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RESEARCH ARTICLE

Biodegradation of 2, 4, 6-Trinitrotoluene (TNT) in Contaminated Soil and Microbial Remediation Options for Treatment

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Abstract

This review paper provides a critical examination on current microbial biodegradation of 2, 4, 6-Trinitrotoluene (TNT) and its metabolites in soil, with focus on: (i) extent of biological degradation of TNT and its metabolites in soil, (ii) factors affecting the TNT transformations, and (iii) microbial bioremediation technologies , and related challenges. This was carried out through an extensive examination of relevant published literature on the topic. The review paper found that the detoxification of TNT contaminated sites by microorganisms based- technologies have been employed but TNT has been proven to resist biological mineralization and undergo biotransformations, leading to immobilization of toxic and unstable transformation products. TNT mineralization is however achievable, but scientific studies are far away from attaining the best desired in situ bioremediation practices and much remains to be delineated. We provide future research directions to design effective bioremediation technologies for solving the problems of TNT and minimize environmental impacts.

Keywords

Bioremediation, Contaminated soil, Microbial degradation, Mineralization, Trinitrotoluene, Trinitrotoluene transformation products

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1 Introduction

In the early 19th century, there was an emergence of manufacturing of organic compounds. Thereafter, chemists synthesized the nitro organic explosives by nitrating the synthesized organic substances [1]. The nitro-aromatic explosives such as 2,4,6-trinitrotoluene (TNT) and 2,4,6-trinitrophenol (picric acid) have been synthesized and widely considered, especially for military purposes because of their highly explosive properties, thermal stability and their insensitivity to shock and friction [2-6]. In addition, they have been used in civilian industries as raw materials for the manufacturing of the pesticides, herbicides, pharmaceutical products, dyes, and explosives, etc. [4, 7, 8].

It was during and post-world war I and II that several million tons of nitro-aromatic explosives were produced, and their usage widely spread around the world [1, 2, 6, 9, 10]. Following 1940, TNT production rates in different countries increased exponentially for over 40 years [10-12]. Consequently, the extensive usage in military applications coupled with improper handling and disposal techniques of these explosives and their transformation products led to increased environmental pollution, particularly soil, sediment, surface and groundwater to the levels that threaten human health and the environment. Over the years, this situation has escalated, thus requiring more attention from the research community [3, 4, 10, 13, 14].

Studies [12, 15-17] have reported that TNT may cause a wide range of adverse health effects on various ecological receptors, namely microorganisms such as *Escherichia coli* K-12 strain AB1157, luminescent bacteria (*Salmonella typhimurium* strain TA1535/pTL210), algae, plants, invertebrates, some vertebrates and human beings. Other studies [17, 18] conducted on animals such as dogs, mice, rats, and frogs indicated that TNT and its transformation products are teratogenic, cytotoxic and may cause cell mutation. However, the carcinogenic effects of TNT on humans still need to be explored [18, 19]. Due to the ecotoxicological effects and persistence of TNT and its transformation products in the environment, the physico-chemical methods [2, 20], and the bioremediation technologies [21-24] have been applied to remediate polluted environment by TNT. While the microbial based- bioremediation technologies offer a number

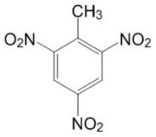
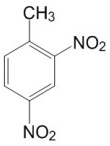
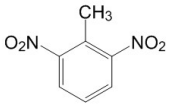
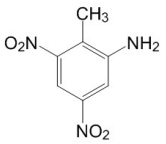
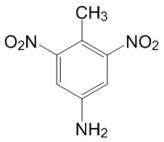
of advantages [14, 21], the associated constraints prevent them from achieving the full contaminant mineralization [7, 14, 25] and its removal from the contaminated environment. Previous research investigations by Gunnison et al. [26] indicated that bioremediation by transformation mechanisms was not beneficial as it leads to the transformation of organic compounds, which are chemically unstable and toxic to the environment.

Since it has been established that TNT was resistant to biological degradation and undergo biotransformation rather than mineralization, important questions have been asked as follows: Why is TNT persistent in the natural environment? Why the technologies used to date have not been able to completely mineralize TNT? What is the current situation? What should be the focus of future research? Therefore, we carried out an in-depth examination of relevant published literature to provide a critical review on microbial degradation of TNT and its main transformation products in soil environment. We endeavoured to carefully examine the extent of TNT mineralization and destination in the substrate, the environmental factors affecting TNT transformation in contaminated soils, made an assessment of the microbial bioremediation technologies currently in use, the challenges encountered as well as the solutions to remediate them. Knowledge compiled in this work constitutes a basic foundation for future research to design effective bioremediation technologies to address the problems of TNT and minimize continuing environmental impacts.

2 The physicochemical properties of TNT and its main transformation products

Table 1 illustrates some important physicochemical properties of TNT and its main transformation products. Studies have reported that TNT has two hydrophobic and hydrophilic properties (octanol-water partition coefficient, log Kow= 1.6 or 1.86), making it to be relatively sorbed in the soil and sediment. Consequently, its relative mobility in the environment might pose serious threats [2, 14], leading to the possibility of contamination of groundwater, especially in vadose zones where the water table is shallow. Other research, however, has put particular emphasis on the hydrophobic character of TNT and strong sorption to soil particles and low mobility [6, 20, 27], which reduces its bioavailability to the bio-degraders [6, 12, 14]. Regarding the main transformation products of TNT (Table 1), many studies have proven that they were more soluble in water than TNT, therefore, have a greater potential for transportation and spreading out into the environment [12]. Soil contaminated with TNT transformation products can exacerbate ground water pollution due to infiltration, leaching of contaminants and other physicochemical processes occurring in the soil. Kalderis et al. [12] and Pichtel [10] reported a strong sorption character and covalent binding of amino derivatives to organic and mineral soil components by the amino groups.

Table 1 TNT and its main derivates physicochemical properties (source: Kalders et al., 2011 and Pichtel, 2012)

Compound name	Chemical Structure	Melting point (°C)	Solubility in water at 20 °C	Boiling point (°C)	Mass density ρ (g/cm^3)	Vapor pressure P (1 bar, 20°C)	Henry's Law constant:kH (bar m^3/mol)	K_{ow}	Molar mass (g/mol)
TNT($C_7H_5N_3O_6$)		80–82	130 mg/L	240 (explodes)	1.5–1.6	7.2×10^{-9}	4.57×10^{-7}-1.1×10^{-8}	1.86	227.13
2,4-DNT (2,4-Dinitrotoluene) ($C_7H_6N_2O_4$)		70	270 mg/L	300 (decomposition)	2.2×10^{-4}	1.86×10^{-7}	1.6-1.99	182.15	182.15
2,6-DNT (2,6-Dinitrotoluene) ($C_7H_6N_2O_4$)		64-66	206 mg/L	300	1.28	7.5×10^{-7}	4.86×10^{-7}-9.26×10^{-8}	2.02	182.15
2-A-4,6-DNT ($C_7H_7N_3O_4$)		176	17 mg/L	-	-	5.3×10^{-8}	1.19×10^{-7}	2.8	197.15
4-A-2,6-DNT ($C_7H_7N_3O_4$)		171	36 mg/L	-	-	2.6×10^{-8}	1.74×10^{-7}	2.62	197.15

Concerning TNT derivatives (Table 1) metabolization, significant research work has been done. Many studies have tested the capability of isolated microorganisms to degrade TNT, analysed its biotransformation products using various methods and tools [28, 29], and studied their toxicity [8, 14, 30, 31]. Alvarez et al. [32] and Gilcrease and Murphy [33] studied the possibilities of further degradation of TNT transformation products by *Pseudomonas aeruginosa* MA01 and *Pseudomonas fluorescens* species. Under anoxic and anaerobic conditions, the two nitro groups of TNT were reduced into high polar compounds aminodinitrotoluenes (ADNTs) and diaminonitrotoluenes (DANT): 2 ADNT or 4 ADNT and 2, 6 DANT or 2, 4 DANT. Further degradation attempts showed that 2, 4 DANT was acetylated into N-acetyl-2, 4-diamino-6-nitrotoluene (4-N-acetylamino-2-amino-6-nitrotoluene: 4-N-AcANT), while 2, 6 DANT was resistant. Similar results of 2, 4-DANT acetylation were later obtained by Sheremata et al. [34]. Other studies[35, 36], indicated that some bacterial species were capable of oxidatively mineralizing the 2, 4-dinitrotoluene (DNT) and 2, 6-dinitrotoluene (DNT) with the release of nitrite. The former DNT isomer was aerobically degraded into a transient product, 4-methyl-5-nitrocatechol (MNC), while the latter was converted into 3-methyl-4-nitrocatechol without further degradation.

