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Microbial transformation of 2,4,6-trinitrotoluene

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The manufacture and decommissioning of explosives has generated, and continues to generate, large quantities of waste material whose primary toxic and mutagenic component is 2,4,6-trinitrotoluene (TNT). The magnitude of this problem has motivated a great deal of research into treatment processes and environmental fate studies, including characterization of microbial transformations of TNT. This work has encompassed studies with mixed cultures and pure cultures of microorganisms derived from either TNT-exposed or unexposed sources, and studies using microorganisms chosen for their known capacities to degrade other pollutants. Several of these studies are discussed with regard to whether they identified a process that may lead to the complete detoxification or mineralization of TNT. Since oxygen can have a significant influence on the types of biochemical reactions that can occur and on the oxidation of intermediates of TNT transformation processes, studies in which oxygen was not excluded are discussed separately from studies conducted under anaerobic conditions.

Keywords: 2,4,6-trinitrotoluene; TNT; biodegradation; bioremediation; munitions

Introduction

2,4,6-trinitrotoluene (TNT), whose annual production is estimated at one thousand tons [24], is the primary explosive used in munitions manufacture. Manufacturing and decommissioning operations have generated and continue to generate large quantities of TNT as a waste product. Much of this waste has been deposited in soil and groundwater through leaching and from disposal in unlined lagoons. The management of munitions waste and the remediation of contaminated sites is critical to public health, since TNT is both mutagenic and acutely toxic [54,61,68]. As public concerns mandate the proper disposal and cleanup of hazardous materials, the destruction of TNT from contaminated media has become an important industrial process that must be evaluated in terms of end-product acceptability as well as cost.

The biological destruction of hazardous organic compounds is often suggested for waste management and remediation processes since it offers the potential for effective removal of the target compound with relatively inexpensive technology. The amenability of a toxic compound to biological remediation depends upon the existence of metabolic activities which can render it non-toxic. The continued presence of TNT in soils contaminated during World War II shows that it can persist in the environment for long periods. Its persistence is not caused by a lack of reactivity in biological systems, for TNT would not be toxic if this were so. The nitro group, due to its electrophilic character, readily oxidizes biological reductants, causing toxicity directly or by formation of other reactive products such as nitroarene radicals [31], and retarding further transformation at high concentrations. Since nitro-substituted compounds are relatively rare in nature, the ability to metabolize them productively is probably correspondingly rare. Microbial activities have been described which lead to the removal of nitro substituents from the aromatic rings of other nitroaromatic compounds and allow metabolism of the remaining carbon skeleton through more conventional pathways. These activities can be classified into the following general categories (Figure 1): i) ring oxygenation followed by release of nitrite [23,55]; ii) nucleophilic attack by a hydride ion to form a hydride-Meisenheimer complex, which may be followed by release of nitrite [29]; and iii) reduction to form a hydroxylamine that is further metabolized [21, 38]. The first type of activity has been described for the metabolism of mono- [23] and dinitrotoluenes [55], however oxygenolytic transformations have not been described for TNT. The increased degree of nitro substitution apparently renders the aromatic ring electron-deficient to the point that it no longer acts as a substrate for the electrophilic oxygenation mechanism. The initial steps of the other types of transformation, ie, hydride-complex formation [46,66] and reduction (numerous references cited below), do seem to operate to various extents in examples of TNT transformation found in the literature. However, cleavage of the aromatic nucleus usually has not been demonstrated, or it occurs at low efficiency [2,8,26]. For substantial mineralization by a single bacterial strain, it has required recruitment of genes from another organism [12]. Apparently the unique substitution pattern of TNT is such that the occurrence of all genes required for productive metabolism (ie, mineralization with energy conservation and carbon assimilation) is rare in a single organism, or else special conditions are required to allow their expression in one organism or consortium of organisms.

