CHAPTER 6

SUMMARY

Ordnance and explosives (OE) including unexploded ordnance (UXO), by their nature present human health and environmental risks. When munitions constituents of OE come into contact with soils, groundwater and air, they may affect humans and ecological receptors through a wide variety of pathways including contamination of groundwater through leaching, exposure to soil and air and various surface water pathways.

High Energy Materials (HEMs) encompass different class of compounds and compositions containing fuel and oxidizer components that react rapidly upon initiation and release large quantities of energy in the form of heat and light. Energetic materials are typically classified as explosives, propellants, pyrotechnics or incendiaries and distinction among these classes are usually in terms of types of products generated and rate of reactions. Explosives are broadly classified based on their chemical structures such as nitrate esters, nitramines, nitroaromatics etc. Propellants include both rocket and gun applications. Most rocket propellants are composites consisting of a polymeric binder filled with ammonium perchlorate (AP) as oxidiser and aluminium powder as fuel. Rocket propellant formulations are also based on nitrate esters usually nitroglycerine (NG), nitrocellulose (NC), nitroguanidine (NQ) or nitramine such as RDX and HMX. Gun propellants usually are single base (NC), double base (NC and NG) or triple base (NC, NG, and NQ) formulations.

A large quantity of energetic materials including nitrate esters, nitarmines, nitroaromatics and their propellant formulations are subjected to disposal either due to expiry of useful life or rejection in manufacturing process on account of Quality Assurance (QA) / Quality Control (QC) checks. Unexploded ordnances (UXO's) occurred during war military training pose serious environmental problems and other potential hazards. The UXO's on prolonged storage contaminate the soil & water due to leaching of explosives materials present in it. Environmental and health problems posed by explosives are massive in scale and need to be studied for their remedial measures. Conventional method of disposal like open burning (OB) /open detonation (OD) is not allowed for high energy hazardous materials including propellants & explosives due to human health risk involved in these operations. The other methods of disposal, which has been studied by various researchers, include thermal processes (incineration & molten salt oxidation), wet air oxidation (WAO), biodegradation and chemical treatment processes.

Bioremediation is a treatment process whereby contaminants are metabolized into less toxic or nontoxic compounds by naturally occurring microorganisms. The microorganisms utilize the contaminants as a source of carbon and energy. The by-products are mainly carbon dioxide and water. Once the microorganisms have consumed all of the contaminants, the microbial population becomes dormant. Bioremediation can take place under aerobic conditions (with oxygen) or anaerobic conditions (without oxygen) in the presence of other suitable electron acceptors such as nitrate, sulfate, or carbonate. The biodegradation method of disposal is found most acceptable for remediation of explosive contaminated soil / water in view of the simplicity of the process and being eco-friendly. In addition biodegradation does not involve processing of hazardous materials at high temperature unlike thermal processes. The potential advantage of biological treatment includes low cost, ease of operation and public acceptance.

Composting is a kind of bioremediation process by which organic contaminants are converted by microorganisms under aerobic and anaerobic conditions to innocuous and stabilized by products. The composting utilizes native aerobic thermophilic microorganisms and thus requires no inoculation. The main advantage of composting is that it generates a macronutrient enriched product that can sustain vegetation. Composting is effective for a wide range of explosive wastes / contaminants. In the present study the native microorganisms present in the compost fillers were utilized for the degradation of explosive contaminants i.e nitrocellulose (NC), nitroglycerine (NG), triple base propellant (TBP) and trinitrotoluene (TNT) by composting. Sugarcane byproducts were used as amendments to the compost matrix for the compost bioremediation of NC, NG, TBP and TNT. Laboratory scale composting process parameters has been optimized and standardized for the effective biodegradation of selected explosives and propellants. Detailed study on compost microflora was carried out for the characterization and identification of native bacterial and fungal species present in explosive incubated compost matrices. Toxicity assessment of end products of composting has also been carried out on matured compost after 15 days of incubation with selected explosives and propellant formulation. Compost enzyme activities were also determined in compost matrices incubated with high concentrations of NC, NG, TBP and TNT. Since most of the processes occurring in soils and compost are microbially mediated and are metabolized by enzymes, therefore, it is reasonable to suggest that the determination

of enzyme activities in soil and compost may be used as a research tool to assess / study microbial functional diversity, biochemical process, microbial ecology and ultimately to provide indicators of soil and compost quality.