3 Soil pollution by TNT and factors affecting its degradation

Pollution of soil by TNT occurs mainly through its production, handling and manipulation process, military activities, improper storage and disposal practices, leakage from explosive remains of war, demilitarization facilities and wastewater contaminated by TNT [12, 18, 20]. These activities may result in severe and long-term environmental pollution, thereby constituting a significant threat to the ecosystem especially in the vicinity areas [10, 12, 20, 37, 38].

Many research studies [12, 38-40] have reported severe contamination of several sites around the world especially in the United States, Europe, Asia and Africa. In 2002, it was estimated that approximately 2,307 sites in the USA were likely to be contaminated by military explosives [41]. Recent data indicated that approximately more than 15 million acres of land [16] in comparison with 4,000,000ha reported in 2006 [38] are contaminated with energetic materials, mainly TNT. In 2003, a survey revealed that out of the 558 sites assessed, 83 necessitated urgent environmental restoration [41] compared to 3,200 sites earmark for the same purpose before 2000 in Germany [40]. Available data from literature indicated that TNT concentration values were variable at various contaminated sites. For instance, the values above 87 g/kg [16] and 10 g/kg [12, 40], 50 to 200 g/kg TNT [38] and 700 g/kg [14] were detected at different soil sites. From the literature, it has been found that over the years, TNT production and soil contamination have increased. Noticeably, the figures given are worrisome and exceptionally far beyond the permissible amounts of remediation goal established by USEPA: 0.0172g/kg of the soil [2], and those of industrial soil screening levels of 0.079 g/kg [18]. Furthermore, concerns have been expressed over the limited number of cleared and fully investigated contaminated sites across the world [10, 12], and the high costs associated with clean-up activities [38]. Moreover, it is estimated that TNT contamination problems would exacerbate in the future because of the demilitarization and disposal of obsolete weapons [12].

Once in the soil, TNT undergoes a series of abiotic and biotic transformations (discussed in Section 5), leading to the formation of its reduction products namely amino groups [10, 12], particularly 2 and 4-ADNT [10]. On the top soil surface, TNT is relatively easy to be abiotically transformed due to the light energy captured, resulting in the formation of aromatic aldehydes, nitrobenzene, azoxydicarobxylic acids and nitrophenols, as an effect of methyl group oxidation and nitro group reduction [10, 12]. Previous studies [34] had indicated that this abiotic reduction is catalyzed by iron compounds, clay minerals or organic compounds which were reported to act as potential electron donors [5, 10, 12]. On the other hand, Kuperman et al.[16] observed that among the abiotic factors that influence the fate of TNT in contaminated soil, photolysis, humidifying and air-drying sequences and temperature are the most critical ones. Studies have also explored the reactions of TNT with soil components. For instance, it was observed that TNT reaction with siloxane surface of clays generated complex compounds which bound covalently [42]. Furthermore, it has been reported that this binding to the sorbent is due to the pronounced electron-withdrawal properties of TNT [34].

Studies [10, 12] have reported that the extent and occurrence of these transformations of TNT in soil depend on a number of factors such as solubility, soil physicochemical properties, temperature, indigenous microflora community, pH, redox potential, pollutant properties, aeration, and the presence of inhibitors. These factors have significant impact not only on the behaviour of TNT in the contaminated soil and the extent of subsequent pollution, but also on the effectiveness of the techniques used for this recalcitrant removal. While processes such as dissolution and adsorption influence the TNT transport in a contaminated soil, others like hydrolysis, photolysis, biodegradation and reduction significantly affect its transformation [10, 12]. It has been indicated that the dissolution of explosive is an important mechanism and rate-limiting step of the contaminant transportation and distribution within the environment [12, 43]. TNT dissolution is dependent on temperature and this dissolution affects adsorption of TNT onto colloidal, humic and mineral materials. In the temperature range of 3 and 33^{o}C, the rate of explosives dissolution including TNT was observed to double as the temperature increased by 10^{o}C. Having a relative water solubility character (Table 1), some particles may dissolve into watered environment during the detonation of TNT, resulting in constant and long-term pollution [10].

Studies have shown that the physicochemical properties of nitroaromatics and contaminated soil significantly influence the extent, rate and speed of TNT sorption by soil and the nature of sorption reactions [10, 12, 16, 34]. Sheremata et al.[34] comprehensively studied the sorption-desorption features of TNT and its main transformation products, namely 4-amino-2, 6-dinitrotoluene (4-ADNT), and 2, 4-diamino-6-nitrotoluene (2, 4-DANT) in various types of soils. They found that in top soil, the sorption capacity (Kd) increased with the number of amino groups, while in illite shale environment, Kd increased with the number of nitro groups. However, in Borden sand, the sorption of the nitroaromatics studied was insignificant. These findings clearly show that TNT is less and more sorbed than its transformation products in topsoil and illite shale environments. In topsoil and illite a substantial sorption-desorption hysteresis of TNT and its transformation products was observed.

The soil structure, its composition as well as its organic and inorganic content have a determinant and significant impact on adsorption constant value (Kd), consequently, affect the adsorption and sorption of explosives [10, 44, 45]. Higher Kd values imply more compound adsorption and sorption in the soil. Studies have proven that Kd values normally decreased with the reduction of the soil total organic carbon content. In a soil containing K^+ and NH_4^+ cations TNT is highly adsorbed compared to the soil dominated by Na^+, Ca^{2+}, Mg^{2+}, and Al^{3+}. Similarly, Larson et al. [46] observed a general trend of explosives sorption effect caused by cations in the soils. More concentration of mono and multivalent cations results in decrease of aqueous concentration of explosives including TNT. Sheremata et al. [34] reported that the strong sorption of TNT transformation product (2, 4-DANT) resulted from the interaction between aluminium ions at the edge sites of the illite and amino groups of 2, 4-DANT. The restriction of the extractability of this compound was attributed to this effect. Many researchers have extensively studied the decisive role of organic carbon for nitroaromatics sorption. Sheremata et al. [34] found that the differences between significant sorption of nitroaromatics (TNT, 4-ADNT and 2, 4-DANT) in top soil and the absence of their sorption in Borden sand were due to the carbon matter content. Similar results were obtained by Larson et al. [46] when comparing the sorption capacity of TNT by different soil types. The richer the soil in total organic carbon the higher the explosive is sorbed by the soil. Other studies [15] have reported that soils with lower organic matter and clay content support the explosive bioavailability and can be used to develop realistic ecological soil- screening level (Eco-SSL) values. In their study, Kuperman et al. [16] observed that clay and organic matter contents of the soil decreased the TNT concentration and consequently reduced reproduction toxicity of TNT to the enchytraeid worm *Enchytraeus crypticus*. Soils containing iron, iron bearing clays and humic acids proved to reduce the effective elution rates of TNT [12, 47]. These inorganic and organic sorptive amendments make the surface area of the non-swelling kaolin clay to adsorb explosives which result into a retention and delay explosive elution [47]. The adsorption of TNT into clay minerals was found to be greater in montmorillonite soil than kaolinite soil [10, 12].

The interactions between soil water content, the redox potential and the biodegradation rate of the common explosives found in soil have been extensively studied [10, 48]. It has been proven that in unsaturated natural environment, the gradual increase in soil water content proportionally decreases the redox potential coinciding with anaerobic conditions which in turn gradually increases the explosive biodegradation rate. The soil water humidity or moisture content of 25 to 85% sustains the in situ microbe activities [10, 48, 49].