This review presents current knowledge about known microbial TNT transformations that is relevant to their evaluation as remediation technologies. These transformations will be divided into 'aerobic' and anaerobic pro-

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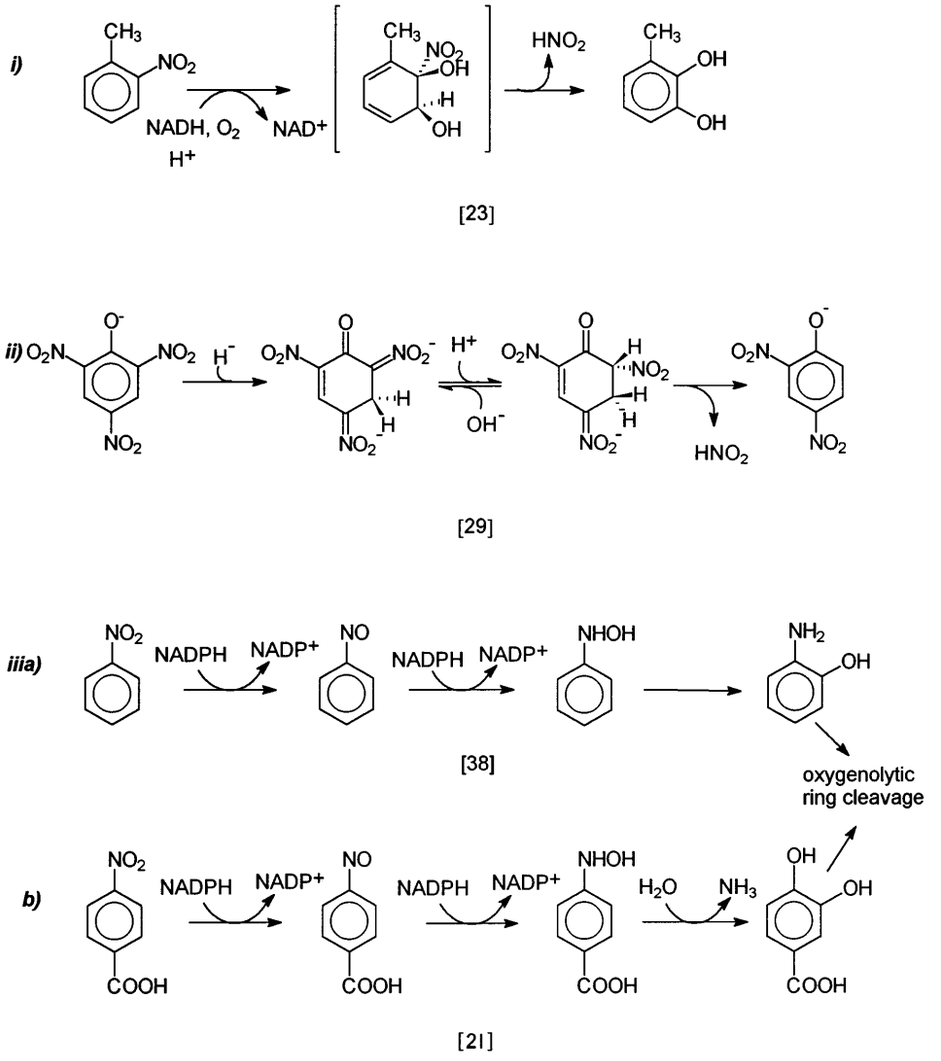

Figure 1 Transformations of aromatic nitro groups leading to assimilation of the respective nitroaromatic compound. References are given in brackets.

cesses, depending on whether or not oxygen was included in the process, rather than actually required for it.

