BIOREMEDIATION OF NITRO AROMATIC EXPLOSIVE CONTAMINANTS 2,4,-DINITROTOLUENE (DNT) AND 2,4,6-TRINITROTOLUENE (TNT) FROM SOIL AND WATER USING SELECTED BIOFUEL CROPS AND NATIVE MICROBES

NANDAN

DEPARTMENT OF CIVIL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY DELHI INDIA

OCTOBER 2017

BIOREMEDIATION OF NITRO-AROMATIC EXPLOSIVE CONTAMINANTS 2,4-DINITROTOLUENE (DNT) AND 2,4,6-TRINITROTOLUENE (TNT) FROM SOIL AND WATER USING SELECTED BIOFUEL CROPS AND NATIVE MICROBES

by
Nandan
Department of Civil Engineering

Submitted

In fulfillment of the requirements for the degree of doctor of philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI
OCTOBER 2017

IN THE SERVICE OF ETERNAL NATURE,

DEDICATED TO MY NATION INDIA

“The power of God is with you at all times; through the activities of mind, senses, breathing, and emotions; and is constantly doing all the work using you as a mere instrument”

Bhagvat Geeta

CERTIFICATE

This is to certify that the thesis entitled "**Bioremediation of Nitro Aromatic Explosive Contaminants, DNT (2,4-Dinitrotoluene) and TNT (2,4,6-Trinitrotoluene) from Soil And Water Using Selected Biofuel Crops and Native Microbes"** being submitted by **Mr. Nandan** to the Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** and is a record of bonafide research work carried by him. He has worked under my guidance and supervision, and has fulfilled the requirements for the submission of this thesis. To the best of our knowledge the results contained in this thesis have not been submitted in part or full to any other University or Institute for award of any degree or Diploma.

Dr.S.Mary Celin
Supervisor
Scientist 'F' & Additional Director,
Centre For Fire Explosive and Environment Safety (CFEES), Defence Research Development Organization, Delhi,
India-110054

ACKNOWLEDGEMENTS

This is a very important moment for me. I am very happy to be able to recognize and acknowledge all of the people that contributed too many different aspects of my PhD research, as well as in the preparation of this dissertation. I would like to begin by acknowledging my supervisor **Prof.Atul K. Mittal (retired professor)** to join Ph.D. in his guidance.His vast knowledge of the subject and logical way of thinking has been of great value for me. His stimulating discussion, constructive criticism and personal guidance on various aspects have provided a great basis for deleaing with the subject problems.I am immensely thankful to him for his wonderfull co-operation, constantencouragement, proper guidance and fruitfull academic discussions for carrying out my research work. I also express gratitude to External supervisor, **DR. S. Mary Celin, Scientist 'F' CFEES, DRDO, India,** who granted me the opportunity to join her research group and provided me with aninteresting project to develop my scientific skills and critical thinking. Their exceptional guidance, as well as trust and encouragement, guided me throughout my research and writing of this thesis, and allowed me to complete the degree successfully.

Next, I would like to extend my gratitude to the my caretaking supervisor and committee members **Prof. K. C. Iyer (Chairperson, SRC), Dr. Arun Kumar (Member, SRC and Caretaking Supervisor)** and **Prof. T. R. Srikrishnan (Member, SRC and Caretaking Supervisor)** for providing valuable suggestions and encouragement for the present Ph.D. research work and for the comments and suggestions to improve my thesis and complete my Ph.D.

I am heartily thankful to **Mr. Sandeep Sahai, Technical Officer 'B'**, **Rashmi Bhalla,Technical Officer 'C' Dr. P.K. Rai, Scientist 'F' and Group Head Environment Safety Group, Prabhanjan Thkaur Scientist 'D', Pankaj Sharma, Scientist 'E',Amit Tyagi, Scientist 'E', Ramesh Singh, Scientist 'D', Dr. Ajay Kumar Shaw, Scientist 'C', Pavan Yadav, Technical Officer 'A', Laxmi Huda, Research Scholar,** and all the staff members atthe CFEES, DRDO Delhi for their assistance and help related to equipment, information, services, and resources. Further I would like to thank to my all seniors/colleagues and other project members.

I owe special thanks to **Dr. Rajeev Narang, Director CFEES, DRDO**, **Dr. Chitra Rajgopal,** (**Former Director CFEES, DRDO) Chief Comptroller, DRDO** and **Harbansh Lal, Retired Former Scientist 'G', CFEES, DRDO** for their assistance and support at various stages of my research experiments.

I am grateful to Centre For Fire Explosive and Environment Safety (**CFEES),** Defence Research Development Organization (**DRDO**) New Delhi, for providing me the junior and Senior Research Fellowship, which enabled me to pursue this dissertation.

Special thanks **to Dr. Sudershan Kumar, Distinguished Scientist and former Chief Controller,R&D (PC & SI), DRDO,** India for being with me during the good and bad times of this journey.Finally, I thank my family members for their support, encouragement, and cooperation. They all contributed in one way and another for allowing me to indulge in the most challenging of my academic endeavors.