The impact of pH on nitro-aromatics degradation is not a new concept. The successful laboratory experiment studies demonstrated that a higher pH >10 leads to the alkaline hydrolysis of nitro-aromatics and TNT deactivation [2, 10]. At a higher pH a complete destruction of 2A-DNT and 4A-DNT was observed. Furthermore, soil pH affects the solubility of toxic metals to the microorganisms as well as the biological functioning of the latter. The pH values of 5.5 to 8.5 have proven to be the optimum for many microbes' survival and good working conditions [49, 50], but some discrepancies may be observed depending on microorganism specificity. Ziganshin et al. [22] have observed that initial pH values of 6.0 and 7.0 influenced the rate and amount of TNT transformation as well as the *Yarrowia lipolytica* AN-L15 activity. However, the same main metabolites, namely Meisenheimer complexes (3H--TNT), 1H-–TNT and dihydride forms, 3,5H-—TNT, 2- Hydroxylaminodinitrotoluenes (2-HADNT), 4- HADNT, 2,4-DNT, 4-ADNT, nitrite, and nitrate were observed at both pH values. In particular, pH influenced the time of appearance and disappearance of transformation products as well as transformation routes of TNT.

The supply of oxygen and nutrients plays a critical role on microorganism's activity and explosive biotransformation. Studies have proven that insufficient O_2 supply and an even nutrient distribution delay the growth of the microorganisms, slowing down TNT biodegradation and impact on its degradation products. Intensive aeration of the incubation medium influenced the biodegrading bacteria [4, 22, 38, 51].

A thorough analysis of the literature indicates that only few studies on microbial degradation of TNT have concurrently investigated the impacts of the factors discussed above. Temperature, pH, redox potential, oxygen and nutrients supplies are the most widely studied factors. Other important factors such as contaminated soil properties, organic carbon content and other pollutants, moisture content, presence of inhibitors or competitors are often left out. Many studies have focused on the capability of an isolated microorganism to degrade TNT, formation of metabolites and their toxicity, and the degradation pathways of TNT. Accordingly, it is important

to carry out an in-depth analysis of the physical, chemical, biological and environmental conditions of the contaminated site prior to undertaking an investigative study for effective and sustainable bioremediation strategies. This recommendation is supported by many studies conducted in the field. Douglas et al. [52] emphasized that TNT degradation in soil depends on biogeochemical parameters. Meanwhile, Muter et al. [51] advocated for a detailed study on environmental conditions during the bio-stimulation process. Khan et al. [14] recommended the inference of physical, chemical and biological properties of the TNT-contaminated environment. Larson et al. [46] suggested a comprehensive understanding of the soil types, their affinity with explosives and contaminant solubility in contaminated soil environment for better approximation of their migration and behavior in the soil. Furthermore, Makris et al. [53] recommended a combination of reductive and holistic approaches where many factors are carefully studied under a controlled environment and applied to large-scale field. This can help to develop the best in situ bioremediation technologies.

4 Ecotoxicity of TNT and its main transformation products

TNT and its transformation products are very toxic to the ecosystem, causing harmful effects to many aquatic and terrestrial living organisms including human beings [1, 5, 30, 37]. The toxicity of TNT on humans has been known since the last century [18] based on the data collected from munitions plant workers [12] during and/or after the 1st world war. Out of the 17,000 people poisoned by TNT, 3% of deaths were reported as a result of inhalation, ingestion or dermal sorption of TNT particulates [18]. Even though there is insufficient evidence of TNT carcinogenicity to humans, the United States Environmental Protection Agency (US EPA) has recently listed TNT as a likely cancer causing to human beings [18, 19]. For the purposes of public health protection, some toxicity thresholds values have been set. The minimal risk concentration was fixed at 0.0005 mg/kg/day while it was established that drinking water containing 1µg/L of TNT represents 1 x 10^{-6} of cancer risk. A residential soil screening level (SSL) was fixed at 19 mg/kg, while 79 mg/kg was set for industrial SSL [18].

Compared with studies on human beings, the potential of TNT and its derivates to exert toxic effects on invertebrates, bacteria, algae, invertebrates, vertebrates and plants [12, 15, 16] has been extensively investigated by many researchers. Studies have shown that these chemicals cause various harmful effects including bacterial cell structure damage or alteration of genetic material (DNA), abnormal organs' functions of various organisms, disruption and damage of the immune and reproduction systems [12, 13, 17-19, 54-56]. A study conducted by Maeda et al.[17] on *Escherichia coli* K-12 strain AB1157, luminescent bacteria (*Salmonella typhimurium* strain TA1535/pTL210) and frogs shown that TNT and its transformation products are cytotoxic and mutagenic, and may cause many other adverse effects. High TNT cytotoxicity led to the death of the luminescent bacteria. In the light of TNT mutagenicity findings, it was observed that when TNT was above 71 picomole (pmol), the luminescence completely disappeared. It was proven that the gradual increase in TNT concentration up to 440 µM caused an extreme decrease in the survival of *E. coli* K-12 strain AB1157. At the dose of 70 pmol, while the mutagenicity of the 2ADNT, 4ADNT and 2, 4-DNT was higher but less than that of the parent product, 2HADNT and 4HADNT shown to be as high mutagenic as or even more than TNT. The surplus legs in the offspring of the frogs were reported to have direct consequence of the frog's genes alteration caused by TNT. It was observed that the mutations leading to birth defects are transferred to the frog offspring by the sperm [17]. TNT toxicity mechanism and that of its metabolic intermediates for some effects has been documented and reported in the literature. Once in the liver, TNT decomposes into several metabolites susceptible to produce very reactive oxygen species which cause lipid peroxidation in the liver and serious damage of the lens leading to cataracts [18]. Research studies [17] have shown that 2HADNT, 4HADNT and tetranitro-azoxytoluenes cause oxidative DNA damage in the presence of NADH and Cu (II). Even though a number of toxicological data of TNT and its derivates for a wide range of ecological receptors such as microorganisms, algae, invertebrates and plants are available [12], there are still gaps to fill in ecological risk assessment [15, 16] and determination of biochemical and molecular pathways for various adverse effects [57].

In recent years, toxicological studies aimed at establishing ecotoxicological benchmarks (Table 2) for ecological soil-screening levels for TNT and its transformations have been undertaken [12, 15, 16]. It is evident that the values and toxicity determined for the same energetic material may differ significantly depending on many factors including soil type and properties, treatment applied to the contaminated soil, validity test criteria, exposure route, time and targeted organ, bioindicator organism, toxicological parameters, toxicological end-point being investigated such as bioaccumulation, reproduction, mutagenicity, growth and survival of organism, germination. Kuperman et al. [16] reported that the ecotoxicological data determined are illustrative of possible exposure to adverse effects in the soil with similar chemical bioavailability conditions. Therefore, they should be used with precautions because the data can overestimate or underestimate the toxicities of TNT and its derivates in the soil that differ significantly in their physicochemical properties. It is imperative to eradicate TNT from the contaminated environment. Moreover, the persistence of TNT in the environment, long life cycle of TNT-containing materials, accumulation and high toxicity of its degradation products are strong enough to justify the urgent need for TNT complete removal. Complete TNT mineralization should therefore be the ultimate goal of any bioremediation study.

Table 2 Some ecotoxicological benchmarks for TNT and its transformation products for some ecological receptors.

