of more recent studies reported the construction of a genetically engineered *Escherichia coli* bioreporter for the detection of 2,4-DNT and 2,4,6-trinitrotoluene (2,4,6-TNT) (Yagur-Kroll et al. 2014, 2015). The bioreporter employs a genetic fusion between two gene promoters *yqjF* and *ybiJ* to the *Photorhabdus luminescens luxCDABE* genes or the green fluorescent protein gene GFPmut2. Both strains were able to detect 2,4-DNT in vapor form, in aqueous solution, and when buried in soil. The bioreporters are induced by degradation products (not identified) of 2,4-DNT and not the compound itself (Yagur-Kroll et al. 2014). A second study by the same group has reported that the detection threshold, response time, and signal intensity were improved by two rounds of random mutagenesis of the *yqjF* promoter region (Yagur-Kroll et al. 2015).

Biodegradation of 2,4- and 2,6-DNT Under Aerobic Conditions

The degradation of 2,4-DNT has been studied very closely by the Spain group at the Air Force Research Laboratory in Florida, USA. Their early studies reported the degradation of 2,4-DNT by a *Burkholderia* strain DNT (formerly *Pseudomonas*). The degradation is initiated by a dioxygenase attack on the 2,4-DNT to produce 4-methyl-5-nitrocatechol (4M5NC) which is later oxidized by a monooxygenase, and further reactions lead to ring fission (Spanggord et al. 1991). The responsible genes were later characterized; it was revealed that the genes for the DNT pathway are organized in three different operons, and the DNT dioxygenase is a multicomponent enzyme system (Suen and Spain 1993). The open reading frames of the genes were similar to those of naphthalene dioxygenase (NDO) from other *Pseudomonas* strains suggesting that these enzymes share a common ancestor (Suen et al. 1996). The 4-methyl-5-nitrocatechol (4M5NC) enzyme was also purified, sequenced, and elucidated as a flavoprotein also sharing properties with other nitrophenol oxygenases (Haigler et al. 1996). The mechanisms of ring fission reaction performed by the 2,4,5-trihydroxytoluene (THT) oxygenase from strain DNT were elucidated as well as the sequence of the THT oxygenase gene *dntD* (Haigler et al. 1999). Thus, it was revealed that it is similar to the catechol 2,3-dioxygenase gene family I and that the native proteins consist of two identical subunits.

Additional strains to the *Burkholderia* sp. strain DNT with the ability to degrade 2,4- and 2,6-DNT were isolated in a later study (Nishino et al. 2000). Burkholderia cepacia JS850 and *Hydrogenophaga paleronii* JS863 were able to degrade 2,6-DNT by a different pathway; 3-methyl-4 catechol was the product of a dioxygenation reaction to 2,6-DNT, which in turn was converted to 2-hydroxy-5-nitro-6-oxohepta-2, 4-dienoic acid, and 2-hidroxy-5-nitropenta-2,4-dienoic acid by an extradiol ring cleavage dioxygenase. The other isolated strains able to degrade 2,4-DNT were designated *Burkholderia cepacia* R34 and P37, *Alcaligenes denitrificans* JS867 and JS871, *Alcaligenes xylosoxidans*, and *Burkholderia cepacia* JS872 (Nishino et al. 1999), and they all used the same pathway as the original isolate but were not able to degrade 2,6-DNT (Nishino et al. 2000). Furthermore it was also shown that the fastest-growing strain JS863 in 2,6-DNT was very similar to *Hydrogenophaga palleroni*; the genus *Hydrogenophaga* is quite versatile since it contains other species capable of hydrocarbon degradation such as benzene (Aburto et al. 2009; Aburto and Ball 2009; Fahy et al. 2006, 2008) and 4-aminobenzenesulfonate (Gan et al. 2011).