Aerobic TNT transformations

Many studies have been conducted to examine the feasibility of removing TNT from waste streams and soil by conventional aerated microbial treatment processes, including composts, activated sewage sludge, and soil slurry reactors [3,11,17,25,27,36,43,67]. In addition, incubations conducted with environmental samples to simulate *in situ* processes or the potential activities of indigenous microbial populations [6,8,9,26,30,39] have generally described a dependence upon added substrates such as carbon, energy, and nitrogen sources, indicating a cometabolic nature for such processes. Mass balances have usually been lacking in these studies, but where examined, evidence for transformations involving nitro group reduction has been found [6,9,17,25,27,30,43,67] with the usual result being the formation of partially reduced products such as aminodinitrotoluene isomers (ADNT), 2,4-diamino-6-nitrotoluene (DANT), and/or azoxytetranitrotoluenes. In addition, more detailed studies of the fate of ^{14}C-labeled TNT in activated sludge and composts have shown accumulation of radioactivity in material apparently derived by covalent attachment to lipid and protein [11], or to humic substances or other natural polymers [43]. It seems that the relatively strong oxidizing character of the TNT molecule leads to its partial reduction whenever it contacts biological reductants. The partially reduced products, nitroso-, hydroxylamino-, and aminonitrotoluenes, have a propensity to react with each other or organics in the surrounding milieu to give covalently bound derivatives. The presence of oxygen probably promotes the coupling reactions (ie, formation of azoxytoluenes) by oxidizing hydroxylamino groups to nitroso groups [46]. Therefore, the combination of fortuitous and possibly inevitable reductions and an aerobic environment would be expected to produce such products, whose decreased solubility may further reduce biological availability. The fate of the covalently derivatized material and its potential toxicity have not been reported. Little or no significant mineralization of TNT has been reported in

the majority of labeling studies done with aerobic mixed culture incubations, indicating that pathways for the complete utilization of TNT, its partially reduced products, and polymeric material are rare in these systems. Some exceptions have been reported recently in soil incubations with low levels of TNT. Bradley *et al* reported up to 10% mineralization of uniformly labeled ^{14}C-TNT in incubations of soil from contaminated and uncontaminated sampling sites with mineralization rates highest in those with uncontaminated soil [9]. An apparent toxicity toward mineralization was observed with higher concentrations of TNT [8]. These authors also showed an antagonistic effect of complex carbon sources toward mineralization, and that optimal mineralization occurred under microaerophilic conditions [8]. A similar effect on mineralization was reported by Jones *et al* [26], who found that when the TNT content in soil was 25 mg kg^{-1}, approximately 10% was mineralized, but higher concentrations were not mineralized. Since some sites contain TNT at concentrations of thousands of mg kg^{-1} [15,16], *in situ* application of these processes for remediation may be infeasible at such sites. The pathways and organisms involved in these examples of TNT mineralization activity have not been reported; however Bradley *et al* did show evidence of reductive transformation in their soil samples using non-labeled TNT [9].

The literature also contained reports of pure culture studies of aerobic TNT transformation, involving organisms either selected for the ability to use TNT as a growth substrate, or chosen from culture collections for their known inherent activities. Although some initial reports on the isolation of organisms using TNT as a sole source of carbon and energy have appeared [37,62,69], these have not shown substantial CO_2 production from TNT [62], and at most only simple reductions have been identified [37,69]. One study noted the appearance of aliphatic material [37], but without tracer studies it is not possible to attribute these to TNT metabolism. Organisms capable of growth on TNT as carbon and energy source were not found when such selection was applied in other studies [7,12,39,53], implying that TNT transformation in aerobic systems is a cometabolic process.

Other reports indicate that organisms could be isolated which are capable of using TNT as a source of nitrogen. By selecting for the ability to use TNT as a source of nitrogen, Jones *et al* [26] isolated a *Pseudomonas* sp from a soil sample shown to mineralize low concentrations of TNT, but only partially reduced transformation products were identified. Naumova *et al* [35] cultured a *Pseudomonas fluorescens* isolate using the partially reduced metabolite, DANT, as the nitrogen source. Ammonia production and cofactor requirements of a cell-free system, as well as previous identification of hydroxylated benzene products, suggested an oxygenolytic pathway. Other predicted products such as hydroxylated aminonitrotoluenes were not identified. In addition, though enzyme activity for the breakdown of hydroxylated benzenes (pyrocatechase, metapyrocatechase, protocatechase) was detected, the organism was not able to use DANT as a source of carbon and energy. This type of pathway could represent an effective degradation of TNT, but again, the counterproductive coupling reactions known to occur when partial reduction proceeds in the presence of oxygen must be minimized. As a followup to the study of Naumova *et al* [35], Gilcrease and Murphy [19] undertook studies using an isolate of *P. fluorescens* from another contaminated site, but were unable to promote the growth of this organism using DANT as the sole nitrogen source. The transformations observed with TNT included the expected reduction to DANT and an acetylation at the 4-amino position of DANT [19]. The product, 4-acetamido-2-amino-6-nitrotoluene, was an apparent dead-end product in their cultures, since it was not transformed further. The implications of such transformation are presently unclear in the absence of any indications of whether this derivatization would enhance the ultimate mineralization of TNT in a mixed culture.

