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BIODEGRADATION OF ENERGETIC COMPOUNDS: APPLICATION TO SITE RESTORATION

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SUMMARY

A multidisciplinary approach involving chemistry, microbiology, ecotoxicology and bioengineering has been undertaken in a joint effort involving the Defence Research Establishment, Valcartier of National Defence Canada (DREV/DND) and the Biotechnology Research Institute of the National Research Council of Canada (BRI/NRC). The aim of this joint collaboration is to study the bioremediation of soils contaminated with energetic compounds such as RDX, TNT, NC and GAP. Contaminated sites were sampled and analyzed for the presence of TNT, RDX and NC. Modified EPA SW 846 Method 8330 was used for the determination of RDX and TNT. As expected, the method was found accurate (≥ 90 % recovery), precise (relative deviation standard ≤ 2 %) and sensitive (detection limit ≤ 0.5 mg/Kg) over a range of concentration from 0.5 to 20,000 mg/Kg of soil dry weight. Labelled -^{14}C energetic compounds were synthesized to monitor their biodegradation. Contaminated soils were screened for microorganism having the ability to mineralize energetic compounds. Bacteria were isolated from RDX contaminated soils based on their ability to use RDX as the sole source of nitrogen under aerobic conditions when amended with a carbon source. Using ^{14}C- labelled RDX, the ability of these isolates to mineralize RDX in liquid medium was verified. Laboratory-scale studies using the isolates to bioremediate RDX contaminated soils indicate that bioaugmentation enhances the rate and extent of RDX biodegradation. TNT contaminated soils exhibited concentration dependent ^{14}C-TNT mineralization activity. GAP is a relatively new energetic compound and might not yet be found as a soil contaminant. However, a soil sampled on a burning range showed some ^{14}C-GAP mineralization activity. NC mineralization studies are planned in the near future.

INTRODUCTION

Contamination of soils and water by energetic organonitro compounds at sites such as firing areas, destruction ranges, explosives dumping grounds and industrial production plant represents a significant worldwide environmental problem. Various solutions may be applied for the remediation of these contaminated sites. One emerging possibility is the use of biotechnological methods. These methods are innovative and inexpensive when compared to conventional methods, and tend to be publicly acceptable. *In situ* bioremediation of soil contaminated with RDX, TNT and related explosives compounds has the potential to be adaptable to specific compounds and environment (Ref. 1.)

In order to support the Canadian Armed Forces regarding their future potential environmental needs related with energetic materials, the Defence Research Establishment, Valcartier from the Department of National Defence (DREV/DND) in collaboration with the Biotechnological Research Institute from the National Research Council of Canada (BRI/NRC) have initiated a biodegradation study of some specific compounds. Of particular concern are contaminants such as 2,4,6 trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX), nitrocellulose (NC), glycidyl azide polymer (GAP) and 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane (HMX) which are derived from manufacturing of explosive munitions.

The three first energetic compounds were selected based on their extensive use in the past and thus on the high probability for their presence as soils or water contaminants. GAP was chosen to study the biodegradability potential of this new energetic product. Some limited biodegradation experiments are also planned with HMX using RDX active strains. Energetic compounds pose serious health and ecological hazards due to their mutagenicity and toxicity, and their tendency to persist in contaminated environments (Ref. 2). TNT, for example, causes liver damage and anemia in humans (Ref. 3), and concentrations above 2 µmol/ml are toxic to fish (Ref. 4). Because of the potential for groundwater contamination, and the subsequent migration of hazardous substances, treatment of the contaminated source is necessary to protect humans, crops and the environment.

An extensive literature search showed that, in general, little is known about the behaviour and biodegradation of energetic chemicals in soil and in the open environment

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(Ref. 5). Due to the xenobiotic nature and toxicity of organonitro compounds, only limited catabolic potential is found in natural microbial communities (Ref. 6). Under laboratory conditions, researchers have identified pure cultures that degrade mononitrobenzoates and mononitrophenols, but comparatively few microorganisms are known to degrade polynitroaromatics (Refs. 7 and 8). While the biodegradation and metabolic fate of TNT has been extensively studied (for a recent review see Ref. 2), comparatively little information is available concerning the metabolic fate and biodegradation potential of energetic compounds such as RDX, HMX, NC, and GAP.

In aqueous systems, RDX was mineralized by mixed populations of microorganisms under anaerobic conditions when supplemental carbon was provided (Ref. 9). Mono-, di-, and tri-nitroso compounds were identified as intermediates in the proposed biotransformation pathway. Such intermediates, however, pose a greater toxicity threat than does RDX itself. Thus, remediation of RDX-contaminated soil requires complete reduction of the toxicity of RDX and its derivatives. To our knowledge, microbial biotransformations of RDX have not been elucidated under aerobic conditions.