Alkaline hydrolysis of nitroaromatics and nitrate esters, as an alternative method for disposal of explosives and its contaminants, has been studied by various research workers. Identification of by-products of hydrolyastes is also attempted. Toxicity studies on hydrolysates and application of hydrolysates as plant nutrients has not been studied much. Very limited technical information is available on plant toxicity assessment of hydrolysates. Therefore, in the present work, plant toxicity studies on hydrolysates and its application as plant nutrients was carried out by using standard protocol for seed germination and plant growth assays on wheat (*Triticum aestivium*) seeds and plant respectively.

Phytoremediation is yet an eco-friendly approach for remediation of contaminated soil and water using plants. Phytoremediation is comprised of two components, one by the root colonizing microbes and other by plant themselves, which degrade the toxic compounds to further non-toxic metabolites. The use of aquatic plants to assimilate and detoxify hazardous substances is one of the promising cleanup methods for explosive contaminated sites. In the present study, two aquatic macrophytes (*Eichhornia* and *Pistia*) and two wetland plants (*Typha* and *Phragmites*) were used in the hydroponic set-up for the phytoremediation studies on nitroglycerine (NG).

Very limited information is available in open literature on bioremediation of HEMs as it is related to defense. The research work carried out in this area is secured as classified information. Therefore, the present study was undertaken. The objective of the present thesis is to carry out studies of various control parameters on development of viable & effective process of bioremediation for remediation of nitrate esters (NC, NG and TBP) and nitro aromatics (TNT) and the findings have been reported accordingly.

The objectives of the thesis include the following:

- Standardization and optimization of compost bioremediation process parameters at laboratory scale for fast and effective biodegradation of NC, NG, TBP and TNT using sugarcane by products as amendments to the compost.

- ❖ Assessment of compost end product of NC, NG, TBP and TNT as plant nutrient by performing seed germination and plant growth studies on wheat (*Triticum aestivium*) seeds and plants grown on matured compost incubated with varying concentrations of selected explosives and propellant formulation.
- ❖ Detailed study on compost microflora. Isolation, characterization and identification of predominant bacterial and fungal species present in explosive incubated compost.
- ❖ Determination of compost enzyme activities before and after incubation with high concentrations of selected explosives and propellant formulation used under the compost bioremediation study.
- ❖ Alkaline hydrolysis of NC, TBP and TNT with varying concentrations of alkali and analysis of hydrolysates.
- ❖ Phytotoxicity assessment of hydrolysates of NC, TBP and TNT as per standard EPA protocol.
- ❖ Composting of hydrolysates and phytotoxicity assessment of composted hydrolysates.
- ❖ Phytoremediation of NG using commonly available aquatic macrophytes and wetland plants.

The compost bioremediation was studied for NC, NG, TBP and TNT in various feedstock concentrations using soil, cow dung, vermicompost and sugarcane-press mud and bagasse as compost fillers. Compost matrix used under the study was designed to achieve the C / N ratio of 33:1. Compost matrices were incubated with varying concentrations of selected explosives and were examined for composting parameters like pH, electrical conductivity, total nitrogen%, total phosphorous% and total potash%, micronutrients and microflora. Activities of compost enzymes viz. urease, amidae, dehydrogenase, phosphatase (acid and alkaline) and L- glutaminase were determined in compost matrices before and after 15 days of incubation with selected concentration of explosives. Analysis of residual parent compound and degraded products in compost matrix were carried out quantitatively as well as qualitatively by using UV- visible spectrophotometry, IR spectroscopy, HPLC and GCMS analysis. In case of NC (feedstock 10,000 ppm) it took 14 days to reduce to below detectable limit i.e <1 ppm. Other researchers reported the incubation period of 70 days for reduction in residual NC to around 59 ppm from the feedstock of about

3039 ppm. Therefore, the composting process for NC developed in the present study is suitable for faster rate of degradation of explosive contaminant. In case of TNT (feedstock 10,000 ppm), the residual TNT was found in compost matrix below detectable limit i.e <4 ppm in 14 days, whereas the similar degradation of TNT has been reported by other researchers in more than 30 days, indicating the merits of the selected matrix over the reported ones. In case of NG (feedstock 1500 ppm), the residual NG was found in compost matrix below detectable limit in 15 days. The degradation of NG by compost bioremediation has not been reported in open literature, indicating the novelty of the findings. In case of TBP composting, more than 30% of the initial TBP remained at the end of the study in the compost bed.