From soil originating at a TNT-contaminated site, Duque *et al* isolated a *Pseudomonas* sp that could use TNT as a nitrogen source [12], and they also obtained variant strains able to utilize this nitrogen source more efficiently. 2,4-Dinitrotoluene, 2,6-dinitrotoluene, and 2-nitrotoluene were identified as intermediates in the transformation of TNT, and toluene and nitrite as products. Another strain that could use toluene as a carbon and energy source was then constructed. This hybrid pathway allows complete mineralization of approximately 50% of the transformed TNT [46], making this the most complete TNT mineralization pathway yet described. To our knowledge, the organism isolated originally represents the first case in which a TNT-metabolizing activity that apparently conferred a selective advantage for growth in a TNT-contaminated environment has been characterized. In this pathway the removal of nitro groups is proposed to proceed via sequential hydride attack. A hydride-Meisenheimer complex of TNT could be detected after extraction with an organic solvent, whereas no such complexes predicted for the dinitro- and mononitrotoluene intermediates were identified [46]. The hybrid organism also showed significant reductive activity, however, and partially reduced products and azoxytetranitrotoluenes accounted for a large proportion of the mass balance. The authors are optimistic that this residual activity can be avoided and that recombinant organisms can be constructed to mineralize TNT aerobically without accumulation of such products [46].

With the reduction of TNT in mind, Alvarez *et al* studied an isolate of *P. aeruginosa* capable of growth on the aromatic amine 2-aminobenzoate for its ability to metabolize TNT and the ADNT isomers [1]. The organism could not use TNT or the ADNT isomers as a carbon or nitrogen source, but was able to co-metabolize high concentrations (100 ppm) of TNT in the presence of other substrates. TNT and both the ADNT isomers were transformed to give similar product profiles: up to 40% was converted into polar products which were not extractable with diethyl ether at any pH tested (pH 2, pH 7, or pH 12). DANT and 4-acetyl-ADNT were dead-end products in the cultures studied. A dependence upon oxygen was observed for production of the polar product fraction, suggesting that aromatic oxygenase activity may be responsible for these transformations. Polar products were also found by Bae *et al*, who studied cometabolic transformation of TNT in aerobic cultures isolated from a contaminated site [2]. These authors found 3% mineralization, and conversion of 80% to

unidentified polar products and 13% to a biomass-associated fraction by an *Enterobacter* sp. Similar product profiles were seen with other isolates. Changes in the character of the TNT molecule leading to dramatic increases in polarity relative to the known reductive products is encouraging, since it may indicate hydroxylation or cleavage of the aromatic ring. Such transformations may make further metabolism by other organisms possible, but additional characterization of the unknown products or actual demonstration of mineralization is necessary before such an assessment can be made.

Vanderberg *et al* studied TNT transformation by the propane-oxidizing bacterium *Mycobacterium vaccae* [64]. Since the monooxygenase of this organism is rather non-specific and has shown hydroxylating activity on a number of aliphatic and aromatic substrates, it was reasoned that novel oxidations of TNT might be possible. This was apparently so, since the novel metabolites amino-dinitrobenzoic acid and a diamino-nitrobenzyl methyl ether were identified. When toluene was included in incubations with TNT, 2-amino-6-nitrotoluene accumulated, indicating removal of the nitrogen at the 4-position. Although mineralization was not observed, a substantial portion of radioactivity from ring-labeled TNT (*ca* 40%) co-migrated with polar lipids in a TLC system, suggesting cleavage of the aromatic ring. This work identified several new transformation reactions that may promote mineralization by other organisms, thus offering promise as a step in TNT remediation.