Fungal technologies (Refs. 3, 10) and composting (Refs. 11, 12) are suggested technologies for RDX, TNT and NC degradation. However, they have proven rather ineffective or too costly to be considered practical for large-scale application. For example, composting has the disadvantage of requiring large quantities of bulking agent with only a small fraction of the total volume composted being contaminated soil, and requiring long incubation times.

Recent studies by Spain (Refs. 13, 14) and research conducted in our laboratories suggests that aerobic bacteria may possess greater capabilities for degrading organonitro compounds than was previously thought. Identification of novel aerobic bacteria that can degrade recalcitrant energetic compounds such as RDX, TNT, GAP, and NC, and characterization of the catabolic pathways involved are imperative to developing *in situ* bioremediation strategies for these hazardous xenobiotics. Moreover, a detailed knowledge of the environmental conditions required to foster optimum rates of biodegradation may lead to the exploitation of these microorganisms for biorestoration of energetic compound-contaminated soils.

The present paper describes the collaborative work done to characterize energetic compounds in pure form and in soils and to verify their potential for biodegradation for future site bioremediation application.

SOIL SAMPLING, EXTRACTION AND ANALYSIS

Three sites potentially contaminated with RDX, TNT and NC were selected for soil sampling: Sites A, B and C. Four samples were collected from Site A, five from Site B and six from Site C. The samples were collected at depths varying from 15 to 45 cm. Soil was collected for general use in polyethylene pails while soil samples to be used for microbiological analysis were collected in sterile tubes. Sites A and B were suspected for specific contamination by TNT, RDX or NC, while Site C was suspected for multi-contamination by various energetic compounds, since the later was previously used as an energetic material burning site.

The analytical method used for the determination of TNT and RDX in soils was based on the work of Jenkins *et al* (Refs. 15-17). The general method consists of extracting 2 g of soil by sonication in acetonitrile followed by the reverse phase HPLC analysis of the extract with methanol/water (50:50%) as the eluent. The technique was evaluated under an interlaboratory study over a wide range of concentrations from 0.5 to 20,000 mg/kg of soil, dry weight of both TNT and RDX. As expected, the technique was found to be accurate (> 90% recovery), sensitive (DL < 0.5 ppm) and precise (RDS $\leq \pm$ 2%). The method used was in fact recently adopted by the U.S. Environmental Protection Agency as the standard method for the analysis of those nitroaromatics and nitramines residues (Ref. 18). Samples contaminated with low to high level of TNT and RDX were actually identified. As expected, Site C shows both TNT and RDX contamination but at a low level.

SYNTHESIS OF ^{14}C-LABELLED COMPOUNDS

The ^{14}C-substrates were all prepared using modified literature methods. RDX was prepared by the Hale process (Ref. 19). This procedure involves the condensation of ^{14}C-formaldehyde with ammonium hydroxide to yield quantitatively ^{14}C-hexamethylene-tetramine (HMTA) which is then nitrated under fuming nitric acid to give ^{14}C-RDX in 60% yield. During the nitration, the opening of the HMTA by the nitrating species involves a loss of three carbons (and one nitrogen) and therefore, a loss of total radioactivity is observed. The syntheses of ^{14}C-TNT was achieved according to the Dorey and Carper method (Ref. 20) using concentrated sulfuric acid 20% oleum instead of 15% oleum as mentioned by the authors. This method consists of nitrating toluene in three steps by increasing the power of the nitrating medium at each step. Concentrated sulfuric acid and fuming sulfuric acid were thus added to dehydrate the medium leading to a more active nitrating species. The toluene was either uniformly labelled at the ring or at the methyl group with carbon-14. There is no loss of carbon in these syntheses, and therefore the total radioactivity obtained is related to the yields.

The ^{14}C-nitrocellulose was obtained by nitration of ^{14}C-cellulose using the procedure of Olsen and Greene (Ref. 21). This method consists of nitrating the cellulose in a mixture of concentrated nitric acid and fuming sulfuric acid. Bacteriological ^{14}C-cellulose was obtained by cultivating *Acetobacter xylinum* on ^{14}C-glucose.

