BIODEGRADATION OF 2,4-DINITROTOLUENE IN THE WASTE STREAMS OF A MUNITIONS PLANT

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ABSTRACT

Abstract — Wastewater from the manufacture of propellants typically contains 2,4-dinitrotoluene (DNT), a suspected animal carcinogen. Previous studies have indicated that DNT is aerobically biodegradable. However, inconsistent removal of DNT during aerobic treatment has been observed at a munitions wastewater treatment plant, necessitating the use of activated carbon pre-treatment. The objective of this study was to evaluate the effect of nutrient and cosubstrate amendments on the rate and extent of DNT removal. Addition of ethanol (100-500 mg/l) and phosphate (0.8-3.3 mg/l) significantly accelerated the rate of aerobic DNT (0.3-5.6 mg/l) biodegradation. Addition of phosphate alone also increased the rate of DNT degradation, but to a lesser degree. The presence of ethyl ether, another substrate commonly found in munitions plant wastewater, had comparatively little effect on the rate of DNT removal. Interruptions in the DNT manufacturing process can result in DNT being absent from the munitions plant wastewater for extended periods. The effect of such interruptions was evaluated in semi-continuously operated reactors, fed daily with phosphate-amended wastewater, at a hydraulic residence time of 3 days. DNT removal resumed without a lag even after it was absent from the feed for periods up to 15 days. During aerobic biodegradation of DNT, reduction to 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene was consistently observed, with reduction at the *para* position predominating. The highest level of aminonitrotoluene formation was 23% of the total DNT degraded. Aminonitrotoluene isomers were consumed shortly after they formed in the semi-continuously operated reactors, confirming the potential for degradation of these metabolites. Although the aminonitrotoluene isomers are not currently regulated, their presence in treated munitions wastewater is a concern due to possible toxicity.

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EXECUTIVE SUMMARY

2,4-Dinitrotoluene (DNT) is a major component in the production of propellant and is therefore found in the waste streams of munitions plants. DNT is a suspected animal carcinogen, and studied plant is regulated to discharge no more than 113 μg/l into the river into which it discharges. It is therefore important to understand the factors that influence the degradation of DNT. The purpose of this study was to characterize the conditions under which DNT concentrations are minimized and to identify ways the plant's treatment facility may be operated and modified to optimize DNT removals.

Batch reactors were designed to simulate the equalization basins at the plant. Wastewater was collected from the end of the equalization basins and the reactors were monitored for approximately 9 days (216 hours). Initial and final samples were collected from the reactors to determine total suspended solids, volatile suspended solids, chemical oxygen demand, ammonia, nitrite, nitrate, phosphate, and sulfate. Concentrations of DNT, aminonitrotoluene (ANT) isomers, and diaminonitrotoluene (DAT) were measured once or twice per day. Reactors were spiked with DNT in aqueous form immediately upon setup along with other agents such as nitrate, phosphate, ethanol, and/or ether.

A control experiment was performed to verify that the reduction in DNT was attributed to biological factors and not abiotic mechanisms, such as sorption or volatilization. Plant wastewater inhibited with 1g/l sodium azide and tap water controls were analyzed. The reactors with plant water inhibited by sodium azide showed less than a ten percent decrease in DNT, which indicates that biodegradation is important and that sorption to biomass and/or other solids did not occur. The reactors with tap water also showed less than ten percent of a decrease in DNT, suggesting that volatilization and sorption to the reactor or aeration pipettes were not factors.

Varying levels of nitrate, phosphate, and ethanol were added to reactors, individually, as well as jointly, to determine their role in the degradation of DNT. Ethanol only and nitrate only made no significant difference in the degradation rate compared to the control. Phosphate, however, significantly increased the reduction in DNT at levels as low as 1.6 mg/l PO_4-P. Phosphate in

addition to 500 mg/l ethanol showed the greatest amount of degradation. In an attempt to minimize the levels of phosphate and ethanol needed to provide sufficient degradation, another experiment was conducted. Levels of phosphate ranging from 0.8 mg/l to 3.3 mg/l PO_4-P, and ethanol values of 100 mg/L and 500 mg/L were considered. DNT degraded fastest in the two reactors with phosphate and 500 mg/l ethanol, but those with phosphate and 100 mg/l ethanol still degraded DNT more than 4.5 times faster than the control.

Another experiment was performed in sealed serum bottles to consider the possibility of ether inhibition. PO_4-P at 0.8 mg/l was added to the bottles, while the ether levels ranged from 0 to 500 mg/l. The degradation rate was not affected by the ether, even at 500 mg/l. This suggests that the mixed culture in the plant's wastewater was more resilient than the enriched cultures previously studied.

Because DNT is not consistently present in the plant's wastewater, it was theorized that the culture in the EQ basin might eventually wash out or have trouble recovering when spiked again with DNT. An experiment was designed to consider varying periods of time in which the reactors would not be fed DNT. The DNT spiking schedule ranged from every 3 days to every 15 days. The degradation rate of DNT in the first reactor was not significantly different than the rate in the other reactors. This alleviated the concern that the microorganisms would not recover when starved of DNT for up to two weeks.

A mass balance for DNT was completed, but some of the DNT degraded was not accounted for in the tests completed. This could be due to complete mineralization of the DNT or formation of byproducts not measured. Samples were analyzed for ANT isomers to support the biodegradation theory and to determine which pathway was being utilized. In most reactors without high levels of ethanol, approximately 20% of the degraded DNT was transformed to the ANT isomers. This suggests that other substrates were responsible for partial reduction before oxidation. The addition of 100 mg/l ethanol with phosphorus showed no increase in the formation of the ANT isomers. When 500 mg/l ethanol was added without phosphate, the formation of ANT isomers increased to almost 24% of the degraded DNT. However, when phosphate was added with 500 mg/l ethanol, the ANT isomers accounted for 35% of the degraded DNT and consumed all initial levels of nitrite, rather than releasing it. This suggests

that the organisms which degrade the ethanol were phosphorus limited. The level of ether also had an impact on the formation of ANT isomers. As the ether level increased to 500 mg/l, the formation of ANT isomers decreased to 15% of the degraded DNT.

A mass balance was also performed on nitrogen to estimate the percentage of DNT mineralized. Two moles of nitrite should be released for each mole of DNT mineralized. However, the mass balance indicated that more than two moles of nitrite per mole of DNT was produced. Nitrate was consumed at approximately the same level of nitrite production, suggesting that denitrification occurred. This implies that there were areas in the reactors where oxygen was not sufficient. Because denitrification was occurring and gases were not being collected, a complete mass balance could not be completed. Nevertheless, as much as 99% of the nitrogen could be accounted for in the reactors that had not been supplied with ethanol and phosphate. In those reactors with phosphate and 500 mg/l ethanol, final nitrogen levels were undetectable. When ethanol only was added or phosphate with 100 mg/l of ethanol, 50% of the initial nitrogen could be accounted for in the final sample. These results support previous studies which suggest that the presence of a cosubstrate encourages an initial reduction, before oxidation occurs. It also agrees with the by-product formation results discussed earlier.

LITERATURE REVIEW

2,4-Dinitrotoluene (DNT) is used in the production of explosives for which it serves as a gelatinizing and waterproofing agent (ATSDR, 1989). It is also utilized as an intermediate in the manufacturing of polyurethane and dyes and is used in smokeless gunpowder (ASTDR, 1989). Treatment of 2,4-dinitrotoluene along with other aromatics has been studied significantly in the past and present. Researchers have considered strategies such as oxidation by Fenton's reaction, abiotic reduction, adsorption, and biological degradation.