Nandan

Abstract

Environmental contamination of explosives, especially by nitro esters and nitro aromatics such as TNT, DNT, RDX, HMX etc., is a matter of ever increasing concern as enormous amounts of these compounds are being produced for military and civilian activities relating to manufacturing of explosives and its use in related processes viz, loading, assembly, packing, firing and training. Due to recalcitrant nature, these chemicals pose threat to human health and environment. It is essential to develop a suitable remediation technology for treating these contaminants. Existing physicochemical techniques based on open burning, oden detonation, adsorption, advanced oxidation process etc., are neither cost-effective nor environmentally safe, as they merely transfer the contaminants from one phase to the other. Realising the need for development of an eco-friendly strategy the present study has been taken up with three different objectives viz., (i) site characterization of the TNT and DNT contaminated soil and water and batch studies on soil adsorption behaviour of TNT and DNT. (ii) Evaluation of the efficiency of native microbes isolated from the contaminated sites in degrading these chemicals and (iii) studies on phytoremediation by energy crops viz., *Azolla microphylla*, *Jatropha curcas* and *Ricinus communis.*

Site characterization studies were aimed to assess the type and extent of explosive contamination in soil and water environment of a selected explosive manufacturing facility in India. These studies proved high levels of explosive contamination in both soil and water profile. pH of the sites was found to be acidic ranging from 1.3 to 5.6 Concentration of TNT in the contaminated site ranged from 20 ppm to 2000 ppm. Studies on soil adsorption behavior of explosives can help in assessing the fate and mass transport characteristics of these chemicals in the subsurface environment, which is essential for risk assessment and implementation of proper remediation technologies. In soil adsorption experiments DNT and TNT of varying concentration in water were added to varying soil quantities. Soil adsorption levels for DNT and TNT were carried out by taking out samples at timely intervals.The equilibrium adsorption data were analyzed usingvarious adsorption isotherm models viz, Frendulich isotherm, Langmuir model, DubninRadushkevich model, BET model and Temkin isotherm model. Adsorption data for TNT is best fitted in the following decreasing order for different studied models: Freundlich> BET >DR > Temkin > Langmuir model, where in DNT

adsorption data is best fitted in the following order, DR >Freundlich> BET > Langmuir >Temkin model. The adsorption affinity of the selected soil as per Freundlich model for both DNT and TNT is less (3.53 mg/g for TNT and 8.16 mg/g for DNT) necessitating the need for an efficient eco-friendly treatment procedure in place.

Native microflora present in the contaminated soil and water samples of the selected explosive manufacturing facility was evaluated for treating DNT and TNT in aqueous phase and soil media. Identification by 16S rRNA gene sequencing was carried out by in collaboration with Institute of Microbial Technology (IMTECH) Chandigarh**.** *Brachybacterium paraconglomeratum, Delftialacustris, Pseudomonas mosselii***,** *Bacillus infantis* were the identified microbial species from the contaminated sites. These selected bacterial strains were tested for tolerance to DNT and TNT **(**100 to 2000 mg/Kg) in soil and for treating synthetic DNT and TNT wastewater (40, 80 and 120 mg/L). Complete removal of both DNT and TNT (99 %) was achieved by the potential bacteria *Pseudomonas mosselii* in 48 hours of treatment time at an initial DNT and TNT concentration of 120 ppm. The order of degradation was found to be *Pseudomonas mosselii* >*Delftia lacustris* > *Brachybacterium paraconglomeratum*>*Bacillus infantis*, for both DNT and TNT respectively.Owing to its superior performance of *P. moselli* was chosen for soil remediation study wherein it was observed that, *Pseudomonas mosellii* could successfully uptake the DNT and TNT from contaminated soils having concentrations up to 2000 mg/kg, the maximum concentration found in the explosive manufacturing site selected in the present study. After 180 days of bacterial incubation, complete DNT and TNT removal (99 %) was observed in all the treatments except control. Concomitant with reduction in DNT and TNT concentration, formation of unidentified putative metabolites was found to occur in both soil and water samples treated by *P.mosselli.*

In phytoremediation studies, pot culture experiments were carried out using *Azolla microphylla* for treating DNT and TNT in aqueous phase at concentrations ranging from 40, 48 and 120 mg/L respectively. *A. microphylla* has the capacity to accumulate DNT and TNT up to an optimum concentration of 80 ppm in aqueous phase in 10 days of exposure. High concentrations beyond 80 mg/L were found to be toxic to its growth and proliferation. In soil bioremediation studies, biofuel crops *Jatropha curcas* and *Ricinus communis,* were evaluated to treat both DNT and TNT in concentrations ranging from 100 to 2000 mg/Kg. Among the two different chosen plants, the

performance of former was better with respect to DNT and TNT removal up to a soil concentration of 1000 mg/Kg (> 95 % removal) in 365 days. Removal efficiency of *Jatropha curcas* for DNT and TNT was found to be in the order of. 1000>500>200>100>1500>2000 mg/Kg Maximum reduction of DNT (97%) and TNT (96%) were found to occur in soils with1000 mg/kg of imparted concentration and minimum reduction in concentration occurred in soils treated with 2000 mg/kg of both DNT and TNT. With respect to *Ricinus communis*, degradation order was found to be 500>200>100> 1000>1500>2000 mg/kg for both DNT and TNT respectively.