The degradation of 2,4-DNT was inhibited by the presence of 2,6-DNT and high concentrations (>100 μM) of either DNT isomer inhibit growth of DNT-degrading strains on simple substrates although concomitant degradation but at lower concentrations has been observed before (Lendenmann et al. 1998). Moreover the degradation pathway for the 2,6-DNT was proposed for the first time: either of the nitro groups is attacked by a dioxygenase to convert 2,6-DNT to 3M4NC with nitrate elimination. This is followed by opening the ring of an extradiol ring cleavage dioxygenase producing 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid, and a

hydrolytic attack would produce 2-hidroxy-5-nitropenta-2,4-dienoic acid with the loss of acetate (Nishino et al. 2000). Further studies by the same group characterized the subsequent steps in the 2,4-DNT degradation pathway in strain R34 (Johnson et al. 2002; Johnson and Spain 2003). The genes located downstream *dntD* include a CoA methylmalonate semialdehyde dehydrogenase (*dntE*), a putative NADH-dependant dehydrogenase (ORF13), and a bifunctional isomerase/hydrolase (*dntG*); other genes within the pathway were also uncovered such as *dntAaAbAcAd*, *dntB*, ORF12, ORF3, ORF10, ORF5 to ORF8, and ORF11. The authors suggest that the pathway evolved recently, since there are still extraneous elements found in the region (Johnson et al. 2002; Johnson and Spain 2003).

Other studies focused to enhance the 2,4-DNT degradation by engineering strains to produce *Vitreoscilla* with the bacterial hemoglobin gene *vgb* (Fish et al. 2000; Lin et al. 2003; Nasr et al. 2001; Patel et al. 2000) since it promotes cell growth, protein synthesis, fermentation, and biodegradation, among other applications (Stark et al. 2011). In all cases, 2,4-DNT degradation was enhanced with the strains containing the *vgb* gene. A three- to fourfold increase in the contaminant degradation was observed for the engineered strain PF6 (Fish et al. 2000), while complete degradation was registered in 3 days when strain YV1 was exposed to an initial concentration of 200 ppm 2,4-DNT (Patel et al. 2000). Also strain YV1 increased the 2,4-DNT degradation 3.5-fold compared to strain DNT when it was grown with co-substrates and limited aeration, and a further strain YV1m also increased 1.3-fold the contaminant degradation compared to YV1 (Nasr et al. 2001). Moreover increased degradation rates were registered in a fed-batch reactor (Lin et al. 2003). Similarly, the transconjugant strain DHK1 was able to degrade 94% of 2,4-DNT (0.55 mM initial concentration) in contaminated soil and allowed alfalfa plants to grow twofold higher than the parent strain in 0.14 mM 2,4-DNT-impacted soil. The strain DHK1 resulted from the addition of the pJS1 DNT-biodegradative plasmid to *Sinorhizobium meliloti* USDA1936 (Dutta et al. 2003).

Another study isolated two consortia capable of 2,4-DNT degradation and with the ability to use the contaminant as sole nitrogen and carbon source from a nitroaromatic-contaminated site (Snellinx et al. 2003). Although the consortia did not share common members, both had two species capable of the contaminant degradation *Variovorax paradoxus* VM685 (consortia 1) and *Pseudomonas* sp. VM908 (consortia 2). The consortia were formed of four and six bacterial species, respectively, and both start the degradation pathway with an oxidation that releases nitrite and forms 4-methyl-5-nitrocatechol (4M5NC) with a gene similar to the *dntAa*; 4M5NC is later metabolized to 2-hydroxy-5-methylquinone (2H5MQ) presumably by a monooxygenase as seen previously for strain DNT. *Pseudomonas marginalis* VM683 in the first consortium and *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905, or *P. viridiflava* VM907 in the second consortium were crucial for the intermediate metabolization, and a similar gene to the *dntD* was also found in these strains. The authors propose an interspecies metabolic interaction for the consortia and suggest that the released nitrite by *Variovorax paradoxus* VM685 may allow nitrogen assimilation by other consortia members (Snellinx et al. 2003).