Biodegradation

Mononitroaromatics and, to a limited degree, dinitroaromatic compounds are degraded oxidatively by aerobic bacteria. As the number of nitro groups on the ring increases, initial reductive reactions are observed (Reiger and Knackmuss, 1995). Biotransformation of 2,4-DNT can occur through oxidative or reductive pathways. In oxidative reactions, molecular oxygen is required along with oxygenase or peroxidase enzymes that aid in ring cleavage, resulting in mineralization (Noguera and Freedman, 1996); the nitro groups are released as nitrite. The reductive pathway is the more common reaction resulting in formation of metabolites such as 2-amino-4-nitrotoluene (2Am4NT), 4-amino-2-nitrotoluene (4Am2NT), 2,4-diaminotoluene (DAT), azoxytoluene isomers, and 4-acetamido-2-nitrotoluene (4Acm2NT) (McCormick *et al*., 1976; McCormick *et al*., 1978). Formation of these metabolites depends on redox conditions. Aminonitrotoluene isomers appear under aerobic, anoxic, and anaerobic conditions. Azoxy compounds are formed only in aerobic systems and reduction to diaminotoluene occurs only in the absence of oxygen.

The initial removal of the nitro groups as nitrite allows for "selective advantage" to organisms which may not completely mineralize nitroaromatics (Bruhn *et al*., 1987). A single enzyme is adequate to give "selective advantage" to microorganisms which use the nitroaromatic compounds as their sole source of nitrogen. Microbes prefer to reduce the nitro groups due to

their electrophilic nature (Reiger and Knackmuss, 1995); thus, aerobic organisms have the ability to reduce nitroaromatic compounds.

There are few instances in which nitroaromatic compounds are completely degraded or utilized as growth substrates (Spain, 1995). This is primarily due to the ability of some microorganisms to reduce the nitro group forming the amino derivatives or condensation products that are resistant to further microbial degradation (McCormick *et al*., 1976).

Aerobic degradation

J. C. Spain (1995) notes that as of 1995, bacteria have been observed to utilize four approaches for aerobically degrading the nitro group of a nitroaromatic compound. The first mechanism is the initial oxidative removal of the nitro group as nitrite in a reaction catalyzed by a monooxygenase enzyme. The second approach utilizes a dioxygenase to eliminate nitro groups forming catechols and nitrite (Spanggord *et al*., 1991). More recently, studies have discovered partial reduction of the nitro group to a hydroxylamino derivative which eventually releases nitrogen as ammonia (Nishino and Spain, 1993). The fourth approach for the initial attack of nitro groups has been studied only with trinitroaromatics. It involves replacement of the nitro group by hydrogen (Reiger and Knackmuss, 1995; Vorbeck *et al*., 1994).

DNT oxidation under aerobic conditions has only been demonstrated in two reports. Spanggord *et al*. (1991) isolated a *Pseudomonas* sp. From a four-member consortium enriched with DNT. DNT was degraded as the sole source of carbon and energy and stoichiometric release of nitrite was observed (2 mol NO_2^-/1 mol DNT) suggesting mineralization. Lendenmann *et al*. (1998) considered transformation of DNT in an aerobic fluidized-bed biofilm reactor. A majority of the nitrogen present in the effluent was in the form of nitrate indicating that nitrite-oxidizing bacteria were present. Ninety percent of the nitrogen was recovered and the authors assumed the remaining ten percent was due to cell synthesis. Nine moles of oxygen are required for complete mineralization of one mole of DNT (assuming nitrifying conditions) which is equivalent to 1.58 mg oxygen/mg DNT. The COD of the biomass formed and oxygen consumed yielded 1.49 mg oxygen/mg DNT. The insignificant difference in these two values suggests mineralization.

The presence of cosubstrates greatly effects the degradation of DNT and determines which metabolites are produced. High concentrations of easily degradable organics support the growth of microorganisms with a variety of metabolic capabilities. Noguera and Freedman (1996) contend that the fate of DNT and the efficiency of its treatment depend on how efficiently DNT-oxidizing microorganisms compete with DNT-reducing microbes and, secondly, the ability of the organisms in the culture to transform and mineralize the reduced DNT products.

Previous studies have considered the significance of solvents such as ethanol and ether on the degradation of DNT. Freedman *et al.* (1996) observed that the presence of ethanol as a cosubstrate resulted in the transient accumulation of the aminonitrotoluene isomers. When DNT was provided as the sole substrate, there was no appearance of these isomers. This implies that biodegradation of ethanol was causing at least a partial reduction of DNT due to cometabolism with ethanol before its oxidation. This was considered by evaluating the nitrite release; when ethanol was added, nitrite levels dropped to 59% of the degraded DNT, confirming that a large amount of DNT was reduced prior to oxidation. The low effluent soluble COD suggests that oxidation of the compounds was completed to mineralization. The authors also recognized that some of the compound removal could be due to formation of insoluble azoxy-type polymers resulting from reactions among aromatics and/or metabolites. However, because DNT is used as a growth substrate, its decrease in concentration is probably due to biological processes thus the reduction in effluent COD (Freedman *et al.*, 1996).

At doses of 142 mg/l, ether has been observed to inhibit the degradation of DNT (Freedman *et al.*, 1996). A study completed by Freedman *et al.* (1996) was performed with cultures enriched from the underflow sludge of the secondary clarifier at a munitions plant. Ether also inhibited degradation of 2Am4NT, but had no significant effect on 4Am2NT or DAT. This implies that the nitro group at the *para* position is affected by the ether and previous studies have suggested that the *para* site is where transformation of many nitroaromatic compounds is initiated (McCormick *et al.*, 1976).

Noguera and Freedman (1996) carried out an experiment with an organism, *Pseudomonas aeruginosa*, isolated from an anoxically enriched culture. *P. aeruginosa* was then grown aerobically and supplied with ammonia, ethanol, and DNT. Most of the DNT was degraded within four days to 53% 4Am2NT, 32% 2Am4NT, and 3% DAT. By the end of the experiment, 2Am4NT had not been removed; however, 4Am2NT and DAT were completely transformed in conjunction with an accumulation with 4Acm2NT and traces of acetomide-aminotoluene. Only 43% of the initial DNT could be accounted for at the end of the experiment.

Anoxic degradation

Under anoxic conditions DNT is initially reduced to 2Am4NT and 4Am2NT (Noguera and Freedman, 1996; Noguera and Freedman, 1997). Anoxically, 2Am4NT persisted while 4Am2NT acetylated to 4Acm2NT (Noguera and Freedman, 1996). When all the DNT was transformed, Noguera and Freedman (1996) observed that 44% remained as 2Am4NT, 36% as 4Acm2NT, while 20% was not accounted for.

Noguera and Freedman (1997) also compared two enriched cultures grown under anoxic conditions: activated sludge from a municipal wastewater treatment plant (WWTP) (unacclimated) and RBC biomass from a munitions plant (acclimated). DNT was consumed only when ethanol was added illustrating the need for an external electron donor. Under these conditions, a negligible amount of DNT was mineralized; however, the RFAAP culture consumed DNT nearly twice as fast as the WWTP culture. The major solvent extractable products were determined to be 6-nitroindazole, 2-nitrotoluene, 4-nitrotoluene, 4Acm2NT, and 4AcmT (Noguera and Freedman, 1997).

Anaerobic degradation

Anaerobic degradation with ethanol as the primary substrate yields the formation of 2,4-DAT from 2A4NT and 4A2NT (Cheng *et al*., 1996). Cheng and colleagues (1996) also observed that without the addition of ethanol as an energy source and/or electron donor, biotransformation of DNT does not occur. At low initial DNT concentrations, reduction of the 4-nitro group is

favorable while reduction of the 2-nitro group is favorable at high initial DNT concentrations (Cheng *et al*., 1996). Initial concentrations of DNT greater than 0.044 mmol/l inhibits its own biotransformation (Cheng *et al*., 1996).