Glycidyl azide polymer is usually obtained by azidation of polyepichlorohydrin which is synthesized by polymerization of epichlorohydrin. ^{14}C-epichlorohydrin was not available commercially and had to be synthesized. Hydrochlorination of ^{14}C-glycerol as the starting material lead to 1,3-dichloro-2-propanol according to the method of Hill and Fisher (Ref. 22). The latter compound was epoxidized under basic conditions to yield ^{14}C-epichlorohydrin which was then polymerized in

accordance with the one-step process developed by Ahad (Ref. 23). The resulting polymer is a low molecular weight ^{14}C-GAP with a specific activity of 226 µCi/g as determined by a scintillation counter. It must be mentioned that not all monomers in the polymer are radioactive since the carbon-14 starting materials were mixed with unlabelled ones during the syntheses.

MICROBIAL ACTIVITY AND MINERALIZATION STUDIES

Total viable bacteria of each contaminated samples were determined by the spread plate technique and the population expressed as colony forming units per gram of soil. All samples showed important significant bacterial populations. Several enrichment cultures were started to isolate bacteria from contaminated soils using energetic substrates as either a carbon or a nitrogen source. In the latter case, glucose was added as a carbon source. Mineralization of ^{14}C-labelled RDX, TNT and GAP have been attempted in microcosms to assess the ability of the indigenous microbial population in contaminated soils to biodegrade energetic compounds.

Soil mineralization studies were done either to study spontaneous mineralization or to perform bioaugmentation studies. In the first case, microcosms were prepared with contaminated soil to obtain an indication of the ability of the indigenous microbial population in contaminated soils to mineralize energetic compounds. In some experiments, agricultural soil or activated sewage sludge was used to dilute the concentration of the energetic compounds contaminated soils. On the other hand, all bioaugmentation studies have been performed in laboratory-scale soil microcosms enriched with ^{14}C-RDX. Aqueous suspension of washed cells of actively-degrading strain was added to the surface of the soil to yield an initial inoculum density of 10^5 or 10^8 CFU per gram of soil. Uninoculated microcosms were included as negative controls.

RDX Mineralization

Soil from the A site (A4), containing 27 000 ppm RDX, supported a substantial indigenous bacterial population (2.55 X 10^7 CFU/g). Enrichment cultures prepared from the A4 sample in a mineral salts medium with RDX as the sole nitrogen source yielded a consortium with the ability to mineralize ^{14}C-RDX. Approximately 40% of the radioactivity was released as $^{14}CO_2$ after an incubation period of 4 days. This suggests that the use of energetic compounds, which are typically rich in nitrogen, as sole nitrogen source in enrichment cultures may favour the selection of microbial consortia capable of metabolizing these compounds by extracting the nitrogen. Two bacterial strains, designated "A" and "C", were isolated and purified from this consortium by their abilities to use RDX (100 ppm) as a sole nitrogen source under aerobic conditions when glucose was provided as a carbon source. The identification of both strain is ongoing. Most of the work accomplished to date has been focused on Strain "A". Experiments to characterize the RDX biodegradation pathway have been initiated by studying the growth of this strain in defined medium with glucose as the carbon source and RDX as the nitrogen source.

Using ^{14}C-RDX, the ability of isolates "A" and "C" to mineralize RDX in liquid culture under aerobic conditions was verified and both organisms mineralized 34% of the RDX within 2 days of incubation. Thus, the enrichment and purification from RDX-contaminated soil of two microbial strains with the ability to mineralize ^{14}C-RDX, when provided as the sole nitrogen source, has firmly established the biodegradation potential of RDX.

Laboratory scale bioaugmentation studies using strain "A" and "C" to bioremediate RDX-contaminated soils are currently in progress. Preliminary results indicated that bioaugmentation of RDX-contaminated soil with an RDX-degrading bacterium failed to enhance important biodegradation of RDX. Several possibilities were considered to establish the reasons for this failure. The results obtained so far suggest that in addition to its toxicity, RDX interaction with soil may influence its biodegradability. Significantly more mineralization was detected in an artificially contaminated soil amended with lower concentrations of RDX and inoculated with strain "A".

It is recognized that microorganisms can often tolerate xenobiotic compounds at low concentrations but not at higher concentrations. An understanding of the toxicity of pure RDX on RDX-degrading isolates both in liquid medium and in soil is critical. The toxicity of RDX to Strain "A" is currently being assessed in detail. Mineralization studies have been set up using increasing concentrations of pure RDX in pure cultures of Strain "A" (0-2000 ppm), and in soil microcosms (0-6000 ppm) bioaugmented with Strain "A". Once the toxicity of RDX on Strain "A" is determined, we may be able to adapt this bacterium to higher concentrations of RDX, specifically those levels likely to be encountered in the natural environment. Equally important is to have a better understanding of the fate of energetic chemicals in the soil environment. For example, the substrate may encounter various other abiotic changes normally determined by the physico-chemical properties of soil. Some of these abiotic processes include inorganic, organic, photolytic, surface-catalyzed, sorptive, and transport processes. This will be the subject of future investigation.