Contam-inant	Organism	Toxicological end point	LOEC mg/kg	EC_{20} mg/kg	EC_{50} mg/kg	Reference
TNT	*Enchytraeus crypticus*(invertebrate)	Adult survival	105	-	-	Kuperman et al. [16]
		Juvenile production	46	38	48	
		Adult survival	105	100	140	Kuperman et al. [42]
		Juvenile production	-	38	48	
	Enchytraeus albidus	Juvenile production	-	59	111	Dodard et al. [59] cited by Kuperman et al. [16]
	E. crypticus	reproduction	-	-	501	Schäfer[60] cited by Kuperman et al. [16]
	Folsomia candida		-	-	64	
	Eisenia andrei	juvenile production	-	52	-	Robidoux et al.[61] cited by Kuperman et al. [16]
	Acheta domesticus	reproduction	-	14	-	Kalderis et al. [12]
2A-DNT			-	1.7	-	
4A-DNT			-	1.9	-	
2,4-DNT			-	0.4	-	
TNT	*Arbacia punctula*	Embryological development	-	-	12	
		Fertilization	-	-	≥103	
2,6-DNT		Embryological development			0.029-36.9	
TNT	*Dinophilus gyrociliatus*					Phillips et al. [15]
	Medicago sativa, *Echinochloa crusgall* L., and *Lolium perenne* L.	-	-	43-62	-	
DNT		-	-	3-24	-	
2-ADNT	*Folsomia candida* (invertebrate)	Adult survival	51	38	65	
		Juvenile production	51	45	51	
2,4-DNT		Adult survival	11.5	12	38	
		Juvenile production	5.2	15	23	
4-ADNT		Adult survival	28	22	55	
		Juvenile production	28	26	47	

LOEC: Lowest-observed-effect concentration, **EC_{20}:** 20% effect concentration; **EC_{50}:** 50% effect concentration

5 Microbial degradation of TNT and transformation products

Over the years, the metabolization and mineralization of TNT by microorganisms have been extensively studied [40]. Many scholars and researchers have considered the microorganisms as successful and promising alternative for explosives degradation [5, 13, 58, 59]. Many bacteria and fungi belonging to different genera of various taxonomic groups [40] have been isolated, studied aerobically and anaerobically for their potential to remediate TNT-contaminated environment. Studies conducted on bacteria, *genus Pseudomonas* [4, 17, 30, 32, 33, 60], and fungi species belonging to basidiomycetes such as *Agaricus aestivalis*, *Agrocybe praecox*, *Clitocybeodora*, *Phanerochaete chrysosporium* (white rot fungus) and *Stropharia* species [10, 61] and other microorganisms such as *Shewanella putrefaciens* CN 32 [5], *Alternaria*, *Aspergillus*, *Penicillium* and *Trichoderma* [55, 62], *Achromobacter spanius* STE 11 [50], *Yarrowia lipolytica* AN-L15 [22], *Bacillus cereuses* [8], *Clavibacter agropyi* (*Corynebacterium*) (R.L1) and *Sphingomonas sanguinis* (R.L2) [56] , *Salmonella typhimurium* [63] have shown their potential of degrading and bioattenuating TNT-contaminated environment, especially soil and aquatic sediments at varying extent [3, 7, 8, 22, 64]. The results of these studies have been compiled in many articles and several reviews [1, 7, 10, 12, 20, 38, 40, 42, 53, 55, 65, 66]. Amongst these bacteria, the most widely studied family of bacterial TNT biodegradation is *Pseudomonadaceae* because of its predominance in TNT contaminated environment [26, 38].

While the use of TNT as the sole nitrogen source by the microorganisms and its incorporation into bacterial cell's structure [17, 50, 56] via passive diffusion [38, 40] seems to be unanimous among the researchers, its use as the sole carbon source is likely to be controversial. Many studies have reported the utilization of TNT as nitrogen source. For instance, *Clavibacterium agropyri* (*corynebacterium*) and *Sphingomonstainas sanguinis* [56], certain fungi and *Pseudomonas sp.* [67] have shown the ability to grow on TNT-medium as a sole nitrogen source and degraded it into its metabolites and nitrite by-products. It has been proven that these microorganisms utilize and uptake nitrogen molecule from TNT to build up their cells and produce the nitrate reductase enzymes responsible for the conversion of TNT nitro groups into amino derivates. It was reported that the nitrogen from TNT is incorporated via the enzymatic activity such as nitrite reductase and glutamine synthetase-glutamate synthase during the addition of hydride ions to the aromatic ring. However, the denitrated transformation products of TNT cannot be used as carbon source [38]. Maeda et al. [17] have also demonstrated that nitrite from TNT can be released as a result of TNT metabolization and utilization by the bacteria as a sole nitrogen source. Other studies [12] reported the utilization of TNT nitro group by *Desulfovibrio sp.* either as an electron acceptor or nitrogen source to convert TNT to toluene.

On the other hand, recent studies have proven that certain bacteria such as *Pseudomonas aeuroginosa* [19], *Pseudomonas putida* strain TP1 and *Pseudomonas aeruginosa* strain TP6 [4], and *Lysobacter taiwanensis* strain [38, 68] are capable to grow in a media containing TNT and use it either as the sole carbon or as carbon and/or nitrogen and energy source to degrade this contaminant through two pathways, namely the nitro group elimination and nitro group reduction. Kalderis et al. [12] have reported that a well-defined sulfate-reducing consortium *Desulfovibrio desulfuricans* strain A, *D. desulfuricans*, strain B, *D. gigas*, and *D. vulgaris* grew anaerobically under various growth conditions including TNT as the sole carbon source and cometabolic condition with pyruvate as co-substrate. Though the growth was observed in all the conditions, the maximum growth was observed under cometabolic conditions. This observation is consistent with the findings by Chien et al. [4] who reported that the utilization rate of TNT by the afore-mentioned bacteria was relatively slower for the effectiveness of practical application. Additionally, they observed that when the bacteria grew on TNT as carbon and/or nitrogen their doubling time was much longer than the one observed when they grew on a complex medium or on glucose as the carbon source and ammonium chloride as the nitrogen source. A study by Gallagher et al. [68] reported that the stable isotope probing analysis revealed the assimilation of 15N and 13C from TNT into bacterial DNA. While this study concluded that the absorption of TNT as primary substrate or co-substrate remained unexplained, Chien et al. [4] have recently reported that the knowledge on dynamics governing the use of TNT as carbon, nitrogen and energy source by the bacteria was still limited. On the other hand, Claus [40] reported that the use of TNT as a source of carbon and energy was extremely unlikely since TNT is transformed and not mineralized and therefore it cannot be used as the sole growth substrate for microorganisms.

Studies have shown that the supply of bacteria growth substrate stimulates the production of the nitroreductase enzymes and increases efficiency of TNT biodegradation [8, 37]. Gunnison et al. [26] demonstrated that the treatment of the contaminated soil by appropriate substrates stimulated the indigenous micro flora to produce the enzymatic degradation activity. Consequently, TNT molecular structure is transformed and possibly being mineralized. The produced nitroreductases catalyse the conversion of nitro groups into nitroso and amino groups [8, 37]. As it has been reported, the problem of this co-metabolic TNT transformation in the presence of organic nutrients, is the accumulation of the dead-end products resulting from nitro group reduction which are often more toxic, and do not undergo further degradation [6, 38]. Similarly, Gunnison et al. [26] have indicated that from the environmental and toxicological view point, these products are unstable and unsafe. They suggested the bioremediation mechanisms aiming at TNT mineralization, leading to the harmless inorganic substances.

5.1 Microbial TNT degradation pathways

5.1.1 Aerobic TNT degradation

Many studies have proven that many isomers of amino-nitro-aromatic compounds such as hydroxyl-aminodinitrotoluenes (HADNT), amino-dinitrotoluenes (ADNT), diamino-nitrotoluenes (DANT), dinitroaniline (DNA), and polynuclear condensation products like azoxytoluenes (Fig. 1), are partially reduced aerobic products of TNT known to be recalcitrant and accumulate in the environment without further mineralization [14, 22, 27, 48, 65]. They present a serious hindrance to the effective bioremediation process [14]. The 4 ADNT and 4 HADNT inhibit the activity of the lignin-peroxidase enzyme (responsible for aromatic ring cleavage) [69]. Given that numerous transformation products are experimentally found, some authors suggested various pathways of TNT biodegradation [70], including converting the nitro groups into nitroso, hydroxylamino and amino groups and direct aromatic ring-reduction subsequent to the addition of hydride-ions, leading to the formation of Meisenheimer complexes (Fig. 1) [40, 50]. The latter are often accompanied by a release of nitrite or nitrate [71]. Recent studies have reported on the co-existence of both pathways in the same cell [40]. The 2-ADNT, 4-ADNT, 2, 4-DANT, tetranitroazoxytoluenes and 2, 6-DANT were reported to be the predominant metabolites of aerobic TNT biodegradation [5, 8, 72]. The degradation of TNT to 4-ADNT pathway was found to be more favorable than the one leading to the formation of 2-ADNT [5]. Studies have shown that the transformation of TNT into amino derivatives is achieved via a non-specific NAD(P)H-dependent nitroreductase enzymes, whose characterization is not well-documented, or a nitrobenzene reductases activity [14, 69]. Aerobically, in a contaminated soil, TNT is transformed into its metabolites via two reductive pathways: the reduction of 1 or 2 nitro groups to a hydroxylamino group and the transformation of TNT into dinitrotoluene, DNT [8]. This metabolite had been previously proven to undergo further degradation and release NO_2^- [36].