The incubation of energetic materials also generates an enriched product that can sustain vegetation as shown by the performance of seed germination parameters on the matured compost incubated with NC, NG, TBP and TNT. In case of NC and TNT incubated matured compost, when subjected to plant growth exhibited significant increase in root/shoot length, biomass weight, total chlorophyll, total protein as well as total carbohydrate as compared to control with unincubated compost matrix. The findings of the present compost bioremediation studies reveal 2.5 - 3.0 times faster rate of degradation of explosive contaminant (NC, NG, TBP and TNT) over conventional composting methods and suitability of composting process for nitrate ester and nitro aromatic class of explosives using the fillers taken in the study, as plant nutrients.

A series of alkaline hydrolysis experiments were conducted at 70°C. Various alkalis to NC, TBP and TNT ratios were studied by varying the concentration of the base. Indian variety of wheat seeds (*Triticum aestivium*) were subjected to seed germination studies on different doses of NC, TBP and TNT hydrolysates. NC hydrolysates prepared from feedstock of 1000 to 5000 ppm with lower base concentration (1, 2 and 5% w/w) were of minimum phytotoxic concern. The higher base concentration (10 % w/w) comparatively, exhibited toxicity due to presence of more dissolved salts in the hydrolysates. Decrease in root length was observed in case of all the feedstock concentrations of NC prepared with 10% alkali. Surface sterilized wheat seeds were grown on highest selected dose of NC hydrolysate (HNC) under the study i.e 16 mL and were examined for metabolic products. Chlorophyll content realized in 'control' was 0.628 mg g of biomass, which increased to 1.08 mg / g of

biomass in NC hydrolysate. The TBP hydrolysate prepared with 1, 2 and 5 % exhibited minimum phytotoxic concern (seed germination > 50%) for all the doses (1, 2, 4, 8 and 16 mL) of hydrolysates prepared with feedstock concentration of 1000 to 5000 ppm. The percent seed germination in case of TNT hydrolysate prepared with 5% alkali was more prominent for higher dose of hydrolysate i.e. 4, 8 and 16 ml compared to hydrolysate prepared with 10 % alkali. For 4 and 8 ml doses of hydrolysate 'minimum phytotoxic concern' (percent seed germination >50%) is exhibited for feedstocks of 1000, 2000, 3000 and 4000 ppm concentration, hydrolysed with 5%, whereas hydrolysate prepared with 10% alkali showed 'minimum phytotoxic concern' only for 1000 and 2000 ppm feedstock concentration for 4 ml dose of hydrolysate and 8 ml dose exhibited no seed germination for all feedstock concentrations i.e. 1000, 2000, 3000, 4000 and 5000 ppm. The improved seed germination was exhibited in case of hydrolysate prepared with 5% alkali.

Hydrolysates of NC, TBP and TNT prepared with 5% alkali (feedstock concentration of 5000 ppm and 10,000 ppm of each) were subjected to composting for the comparative phytotoxicity assessment of hydrolysates as such and composted hydrolysates after 15 days of incubation in compost matrix. Seed germination was carried out on NC, TBP and TNT composted matrices and significant increase in biomass weight and chlorophyll content was observed as compared to control (unicubated compost matrix). In case of composted hydrolysate of NC (CHNC), when wheat seeds were subjected to germination studies, wheat plant showed significant increase in total chlorophyll by 57%; total carbohydrate by 29.5%; total protein by 78.5% as compared to unincubated compost. In case of Composted hydrolysate of TNT (CHTNT), total chlorophyll is increased by 82 % while in case of CHTBP (composted hydrolysate of TBP) total chlorophyll is increased by 52%. Among all the three composted hydrolysates, CHNC showed maximum increase in biomass, total chlorophyll and total carbohydrate as compared to unincubated compost while maximum increase in total protein was observed in case of CHTNT.

All the five compost fillers individually and final compost mixture used in the compost bioremediation study were subjected to isolation, characterization and identification of dominant native bacterial and fungal species present in compost (explosive incubated as well as unincubated compost) by employing the Dilution plate method. Using slide culture method all predominant fungi population was isolated.