Streptomyces species have been examined for TNT transformation because of their prevalence in composts. Funk *et al* studied TNT transformation by *Streptomyces chromofuscus* A11 and found transient appearance of reductive intermediates, but the end products were not determined [17]. Pasti-Grigsby *et al* tested *Streptomyces* spp obtained from TNT-contaminated soil and from unexposed environments and found that no significant resistance to TNT inhibition of growth could be attributed to the previous exposure to TNT [41]. No novel TNT transformation activities could be attributed to the selected strains either, since only partial reductions to form amino-nitrotoluenes were observed as well as conversion to insoluble material.

A combined chemical/biological process was tested with the intent of causing the breakdown of TNT into products more degradable by bacteria. Using UV irradiation plus ozone treatment, Kearney *et al* saw a 25% mineralization of TNT upon subsequent incubation with a strain of *Pseudomonas putida* adapted to growth with aromatic substrates [28], a marked improvement over incubations without the pretreatment.

Fungi have also been examined for their metabolism of TNT. Parrish found that of 190 fungi from 98 genera, 183 could transform 100 ppm TNT to reduced products [40]. Because the ligninolytic basidiomycete *Phanerochaete chrysosporium* is known to produce non-specific peroxidases capable of catalyzing the oxidation of many xenobiotic compounds [42], Fernando *et al* tested it for metabolism of TNT [14]. It was found that under conditions promoting the expression of the peroxidases, approximately 35% of the radioactivity from ring-labeled ^{14}C-TNT (initial concentration, 1.3 ppm) was trapped as CO_2. Of the remaining radioactivity, 25% was water-soluble material, and 16% was extracted into methylene chloride and eluted from an HPLC system as material more polar than TNT [14]. When higher concentrations of TNT were used in aqueous or soil incubations, mineralization and overall transformation were less extensive over the 90-day time periods studied. These data indicating that *P. chrysosporium* is capable of extensive degradation of TNT and stimulated work characterizing the process and devising technology to exploit it [60,63]. Spiker *et al* found that TNT was inhibitory to spores of *P. chrysosporium* at concentrations greater than 5 ppm [56]. This toxicity is apparently related to the activity of TNT as an oxidant, since reduction to aminodinitrotoluenes relieved toxicity [57]. The activities of reduction and subsequent oxidation by *P. chrysosporium* have been further characterized. Mineralization has been found to be correlated with expression of ligninolytic activity [33,58]. Aromatic nitroreductase activity by *P. chrysosporium* was found to be membrane-bound in two cases [48,58], and in the soluble fraction in another [34]. However, conditions of cell disruption were noted to be important for maintaining membrane association [48]. One report [58] found the maintenance of a membrane potential to be required for nitroreductase activity, while another did not [48]. The intermediacy of nitroso- and hydroxylamino intermediates indicates that these are also substrates of the reductase and that reduction is stepwise [10,33,48]. The transient accumulation of 4-hydroxylamino-2,6-dinitrotoluene (4HADNT) has been observed [33], and this reductive intermediate inhibits lignin peroxidase activity [10,33], causing an inhibition of TNT mineralization [33]. 4HADNT was also oxidized by lignin peroxidase [10,33] and MnO_2 [34], causing a futile cycle of reduction/oxidation and formation of azoxytetranitrotoluenes.

Once TNT has been reduced to give 4-amino-2,6-dinitrotoluene (4ADNT), additional derivatization and reduction reactions take place before oxidation by the ligninolytic systems. 4ADNT is formylated to give 4-formamido-2,6-dinitrotoluene, which is reduced to give 2-amino-4-formamido-6-nitrotoluene [34]. This can be slowly transformed to DANT [34], which accumulates only under non-ligninolytic conditions [57], but which under ligninolytic conditions was a superior substrate for further transformation [34].