5.1.2 Anaerobic TNT degradation

The nitro group reduction occurs via a two-electron transfer's step, which is favoured during bacterial reduction or through a single-electron transfer's process. A series of derivate compounds such as 2-ADNT, 4-ADNT, 2, 4- diamino-6-nitrotoluene (2,4-DANT), 2,6-diamino-4-nitrotoluene (2,6-DANT), triaminotoluene (TAT) and nitroso compounds (Fig. 1), as the main products have been experimentally found [7, 13, 14, 42]. The formation of 2, 4, 6- triaminotoluene (TAT) through which TNT is mineralized, has been observed [3, 10, 12, 30]. However, this mineralization was reported to be controversial [14]. Hawari et al. [73] had previously proven that the disappearance of TAT was only due to irreversible and strong binding to the soil matrix

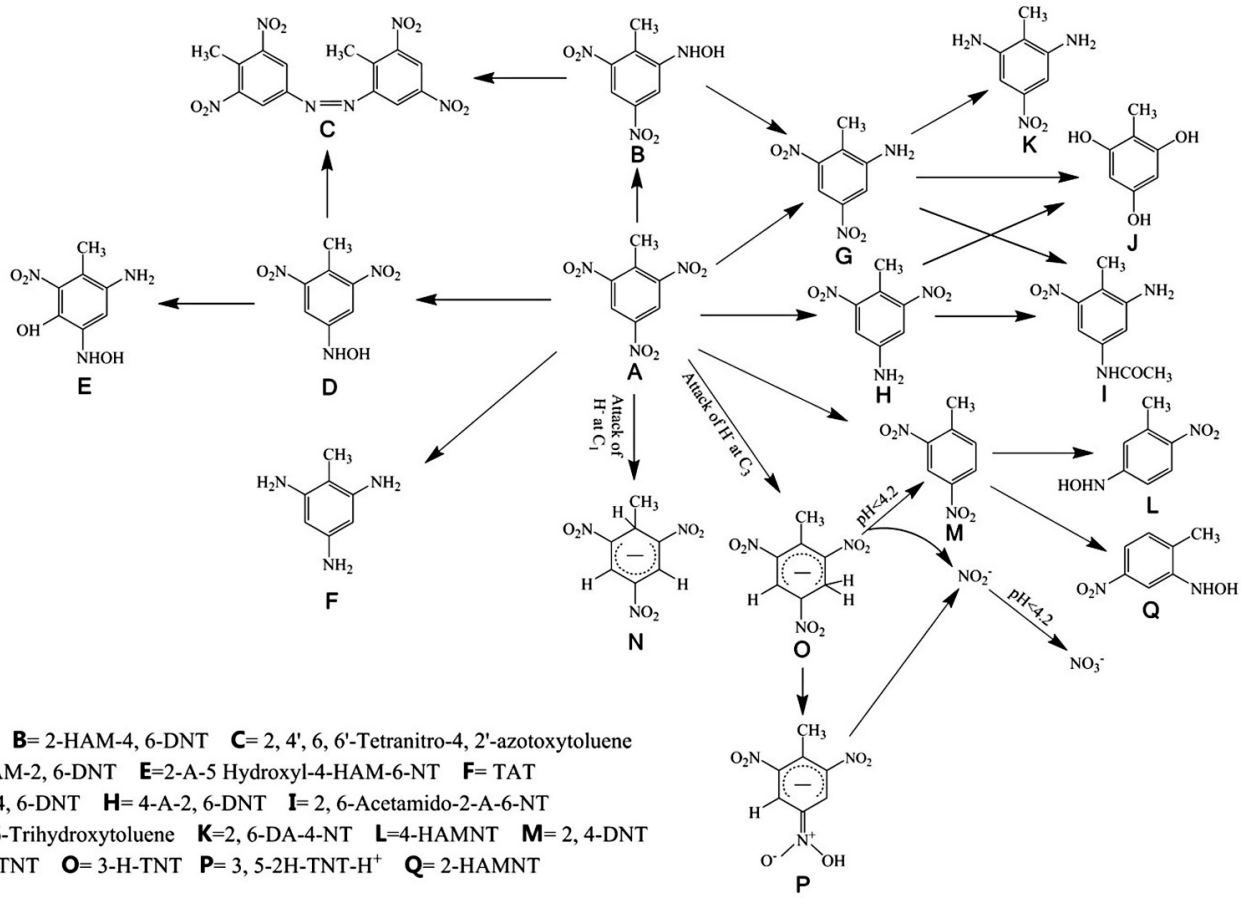


Fig. 1 Aeorbic and anaerobic TNT metabolization. It is important to note that only initial and final transformation products are represented here. 1-H-TNT, and 3, 5-2H-TNT-H$^+$are the Meisenheimer complexes (adapted from Kalderis et al., 2011 and Ziganshin et al., 2010).

by a range of various mechanisms [14, 20], including ionic interaction with clay minerals and humic materials structure: interlamellar adsorption and humification. Sheremata et al. [34] found that these interactions of nitro-aromatics sorption by clay and soil organic matter were mediated by the nitro and amino groups respectively. The negative charges of reactive sites of the carboxylic and phenolic acids in humic acid interacted with partial positive charges of amino groups. Compared to other soil types, it is possible that the small fraction of TNT recovered from the Richfield sandy loam (high content of clay and organic matter), [16] resulted from this kind of interactive forces.

In comparison to anaerobic bioremediation, aerobic is promising and had been extensively studied because of the widespread and easy maintenance of aerobic bacteria and satisfactory results of TNT reduction [50, 56]. In addition, some of the aerobic sub-pathways lead to the tetranitroazoxytoluenes, which are the condensed di-aromatic dead-end products and less toxic than the mono-aromatics formed under anaerobic environment [14]. On the other hand, under anaerobic conditions at low redox potential, TNT reduction is more important than in aerobic environment [10]. Full mineralization of TNT can be achieved via the sub-pathways involving NADH nitroreductases although the intermediates formed are more toxic than the ones obtained through the sub-pathways that involve the nitrobenzene reductase [14].

Even though TNT microbial degradation has been studied over the years, the major challenge is still its recalcitrance and refractory to biological degradation, chemical oxidation and hydrolysis. The symmetrical arrangement of three nitro-groups and methyl group on the aromatic ring coupled with strong electron-withdrawing properties of the nitro group limit the attack of the aryl group by dioxygenase enzymes. This prevents the aromatic ring with electron shortage to act as an electrophilic oxygenation mechanism, hindering its mineralization and removal from the contaminated sites [2, 4, 20, 40, 42]. The resistance of TNT to complete mineralization is also due to easy reduction of nitro groups into amino groups and ultimate chemical misrouting reactions of its intermediates, in particular, TAT [34, 65]. Furthermore, it was reported that the non-mineralization of TNT is a direct consequence of irreversible sorption by soil of this explosive and its transformation products [34]. This situation restricts their bioavailability for the subsequent further biodegradation. Consequently, desorption studies are required but their long time period requirement and small amount of extractable fraction were reported to have negative impacts on the effectiveness of some remediation alternatives [34]. Previous studies had proven that the resistance of TNT mineralization resulted from the lignin-peroxidase inhibition by the nitroreductase enzymes'effect: accumulation of HADNT and ADNT. The latter enzymes are responsible for nitro group reduction while the former catalyze the oxidation and aromatic ring cleavage [69].