Sequential anaerobic-aerobic degradation

Sequential treatment of xenobiotics has been studied most recently. Maloney et al. (1998) observed the results of sequential treatment of DNT in an anaerobic fluidized-bed reactor (FBR) followed by a rotating biological contactor (RBC). Build-up of DNT on the carbon from the anaerobic FBR was observed without the addition of substrate; however, no DNT was present on the carbon when cosubstrate was present (Maloney *et al*., 1998). Thus, DNT was simply being sorbed by the carbon without substrate present and degraded rather than sorbed when substrate was added. DNT was stoichiometrically converted to DAT in the presence of ethanol which decreases as the substrate level decreases. The pilot scale project did not perform as well as the bench-scale project which consisted of an anaerobic FBR followed by an activated sludge reactor. The activated sludge reactor removed all of the DAT and oxidized all ammonia present. Mineralization of DNT in this system is suspected since previous studies have suggested that DAT is mineralized under aerobic conditions (Freedman *et al*., 1996). Also, influent and effluent nitrogen levels were approximately the same further supporting mineralization. Abiotic condensation reactions were ruled out since it would be unlikely that much DNT would come in contact with DAT for any length of time in the aqueous phase using the FBR (Maloney *et al*., 1998).

It appears from this work (Maloney *et al*., 1998) that a sequential anaerobic-aerobic system is the ideal process for removing DNT from wastewater. Although the pilot-scale study did not produce the same encouraging results as the bench-scale tests, it should be noted that the RBC in the pilot study was not designed for the project; it was simply available at the time of the study. If it had been properly designed, it may have performed as well as the activated sludge reactor in mineralizing DAT.

Cometabolism

Cometabolism can be defined as the transformation of a nongrowth substrate by cells that are growing in the presence of growth substrate or by resting cells in the absence of growth substrate (Criddle, 1993). As mentioned previously, DNT is not degraded in the absence of ethanol under anoxic or anaerobic conditions. Ethanol serves as the growth substrate and DNT is then reduced to DAT which is extremely stable in the absence of oxygen. Aerobically, DNT can be degraded without ethanol present. In the competitive microbial environment, the organisms which readily degrade ethanol out-compete the others when ethanol is present and reductively cometabolize the DNT.

Cometabolism of xenobiotics has been studied especially with chlorinated compounds. Methane, propane, ammonia, and toluene (or phenol) are some of the substrates which support the cometabolism of several chlorinated aliphatic hydrocarbons (Hamamura *et al.*, 1997). In chloroform cometabolism, butane and propane are effective growth substrates (Hamamura *et al*., 1997). Trichloroethylene (TCE) can be cometabolized by several bacterial species, including those grown on ammonia, cumene, 2,4-diphenoxyacetic acid, isoprene, methane, phenol, propane, and toluene (Ely *et al*., 1997).

Cometabolism allows the biodegradation of compounds to occur that otherwise would break down very slowly in the environment. It also can reduce the residual contaminant concentration that typically would be achieved.

Sorption

Adsorption of DNT on activated carbon proves to be a feasible treatment for complete removal. Ho and Daw (1988) observed that equilibrium loadings up to 800 mg DNT/g of dry carbon were possible at influent concentrations of 100 mg/L DNT. The formation of 2,4-dinitrobenzyl alcohol, 2,4-dinitrobenzaldehyde, 2,4-dinitrobenzoic acid, and 2,4-dinitrobenzoate was also detected suggesting that oxidation was occurring after adsorption (Ho and Daw, 1988). Due to

the costs associated with activated carbon and its regeneration, this treatment has not been considered.

Photooxidation

Photooxidation is a synergistic combination of ultraviolet (UV) irradiation and an oxdizing agent, typically hydrogen peroxide or ozone. This method has been clearly illustrated in mineralization of TNT (Andrews *et al*., 1977). Ho (1986) determined from her results that the following pathways will result from photooxidation with hydrogen peroxide: 1) side-chain oxidation which converts DNT to 1,3-dinitrobenzene; 2) hydroxylation of benzene ring which 1,3-dinitrobenzene to hydroxynitrobenzene derivatives; 3) benzene ring cleavage of these hydroxynitrobenzene derivatives which produces lower molecular weight carboxylic acids and aldehydes; and, 4) further photooxidation which eventually converts the lower molecular weight acids and aldehydes to carbon dioxide, water, and nitric acid.

Supercritical water oxidation

Above its vapor-liquid critical point of 374° C and 221 bar, water is an excellent solvent for organics and becomes completely miscible with oxygen (Li *et al*., 1993). Treatment of DNT using supercritical water oxidation results in complete mineralization (Li et al., 1993). Li and colleagues (1993) determined that organic destruction efficiency of greater than 99% occurs at 450° C or higher within one minute. Hydrogen peroxide and oxygen were both considered as solvents. At 200° - 300° C, hydrogen peroxide was more effective, while oxygen served as the better oxidant from 400° - 500° C (Li *et al*., 1993). Obviously, the cost of such a system would be a major factor in considering a full-scale operation.

Sequential ozonization-biodegradation

Another sequential treatment system was considered recently by Saupe and Wiesmann consisting of a semi-batch ozonization-biodegradation. Complete removal of DNT along with 75% removal of DOC occured at a specific ozone dose of 4 g ozone/g DNT at pH 7 (Saupe and

Wiesmann, 1998). However, a continuous chemical-biological setup resulted in no benefits; the ozone levels had to be increased by a factor of 2.5 to 3.0 to achieve the same level of DNT removal as the batch system (Saupe and Wiesmann, 1998).

Oxidation using Fenton's reagent

Fenton's reagent is described as a combination of hydrogen peroxide and a ferrous salt to form hydroxyl radicals which play a major part in oxidation of organics (Mohanty and Wei, 1993). Fenton's reagent was capable of oxidizing DNT at a molar ratio of 20:1:2.5 (hydrogen peroxide:DNT:Fe^{2+}) at room temperature (Mohanty and Wei, 1993). Temperatures greater than 30° C drastically decreased the contact time needed, as did adding Fe^{3+} in addition to Fe^{2+}. Additionally, the presence of oxygen in the reaction mixture decreased the concentration of end products (Mohanty and Wei, 1993). Mineralization is implied in this system when sufficient oxygen is present.

Toxicity

Listed as a priority pollutant by the U.S. Environmental Protection Agency (Keither and Telliard, 1979), DNT is a suspected animal carcinogen. It can enter the body through inhalation of vapors or dust particles, ingestion of contaminated food or water, and absorption through the skin (Priority Toxic Poll., 1980). Although it can spontaneously decompose at elevated temperatures, DNT is relatively stable at temperatures less than 200° C (Priority Toxic Poll., 1980). EPA's web site states that animals acutely exposed to DNT by ingestion develop cyanosis and ataxia; whereas, chronic oral exposure affects the liver and kidneys, and decreases fertility of animals (1998). DNT also induces mammary tumors in mice (Dangerous Prop., 1994). Chronic inhalation exposure of DNT in humans affects the central nervous system, resulting in symptoms such as: nausea, headaches, dizziness, and insomnia (EPA web site, 1998). It is also important to note that the activity 2,6-DNT is approximately 10-fold greater than 2,4-DNT (Rickert *et al*., 1985). The $1/ED_{10}$ (measure of carcinogenic potency of a chemical) value for 2,4-DNT is 3.8 mg/kg/d (EPA web site, 1998). The LD_{50} for oral exposure in mice is 268 mg/kg and for subcutaneous exposure in mammals is 50 mg/kg (Dangerous Prop, 1984).