The current study revealed the potential of *Pseudomonas moselli* for treating DNT and TNT. The viability of *Azolla microphylla* as a potential wetland plant species has been proven for DNT and TNT concentrations up to 80 mg/L. Soil remediation by the potential phyto remediator *Jatrpha curcas* was proven to be successful up to soil DNT and TNT contamination levels of 1000 mg/Kg.

सार

"नाइट्रो एरोमेटिक विस्फोटक प्रदूषकों 2,4,डायीनायीत्रोताल्यूयीन (डीएनटी) और 2,4,6-त्रायीनायीत्रोताल्यूयीन(टीएनटी) के दवारा प्रदूषित मिट्टी और पानी का जैवऊर्जा युक्त पौधो और सूक्ष्मजीवों का उपयोग करके संशोधित करना"

विस्फोटकों के दवारा उतपन्न पर्यावरण प्रदूषक , विशेष रूप से नाइट्रो एस्टर और नाइट्रो एरोमैटिक्स के दवारा जैसे टीएनटी, डीएनटी, आरडीएक्स, एचएमएक्स इत्यादि बढ़ती हुई चिंता का विषय है। विस्फोटकों के निर्माण से संबंधित सैन्य और नागरिक गतिविधियों के लिए और संबंधित प्रक्रियाओं में इसका उपयोग, जैसे लोडिंग, निर्माण, पैकिंग, फायरिंग और प्रशिक्षण में इन यौगिकों की भारी मात्रा में उत्पादन किया जा रहा है। अव्यवहारिक प्रकृति के कारण, ये रसायन मानव स्वास्थ्य और पर्यावरण के लिए खतरा पैदा करते हैं। इन दूषित पदार्थों के इलाज के लिए एक उपयुक्त शोधन प्रौदयोगिकी को विकसित करना आवश्यक है। ओपन बर्निंग, ओपन विस्फोट, सोखना, उन्नत ऑक्सीकरण प्रक्रिया आदि पर आधारित मौजूदा भौतिक रसायनिक तकनीक न तो लागत प्रभावी हैं और न ही पर्यावरणीय रूप से सुरक्षित हैं, क्योंकि वे केवल एक चरण से दूसरी तक दूषित पदार्थों को स्थानांतरित करते हैं। पर्यावरण अनुकूल रणनीति के विकास की आवश्यकता को महसूस करते हुए वर्तमान अध्ययन को तीन अलग-अलग उद्देश्यों में बता गया है जैसे, (i) टीएनटी और डीएनटी दूषित मिट्टी और पानी की साइट के लक्षण वर्णन और टीएनटी और डीएनटी के मिट्टी सोखने के व्यवहार पर पानी और बैच अध्ययन। (ii) इन रसायनों के प्रभाव को कम करने में प्रदूषित साइटों से पृथक देशी रोगाणुओं की दक्षता का मूल्यांकन तथा (iii) ऊर्जा फसलों दवारा फाइटोरिडिएशन पर अध्ययन, जैसे अज़ोला माइक्रोफ़ाली, जेट्रोफा क्यूकास और रीसिनस कम्युनिस के दवारा.

भारत में एक चयनित विस्फोटक निर्माण प्रसंस्करण प्रणाली के मिट्टी और पानी के वातावरण में विस्फोटक संदूषण के प्रकार और सीमा का आकलन करने के लिए, साइट वर्णनीकरण, प्रथम अध्ययन का उद्देश्य था।इन अध्ययनों ने मिट्टी और पानी दोनों में विस्फोटक संदूषण के उच्च स्तर को प्राप्त किया गया है। साइटों के पीएच 1.3 से 5.6 तक अम्लीय तथा टीएनटी की मात्रा 20 पीपीएम से 2000 पीपीएम तक संक्रमित स्थल में पाए गए थे।विस्फोटकों के मिट्टी दवारा अवशोषित होने के व्यवहार पर अध्ययन एवं उपसतह वातावरण में इन रसायनों के सामूहिक परिवहन विशेषताओं का आकलन करने में मदद करता है , जो जोखिम मूल्यांकन और उचित उपचार तकनीक के कार्यान्वयन के लिए आवश्यक है।मृदा सोखने के प्रयोगों में पानी में डीएनटी और टीएनटी की भिन्न मात्रा को अलग-अलग मिट्टी की मात्रा में मिलाया जाता है। डीएनटी और टीएनटी के लिए मृदा दवारा अवशोषित होने के स्तर का आकलन विभिन्न समय-अंतराल पर नमूने लेकर किया जाता है। डेटा का विश्लेषण विभिन्न प्रकार के समताप मॉडल के माध्यम से किया गया जैसे, फ्रेंडलीच इसोथर्म, लैंगमुइर मॉडल, दबनीन रोदुकेविच मॉडल, बीईटी मॉडल और टेम्किन इसोथर्म मॉडल।