A later study reported for the first time the degradation of 2,3- and 2,5-DNT by performing a saturation mutagenesis on codon I204 of the *Burkholderia* sp. strain DNT alpha subunit (DntAc). Oxidation of the other isomers 2,4- and 2,6-DNT was also enhanced, and their transformation was twofold faster than the wild type (Leungsakul et al. 2005). Moreover, it was revealed that the dioxygenases from *Burkholderia* sp. strain DNT and *B. cepacia* R34 are more closely related than originally reported.

Bioremediation with bacteria-laden agar, physical absorption of DNT by agar, and photocatalysis by UV light were evaluated as methods for the 2,4-DNT removal from concrete (Phutane et al. 2007). While photocatalysis only achieved 50% removal, 80% removal and desorption were observed with the sterile agar permitting separate further biodegradation; the bioremediation method was able to reach efficiency above 95% at optimum conditions but is also rate limiting. Another study tested photocatalysis on both isomers 2,4-DNT and 2,6-DNT. They reported a photolysis half-life of 15 and 100 h in salt and freshwater, respectively, for 2,4-DNT, while the photolysis half-life in fresh and seawater was 20 and 5 h, respectively (O'Sullivan et al. 2010).

The aerobic degradation of both DNT isomers 2,4 and 2,6 with concentrations up to 45 mg l^{-1} was evaluated in continuous packed bioreactors (poraver and fireclay). Higher removal efficiencies (above 90%) were observed in the poraver reactor than the fireclay (65%); a more efficient degradation of 2,4-DNT than 2,6-DNT was also observed in both reactors. The reactors were inoculated with adapted microorganisms that included eight Gram-negative and one Gram-positive bacterial strains that had been isolated previously (Páca et al. 2008). The strains were *Pseudomonas putida* A1, *Pseudomonas veronii* B1, *Pseudomonas* sp. C1, *Chryseobacterium* sp. D1, *Stenotrophomonas* maltophilia D2, *Sphingobacterium multivorum*, *Sphingomonas* sp. PCN3, and *Paenibacillus glucanolyticus* D1/B8. After 8 months of operation of the bioreactors, the microbial communities changed to *Brevundimonas* sp., *Achromobacter xylosoxidans* ssp., *A. denitrificans*, *Pseudomonas aeruginosa*, and *Bacillus cereus* in the poraver reactor, while a wider diversity was observed in the fireclay reactor since non-degrading strains as well as fungal strains were also registered such as *Cryptococcus humicolus*, *Pichia guilliermondii*, *Haplosporangium*, and *Stachybotrys*. The bacterial strains were *Sphingomonas* sp., *Chryseobacterium indologenes*, and *Pseudomonas* sp. (Paca et al. 2009).

The addition of persulfate activated with zerovalent iron was successful for the degradation of 2,4-DNT (50 mg l^{-1} initial concentration). The rate of DNT degradation is increased by the addition of zerovalent iron but not with Fe^{2+}, and the reduction products of DNT were preferentially oxidized by persulfate than DNT, which suggests that a sequential zerovalent reduction and persulfate oxidation may be highly effective in the degradation of 2,4-DNT and other nitroaromatics (Oh et al. 2010). A study established the lowest concentrations of the dinitrotoluene isomers to allow sustained growth of DNT-degrading microbes. These values were 0.054 and 0.057 μM in chemostat and column systems, respectively, for 2,4-DNT, while for 4,6-DNT they were 0.039 and 0.026 μM for chemostat and columns systems, respectively (Han et al. 2011). The authors state that these limits are below the regulatory requirements, and bioremediation strategies such as monitored natural attenuation could be used. Further bacterial strains capable of dinitrotoluene degradation

were isolated from a nitroaromatic-contaminated soil from an ammunition plant in the Czech Republic. The strains were *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, *Achromobacter* sp. NTD3, and *Bacillus cereus* NTD4, and they were tested as individual strains and as a mixture in the degradation of 2,4-DNT. The strain mixture had shorter lag periods and degraded the contaminant nearly 50 times faster than any individual strain; moreover it could degrade a wide spectrum of nitrotoluenes over a wider concentration range (Hudcova et al. 2011).