Information about the toxicity of DNT byproducts is very limited. 2-Amino-4-nitrotoluene is an experimental carcinogen and mutagen, and diaminotoluene is a known animal carcinogen (National Cancer Institute, 1978; Crabtree *et al.*, 1991). No known toxicity studies have been completed for 4-amino-2-nitrotoluene.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). (1989) *Toxicological Profile for 2,4-Dinitrotoluene and 2,6-Dinitrotoluene*. U.S. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA.

Andrews, C. C., Klausmeier, R. E. and Osmon, J. L. (1977) The effect of UV light on TNT and other explosives in aqueous solution. WOEC/C 77-32, AD-A036132, Naval Weapons Support Center, Crane, IN.

Bruhn, C., Lenke, H. and Knackmuss, H.-J. (1987) Nitrosubstituted aromatic compounds as nitrogen source for bacteria. Applied and Environmental Microbiology **53**, 208-210.

Cheng, J., Kanjo, Y., Suidan, M. T. and Venosa, A. D. (1996) Anaerobic biotransformation of 2,4-dinitrotoluene with ethanol as primary substrate: Mutual effect of the substrates on their biotransformation. *Water Research* **30** (2), 307-314.

Crabtree, H. C., Hart, D., Thomas, M., Witham, B., McKenzie, I. And Smith, C. (1991) Carcinogenic ranking of nitroaromatic amines and nitro compounds. *Mutation Research* **264**, 155-162.

Criddle, C. S. (1993) The kinetic of cometabolism. *Biotechnology and Bioengineering* **41**, 1057-1065.

Dangerous Properties of Industrial and Consumer Chemicals. (1994) N. P. Cheremisinoff, J. A. King and R. Boyko (eds.) p. 305-306, Marcel Dekker, Inc., New York.

Dangerous Properties of Industrial Materials, 6th ed. (1984) N. I. Sax (ed.) p. 1220, VanNostrand Reinhold Company, New York.

Ely, R. L., Williamson, K. J., Hyman, M. R. and Arp, D. J. (1997) Cometabolism of chlorinated solvents by nitrifying bacteria: Kinetics, substrate interactions, toxicity effects, and bacterial response. *Biotechnology and Bioengineering* **54**(6), 520-534.

Environmental Protection Agency. (1998) http://www.epa.gov/ttnuatw1/hlthef/dini-lue.html.

Freedman, D. L., Shanley, R. S. and Scholze, R. J. (1996) Aerobic biodegradation of 2,4-dinitrotoluene, aminonitrotoluene isomers, and 2,4-diaminotoluene. *Journal of Hazardous Materials* **49**, 1-14.

Hamamura, N., Page, C., Long, T., Semprini, L. and Arp, D. J. (1997) Chloroform cometabolism by butane-grown CF8, *Pseudomonas butanovora*, and *Mycobacterium vaccae* JOB5 and methane-grown *Methylosinus trichosporium* OB3b. *Applied and Environmental Microbiology* **63** (9), 3607-3613.

Ho, P. C. (1986) Photooxidation of 2,4-dinitrotoluene in aqueous solution in the presence of hydrogen peroxide. *Environmental Science and Technology* **20** (3), 260-267.

Ho, P. C. and Daw, C. S. (1988) Adsorption and desorption on activated carbon. *Environmental Science and Technology* **22** (8), 919-924.

Keither, L. H. and Telliard, W. A. (1979) Priority pollutants. I. A perspective view. *Environmental Science and Technology* **13**, 416-423.

Lendenmann, U., Spain, J. C. and Smets, B. F. (1998) Simultaneous biodegradation of 2,4-dinitrotoluene and 2,4-dinitrotoluene in an aerobic fluidized-bed reactor. *Environmental Science and Technology* **32** (1), 82-87.

Li, L., Glonya, E. F. and Sawicki, J. E. (1993) Treatability of DNT process wastewater by supercritical water oxidation. *Water Environment Research* **65** (3), 250-257.

Maloney, S. W., Engbert, E. G., Suidan, M. T. and Hickey, R. F. (1998) Anaerobic fluidized-bed treatment of propellant wastewater. *Water Environment Research* **70** (1), 52-59.

McCormick, N. G., Cornell, J. H. and Kaplan, A. M. (1978) Identification of biotransformation products from 2,4-dinitrotoluene. *Applied and Environmental Microbiology* **35** (5), 945-948.

McCormick, N. G., Feeherry, F. E. and Levinson, H. S. (1976) Microbial transformation of 2,4,6-trinitrotoluene and nitroaromatic compounds. *Applied and Environmental Microbiology* **31**, 949-958.

Mohanty, N. R. and Wei, I. W. (1993) Oxidation of 2,4-dinitrotoluene using Fenton's reagent: reaction mechanisms and their practical applications. *Hazardous Waste and Hazardous Materials* **10** (2), 171-183.

National Cancer Institute. (1978) National Cancer Institute, Bioassay of 5-Nitro-*o*-toluidine for Possible Carcinogenicity. National Cancer Institute of Health Carcinogenesis Technical Report Series No. 107, DHEW Publ. N (NIH), Bethesda, MD, 78-1357.

Nishino, S. F. and Spain, J. C. (1993) Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Applied and Environmental Microbiology* **59**, 2520-2525.

Noguera, D. R. and Freedman, D. L. (1996) Reduction and acetylation of 2,4-dinitrotoluene by a *Pseudomonas aeruginosa* strain. *Applied and Environmental Microbiology* **62** (7), 2257-2263.

Noguera, D. R. and Freedman, D. L. (1997) Characterization of products from the biotransformation of 2,4-dinitrotoluene by denitrifying enrichment cultures. *Water Environment Research* **69** (3), 260-268.

Priority Toxic Pollutants: Health Impacts and Allowable Limits. (1980) M. Sittig (ed.) Noyes Data Corporation, Park Ridge, New Jersey.

Reiger, P.-G. and Knackmuss, H.-J. (1995) Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds in contaminated soil. P. 1-18. *In* J. C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*. Plenum Press, New York.

Rickert, D. E., Chism, J. P. and Kedderis, G. L. (1985) Metabolism and carcinogenicity of nitrotoluenes, p. 563-571. *In* J. J. Kocsis, D. J. Jollow, C. M. Witmer, J. O. Nelson and R. Snyder (eds.), *Biological Reactive Intermediates III: Mechanisms of Action in Animal Models and Human Disease*, Vol 197. Plenum Press, New York.

Saupe, A. and Wiesmann, U. (1998) Ozonization of 2,4-dinitrotoluene and 4-nitroaniline as well as improved dissolved organic carbon removal by sequential ozonization-biodegradation. *Water Environment Research* **70** (2), 146-154.

Spain, J. C. (1995) Bacterial degradation of nitroaromatic compounds under aerobic conditions, p. 19-35. *In* J. C. Spain (ed.), *Biodegradation of Nitroaromatic Compounds*. Plenum Press, New York.

Spanggord, R. J., Spain, J. C., Nishino, S. F. and Mortelmans, K. E. (1991) Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. *Applied and Environmental Microbiology* **57** (11), 3200-3205.