टीएनटी का मिट्टी में अवशोषण डेटा विभिन्न अध्ययन मॉडलों के निम्न घटते क्रम में सबसे अच्छा प्राप्त हुआ ।फ्रीडैलिच> बीईटी> डीआर> टेम्किन> लैंगमुइर मॉडल, जहां डीएनटी अवशोषण डेटा निम्न क्रम में सबसे अच्छा प्राप्त हुआ, डीआर> फ्रींडिलिच> बीईटी> लैंगमूयर> टेम्किन मॉडल। डीएनटी और टीएनटी दोनों के लिए फ्रींडिलिच मॉडल के अनुसार चयनित मिट्टी में अवशोषण संबंध (टीएनटी के लिए 3.53 मिलीग्राम / ग्राम और डीएनटी के लिए 8.16 मिलीग्राम / ग्राम) एक प्रभावी पर्यावरण अनुकूल उपचार प्रक्रिया की आवश्यकता को दर्शाते हैं।

सुक्छम्जीवो के दवारा विस्फोटक प्रदुषकनो का अध्धयन इस भाग में किया गया, जहाँ विस्फोटक विनिर्माण प्रसंकरण के प्रदूषित मिट्टी और पानी के नमूनों में उपस्थित मूल माइक्रोएफ्लोरा का मूल्यांकन जलीय और मिट्टी मीडिया में डीएनटी और टीएनटी के् इलाज के लिए किया गया। 16 एस आरआरएनए जीन सीक्वेंसिंग की पहचान इंस्टिटयूट ऑफ माइक्रोबियल टेक्नोलॉजी (आईएमटेक) चंडीगढ़ के सहयोग से की गई थी। ब्रैकीबैक्टीरियम पेराकॉन्ग्लोमेराटम, डेल्फिआ लाकास्ट्रिस, स्यूडोमोनस मॉस्सेल, बैसिलस इन्फैंटिस आदि दूषित साइटों से पहचान की गई माइक्रोबियल प्रजातियां हैं। इन चयनित बैक्टीरियल उपभेदों को मिट्टी में डीएनटी और टीएनटी (100 से 2000 मिलीग्राम / किग्रा) के लिए एवं अपशिष्ट जल में सिंथेटिक डीएनटी और टीएनटी (40, 80 और 120 मिलीग्राम / एल) के उपचार के लिए परीक्षण किया गया है। डीएनटी और टीएनटी (99%) दोनों को संसोधन के लिए बैक्टीरिया स्यूडोमोनस मॉस्सेली का टीएनटी और डीएनटी की 120 पीपीएम के मात्रा में 48 घंटे के दवारा संसोधन प्राप्त किया गया है।संसोधन का क्रम क्रमसः स्यूडोमोनस मॉस्सीली> डेल्फीटा लैकस्ट्रिस> ब्रैकीबैक्टीरियम पेराकॉन्ग्लोमेरेटम,> बैसिलस इन्फैंटिस, पाया गया जो की डीएनटी और टीएनटी दोनों के लिए है। मॉस्यूडोमोनस मॉस्सीली के अपने उत्कृष्ट प्रदर्शन के कारण मृदा उपचार अध्ययन के लिए चुना गया था जिसमें यह देखा गया है कि स्यूडोमोनस मॉस्सीली, 2000 एमजी / किग्रा तक सांद्रता वाले दूषित मिट्टी से डीएनटी और टीएनटी को सफलतापूर्वक अवशोषित कर के संशोधित कर सकता है जो कि वर्तमान अध्ययन् में चयनित विस्फोटक निर्माण स्थल में पायी गयी अधिकतम सांद्रता है। बैक्टीरिया के दवारा शोधन के 180 दिनों के बाद, पूर्ण डीएनटी और टीएनटी को (99%) विघटन प्राप्त हुआ। डीएनटी और टीएनटी मात्रा में कमी के साथ सुक्छम्जीवो के चयापचय की क्रिया के दवारा सम्मीलित अज्ञात पदार्थ भी पाए गए.

पौधो के दवारा विस्फोटक संसोधन के अध्ययन में, गमले के प्रयोग के दवारा प्रयोग क्रमशः 40, 80 और 120 मिलीग्राम / एल तक सांद्रता के जल में डीएनटी और टीएनटी के इलाज के लिए अज़ोला मिक्रोफिला का उपयोग किया गया। ए. मिक्रोफिला में डीएनटी और टीएनटी को 80 पीपीएम के सांद्रता तक विघटित करने की क्षमता होती है जो की १० दिनों में प्राप्त हुई । 80 मिलीग्राम / एल से अधिक उच्च सांद्रता, ए. मिक्रोफिला के विकास और प्रसार के लिए विषाक्त पाया गया है। मिट्टी के जैव चिकित्सा / शोधन अध्ययन में, जैव ईंधन फसलों जेट्रोफा कर्कस और रिसीनस कम्युनस का मूल्यांकन 100 से 2000 मिलीग्राम / किग्रा तक की सांद्रता में डीएनटी और टीएनटी दोनों के इलाज के लिए किया गया था। दोनों चुनें हुआ पौधों के दवारा, 365 दिनों में 1000 एमजी / किग्रा (> 95% हटाने) की मिट्टी की मात्रा तक डीएनटी और टीएनटी हटाने में बेहतर था। डीटीटी और टीएनटी के लिए जटरोफा कर्कस के दवारा निकासी की क्षमता 1000>