The measurements of the fungal structures were performed in lacto-cotton blue. Morphotaxonomical identification of all the 10 fungal isolates got confirmed from the NFCCI (National fungal culture collection of India) and were identified as *Aspergillus niger*, *Penicillum sp, Aspergillus terreus, Rhizopus stolonifer, Paecilomyces varioti and Aspergillus flavus.*

Using serial dilution method the predominant bacterial population was isolated on nutrient agar medium. Gramstaining, motility, cell shape, density, elevation, configuration and pigments were observed under the microscope. Biochemical characterization of bacterial isolates was also carried out. Growth at different temperature, pH and different conc. of NaCl was also observed. 16- S ribosomal identification revealed the identification of first isolate (VRI-1) as *Micrococcus sp.*, second isolate (VRI-2) as *Exiguobacterium sp.*, third isolate (VRI-3) as *Cellulomonas hominis* and the fourth isolate (VRI-4) as *Bacillus cereus.*

Phytoremediation of explosive grade nitroglycerine was studied in hydroponic culture. Based on factors like easy and fast propagation of plants, high biomass of plants and their common availability, four aquatic plants were selected for this study. Two commonly available aquatic macrophytes, *Eichhornia crassipes* (Water hyacinth), *Pistia stratiotes* (Water lettuce) and two wetland plants, *Typha latifolia* (broadleaf cattail) and *Phragmites australis* (common reed) were selected for phytoremediation studies on nitroglycerine using hydroponic culture. All the plants were hydroponically cultivated in Hoagland's solutions.

The presence of NG in the leaves was clear evidence that NG was taken up and translocated to the leaves by the macrophytes. Substantial transformation of the NG occurred as evident from IR, HPLC and GCMS spectra of leaves and root extracts, either by bacteria in solution or in plant itself. In experimental setup containing *Typha latifolia* (broadleaf cattail) plants, there was significant loss of NG from solution, yet no accumulation in the tissues was observed. Assuming the microbial cultures in all the experimental setup were not appreciably different than those in other setups of plants, it is likely that enzymes within the *Typha latifolia* tissues were especially effective at transforming NG. Phytoremediation studies on NG showed the potential of aquatic herb *Typha latofolia* towards the tolerance as well as for the transformation of NG as evident from the IR and HPLC spectra of *Typha latofolia* plant extracts. Trials with killed plant roots showed high concentration of NG

absorbing to the dead roots. Based on the observation that NG concentrations in the dead roots were significantly higher than NG concentrations in the live roots, further indicates that enzymes within all of the live macrophytes were actively transforming the NG.

The values of peroxidase activity indicates high antioxidant capacity of *Phragmites australis* (common reed) in the presence of oxidative stress produced in condition of NG retention. There was a significant ($p<0.05$) reduction in chlorpphyll a, chlorpphyll b, total chlorophyll, chlorophyll a:b, carotenoides, total proteins, total carbohydrates and free reducing sugar contents as a result of the growth of the *Eichhornia crassipes* (Water hyacinth) plant in the NG and a significant increase ($p<0.05$) was noticed in the free amino acids level. This indicates that the NG has phytotoxic effects on the plants. The reduction in carbohydrate content in leaves of *Eichhornia crassipes* plant is due to contaminant (NG), which has exhibited decreased photosyntheis resulting in less formation of carbohydrate. On the other hand, a decrease in proteins could be attributed to both breakdown of existing proteins and reduced de novo synthesis. Such a reduction in protein content may be utilized as a suitable bio indicator of NG pollution.

In the present research work, NC, NG, TBP and TNT were successfully degraded into non- hazardous and non-explosive products by compost bioremediation and the end products of compost bioremediation showed application as plant nutrient as well. The data generated on plant performance parameters like % seed germination, root-shoot length, biomass, carbohydrate and protein content under seed germination study on explosive incubated compost and hydrolysate incubated compost are not reported. Therefore, phytotoxicity studies, in conjunction with the appropriate controls and analytical evaluations were carried out to establish composting and alkaline hydrolysis as an alternative technology for explosive contaminants. Detailed study carried out on compost microflora is of vital importance as bacterial isolates reported in this study has not been reported for the biodegradation of nitrate esters as well for the other group of explosives and therefore the findings of this study opens the scope for the further assessment of biodegradation potential of these isolates towards such explosives. No reports are available on phytoremediation of NG using aquatic macrophytes used under this study.

The findings of the present studies have practical utility,

- For the disposal of explosives on large scale and as a cost effective and eco-friendly alternative treatment and disposal method for high energy materials by applying composting and phytoremediation process as developed in the present study.
- As plant nutrient in form of compost end products resulting from compost bioremediation process of high energy materials developed in the present study.