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

AEROBIC BIOLOGICAL TREATMENT OF 2,4-DINITROTOLUENE IN MUNITIONS PLANT WASTEWATER

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Abstract — Wastewater from the manufacture of propellants typically contains 2,4-dinitrotoluene (DNT), a suspected animal carcinogen. Previous studies have indicated that DNT is aerobically biodegradable. However, inconsistent removal of DNT during aerobic treatment has been observed at a munitions wastewater treatment plant, necessitating the use of activated carbon pre-treatment. The objective of this study was to evaluate the effect of nutrient and cosubstrate amendments on the rate and extent of DNT removal. Addition of ethanol (100-500 mg/l) and phosphate (0.8-3.3 mg/l) significantly accelerated the rate of aerobic DNT (0.3-5.6 mg/l) biodegradation. Addition of phosphate alone also increased the rate of DNT degradation, but to a lesser degree. The presence of ethyl ether, another substrate commonly found in munitions plant wastewater, had comparatively little effect on the rate of DNT removal. Interruptions in the DNT manufacturing process can result in DNT being absent from the munitions plant wastewater for extended periods. The effect of such interruptions was evaluated in semi-continuously operated reactors, fed daily with phosphate-amended wastewater, at a hydraulic residence time of 3 days. DNT removal resumed without a lag even after it was absent from the feed for periods up to 15 days. During aerobic biodegradation of DNT, reduction to 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene was consistently observed, with reduction at the *para* position predominating. The highest level of aminonitrotoluene formation was 23% of the total DNT degraded. Aminonitrotoluene isomers were consumed shortly after they formed in the semi-continuously operated reactors, confirming the potential for degradation of these metabolites. Although the aminonitrotoluene isomers are not currently regulated, their presence in treated munitions wastewater is a concern due to possible toxicity.

Key Words – dinitrotoluene, munitions wastewater, aerobic biodegradation, 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, phosphorus

INTRODUCTION

The manufacture of energetic organic compounds results in generation of wastewater containing a number of hazardous compounds, including 2,4-dinitrotoluene (DNT). DNT is a major component in the production of propellants, although its most widespread industrial use is in production of toluene diisocyanate and methylene diphenyldiisocyanate. DNT is a suspected animal carcinogen and is one of several aromatic compounds on the U.S. Environmental Protection Agency's Draft Drinking Water Contaminant Candidate List, for possible regulation under the Safe Drinking Water Act (Pontius, 1997). Removal of DNT from manufacturing wastewater is therefore a significant environmental concern.

Previous laboratory studies have demonstrated that DNT is biodegradable through oxidative and reductive pathways (Kaplan, 1996). In oxidative reactions, molecular oxygen is required along with oxygenase or peroxidase enzymes that initiate ring cleavage. The reductive pathway results in formation of metabolites such as 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,4-diaminotoluene (DAT), azoxytoluene isomers, and 4-acetamido-2-nitrotoluene (McCormick *et al*., 1974 & 1978; Noguera and Freedman, 1996). Formation of these metabolites depends, in part, on redox conditions. Aminonitrotoluene (ANT) isomers appear under aerobic, anoxic, and anaerobic conditions. Azoxy compounds are formed only in aerobic systems, while stoichiometric reduction of DNT to DAT has been shown only under methanogenic conditions (Berchtold *et al.*, 1995). 2A4NT is an experimental carcinogen and mutagen and DAT is a known animal carcinogen (National Cancer Institute, 1978; Crabtree *et al*., 1991). The presence of these metabolites may therefore effect the toxicity of biologically treated wastewater.

The use of DNT as a growth substrate under aerobic conditions has been demonstrated with a *Pseudomonas* sp., and a pathway leading to mineralization has been proposed (Spanggord *et al*., 1991). Stoichiometric release of nitrite was observed when ammonium was provided as

the nitrogen source for cell synthesis. Using a mixed culture derived from a wastewater treatment plant at a munitions facility, Freedman *et al.* (1996) also observed aerobic biodegradation of DNT and stoichiometric release of nitrite when it was provided as the sole organic substrate. However, munitions wastewater typically contains a mixture of organic contaminants, including ethanol, ether, and acetone, which are present at much higher levels than DNT. When DNT was biodegraded in the presence of ethanol, Freedman *et al.* (1996) showed that more than half of the energetic compound was reduced to an ANT isomer prior to ring fission. This confirmed the importance of DNT reduction during aerobic biodegradation of DNT. Ether had an inhibitory effect on DNT biodegradation, while ethanol accelerated it.

In spite of promising laboratory results, inconsistent performance with DNT removal has been observed at a munitions wastewater treatment plant, which was the subject of this research. This industrial treatment plant must meet a DNT discharge limit of 113 µg/l. The majority of their by-product DNT is currently removed by passing concentrated wastewater (from tanks referred to as "water drys") through an activated carbon column. However, some DNT still makes its way to the biological treatment plant due to break-through from the carbon column and leaks from various processes. The first unit operation at the treatment plant is an aerated equalization (EQ) basin, followed by rotating biological contactors and secondary clarifiers. Most of the DNT is removed in the EQ basins, although DNT levels above 113 µg/l have been recorded in the treatment plant effluent.

The objective of this study was to evaluate the effect of nutrient and cosubstrate amendments on the rate and extent of DNT removal in the munitions plant wastewater. The influent typically contains very high levels of organics such as ethanol and ether, significant amounts of nitrate, and low levels of DNT. In batch-fed laboratory reactors, addition of phosphate along with ethanol significantly improved the rate of DNT removal. When the reactors were operated semi-continuously at a 3 d retention time, withholding DNT from the

influent for more than two weeks did not result in a lag in DNT removal when it was added back to the feed.

MATERIALS AND METHODS

Chemicals and Analytical Methods

All reagents were obtained at the highest purity commercially available. Methanol, DNT, 2A4NT, and 4A2NT were purchased from Aldrich; ethanol, ethyl ether, acetone, potassium phosphate, and sodium nitrate were purchased from Fisher Scientific.

Measurement of anion concentrations (nitrite, nitrate, phosphate, and sulfate) was performed on a Dionex 2010i Ion Chromatograph with an AS4A-CS column (4 mm i.d.) or a Dionex DX-120 with an AS9-SC column (4 mm i.d.). Both instruments use a conductivity detector with 2 ml/min of 1.7 mM $NaHCO_3$ and 1.8 mM Na_2CO_3 eluent. Chemical oxygen demand (COD) was measured according to Standard Method 5220 (Amer. Pub. Health Assoc., 1995). Ammonia-nitrogen (NH_3-N) was analyzed using methods 4500-NH3 B and C (Amer. Pub. Health Assoc., 1995). Volatile suspended solids (VSS) were determined using methods 2540 D and E (Amer. Pub. Health Assoc., 1995).

DNT, ANT isomers, and DAT were analyzed on a high performance liquid chromatograph (Hewlett Packard 1090) with a diode array detector set at 246 nm. The mobile phase consisted of 50% water and 50% methanol (v/v) at 1.5 ml/min. Samples (250 µl) were injected on an Alltech RP-18 Aquapor guard column (7 µm) followed by an Alltech Econosphere C-18 column (250 mm x 4.6 mm i.d., 5 µm) held at 40° C. Samples and standards were prepared by mixing 2 ml with 2 ml of methanol and filtering through a Whatman 0.45 µm PTFE filter.

Due to matrix interferences in the wastewater, DAT did not consistently elute from the liquid chromatograph, making its identification and quantification unreliable. Data for DAT are

therefore not reported. This was not considered a significant problem, however, since no previous studies have shown DAT formation under aerobic conditions.

Retention times for DNT, 2A4NT and 4A2NT were 5.0, 3.5, and 3.3 min, respectively. After installing a new detector, the retention times for DNT, 2A4NT and 4A2NT changed to 4.3, 3.3, and 3.1 min, respectively. At higher concentrations, the peaks for the ANT isomers overlapped somewhat, occasionally making it necessary to manually integrate these responses.