500> 200> 100> 1500> 2000 मिलीग्राम / किग्रा के क्रम में पाया गया है। डीएनटी (97%) और टीएनटी (96%) की अधिकतम कमी 1000 मिलीग्राम / किग्रा मात्रा वाली मिट्टी में और न्यूनतम कमी डीएनटी और टीएनटी दोनों में 2000 मिलीग्राम / किग्रा मात्रा वाली मिट्टी में पाया गई है। रिसिनस कम्युनिस के संबंध में, संशोधन का क्रम क्रमशः डीएनटी और टीएनटी दोनों के लिए 500> 200> 100> 1000> 1500> 2000 मिलीग्राम / किग्रा पाया गया है।

वर्तमान अध्ययन में डीएनटी और टीएनटी के संशोधन के लिए स्यूडोमोनस मोसेली की सकारात्मक क्षमता का पता चला है। अज़ोला माइक्रोफ़िला की क्षमता जलीय पौधे की प्रजाति के रूप में व्यवहार्यता डीएनटी और टीएनटी सांद्रता के लिए 80 मिलीग्राम / एल तक सिद्ध हुई है। संभावित जैवऊर्जा पौधे जट्रोफा कर्कस और रिसिनस कम्युनिस के दवारा मिट्टी में डीएनटी और टीएनटी के 1000 एमजी / किग्रा के स्तर का संसोधन मिट्टी में सफलता से प्राप्त हुआ।

CONTENTS

DISCRIPTION	PAGE NUMBER
Certificate	
Acknowledgements	i
Abstract	iii
Contents	vi
List of figures	xii
List of tables	xv
Abbreviation	xvii
Chapter 1. Introduction	
1.1. Introduction and background	2
1.2. Remediation of explosive contaminants	2
1.2.1 Treatment technologies for waste solids / sludge containing explosives	3
1.2.1.1. Open dumping:	3
1.2.1.2. Combustion based techniques	3
1.2.1.3. Soil chemical treatment.	3
1.2.2 Treatment technologies for wastewater containing explosives:	4
1.2.2.1. Alkaline hydrolysis	4
1.2.2.2. Advance oxidation process.	4
1. 2.2.3. Activated carbon adsorption.	4
1.3. Technology gap in treating explosive contaminated soil and water media:	5
1.4. Objective	6
1.5. Refrences	6
Chapter 2: Site characterization and adsorption study of target contaminants DNT and TNT on soil:	
2.1. Introduction and literature review	13
2.1.1. Site Characterization study of soil and water contaminated with explosive waste	13

2.1.2. Adsorption of explosive and importance of the the site characterization study. 14
2.1.3.Environmental Fate and mass transport of DNT and TNT in to the soil 14
2.2. Material and method 15
2.2.1.Environmental site assessment of the sites taken-up in the present study 15
2.2.1.1.Collection of soil sample from industrial facility 16
2.2.1.2. Characterization of samples collected from Site I and Site II 16
2.2.1.3. Instrumentation parameters. 17
2.2.2. Equilibrium and kinetic studies on the adsorption of DNT(2,4- dinitrotoluene) and TNT (2, 4, 6-trinitrotoluene) on garden soil 17
2.2.2.1 Adsorbate. 17
2.2.2.2. Adsorbent (soil) and its characterization 18
2.2.2.3. Experimentation procedure 18
2.2.2.4. HPLC analysis 18
2.2.2.5. Adsorption Equilibrium studies 18
2.2.2.5.1.Langmuir Adsorption Isotherm for TNT 20
2.2.2.5.2.Freundlich Adsorption isotherm for TNT 21
2.2.2.5.3.Dubinin–Radushkevich(D-R) isotherm model for TNT 22
2.2.2.5.4. BET model for TNT 23
2.2.2.5.5. Temkin adsorption isotherm for TNT 24
2.2.2.5. Calculation for Standard error/ standard deviation 25
2.3. Result and discussion. 25
2.3.1. Physicochemical analyses of soil and water samples at selected sites. 25
2.3.2. Kinetics of the Sorption of DNT and TNT from the solution phase on to the soil 27
2.3.3. Equilibrium studies for the sorption of TNT from the solution phase on to the soil. 29
2.3.3.1. Langmuir model for TNT 31
2.3.3.2. Freundlich model for TNT adsorption. 32
2.3.3.3. BET model for TNT adsorption 33
2.3.3.4. DR-model for TNT adsorption 33
2.3.3.5. Temkin model for TNT adsorption 34
2.3.4. Adsorption isotherms modeling for sorption of DNT into soil. 37

2.3.4.1. Langmuir model for DNT adsorption in soil 39
2.3.4.2. Freundlich model for DNT adsorption in soil 3940
2.3.4.3.BET model for DNT adsorption in soil 39
2.3.4.4. DR model for DNT adsorption in soil 39
2.3.4.5. Temkin model for DNT adsorption in soil 40
2.5. Conclusion 41
2.6. References 42