Degradation of 2,4-DNT Under Anaerobic Conditions

The degradation of 2,4-DNT has been observed in anaerobic conditions in several studies (Cheng et al. 1996, 1998; VanderLoop et al. 1999; Wang et al. 2011; Yang et al. 2009) and by individual strains such as *Pseudomonas aeruginosa* (Noguera and Freedman 1996), *Lactococcus lactis* (Shin et al. 2005), *Clostridium acetobutylicum* (Hughes et al. 1999) by the lignin-degrading fungus *Phanerochaete chrysosporium* (Valli et al. 1992), and more recently marine *Shewanella marisflavi* (Huang et al. 2015). The white rot basidiomycete *Phanerochaete chrysosporium* was able to degrade 2,4-DNT under ligninolytic conditions. The enzymes involved are lignin peroxidase (LiP), manganese peroxidase (MnP), and crude extracellular extracts, also the first intermediates in the degradation pathway include 2-amino-4-nitrotoluene and 4-nitro-1,2-benzoquinone, and the authors proposed the removal of both aromatic groups before ring cleavage (Valli et al. 1992). A later study reported the isolation of a *Pseudomonas aeruginosa* strain from a propellant manufacturing wastewater treatment plant capable of degrading 2,4-DNT. The strain reduced both DNT nitro groups, and the main metabolites 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, and 2,4-diaminotoluene were formed among others (Noguera and Freedman 1996). Further studies revealed the degradation of 2,4-DNT with different substrates such as ethanol, methanol, and acetic acid (Cheng et al. 1996, 1998). Moreover a *Clostridium acetobutylicum* strain was exposed to both isomers since it had been shown to transform nitroaromatics (Hughes et al. 1998), and they were biotransformed to dihydroxylamino intermediates not previously seen before (Hughes et al. 1999). *Lactococcus lactis* strain 27 was isolated from the intestines of earthworms and could also biotransform the four dinitrotoluene isomers – 2,3-, 2,4-, 2,6-, and 3,4-DNT – into the corresponding aminonitrotoluenes. However, no simultaneous or sequential reduction of two nitro groups of the dinitrotoluene was observed; furthermore the authors warn that production of dinitroazoxytoluenes may increase the environmental risk (Shin et al. 2005).

Another study reported a 99% removal efficiency of 2,4-DNT with an initial concentration of 95.5 mg l^{-1} in a treatment combining aerobic and anaerobic filters. The microorganisms recovered from the anaerobic filters by DGGE were highly similar to *Pseudomonas* sp. SRU_14, *Riemerella* sp. clone GI7-5-G11, *Chryseobacterium* sp. (FJ 202055), Flavobacteriaceae family (EU 839047), and

Agrobacterium sp. (CCAM 010004), and the main metabolites were also 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, and 2,4-diaminotoluene (Wang et al. 2011). In anaerobic conditions, the obligate marine *Shewanella marisflavi* EP1 has been shown to degrade 2,4-dinitrotoluene in 24 h with the transformation product 2,4-diaminotoluene via 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene as intermediates. Flavins, dicumarol, and Cu^{2+} enhanced the degradation of DNT, suggesting they are involved in the electron transfer process. EP1 oxidize lactate to reduce 2,4-DNT as electron acceptor via a respiration process that supports anaerobic growth (Huang et al. 2015).

The majority of the studies conducted in anaerobic conditions reported 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), and 2,4-diaminotoluene (2,4-DAT) as the main intermediates in the degradation process, suggesting the presence of a common degradation pathway.