Bioreactors and Experimental Design

Six batch reactors were set up to simulate the EQ basins at the munitions plant. Each consisted of a 500 ml Wheaton glass serum bottle aerated with an aquarium air pump. Tubing from the pump was connected to a glass Pasteur pipette; no plastic parts were in contact with the wastewater, in order to decrease the potential for sorption. The reactors were covered with aluminum foil to prevent photodegradation. Wastewater was collected from the outlet end of the EQ basins at the plant and 500 ml was put into each reactor.

During this study, samples of the plant wastewater taken from the EQ basins contained 0.062 – 0.24 mg/l of DNT. In order to simulate likely conditions in the basins if no pretreatment of DNT was applied, the wastewater was spiked with DNT just prior to starting the reactors, to an initial concentration of 0.5 – 5.5 mg/l. This was done using DNT-saturated wastewater, which was prepared by slowly heating raw wastewater with solid DNT until the DNT had dissolved. Nitrate (as KNO_3), phosphate (as Na_3PO_4), ethanol, and/or ethyl ether were added at the same time, depending on the treatment.

For the experiments conducted with ethyl ether, it was necessary to seal the reactors with septa to prevent rapid losses due to volatilization. The wastewater was purged with air before sealing and sufficient head space was provided to keep the system aerobic, even at the highest COD. Mixing was achieved on a shaker table. To minimize diffusive losses of ether through the

septa, the bottles were stored on the shaker table in an inverted position, so that the liquid remained in contact with the septa.

In order to test the effect of intermittent DNT loading on its removal efficiency, five reactors (open to the atmosphere) were set-up and operated in a semi-continuous batch mode, at a 3 d hydraulic residence time. This is approximately the residence time in the EQ basins. One third of the reactor volume was replaced daily with EQ basin wastewater that was collected on day 0 and day 17 of the experiment. The wastewater was stored at 4 °C between feedings. Phosphate was added daily (0.8 mg/l PO_4-P) along with the replacement wastewater. Addition of DNT ranged from once every 3 days to once every 15 days, over a period of 32 days.

RESULTS

Biotic Versus Abiotic DNT Removal

An experiment was performed to assess the role of biotic and abiotic processes in disappearance of DNT from the munitions plant wastewater. One set of duplicate reactors was set up with wastewater, another set received wastewater plus 1 g/l sodium azide to inhibit biological activity, and a third set contained only tap water (Fig. 1). The reactors with wastewater showed a significant decrease in DNT. Based on an initial VSS level of 65 mg/l, the highest specific rate of degradation in these reactors was 0.012 mg DNT mg VSS^{-1} d^{-1}. Addition of sodium azide inhibited DNT removal to less than 10% of the initial amount added. The two reactors with only tap water also showed less than a 10% decrease in DNT, confirming that volatilization and sorption were insignificant factors in disappearance of DNT from the aqueous phase. The duplicates within each treatment behaved very similarly.

Effects of Nitrate, Phosphate, and Ethanol

The effects of varying amounts of nitrate, phosphate and ethanol on the rate of DNT biodegradation are shown in Figure 2. Ethanol alone (reactor E) and nitrate alone (reactor D) made no significant difference in the degradation rate compared to wastewater alone (reactor A). The highest specific rate of degradation in these reactors was approximately 0.017 mg DNT mg VSS^{-1} d^{-1}, similar to the duplicate wastewater-only reactors shown in Figure 1. Addition of phosphate (reactors B and C) significantly increased the rate of DNT disappearance, to as high as 0.046 mg DNT mg VSS^{-1} d^{-1}. The highest rate of degradation (0.086 mg DNT mg VSS^{-1} d^{-1}) occurred in wastewater amended with ethanol, phosphate, and nitrate (reactor F).

Analysis of final nitrogen levels in the six reactors (Table 1) indicated that a residual amount was present in all cases except for reactor F, which still managed to achieve the highest degree of COD removal and the fastest rate of DNT biodegradation. This suggested that nitrogen was not a limiting nutrient. For this reason, nitrate was not added to the wastewater in all subsequent experiments, even with high ethanol or ether additions. Residual phosphate was present in the three reactors to which it was added, while none was detected in the wastewater.

The next experiment further evaluated the role of phosphate and ethanol in enhancing DNT biodegradation. Reactors were amended with 0.8, 1.6, or 3.3 mg/l of PO_4-P and 100 or 500 mg/l of ethanol. As shown in Figure 3, all of the treatments consumed DNT faster than the unamended control (reactor A), which exhibited a maximum rate of 0.011 mg DNT mg VSS^{-1} d^{-1}. Addition of 100 mg/l of ethanol increased the maximum specific DNT removal rate approximately three fold (reactors B, C, and F). The highest specific rate of DNT removal occurred in Reactor E (0.080 mg VSS^{-1} d^{-1}), which was amended with 500 mg/l of ethanol and 1.6 mg/l of phosphate. COD removal was close to or above 90% in all of the amended reactors, with slightly higher removal percentages occurring in the reactors with the fastest DNT removal

rates (D and E). Residual amounts of nitrogen were present in all of the reactors, although residual phosphate was present only when 1.6 or 3.3 mg/l was added (Table 2).

Sulfate was monitored during both of the experiments described above. Levels ranged from 102 – 554 mg/l and remained unchanged throughout the incubation period.

Effect of Ether on DNT Biodegradation

Since ether is a potentially significant source of COD in the munitions plant wastewater, its effect on DNT degradation was also assessed. Experiments were conducted in sealed reactors to prevent volatilization losses, with a surplus of oxygen in the headspace to satisfy the total COD. Phosphate (0.8 mg/l of PO_4-P) was added to each reactor. In all of them, DNT degraded faster than in previous tests (i.e., in 3 days versus approximately 7), most likely due to the comparatively high initial VSS level of 118 mg/l (Fig. 4). Taking the initial VSS into account, the highest specific rate of DNT removal in the control (reactor A, 0.8 mg/l of PO_4-P added) was 0.010 mg DNT mg VSS^{-1} d^{-1}, similar to what was observed in the unamended controls shown in Figures 1-3. Slightly higher specific rates occurred with low amounts of ether (25-100 mg/l), and slightly lower rates with higher ether levels (250-500 mg/l). Thus, unlike ethanol, the presence of ether did not significantly alter the specific rate of DNT degradation.

In the reactors amended with ethyl ether, COD removal ranged from 51-87%. Since the majority of the initial COD consisted of ether (rather than other organics in the wastewater or the added DNT), COD removal is representative of the amount of ether that was oxidized. Thus, the rate of DNT removal did not increase significantly even though more than half of the cosubstrate was mineralized.

Effects of Intermittent Loading of DNT

Because DNT is not consistently present in the plant's wastewater, the effect of intermittent loadings on its removal efficiency was examined using varying intervals of DNT addition. All five reactors were wasted and fed wastewater (supplemented with 0.8 mg/l of PO_4^{3-}P) on a daily basis, at a 3 d hydraulic residence time. DNT was added to the first reactor once every 3 days. The second reactor received DNT every 6 days, the third every 9 days, the fourth every 12 days, and the fifth every 15 days.