Despite this recalcitrance, to some extent TNT mineralization has been achieved [8, 50, 61, 74, 75] by bacterial consortia and many white-rot fungi [40]. Laboratory studies on fungi species belonging to basidiomycetes such as *Agaricus aestivalis*, *Agrocybe praecox*, *Clitocybeodora*, *Phanerochaete chrysosporium* (white rot fungus) and *Stropharia* species have proven to achieve TNT mineralization with range rates varying between 10 and 40% [10, 40, 61]. Less than 2% of TNT was mineralized by *Pseudomonas aeruginosa* strain MX and *Pseudomonas savastanoi* [14, 22]. *Pseudomonas* hybrid strain was reported to mineralize TNT [12] but no figure was provided. In their study, Brandon and Boopathy [75] observed 6.5% of TNT mineralization. Highest TNT mineralization level of 39.0% by the *Phanerochaete chrysosporium* strain BKM-F-1767 was observed in a study by Hodgson et al. [76]. The use of a nonionic surfactant (Tween 80) not only enhanced the TNT desorption and removal rates, but also stimulated the growth of *P. chrysosporium.* The same study by Hodgson et al. [76] also reported the findings of previous studies on significant TNT mineralization rates of 19.6% by the wood-decaying fungus *P. chrysosporium*.

Recently, Boopathy[77] has studied the anaerobic biodegradation of 2, 4, 6-trinitrotoluene (TNT) under sulfate and nitrate-reducing environments and proved that more than 90% of TNT was mineralized into acetic acid, CO_2, and ammonia. The results of this research study corroborated earlier reported predictions that sulfate-reducing and methanogenic bacteria might metabolize nitroaromatic compounds under anaerobic conditions by conditions that appropriate electron donors and acceptors are available [12]. Previously, Ziganshin et al.[22] had reported for the first time TNT aromatic ring reduction as the main pathway (70%) with the production of NO_3^- as a major product resulting from denitration of TNT hydride complexes (Fig.1). The reduction products, the hydroxylamino-and aminoaromatics and 2,4-DNT accounted for 30%. An acidic medium tolerant yeast *Yarrowia lipolytica* AN-L15 metabolized TNT into nitrite and nitrate ions via the formation of the hydride complexes as a result of the hydride ion addition to the aromatic ring. However, the enzymatic properties of this microorganism and TNT destruction mechanism have not been studied. It was reported in the literature that a purified pentaerythritol tetranitrate (PETN) reductase belonging to the family of flavoproteins, the old yellow enzyme (OYE) is known to catalyze this kind of reaction [14, 22]. Claus [40] has recently reported that the main types of enzymes involved in TNT transformation are the nitroreductases and laccases catalysing the initial and secondary TNT transformation respectively. The nitro-reductases (related to OYE) which are abundant in bacteria (fungi) and superior organisms such as plants, and animals, catalyse the nitro group reduction to amino groups (activity of Oxygen-insensitive Type I Nitroreductases). However, the nitro-reductases of *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas*

fluorescens and *Pseudomonas putida* were also reported to catalyse the nucleophilic addition of hydride ions to the TNT aromatic ring (activity of Type II Hydride Transferases), yielding the Meisenheimer complexes and nitrites [38, 40]. In addition to these two main enzyme classes of nitroreductases, an iron hydrogenase from *Clostridium acetobutylicum* has proven to reduce TNT to its dihydroxylamino derivate in hydrogen depending condition. The laccases were proven to catalyze the secondary transformation of TNT metabolites [40].

From the available literature, it is noticed that earlier studies on TNT biodegradation focused on isolation and identification of indigenous microorganisms capable to transform TNT, enzymatic pathways and metabolites formation [74]. Aerobic and anaerobic bio-transformations of TNT have both led to the immobilization of amino derivative products and not the mineralization of TNT and their fate remains doubtful. Several authors believe that effective TNT biotransformation pathways could lead to the diversion of amino derivatives formation and/or the destruction of TNT aromatic ring. Previous studies have shown that for the aromatic ring to be cleavaged, the microorganism must entirely remove or transform the NO_2 groups by nitroreductase activity and then follow the oxidation and subsequent cleavage of aromatic ring, which is a lignin-peroxidase activity [69]. This lignin-peroxidase is a ligninolytic enzyme system of white-rot fungi [40]. Earlier research work has indicated that TNT can be mineralized via biodegradable transformation products [26, 78, 79] or using fungi whose enzymatic system has powerful enzymes such as the lignin peroxidase system. Therefore, further extensive research to comprehend the biochemical properties, enzymatic activities and optimum conditions of all enzymes involved could contribute to the existing knowledge and help to solve problems of TNT. Furthermore, it was reported that there are limited studies on denitrating bacteria and many successful studies are still lacking [14, 21, 22]. Moreover, further techniques on TNT degradation pathways need to be developed [14]. Earlier studies have indicated that complete mineralization of TNT can only be achieved if the degradation pathways are explored [26], potent TNT degrading enzymes are identified and biochemistry behind its metabolism is understood [66]. Hence, it can be suggested that the deciphering of the enzymatic physiology and advanced characterization as well as optimum working environmental conditions of the PETN reductases, hydrogenases, ring hydroxylating dioxygenases [31], and lignin-peroxidases involved in TNT degradation can give new directions and starting points for novel perspectives in developing new technologies for effective TNT bioremediation.

6 Microbial uses related challenges and remediation

The microbial based -bioremediation technologies offer a number of advantages: TNT degrading bacteria are spread out and predominant in environment and they are important elements in biochemical cycling [14, 21]. However, some associated constraints preventing them from achieving the full contaminant mineralization are documented. These are, but not limited to, the exponential increase of contaminants in the environment beyond the degrading capacity of the microbes, presence of inhibitors, heavy metals and other harmful substances, long time requirements for completing the biodegradation process, lack of specific enzymes required for the xenobiotic mineralization, the low TNT bioavailability and its high concentration and toxicity to microorganisms, and the competition with other bacteria over nutrients and space [7, 14, 25].

To overcome these challenges, various studies have called for engineering of the microorganisms' genes and/or bio-augmentation application to detoxify specific contaminants and improve qualitatively and quantitatively the microorganisms' performance [7, 49]. Suggestions were also made on the use of surfactants to enhance the availability of contaminants to the bio-degraders [80]. Other researchers [20, 30] have emphasized on the promotion of naturally occurring microorganisms rather than engineering specific strains, emphasizing that the latter lack survivability in the field despite impressive results at laboratory scale. On the other hand, the use of genetically modified plants as effective in situ bioremediation has been criticized and conflicted with ethical and environmental consideration [50]. Other studies [14, 42] recommended the addition of specific nitro-aromatic-mineralizing bacteria other than the native ones or bacterial consortia to enhance the work performed by the indigenous microflora, thus mineralizing effectively the transformation products formed during the incubation period. Khan et al. [14], Bernstein and Ronen [3] and other researchers have recommended the use of stable isotope probing (SIP) technique and its combination with gene-targeted metagenomics. This combination could help to isolate a metagenome of active TNT-metabolizing populations under various environmental conditions, identify specific biodegradative genes of the microorganisms and better understanding of the mechanisms of degradation pathways. In addition, the SIP technique provides knowledge on explosives' behavior in a contaminated environment for effective bioremediation [3, 14, 68, 81]. For the purposes of monitoring the in situ TNT degrading microbes, suggestions were also made on the use of old yellow enzyme (OYE) family of flavoproteins as the biomarkers. These are largely spread in various organisms such as bacteria, fungi, and plants [14]. A detailed study on the physical factors, pollutant biodegradability and its chemical reactivity, phase-distribution, in situ inhibitors, oxygen competitors, leaching possibility, indigenous micro flora community, and knowledge on TNT transformation products toxicity is very critical for successful bioremediation [14, 49]. An in-depth understanding of ecology of TNT biodegrading microorganisms [14] and the use of reductive and holistic approaches [53] are critical for sustainable bioremediation and effective in situ bioremediation technologies.