Anaerobic transformations of TNT

Anaerobic processes, which have also been described for TNT transformation, have the potential advantages of rapid reduction at low redox potential, and minimization of oxidative polymerization reactions due to the absence of oxygen. In addition, azoxy- and azo-coupling products would probably not be stable under highly reducing conditions and would be reductively converted to the more soluble amine monomers [22,49]. If amines are thereby stabilized with reducing conditions, more time is allowed for biological or chemical removal of nitrogen from the aromatic ring. Complete reduction of the nitro groups of TNT would give triaminotoluene (TAT), a reactive chemical itself subject to additional transformations, particularly autoxidation [44]. With these factors in mind, Funk *et al* examined TNT trans-

formation in anaerobic systems using mixed cultures derived from sewage sludge and soil [15,18]. pH is critical in preventing polymerizations and neutral to slightly acidic conditions (pH 6.5–7.0) are optimal. TAT was identified in anaerobic soil cultures, and aromatic products lacking nitrogen substitution were detected in cultures of anaerobes deliberately adapted to munitions [18]. The only mass balance experiments performed with soils under the optimized conditions showed that transformation into soluble products was promoted, but this was accounted for by incomplete reduction to ADNT and DANT. Further characterizations, including tracer studies to confirm deamination reactions, are necessary.

Using anaerobic incubations with sewage sludge cultures fed glucose [49], Rieger and Knackmuss showed rapid, nearly stoichiometric reduction of the nitro groups of TNT. These studies showed production of TAT, followed by its slow disappearance. The authors described several possible fates of TAT in various chemical environments, noting that biological transformations were only postulated, not characterized. The irreversible binding of TAT to humic substances and charged soil particles was recognized as the highly likely fate for TAT. The potential for release of toxic products from this immobilized material is not known, requiring further assessment of the remedial effectiveness of this result. A remediation process based on this presumed immobilization has been described and tested with soil at a pilot scale (30 tons) [59].

Roberts and Pendharkar described the results of different anaerobic enrichments and the consequent types of TNT transformation activities [50] and concluded that a yeast extract/TNT medium yielded the most effective TNT transformation activity, with small amounts of *p*-cresol detected from cultures derived in this manner. Again, it was suggested that anaerobic transformations can lead to deamination and the removal of the polymerization-sensitive attributes of the TNT transformation congeners, but confirmation by tracer studies is required.

In an anaerobic/aerobic sequence of reactors tested by VanderLoop *et al* [65], TNT was adsorbed onto granular activated carbon and the reactor was fed with ethanol and ammonium. Ammonium, nitrate, and biomass (estimated as 12% of the volatile suspended solids) were quantified in effluent streams, yielding a minimum recovery of 80% of influent TNT nitrogen. However, because of statistical uncertainties and limited chemical analyses, it cannot be unequivocally determined that TNT nitrogen was not bound in polymeric material.

The rapid and complete reduction of TNT has also been indicated in pure culture studies of anaerobic bacteria. McCormick *et al* showed that TNT nitroreductase activity was present in a number of bacteria [32]. Using cell suspensions and cell extracts of the obligate anaerobes *Veillonella alkalescens* and *Clostridium pasteurianum* with hydrogen as reductant, they showed complete reduction of the TNT nitro groups to give TAT. *V. alkalescens* cultures did not carry out as complete a reduction as the cell suspensions or cell-free extracts, giving partially reduced products. Cultures of *C. pasteurianum* transformed TNT into undetected products rather than TAT. Hydrogenase and ferredoxin-like material were associated with the non-specific nitroreductase activity of *V. alkalescens* cell-free extracts, providing the first evidence that low-potential bacterial redox proteins were capable of completely reducing the nitro groups of TNT.

Preuss *et al* studied pure cultures of a *Desulfovibrio* sp selected for using TNT as sole nitrogen source, finding that the organism transformed TNT to TAT [44]. This work also included biochemical studies showing that the reduction of the third nitro group of TNT (the nitro group of DANT) was carried out by ferredoxin-reducing enzymes and probably dissimilatory sulfite reductase. The reactivity of TAT in physiological situations was also described, including an apparent hydrolysis occurring spontaneously in acidic solution (pH 2–5) which releases approximately one mole of ammonia per mole of TAT transformed. A denitrifying organism could also carry out a transformation of TAT under conditions of neutral pH, suggesting that biological transformation of TAT is also possible under conditions in which it is most stable chemically. The products of TAT transformation were not identified.