Having established that RDX is a biodegradable pollutant, and that bioaugmentation of RDX-contaminated soil enhances the rate and extent of biodegradation, a better understanding of the parameters (including soil/contaminant interactions) affecting RDX degradation kinetics in soil must now be developed. The fact that we observe more mineralization when the soil is artificially contaminated signifies that the soil/contaminant interactions are not as severe as in an aging soil that has been contaminated for several years. In this latter case severe adsorption becomes a problem on bioavailability.

TNT Mineralization

TNT appears to be a much more recalcitrant compound than RDX. A review of the relevant literature shows that TNT is quite resistant to biodegradation by most bacteria and fungi. Typically, biotransformations of TNT do not result in cleavage of the aromatic ring, and degradation to CO_2 does not occur.

Viable heterotrophic bacterial populations are lower in TNT contaminated soils than in the RDX-contaminated soil. Soil microcosms were set up with soils B4 (25 ppm), B3 (4700 ppm), and B5 (12 000 ppm) to assess [^{14}C] TNT mineralization by indigenous soil bacteria. Soil B4 has a slow but steady [^{14}C] TNT mineralization activity showing 8-10% of the radiolabel evolved as $^{14}CO_2$ after incubation for 100 days. The addition of glucose (250 ppm) as a supplementary carbon source, or yeast extract (50 ppm) as a supplementary nitrogen source did not enhance this rate of mineralization.

The effect of increasing TNT concentration on this activity has confirmed that the low level of mineralization observed in this soil is a real phenomenon as concentrations of 100 ppm TNT and higher result in complete abrogation of the mineralization activity. In light of this, soil B4 may also be inhibitory to the activity of its indigenous microbial population. When the TNT concentration in soil B4 was decreased to 2.5 and 10 ppm by diluting this soil with an agricultural soil, TNT mineralization activity was somewhat enhanced.

We therefore conclude that TNT is an extremely toxic compound with at least limited biodegradation potential. However, despite spontaneous TNT mineralization, soil B4 has as yet yielded no microbial populations with the ability to mineralize [^{14}C] TNT under aerobic conditions. Also the severe interactions between TNT and its intermediate metabolites with soil may limit its mineralization potential in a soil environment. An understanding of the mechanisms of transportation and transformation of TNT and its initial metabolites in the soil environment is critical in optimizing its mineralization efficiency. New enrichment cultures of soils will be set up providing TNT as sole carbon or nitrogen source at low TNT level. Finally, the biodegradation of TNT might also be evaluated under denitrifying and anaerobic conditions.

GAP Mineralization

Although it remains unknown whether any of the soils we are presently working with are contaminated with GAP, six soil samples (C1 to C6) obtained recently were all found to harbour significant numbers of viable bacteria (10^3-10^6 CFU/g). In addition, all soil samples displayed ^{14}C-GAP mineralization activity. Soils C4 and C5 were identified as possessing the greatest potential for isolating GAP-degrading bacteria. Enrichment cultures were recently prepared using a mixture of these two soils with GAP (100 ppm) provided as either sole carbon or nitrogen source. At present, the fourth subcultures are incubating; there is significantly more microbial growth in the enrichment culture with GAP provided as carbon source. [^{14}C] GAP mineralization tests are in progress, but data are not available yet concerning the ability of these cultures to degrade GAP.

NC mineralization

Two soils possibly contaminated with NC were collected, but the analytical procedures are not yet in place in our laboratories to confirm this. These soils, B1 and B2, harbour significant heterotrophic bacterial populations (5.43×10^7 and 5.63×10^6 CFU/g, respectively). Enrichment cultures were set up using these soils where NC was supplied as either sole carbon or nitrogen source. At various stages of subculture, samples of the enrichment cultures were preserved at -80°C for subsequent analysis. ^{14}C-NC was recently synthesized and the mineralization studies will be conducted soon.

ECOTOXICOLOGICAL STUDIES

Ecotoxicological evaluation of the four energetic compounds is essential in order to allow a future ecotoxicological follow-up of soil remediation. Therefore, different biotests were developed for analyzing pure compounds in the liquid phase. Partial results were obtained up to now and among the energetic compounds studied, TNT showed an important toxicological effect however it did not have a genotoxic effect. RDX showed an acute and a chronic toxicity but less important than TNT, and RDX did not reveal a detectable genotoxicity. According to the biotests, NC is not a potentially dangerous product for the environment since it is non genotoxic and very weakly toxic. GAP or GAP by-products were lightly genotoxic. Future work is needed to complete the ecotoxicity evaluation.