7 Bioremediation technologies

Growing concerns over the human health and ecological threats have led many researchers to devote their efforts on the toxicity, ecotoxicity and remediation of TNT contaminated environment. The bioremediation process uses microorganisms, most often bacteria either indigenous or isolated to transform environmental contaminants into less harmful substances during microorganisms' metabolic process [5, 58, 59]. Bioremediation has become an attractive, cost-effective and environmentally friendly technology [7, 13] for remediation of contaminated sites [48, 82]. Several bioremediation treatment processes are based on reduction reactions, leading to the immobilization of TNT and its transformation products into soil organic materials [40]. In situ and ex situ bioremediation methods have been widely used. In in situ bioremediation, the contaminated material is not removed from the site under investigation [7, 25, 49]. A variety of in situ bioremediation methods mainly natural attenuation, bio-stimulation, and bio-augmentation [40, 49, 83] have been used and verified with relative success [40]. The ex situ methods are off-site treatment of the contaminated soil by the microbial community [40]. A portion of a contaminated soil is excavated, transported and studied in bioreactors under controlled factors such as temperature, pH, light, oxygen and nutrient supply [7, 42]. Soil slurry reactors, land farming and soil composting are the most ex situ bioremediation methods [12, 25, 40, 84] that have been employed for many years. Although they have been widely used, studies have shown that ex situ bioremediation treatments are not economically feasible and applicable to large-scale remediation of TNT-contaminated environment [53]. In comparison with in situ bioremediation methods, additional costs are mainly related to excavation and transportation employed [7, 42]. Furthermore, there are discrepancies between laboratory degradation rates' success and the ones observed at the field level [53]. This can be explained by the differences in contaminant's concentration rates and the easy control of other environmental factors and working conditions at lab scale.

7.1 Natural attenuation

It is a natural process which depends on natural conditions and indigenous microorganisms [7, 48]. Among the bioremediation methods, natural attenuation is the simplest and cheapest. However, the efficiency of the indigenous microflora may take long time, sometimes decades [40]. Common basic methods are often used to measure the magnitude and effectiveness of the natural attenuation: the contaminant disappearance, the reduction in concentration of electron acceptor (O_2, NO_3^-, SO_4^{2-}) and transformation by-products mainly CO_2 [48, 49].

7.2 Land farming technology

The contaminated soil is mixed with uncontaminated soil and nutrients or co-substrate such as molasse, starch, and moisture ranging from 45 to 85% of the water-holding capacity of the soil. Periodic mechanical turning of the mixture is required to increase aeration and maximize microbial contaminant degradation [77]. Brandon and Boopathy [75] utilized this technique together with soil slurry reactor method to evaluate their efficiency for the treatment of soil highly contaminated with explosives, including TNT. Although the method showed TNT removal efficiency of more than 80% and 6.5% of TNT mineralization, soil slurry reactor method was the most effective (≈99%), and the biodegradation rates were much faster than land farming. This technology was recommended for full-scale applications, but the process was too slow and efficiency time could go up to 6 month study [75, 85]. However, leakage issues may be associated with utilization of this technique and therefore contaminate groundwater. Hence, studies recommended that the land farming be used in a constructed cell with liner [12].

Due to some limitations of land farming technology, studies suggested that phyto-remediation, rhizoremediation or transgenic plants may be used as an alternative to land farming [7]. Makris et al.[53] have called for the use of stimulative phytoremediation technologies because of their large field scale applications, high TNT degradation efficiency, low cost, eco-friendly and public acceptance [53]. Moreover, plant-based remediation technologies are suggested based on TNT relative high hydrophilicity, which makes it available for plant uptake [14].

7.3 Bio-stimulation

The use of this technology is dictated by the inefficiency of passive bioremediation due to the limited nutrients and electron acceptors. Therefore, the indigenous microorganisms are stimulated by creating conducive environment and forced to utilize the recalcitrant as carbon and energy source. Oxygen, nutrients and moisture are supplied alongside with the control of temperature, redox potential and pH [7, 30, 40, 49, 82]. Muter et al. [51] reported that the biostimulation method is the most widely used bioremediation procedure and core soil remediation technology. It shown to enhance significantly the microbial degradation of TNT and has been in use for many years. Earlier research studies by Gunnison et al. [26] had described this stimulating activity as "soil priming", where the choice of the substrate is based on its structural similarity with TNT or on evidence demonstrated by the previous studies. Boopathy et al. [25] had proven the feasibility of this technology, and biostimulated the indigenous bacteria by adding molasse to the TNT contaminated soil. Byung-Taek et al. [30] aerobically biostimulated the bacterial growth and TNT biotransformation by *Pseudomonas aeruginosa* strain using yeast extract. Fahrenfeld et al. [13] obtained satisfactory results using lactate and ethanol to anaerobically study aquifer sediments TNT biodegradation. Recently, the study on liquid and soil media confirmed that nutrients have a stimulating effect on microbial activity and on TNT biodegradation as well. The study demonstrated that enzymatic activity significantly increased with increasing

nutrient concentrations, thus, affecting the overall effectiveness of nitro-aromatic compounds' reduction [51, 83]. However, a careful monitoring of CO_2 and O_2 levels to efficiently control TNT degradation limiting factors such as water, nutrients and oxygen was recommended [25, 30, 49].

7.4 Bio-augmentation

The principle of this technology relies on the use of selected specific exogenic microorganisms through enrichment culture or genetically modified microorganisms to improve the effectiveness of natural attenuation and bio-stimulation technologies [49]. Therefore, initially tested or known for their contaminant degradation potential, specific potent microorganisms are added to the TNT-contaminated soil to further accelerate the biodegradation of pollutants [40, 86]. Muter et al. [51] added to the soil contaminated with TNT (500 mg/kg) a bacteria consortium (AM 06 consortium) consisting of seven genera, namely *Klebsiella, Raoultella, Serratia, Stenotrophomonas, Pseudoxanthomonas, Achromobacter* and *Pseudomonas* that had previously demonstrated the ability to degrade TNT. After 14 days incubation, the bacteria consortium had shown significant effects, particularly after the addition of 50% and 100% of nutrients to the contaminated soil. However, some limitations were reported to be associated with this method. Xin et al. [86] have recently reported that growing competent microorganisms is costly and these exogenic microorganisms are weak competitors against indigenous ones, limiting the future application of this technology compared to the biostimulation technique. This is probably the fundamental reason that motivated other researchers to suggest the coupling of biostimulation and bioaugmentation to speed up TNT degradation and enhance bioremediation efficiency [53]. However, combination studies of these two methods are still limited.

7.5 Soil slurry

The general principle is that the mixture of soil-water ratio (1:1 w/w or 40% or 15 to 60% w/v) and co-substrate is filled in a bio-slurry reactor, stirred and completely mixed, and successively treated anaerobically and aerobically [7, 12, 20, 25, 75, 80, 87]. The amount of contaminated soil is a key factor as it determines the mixing energy required, aeration effectiveness in aerobic slurry bioreactors and the size of by-stream post-treatment installations [80]. Strict anaerobic and aerobic conditions are often maintained by regular flushing nitrogen and oxygen gas into the respective incubation microcosms [87]. Aerobic slurry bioreactors have been prevailing in a larger scale application. Molecular oxygen (in aerobic biodegradation process), nitrates and some metals (in anoxic process) and sulfate-reducing, methanogenic and fermentation (in anaerobic biodegradation conditions) are the main electron acceptors commonly used during the biodegradation process [80]. The incubation in anoxic conditions aims at avoiding aerobic polymerization, whereas aerobic treatment strengthens and intensifies the irreversible transformation products binding [1, 42]. The addition of carbon source like molasse, pyruvate, starch, sucrose, lactate, glucose, ethanol, and citric acid not only increases the bacterial cell numbers, their effectiveness by producing TNT degrading enzymes and shortens the degradation time [13, 20, 25, 50, 77], but also removes oxygen during aerobic treatment and serves as electron donor for nitro group reduction. Similarly, Muter et al.[51] have observed a proportional increase of FDA (Fluorescein diacetate) hydrolysis with the rise of nutrients' concentration in the presence of TNT. This resulted in high rates of nitroaromatic degradation due to the stimulation of bacterial enzymatic activity. In parallel with this bacterial stimulative effect by TNT, the observed inhibition of the FDA hydrolysis activity has also been attributed to this explosive. Moreover, Erkelens et al. [31] found that TNT causes the production of bacterial nitroreductases, pnr genes (A and B) reputed for TNT reduction. Contrary to the carbon source supply effects, other study by Gumuscu and Tekinary [50] found that the supply of additional nitrogen source showed an inverse effect and decreased the TNT biotransformation. On whether this nitrogen source acted as a TNT competitor or not, this has not been scientifically proven.