Chapter-3: **Microbial remediation study of DNT and TNT in soil and aqueous phase using selected microbes isolated and identified from an actual explosive contaminated site:**

3.1.Introduction and literature review 53
3.1.1. Microbial identification by 16S r RNA gene sequencing 54
3.1.2.Microbial remediation of water contaminated with explosive chemicals 55
3.1.3. Microbial remediation of soil contaminated with nitro aromatic contaminant 57
3.2. Materials and method 60
3.2.1.1.Collection of soil and water sample from explosive manufacturing facility 60
3.2.1.2. Isolation and identification of native microbes from actual explosive contamina 60
ted site.
3.2.1.3. Procedure for Serial Dilutions 61
3.2.1.4. Conservation of isolate 61
3.2.1.5. Identification of isolated microbes. (Molecular systematic analysis: DNA extraction and PCR amplification) 62
3.2.1.5.1. DNA extraction 62
3.2.1.5.2. Protocol 62
3.2.1.6. PCR amplification, sequencing of the 16S rDNA and identification (work carried out in collaboration with CSIR- IMTECH, Chandigarh). 62
3.2.1.7.Sequencing of 16S rDNA 63
3.2.1.8.Identification through EzTaxon server 64
3.2.2. Biodegradation experiments 65

3.2.2.1.Chemicals 65
3.2.2.2. Microbial inoculation experiments 65
3.2.2.3. Aqueous phase biodegradation studies. 65
3.2.2.3.1.Instrumentation and analysis 66
3.2.2.3.2.Microbial growth monitoring 66
3.2.2.3.3.Physicochemical characterization of contaminated water solution 66
3.2.2.3.4. DNT and TNT biodegradation –Reaction kinetics 66
3.2.3.Soil biodegradation study/Soil preparation for biodegradation and removal of DNT and TNT from Soil 67
3.2.3.1. Physicochemical characteristics of soil 67
3.2.3.2. Immobilization of bacteria. 68
3.2.3.3. Estimation of CFU in soil 69
3.2.3.4. Extraction and analysis of DNT and TNT by HPLC. 69
3.2.3.5.Calculation of standard error / deviation 70
3.3. Results and discussion. 70
3.3.1. Isolation and identification of native microbes from actual explosive contaminated site 70
3.3.1.1. Isolation of bacteria 70
3.3.1.2. Identification of bacteria - Phylogenetic analysis 71
3.3.2. Aqueous phase studies on DNT and TNT biodegradation 76
3.3.2.1.Effect of DNT and TNT on bacterial growth in suspension cultures 76
3.3.2.2.Biodegradation of DNT and TNT by selected bacterial isolates 78
3.3.2.3.Reaction kinetics of DNT and TNT biodegradation 85
3.3.2.4. Physicochemical parameters 88
3.3.2.4.1 pH content 89
3.3.2.4.2. Nitrate Nitrogen content 91
3.3.3.Microbial degradation of DNT and TNT from soil using selected microorganism *Pseudomonas mosselii* 91
3.3.3.1 DNT and TNT degradation in soil 91
3.3.3.2. Soil physicochemical parameters 94
3.3.3.2.1.Soil pH 94

3.3.3.2.2.Soil electrical conductivity 95
3.3.3.2.3.Soil nitrate nitrogen 95
3.3.3.3.Total microbial density 96
3.4. Conclusion 97
3. 4.1. Aqueous phase biodegradation study by free cell culture of selected native microbes 97
3. 4.2. Soil degradation of DNT and TNT by *Pseudomonas mosselii* 98
3.5. References 99

Chapter 4: Phyto-remediation of DNT and TNT in soil and aqueous phase using selected biofuel crops:

4.1. Introduction and Literature review 113
4.1.1.Literature review on the application of *Azolla microphylla* for phytoremediation 115
4.1.2.Literature review on the application of *Jatropha curcus* for phytoremediation 116
4.1.3.Literature review on the application of *Ricinus communis* for phytoremdiation 118
4.2. Materials and Methods. 119
4.2.1. Chemicals 120
4.2.2.Aqueous phase studies 120
4.2.2.1.Experimental set up 120
4.2.2.2.Analysis of physicochemical parameters and HPLC analysis 120
4.2.2.3.Withering studies 121
4.2.3.Phytoremediation of DNT and TNT from soil using *Jatropha-curcas* and *Ricinus communis* 121
4.2.3.1. Green house Pot culture experiment using *Jatropha curcus* and *Ric -inus communis.* 121
4.2.3.2. Soil sampling, Extraction and analysis of DNT and TNT in soil 121
4.2.3.3. Physicochemical characteristics of soil 124
4.2.3.4. Monitoring of plant growth parameters 124
4.2.3.4.1. Biometric observations 124
4.2.3.4.2. Bio-chemical analyses of plant 125