CONCLUSION AND FUTURE WORK

An interlaboratory study demonstrated that the method used to extract and analyze TNT and RDX was accurate, sensitive and precise. These two contaminants were found in soils from the three sites at concentrations ranging from low to high levels, either as the sole contaminant or as a combination. Contaminated soils, even those with high level of contamination supported substantial indigenous bacterial population. Enrichment culture techniques were employed to isolate indigenous microbes with the potential to degrade energetic compounds. Microcosm mineralization experiments were done in liquid medium with these enrichment cultures and ^{14}C labelled energetic compounds that were expressly synthesized for that purpose. Labelled RDX, TNT and GAP were prepared with good yields starting respectively with labelled formaldehyde, toluene and glycerol. Labelled NC was prepared from the microbial polymerization of labelled glucose which was then nitrated to ^{14}C-NC.

The present study showed that RDX was mineralized under aerobic conditions when used as a nitrogen source using isolates from RDX contaminated soil. Two RDX mineralizing pure cultures were obtained and preliminary characterization of the two strains were done. Some preliminary bioaugmentation studies using actively-degrading strains indicated mineralization of RDX when amended in agricultural soil. TNT biodegradation has been demonstrated to be highly concentration dependent and TNT toxicity appears to be a key factor. Low levels of TNT mineralization were detected in contaminated soils. Low level of mineralization of GAP were obtained by enrichment cultures from a mixture of soil contaminated with energetic compounds. Also, a multi-contaminated soil showed some microbial activity toward GAP. More work will be conducted to optimize the mineralization of the above substrates for future site decontamination. RDX small scale site decontamination is planned. Work will be conducted on quantitative

evaluation of NC in soils and on NC biodegradation with labelled ^{14}C-NC. Future work include the ecotoxicity evaluation of explosives and their metabolites.

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Discussion

QUESTION BY J.M. TAUZIA : Quelle relation existe-t-il entre la vitesse de dégradation de la Nitrocellulose et son taux d'Azote ?

ANSWER : On vient de commencer le travail avec la Nitrocellulose. On va déterminer "la correspondance" ou la corrélation quantitative entre le taux d'azote et le taux de neutralisation de NC. Cette étude peut servir notre projet parce que le taux d'azote de la NC explosive est d'environ 13 %.

QUESTION BY J.M. TAUZIA : Peut-on utiliser les technologies biologiques pour purifier les "eaux rouges" (Pink Water) qui proviennent de la synthèse du TNT ?

ANSWER : Nowadays our research is made on contaminated soil. Our future developments in the project may involve waste water or more specifically "pink water" treatment. We have established expertise in waste water treatment under anaerobic conditions. Several industrial products such as PCP, TCE, have been biodegraded. Further information on waste treatment may be obtained by consulting Dr S. GUIOT.

QUESTION BY T. ROSENDORFER : Cost for incineration compared to biodegradation seems too high. What is included in this price or how is this price calculated ?

ANSWER : The values shown in my presentation is what has been published by other researchers. These figures do not represent our calculations. We have not done that yet ! One source of information : Gregory D. Sayles (US EPA) and Makram T. Suidan, In : Biological Treatment of Industrial and Hazardous Wastewater, (Biotreatment of Industrial and Hazardous Waste, Ed Morris A. Levin and Michael AS. Gealt, McGraw Hill, Inc, Ch 11, 1993).

QUESTION BY T. ROSENDORFER : How do you correlate the poor migration of explosives in the soil, so that the microbes will find the explosives ?

ANSWER : Soil/contaminant interactions (adsorption/desorption) is always a problem that we have to consider when working with soil. Absorption often determines bioavailability and in turn biodegradation kinetics. We have in the past looked at the mechanisms of interactions between the contaminant (more specifically nitro PAHs and amino-PAHs, Hawari et al., Water Research 1994) and soil components so that we can understand and optimize biodegradation in soil.

QUESTION BY T. ROSENDORFER : What cosubstrates are used in addition ?

ANSWER : As explained in our paper when the explosive is used as N-source a cosubstrate is used to serve as C-source. For example, used as C-source (e. g. glucose, sucrose, succinate, etc). Some explosives were also used as C-source.

QUESTION BY H. SCHUBERT : Do you work with HMX ?

ANSWER : We have not started with HMX yet. It is a target on our agenda.