DNT Isomer Degradation Pathway

Aerobic Conditions

2,4-DNT Degradation Pathway

When there is oxygen available, the degradation follows an oxidative pathway, which involves a dioxygenase, a reductase, a monooxygenase, a dehydrogenase, and a hydrolase. The degradation starts with a dioxygenase (*dntA*) catalyzing the oxidation of 2,4-DNT to 4-methyl-5-nitrocatechol (2M5NC) and the release of nitrite. A monooxygenase (*dntB*) removes the second nitro group producing 2-hydroxy-5-methylquinone (2H5MQ) that is later reduced to 2,4,5-trihydroxytoluene (2,4,5-THT) by the reductase *dntC*. Another dioxygenase (*dntD*) catalyzes the meta cleavage of 2,4,5-THT producing 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA). The degradation pathway continues with a CoA-dependant methylmalonate semialdehyde dehydrogenase (*dntE*) and a bifunctional isomerase/hydrolase (*dntG*) catalyzing the further reactions to produce propionyl CoA and pyruvate; these compounds enter the central metabolic pathways of the cell. This pathway has been extensively studied by the research group at the Air Force Research Laboratory in Florida, USA (Haigler et al. 1994, 1996, 1999; Johnson et al. 2000, 2002; Johnson and Spain 2003; Nishino et al. 2000; Spanggord et al. 1991; Suen et al. 1996; Suen and Spain 1993), and it has also been revealed that it evolved from those of naphthalene biodegradation routes (de las Heras et al. 2011).

The degradation of the 2,6-DNT isomer has been reported for several studies, and a degradation pathway was proposed by Nishino and collaborators (Nishino et al. 2000). The pathway also initiates with a dioxygenase attack converting the 2,6-DNT to 3-methyl-4-nitrocatechol and nitrite is released. An extradiol ring cleavage attacks the 3-methyl-4-nitrocatechol yielding 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid which in turn is converted to 2-hydroxy-5-nitropenta-2,3-dienoic

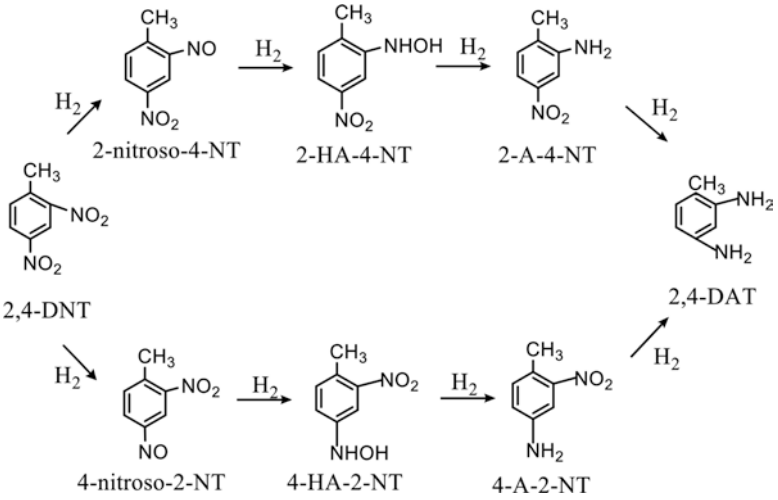


Fig. 1 Anaerobic DNT degradation pathway (Redrawn from Wang et al. (2011))

acid. The strains *Hydrogenophaga palleroni* JS863, Burkholderia cepacia JS850, and *Pseudomonas putida* JS881 were able to degrade 2,6-DNT, and the main intermediates were 3M4NC and HNOHA (Nishino et al. 2000).

Anaerobic Conditions

During anaerobic conditions a reduction occurs; the end product is 2,4-diaminotoluene via the formation of two metabolites: 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. These intermediates have been observed in all the degradation studies with the lack of oxygen (Cheng et al. 1996, 1998; Huang et al. 2015; Wang et al. 2011) (Fig. 1).

Future Works

The number of individual strains and consortia able to degrade the dinitrotoluene has increased in the recent years. Furthermore engineered strains have enhanced the contaminant removal, and the degradation pathways in the presence and absence of oxygen have been elucidated. Thus, future efforts could focus on metagenomics studies performed in dinitrotoluene-degrading consortia in order to understand the function of each of the consortium members.

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