4.2.3.4.3.Pigment Concentration 125
4.2.3.4.4.Nitroreductase activity 126
4.2.3.4.5. Calculation of standard error / deviaiton 126
4.3.Results and discussion 126
4.3.1. Phytoremediation of DNT and TNT from water using *A.microphylla* 126
4.3.1.1. Analysis of physicochemical parameters 126
4.3.1.1.1. pH content 126
4.3.1.1.2. Nitrate nitrogen content of water sample 127
4.3.1.2. DNT and TNT degradation by *A.microphylla* 128
4.3.1.3. Withering of *Azolla* in aqueous phase 129
4.3.2.Phytoremediation of DNT and TNT using *Jatropha curcus* and *Ricinus communis* 131
4.3.2.1. DNT and TNT degradation study by using *Ricinus communis* and *Jatropha curcas* 131
4.3.2.2. Change in soil physicochemical parameters treated with DNT and TNT 138
4.3.2.2.1.Nitrate nitrogen 138
4.3.2.3.Biometric observations 139
4.3.2.4. Monitoring of selected biochemical parameters 143
4.3.2.4.1.Cotents of chlorophyll a,b, total chlorophyll and carotenoid in leaves of *Jatropha curcas* and *Ricinus communis* 143
4.3.2.4.2. Nitroreductase activity 146
4.4.Conclusion 148
4.5.Refrences 150

Chapter 5 :Conclusion 169
Apendix I: Publications and presentations based on thesis
Appendix II: Brief Biodata of Author

List of Figures

Figure no.	Details .	Page no.
2.1	TNT adsorption in different doses of soil	28
2.2	DNT adsorption in different doses of soil	28
2.3	Linearized form of sorption equilibrium models for TNT adsorption in soil (a) Langmuir Model, (b) Freundlich Model, (c) D-R model, (d) BET model, (e) Temkin model	30
2.4	Linearized form of sorption equilibrium models for DNT adsorption in soil (a) Langmuir Model, (b) Freundlich Model, (c) D-R model, (d) BET model, (e) Temkin model	38
3.1	Serial dilution plate technique	61
3.2	Agar cubes	68
3.3	Addition of Agar cubes in soil.	68
3.4	Pot culture study for microbial remediation of DNT and TNT from soil	69
3.5	Microbial remediation study of DNT and TNT in aqueous phase	69
3.6	Neighbour-joining tree based on 16S rRNA gene sequences showing relationships of isolate $S4A^T$ with closely related species of genus *Brachybacterium.* Bar 0.01 indicates substitutions per nucleotide position. *Nesterenkonialutea* (YIM 70081^T) was used as an out group.	72
3.7	Neighbor-joining tree based on 16S rRNA gene sequences showing relationships of isolate $S3A^T$ with closely related species of genus 22 Bacillus. Bar 0.01 indicates substitutions per nucleotide position. Domibacillusindicus ($SD111^T$) was used as an out group.	73
3.8	Neighbor-joining tree based on 16S rRNA gene sequences showing relationships of isolate W1-$P5^T$ with closely related species of genus *Pseudomonas.* Bar 0.005 indicates substitutions per nucleotide position. *Azomonasagilis* ($NCIB11693^T$) was used as an outgroup.	74
3.9	Neighbor-joining tree based on 16S rRNA gene sequences showing relationships of isolate S5-$P1^T$ with closely related species of genus *Delftia*. 24 Bar 0.005 indicates substitutions per nucleotide position. *Polaromonasaquatica* $(CCUG\ 39402)^T$ was used as an out group.	75
3.10	Growth pattern of isolated bacteria viz., *Pseudomonas mosselii* (PM) *Delftia lacustris*(DL) ,*Brachybacterium paranconglomeratum* (BP 26 and *Bacillus infantis* (BI) in different TNT concentration, a (40mg/L),b(80mg/L), c (120mg/L)	77
3.11	Growth pattern of isolated bacteria viz., *Pseudomonas mosselii* (PM) *Delftia lacustris* (DL), *Brachybacterium paranconglomeratum*(BP), 27 and *Bacillus infantis* (BI) in different DNT concentration, a (40mg/L), b(80mg/L), c (120mg/L)	78
3.12	Degradation of TNT by free cell culture of bacterial isolatesviz., *Pseudomonas mosselii* (PM) *Delftia lacustris* (DL)*Brachybacterium*	80