Anaerobic bio-slurry reactors supplemented with municipal anaerobic sludge offered favourable conditions for TNT degradation into its metabolites which were irreversibly bound onto soil matrix[87]. Hodgson et al. [76] reported significant TNT desorption and removal rates subsequent to the use of the non-ionic surfactant Tween 80 in an aerobic bacterial slurry. Robles-González et al. [80] reviewed the slurry bioreactors (SB) and their application to the bioremediation of soils and sediments contaminated with recalcitrant and toxic compounds. This study found that the SB is an effective ad situ and ex situ technology for bioremediation of soils polluted with recalcitrant and explosive compounds if environmental conditions such as pH, dissolved oxygen, temperature, and concentration of inorganic nutrients are strictly controlled. Furthermore, the same study reported that significant removal of explosives such as TNT and its intermediates (2, 4 and 2,6-DNT) from highly polluted soils was achieved using SB technique. Almost all 2, 4 and 2, 6-DNT were removed from the contaminated soils in 2 days. The concentrations ranged between 1.125 and 14.715 g/kg for 2, 4- DNT and 4.8 and 8.940 g /kg for 2, 6-DNT. Regarding the TNT removal, in 4 months and half of the treatment more than 90% was removed from the contaminated soil, whose concentration in TNT was in the range of 0.4 and 1.2 g/kg [80].

Depending on whether the aim study is on TNT mineralization or its transformation products binding to the soil matrix, two methods can be used. In the first method, a consortium of facultative anaerobic microorganisms transforms the xenobiotic into less toxic and sometimes non-aromatic compounds susceptible to be aerobically mineralized. With this method,

approximately 25% of TNT mineralization can be achieved [75], while in the second method the incorporation of 84 to 99% of TNT transformation products to the soil matrix after anoxic-aerobic treatment is attainable [42, 75]. In soil slurry method, TNT reduction products, especially TAT, 4-ADNT, 2, 4- DANT and azoxy compounds were observed and were the same as the ones obtained in the composting technology [1, 20]. Shen et al. [87] observed the 2-ADNT and 4-ADNT as the main products of aerobic incubation, while in addition to these metabolites, 2, 6-DANT and TAT (detected at the later stage) were identified in anaerobic bioslurry reactors. In this kind of nitro-aromatic treatment, two types of microorganisms are often used: free cells and immobilized cells systems [7]. In the case of immobilized cells, the cells are entrapped and adhered to the surface of the carrier or various matrices such as agarose, clay, alginate, and charcoal to provide an alternative to overcome the constraints encountered with the free cell system, thus providing high loading rate. Immobilized bacterial cell–based technology exploits very high selected potent TNT degrading bacteria, which are injected into a specific bioreactor like biological filters designed to offer optimal conditions for bioremediation purposes [7, 88]. Many successful studies of cells such as *Bacillus sp.* YRE1[40], *Arthrobacter sp.* [71] immobilized on charcoal or polystyrene showed satisfactory results. Soil slurry was recommended as suitable technology for treating highly contaminated soil [7], and offers many advantages: simple operating conditions, requires only mixing, supply of air and a carbon source like molasse. Robles-González et al. [80] reported that the slurry bioreactors are most often used to determine the feasibility and estimate the real prospective of a biological remediation approach for the final restoration of the contaminated soil. However, some disadvantages are associated with the use of this technology: the threshold concentration of the contaminant hinders the resistance and survival of microbes, and the reduction of the pollutant is limited and other pollutants such as heavy metals and co-contaminants can inhibit the effectiveness of the free cells, high costs resulting from soil excavation and pre-treatment (crushing, sieving, and screening), construction of lined lagoons, mixing equipment and materials [1, 42, 80]. Even though the soil slurry reactor was recommended for effective and fast remediation of TNT contaminated sites [3], it does not mineralize TNT, but rather leads to the incorporation of irreversible compounds strongly bound by covalence to the humic substances [20, 65] as it is the case for composting technology [30]. Shen et al. [87] obtained poor results, with less than 2% of TNT mineralization in either aerobic or anaerobic conditions. Its metabolites were resistant to further microbial degradation and mineralization and were bound into the soil matrix [87].

7.6 Composting technology

The technology has been used as the first biological treatment process and proved to be effective for remediating highly munitions contaminated soils [12, 75]. Basically two systems have proven efficient: windrow and anaerobic-aerobic composting systems. Both organic materials and TNT are biologically transformed and reduced, resulting in the production of organics or inorganic by-products and heat. The toxicity tests with bacterial tester strains, aquatic invertebrates, earthworms, and rats have proven that after composting there is almost a full reduction in the toxicity and mutagenicity of the contaminated soil and leachates [1, 20, 42, 75, 84], even though Heiss and Knackmuss [65] reported higher ecotoxicity of the composted soil. However, the technology requires long time incubation, high costs and a lot of organic inputs. In addition, there is a limited knowledge on microorganisms and biological systems involved. At the end of the process, the chemicals are sometimes not easy to identify and characterise [7, 20, 75]. Furthermore, the technology is not applied to large scale contaminated site [30], a smaller percentage of TNT is fully mineralized [30, 75, 89, 90], and the effectiveness is limited to the sites with shallow and moderately contaminated environment. Moreover, the major limitations are the contaminant toxicity to the degraders and eventual transfer to the food chain network [7, 20].

8 Future research directions

Based on the analysis of the relevant published literature above, it is clear that microbial degradation of TNT has been widely studied. However, the studies are somewhat limited to attaining the best achievable in situ bioremediation practices and more remains to be explored to find appropriate technologies to address the concerns that still exist and to completely eradicate this recalcitrant. Thus, the following recommendations for future studies have been made:

i. Future studies should aim at mineralizing TNT and complete removal from contaminated soil.
ii. The organic substrate acts as an electron donor, reduces TNT nitro groups to amino derivatives and stimulates the production of the nitro-reductase enzymes known to inhibit the enzymatic aromatic ring cleavage. Future research should find alternatives to the organic substrate to cope with this challenge.
iii. For effective and sustainable remediation strategies of the contaminated environment, a combination of reductive and holistic approaches is required.
iv. Further extensive research studies should focus on deciphering the biochemical properties and physiological activity of TNT degrading enzymes, elucidating the degradation pathways and identification of many microorganisms capable of mineralizing this xenobiotic.
v. Effective TNT mineralization should involve a total elimination of TNT amino groups or deactivate the reductive

biotransformation pathways and promote the ones achieving total destruction and cleavage of aromatic ring.

vi. Future studies to re-examine recent and early successful studies' findings and recommendations to address the gaps and/or the limitations.

vii. Many sites around the world are highly contaminated and yet isolated microorganisms are capable of degrading a small amount of TNT at lab scale. Therefore, one of the major challenges is to find an appropriate biotechnology capable of decontaminating highly contaminated sites and applicable to a larger scale.

9 Conclusions

The present review paper focused on: (i) the extent of biological degradation of TNT and its transformation products and the fate of TNT degradation products in a soil environment, (ii) the factors affecting the TNT transformation, (iii) the microbial bioremediation technologies in use, and (iv)the challenges encountered during the bioremediation process and their remedy. It was found that the aerobic and anaerobic biotransformation of TNT leads to the immobilization of toxic transformation products, which are difficult to remove from the contaminated environment and the fate is uncertain. The resistance of TNT to completely mineralize is essentially due to: (i) its structure, (ii) easy reduction of nitro groups and ultimate chemical misrouting reactions of its intermediates, (iii) the characteristics of the contaminated soil and (iv) and lack of specific TNT degrading enzymes and/or the lignin-peroxidase inhibition by the nitroreductase enzymes. However, TNT mineralization is achievable, but only fewer studies have been successful and therefore more studies are still needed [76]. The bioremediation technologies, their challenges and limitations as well as the remedial strategies have been thoroughly reviewed. Given the toxicity of TNT and its transformation products, their persistence in environment and TNT resistance to mineralization, the eradication of this recalcitrant is imperative. Thus, future research studies should look carefully and primarily at the reviewed and highlighted issues, and then find appropriate biotechnologies that could be widely applicable. This review paper constitutes a compilation of current knowledge on the topic and basic foundation for future research to design effective bioremediation technologies for providing solutions of TNT in contaminated soil environment and minimize environmental impacts.

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