As shown in Figure 5, DNT was completely removed within two days after adding it, regardless of the time between DNT additions. Since most of the DNT degradation typically occurred within 1 day of adding it, relatively little was removed during wasting (shown as vertical lines in Fig. 5). Even in the reactor that only received DNT once every 15 days, the rate of disappearance was comparable to DNT removal in the reactor that received DNT once every three days. Thus, long gaps between additions of DNT did not effect the rate of DNT consumption, at least when the wastewater was supplemented with 0.8 mg/l of phosphate. In reactor A, which was fed DNT most often, the highest specific rate of DNT removal was approximately 0.007 mg DNT mg VSS^{-1} d^{-1} (based on an initial VSS level of 205 mg/l), similar to the unamended controls shown in Figures 1-3 and the phosphate-only control reactor in Figure 4.

4A2NT and 2A4NT Formation

In all of the experiments conducted, disappearance of DNT was accompanied by an accumulation of ANT isomers. The highest percentages observed in live bottles ranged from 4.8-23%, expressed as moles of 2A4NT + 4A2NT per mole of initial DNT consumed (Table 3). In general, the faster the rate at which DNT disappeared in a given experiment, the higher the level of ANT isomers formed. For example, in Figure 2, DNT consumption was fastest in

reactor F, in which the ANT level peaked two to three times above ANT in the other reactors. In Figure 3, DNT consumption was fastest in reactors D and E, which also yielded the highest peak ANT levels.

Of the two ANT isomers, 4A2NT was always present at a higher concentration than 2A4NT, and it was often the only isomer observed. During one of the experiments (Fig. 1), a low level of 4A2NT (0.21 mg/l) was measured in the munitions plant wastewater, while 2A4NT was not observed. When 2A4NT did appear during DNT degradation, it never represented more than 42% of the total ANT.

It is of interest to note that the highest percentage of ANT formation occurred in the azide control reactors (Fig. 1), although only a small amount of DNT was transformed. Azide inhibits the activity of cytochromes in membrane-bound electron transfer. These results suggest that electron carriers other than cytochromes are involved in reduction of DNT, and that reduction was able to proceed to some extent even in the absence of terminal electron transport.

For the results shown in Figures 1-4, the ANTs that formed during DNT degradation were still present at the termination of the experiment, although at levels below their peaks. The presence of ANTs was consistent with the yellowish tint that developed in the wastewater as DNT degraded, since 2A4NT and 4A2NT are both highly colored. Persistence of 4A2NT or 2A4NT in the wastewater represents a concern, even though neither is currently regulated. During the intermittent loading experiment shown in Figure 5, monitoring of the reactor effluent did continue after the DNT was consumed. In all of these reactors, once the DNT disappeared, the ANT isomers subsequently declined below the detectable limit. Consistent with this was a lack of yellow coloration in the treated wastewater. This suggests that degradation of 4A2NT and 2A4NT can be expected with sufficient residence time.

DISCUSSION

The results of this study demonstrated that aerobic biodegradation of DNT was significantly enhanced by the addition of phosphate and ethanol to munitions plant wastewater. Addition of phosphate alone also improved DNT removal, but to a lesser degree. Differences among experiments in the time required to consume DNT were mainly related to differences in initial VSS concentrations. When normalized for this factor, maximum specific removal rates were consistently close to 0.011 mg DNT mg VSS^{-1} d^{-1} in unamended wastewater. DNT removal rates were nearly four times higher with the addition of 1.6-3.3 mg PO_4^{3-}—P/l, and nearly eight times higher in wastewater amended with phosphate and ethanol.

The mechanism by which phosphate enhanced DNT removal is not yet known. Given the nearly complete lack of phosphate in the munitions plant wastewater, adding this essential nutrient most likely stimulated microbial activity, including the organisms responsible for DNT degradation. However, addition of phosphate alone did not significantly improve COD removal efficiency over the unamended control (reactors B and C versus A, Table 1), even though DNT removal rates were higher in the phosphate-amended reactors.

Only one organism has been identified so far that can use DNT as a growth substrate. Spanggord *et al*. (1991) isolated a *Pseudomonas* species using DNT as a sole organic substrate and a basal salts medium containing an excess of phosphate and ammonium. This isolate apparently uses a dioxygenase attack on the aromatic ring, forming 4-methyl-5-nitrocatechol prior to fission and stoichiometric release of 2 mol NO_2^- per mol DNT.

The results of this study demonstrated that direct oxidation is not the only catabolic pathway involved in DNT degradation in the munitions plant treatment system. Formation of 4A2NT and 2A4NT indicates that reduction is also an important pathway. 4A2NT was the predominant isomer formed, consistent with other studies that have noted a preference for reduction at the *para* position (McCormick *et al*., 1978; Noguera and Freedman, 1996 and 1997).

Reduction presumably precedes ring cleavage or some other transformation of the ANT isomers, since both were consumed during operation of the reactors in a semi-continuous mode (Fig. 4). Accumulation of ammonium in the reactors (Tables 1 and 2) may be a consequence of deamination of the ANTs, since no ammonium was present in the wastewater. Assuming complete reduction of 5 mg/l of DNT to ANTs, a maximum of 0.38 mg/l of NH_4^+-N would form. This is consistent with final results from most of the reactors, although higher amounts of ammonium did accumulate in several cases. The source of this ammonium is not yet known.

Additional work is needed to confirm the fate of 4A2NT and 2A4NT. While neither is currently regulated, their presence in the wastewater effluent raises concerns over potential toxicity. In addition, the formation of other metabolites is possible under aerobic conditions, including 4-acetamido-2-nitrotoluene, 4,4'-dinitro-2,2'-azoxytoluene, and 2,2'-dinitro-4,4'-azoxytoluene (McCormick *et al*., 1978). The presence of these compounds in treated munitions wastewater should also be assessed.

The factors that influence the rate and extent of DNT reduction under aerobic conditions are not well established, although the presence of readily degradable organic substrates appears to play a role. Within each experiment of this study, the extent of DNT reduction to ANTs increased when increasing amounts of ethanol were added, as long as enough phosphate was also present (Fig. 2, reactor F, and Fig. 3, reactors D and E). Accumulation of ANTs also correlated with increases in the maximum specific rates of DNT removal. DNT accounted for a relatively small fraction of the initial COD, ranging from 3% in unamended wastewater to only 0.5% when 500 mg/l of ethanol was added. Thus, the majority of electron flow in the reactors involved substrates other than DNT, and the amount of electron equivalents needed for DNT reduction to ANTs was comparatively insignificant.

Freedman *et al*. (1996) also observed DNT reduction in an enrichment culture amended with ethanol as a cosubstrate and ammonium as a nitrogen source for cell synthesis. Based on

the stoichiometry of nitrite release, they estimated that as much as 82% of the DNT was reduced to ANT prior to oxidation. In this study, direct measurement of 4A2NT and 2A4NT confirmed their formation, with the highest level reaching 23% of the total DNT consumed in live bottles (Table 3). Higher amounts may have formed but transformation of the ANTs probably limited their accumulation. Since ammonium was not added to the wastewater, consumption of nitrite and nitrate for cell synthesis prevented use of the inorganic nitrogen data for estimation of how much DNT was reduced to ANTs. As expected, nitrogen consumption was highest in the reactors amended with the highest ethanol levels (Table 1, reactors E and F; Table 2, reactors D and E).

Unlike ethanol, the presence of ethyl ether as a cosubstrate had little effect on the specific rate of DNT removal. This experiment was conducted under the most conservative conditions, i.e., volatilization of the ether was prevented by using sealed reactors. In the aerated EQ basins at the munitions plant, a considerable amount of volatilization would be expected. COD consumption in the reactors indicated that 50-87% of the ether was oxidized. Thus, as with ethanol, there was an abundance of electron flow associated with cosubstrate oxidation. This did increase the percent formation of ANTs somewhat (Table 3, reactors B-F versus A), but did not significantly alter the DNT removal rates. A better understanding of DNT reduction in mixed cultures with multiple substrates is needed to explain why ethanol oxidation increases rates, while ether does not.