Boopathy and Kulpa, in examining the transformation of TNT by a *Desulfovibrio* strain in different nutrient conditions [5], found that this organism used TNT as its sole source of nitrogen, forming toluene from TNT after 45 days of incubation. The transformation was presumed to proceed via TAT, which was not identified, and toluene derived by reductive deamination. The kinetics of growth and stoichiometry of toluene production were peculiar, however, since at a point during exponential growth, an amount of DANT-nitrogen in excess of that added as TNT, but no toluenes with fewer nitrogen substituents, were reported and thus no nitrogen removal for growth was evident. The amount of toluene-carbon reported at the end point was also in excess of that added as TNT. The process of reductive deamination would represent a complete removal of the problematic substituents if such activity could be directed toward TAT or any of the aminonitrotoluenes. Aromatic reductive deamination activity has been described for aniline; it requires carboxylation and acetylCoA activation to proceed [51]. As pointed out previously [45], the steric and electron density characteristics of TAT and the TNT reductive transformation congeners make them very different substrates, unlikely to be acted upon by previously identified reductive deaminases.

Boopathy *et al* also looked at TNT transformation by a *Methanococcus* sp and showed incomplete nitro group reduction [4]. Gorontzy *et al* examined the ability of members of three genera of methanogens to reduce other nitroaromatic compounds and noted that cell lysis occurred, but that nitro-reduction reactions took place regardless [20]. These studies have not identified any special capabilities for transformation of nitroaromatics in methanogens.

Regan and Crawford [47], Shin and Crawford [52], and Ederer *et al* [13] have studied cometabolic TNT transformation by *Clostridium bifermentans* isolated from a long-term bioreactor fed munitions compounds. These studies showed a capacity for rapid reduction of TNT nitro groups in the isolates. No apparent adaptation was evident, and other clostridia not closely related to the isolates showed indistinguishable activity, suggesting that this activity is widespread among clostridia [13]. The reduction of the

nitro groups resulted in substantial TAT production in cell suspension experiments. Another as yet unidentified transformation product of TAT is also found in cell suspension and culture experiments [13]. The significance of this process and the exact role of this organism in the bioreactor is not known.

Figure 2 attempts to summarize several schemes for biological detoxification of TNT, including the predominant nitro-reduction reactions and several side reactions. Most of these routes are incompletely characterized and some are speculative. Some of the end points, such as polymerized materials, are uncharacterized in terms of their toxicity or potential for conversion to toxic products, and thus cannot yet be considered as points of permanent remediation. In white-rot fungi, the process shows a high degree of mineralization, which is probably the best measure of the extent of transformation. Should some assurance be gained that the remaining organic material is not a potential hazard, an effective bioremediation process using white-rot fungi may be developed, perhaps including a pre-treatment to give reduction products which are more readily mineralized [34]. Aerobic bacterial processes have not yet shown such extensive mineralization without genetic engineering, especially with high concentrations of TNT, and they also