The intermittent loading experiment (Fig. 5) came closest to simulating what might be expected in the munitions plant EQ basins, since the reactors were operated in a semi-continuous mode. Consistently good removal of DNT occurred, in spite of extended periods when none was present in the wastewater. Results from the other experiments suggest that the addition of phosphorus was critical to efficient DNT removal, although a companion study with no phosphate added is still needed to confirm this. The concentrations of DNT consumed suggest

that pretreatment of the wastewater to reduce DNT levels with activated carbon are not necessary to meet the discharge limit of 113 µg/l.

Another approach to removing DNT from munitions plant wastewater is sequential anaerobic/aerobic treatment (VanderLoop *et al*., 1994; Berchtold *et al*., 1995; Maloney *et al*., 1998). In this process, concentrated wastewater from the "water drys" operation is fed to an anaerobic fluidized-bed granular activated carbon reactor, where catabolism of ethanol and ether drives the stoichiometric reduction of DNT to DAT. One of the principal reasons for converting DNT to DAT is a presumption that DAT is more readily degradable under aerobic conditions. Berchtold *et al*. (1995) reported mineralization of DAT in a laboratory activated sludge reactor (based on a nitrogen balance, not $^{14}CO_2$ formation), although they did not conduct a similar test with DNT. Freedman *et al*. (1996) compared the relative biodegradability of DNT, DAT, 4A2NT, and 2A4NT in enrichment cultures developed with inoculum from a munitions plant. Removal of DNT (along with ethanol as a cosubstrate) was more difficult to establish than for DAT, 4A2NT, and 2A4NT, but once the culture was acclimated, DNT removal progressed as readily as with the other compounds.

At this point, it remains questionable whether or not DAT is more readily degradable than DNT under aerobic conditions. Maloney *et al*. (1998) reported inconsistent removal of DAT when effluent from their anaerobic fluidized bed reactor was fed to a rotating biological contactor, in contrast to their previous laboratory studies (Berchtold *et al*., 1995). The critical litmus test for any DNT treatment process is the extent to which effluent toxicity is reduced, not just the ability to meet a discharge permit limit for DNT. This will most likely require removal of DNT metabolites. The results of this study suggest that consistent removal of DNT, 4A2NT, and 2A4NT may be achievable by direct aerobic treatment, as long as sufficient phosphate is available to enhance degradation. Additional testing is needed to confirm the role of phosphate and the fate of all possible DNT metabolites.

CONCLUSION

Addition of ethanol (100-500 mg/l) and phosphate (0.8-3.3 mg/l) to munitions plant wastewater significantly accelerated the rate of aerobic DNT (0.3-5.6 mg/l) biodegradation. Addition of phosphate alone also increased the rate of DNT degradation, but to a lesser degree. Background levels of nitrate were sufficient to provide enough nitrogen, even at the highest COD levels tested. The presence of ethyl ether, another substrate commonly found in munitions plant wastewater, had comparatively little effect on the rate of DNT removal.

Interruptions in the propellant manufacturing process can result in DNT being absent from the munitions plant wastewater for extended periods. The effect of such interruptions on the performance of DNT removal was evaluated in semi-continuously operated reactors, fed daily with phosphate-amended wastewater, at a hydraulic residence time of 3 days. DNT removal resumed without delay even after it was absent from the feed for periods up to 15 days.

During aerobic biodegradation of DNT, reduction to 4A2NT and 2A4NT was consistently observed, with 4A2NT being the predominant isomer. The highest level of ANT formation was 23% of the total DNT consumed. In general, the faster the rate of DNT degradation (related to the presence of ethanol and phosphate, as well as initial VSS levels), the higher the amount of ANT that accumulated. In batch degradation experiments, ANT isomers declined but residual amounts persisted after the disappearance of DNT. However, in the semi-continuously operated reactors used to test the effect of intermittent DNT loadings, ANT isomers were consumed shortly after they formed, confirming the potential for degradation of these metabolites on a consistent basis. Although 4A2NT and 2A4NT are not currently regulated, their presence along with other possible metabolites in treated munitions wastewater is a concern due to potential toxicity.

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REFERENCES

American Public Health Association, American Water Works Association and Water Environment Federation. (1995) *Standard Methods for the Examination of Water and Wastewater*, 19th ed. A. D. Eaton, L. S. Clesceri and A. E. Greensberg (eds.) American Public Health Association, Washington, D.C.

Berchtold, S. R., VanderLoop, S. L., Suidan, M. T. and Maloney, S. W. (1995) Treatment of 2,4-dinitrotoluene using a two-stage system: Fluidized-bed anaerobic granular activated carbon reactors and aerobic activated sludge reactors. *Water Environment Research* **67**, 1081-1091.

Crabtree, H. C., Hart, D., Thomas, M., Witham, B., McKenzie, I. And Smith, C. (1991) Carcinogenic ranking of nitroaromatic amines and nitro compounds. *Mutation Research* **264**, 155-162.

Freedman, D. L., Shanley, R. S. and Scholze, R. J. (1996) Aerobic biodegradation of 2,4-dinitrotoluene, aminonitrotoluene isomers, and 2,4-diaminotoluene. *Journal of Hazardous Materials* **49** (1), 1-14.

Kaplan, D. L. (1996) Biotechnology and bioremediation for organic energetic compounds, p. 373-416 in P. Marinkas (ed.), *Organic Energetic Compounds*, Nova Science Publishers, Inc., New York.

Maloney, S. W., Engbert, E. W., Suidan, M. T. and Hickey, R. F. (1998) Anaerobic fluidized-bed treatment of propellant wastewater. *Water Environment Research* **70** (1), 52-59.

McCormick, N. G., Cornell, J. H. and Kaplan, A. M. (1978) Identification of biotransformation products from 2,4-dinitrotoluene. *Applied and Environmental Microbiology* **35** (5), 945-948.

McCormick, N. G., Feeherry, F. E. and Levinson, H. S. (1974) Microbial transformation of 2,4,6-trinitrotoluene and nitroaromatic compounds. *Applied and Environmental Microbiology* **31**, 949-958.

National Cancer Institute. (1978) National Cancer Institute, Bioassay of 5-Nitro-*o*-toluidine for Possible Carcinogenicity. National Cancer Institute of Health Carcinogenesis Technical Report Series No. 107, DHEW Publ. N (NIH), Bethesda, MD, 78-1357.

Noguera, D. R. and Freedman, D. L. (1997) Characterization of products from biotransformation of 2,4-dinitrotoluene by denitrifying enrichment cultures. *Water Environment Research* **69** (3), 260-268.

Noguera, D. R. and Freedman, D. L. (1996) Reduction and acetylation of 2,4-dinitrotoluene by a *Pseudomonas aeruginosa* strain. *Applied and Environmental Microbiology* **62** (7), 2257-2263.

Pontius, N. L. (1997) SDWA mandates drinking water contaminant candidate list. *Opflow* **23** (9), 8-9.

Spanggord, R. J., Spain, J. C., Nishino, S. F. and Mortelmans, K. E. (1991) Biodegradation of 2,4-dinitrotoluene by a *Pseudomonas* sp. *Applied and Environmental Microbiology* **57** (11), 3200-3205.

VanderLoop, S. L., Suidan, M. T., Moteleb, M. A. and Maloney, S. W. (1994) Biodegradation of 2,4-dinitrotoluene in a two stage system, pp. 710-717 in *Proceedings of the 1994 National Conference on Environmental Engineering*, American Society of Civil Engineers, New York.

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