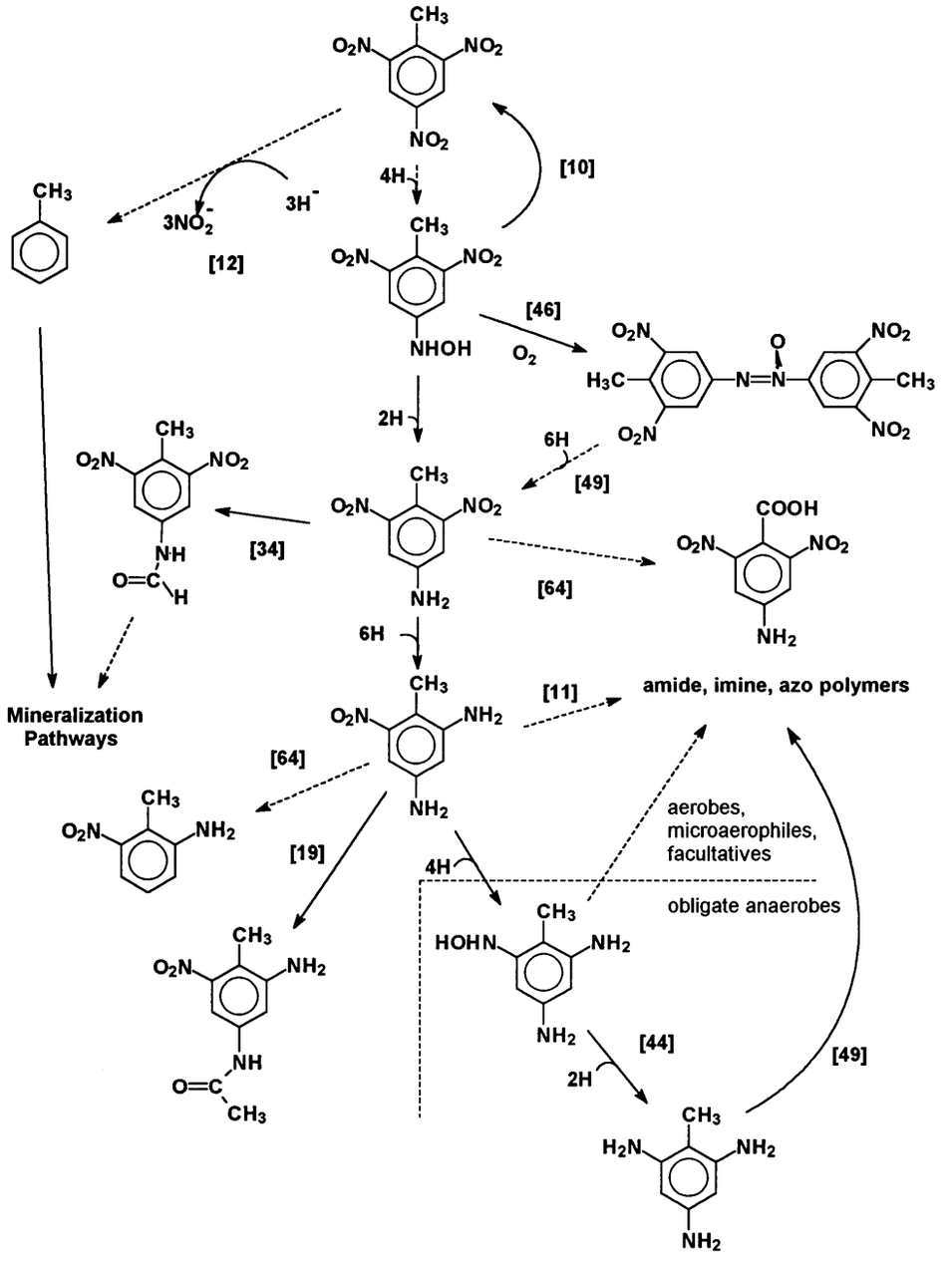

Figure 2 Transformation pathways of TNT. All structures shown have been identified in various examples of TNT transformation. Solid arrows connect compounds occurring in a known sequence. Dotted arrows are speculative pathways in that intervening metabolites have not been identified or the exact source of the product compound is not known. References are given in brackets.

lack complete characterization of the organic products. The isolation of an organism that can remove the nitro groups of TNT [12] is promising, particularly since the end product, toluene, is readily degraded. The dead-end metabolites of this and most other aerobic, reductive transformations may be degraded by white-rot fungi as mentioned above. Further characterization of products of other aerobic processes [1,2,64] may also allow their use alone or as part of a multi-step process or consortium depending on whether the end products prove to be environmentally acceptable or to serve as substrates of mineralization pathways. Treatment by strict anaerobes would be an even simpler option, requiring no aeration and posing little problem with toxicity to the active microorganisms. Should TAT fixation prove to be an effective solution to its detoxification, or if processes leading to further metabolism and mineralization of TAT are demonstrated, these developments would offer a cost-effective solution to the problem of TNT contamination.

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