	paranconglomeratum(BP),and *Bacillus infantis* (BI) in different TNT concentrations:(a) 40mg/L,(b) 80mg/L, (c) 120mg/L	
3.13	Degradation of DNT by free cell culture of bacterial isolates viz., Pseudomonas mosselii (PM) *Deltfia lacustris*(DL)*Brachybacterium paranconglomeratum* (BP),and *Bacillus infantis* 30 (BI) in different DNT concentrations:(a) 40mg/L, (b) 80mg/L, (c) 120mg/L .	81
3.14	Natural degradation of DNT and TNT in control	82
3.15	Overlaid HPLC chromatograms showing DNT and TNT removal by *Pseudomonas mosselii*	82
3.16	Overlaid HPLC chromatograms showing DNT and TNT removal by *Delftia lacustris*	83
3.17	Overlaid HPLC chromatograms showing DNT and TNT removal by *Bacillus infantis*	83
3.18	Overlaid HPLC chromatogram showing DNT and TNT removal by *Brachybaterium paranconglomeratum*	84
3.19	Model of TNT transformation and entrapment by bacterial cell.	85
3.20	First order kinetic model for degradation of 120 ppm TNT concentration by using free cell culture of selected microbe	87
3.21	First order kinetic model for degradation of 120 ppm DNT concentration by using free cell culture of selected microbe	88
3.22	Removal of TNT from soil by immobilized cells of *Pseudomonas mosselii*	93
3.23	Removal of DNT from soil by immobilized cells of *Pseudomonas mosselii*	93
3.24	Overlaid HPLC chromatogram showing DNT and TNT reduction from soil by using *Pseudomonas moselli.*	94
4.1	Soil with TNT solution preparation	122
4.2	Contaminated soil solution preparation	122
4.3	Planting of trees in contaminated soil	122
4.4	Soil Remediation study	122
4.5	Soil and contaminated TNT solution	123
4.6	Contaminated soil solution preparation	123
4.7	Soil contaminated with DNT and TNT	123
4.8	Contaminated soil solution	123
4.9	Pots prepared for study	123
4.10	Set up of phytoremediation study	123
4.11	Withering of *Azolla* biomass, Control	130
4.12	Withering of *Azolla* biomass in 40 ppm (a)TNT concentration (Initial) (b)TNT concentration (final)	130
4.13	Withering of*Azolla* biomass in 80 ppm(a)TNT concentration (Initial) (b)TNT concentration (final)	130
4.14	Withering of *Azolla* biomass in 120 ppm(a) TNT concentration (Initial) (b)TNT concentration (final)	130
4.15	Pathways of organic contaminant transformation in plant cells	133
4.16	Reduction in concentration of DNT in soils planted with *Jatropha curcas*	135

4.17	Reduction in concentration of TNT in soils planted with *Jatropha curcas*	136
4.18	Reduction in concentration of DNT in soils planted with *Ricinus communis*	136
4.19	Reduction in concentration of TNT in soils planted with *Ricinus communis*	137
4.20	Overlay of Chromatogram showing TNT reduction in soil by using *Jatropha curcas* in 1000 ppm concentration.	137
4.21	Overlay of Chromatogram showing DNT and TNT reduction in soil by using *Ricinus communis* in 500 ppm concentration.	138
4.22	Shoot length of *Jatropha curcas* grown in soils treated with varying concentration of DNT and TNT.	141
4.23	Number of leaves of *Jatropha curcas* grown in soils treated with varying concentrations of DNT and TNT	141
4.24	Shoot length of *Ricinus communis* grown in soils treated with varying concentrations of DNT and TNT	142
4.25	Number of leaves of *Ricinus communis* grown in soils treated with varying concentrations of DNT and TNT	142
4.26	Carotinoids in leaves of *Jatropha curcas* plant species.	145
4.27	Carotinoids in leaves of *Ricinus communis* plant species	146

List of Tables

Table no.	Details	Page no
2.1	Soil and water samples collected from different zones of TNT manufacturing site	16
2.2	HPLC operating parameters for different explosive components	17
2.3	Isotherm models used for DNT and TNT adsorption and their properties	19
2.4	Concentration of TNT and Nitrate nitrogen in soil samples at selected site.	26
2.5	Physichochemical analyses of soil and water samples at selected site.	26
2.6	Lifetime Health Advisory of explosive nitrobodies for Drinking Water	27
2.7	Risk based concentrations of explosive nitrobodies in soil	27
2.8	Equilibrium Isotherm parameters for TNT adsorption in CFEES soil.	31
2.9	Sorption isotherm parameters for the sorption of TNT onto the soil	34
2.10	Equilibrium isotherm parameters for DNT adsorption in CFEES soil.	39
2.11	Equilibrium isotherm parameters for DNT adsorption onto the soil.	40
3.1	Componenets of PCR amplification	63
3.2	The purified DNA was used for sequencing of either partial or full 16S rDNA.	64
3.3	Colony Forming units of Explosive contaminated site.	70
3.4	Phylogenetic tree.	71
3.5	Degradation rate of of 120 ppm TNT by free cell culture of selected microbes, kinetic equations degradation rate constant(k),R^2 ,Degradati -on half life ($t_{1/2}$), and percentage degradation (%D).	87
3.6	Degradation rate of 120 ppm DNT by free cell culture of selected microbes, kinetic equations degradation rate constant (k),R^2 ,Degradat-ion half life ($t_{1/2}$),percentage degradation (%D).	88
3.7	Changes in pH in water during the process of microbial remediation	89
3.8	Changes in nitrate-nitrogen content in water during the process of microbial remediation by *Pseudomonas moselii* (Mean of three replicates ± SD)	89
3.9	Changes in nitrate-nitrogen content in water during the process of microbial remediation by *Brachybacterium paranconglomeratum* (Mean of three replicates ± SD)	90
3.10	Changes in nitrate-nitrogen content in water during the process of microbial remediation by *Delftia lacustris* (Mean of three replicates ± SD)	90
3.11	Changes in nitrate-nitrogen content in water during the process of microbial remediation by *Bacillus infantis.* (Mean of three replicates ± SD)	90
3.12	Physicochemical parameters of soil	96

4.1	pH values observed during the aqueous phase phytoremediation of DNT and TNT by *A.microphylla* (Mean of three replicates ± SD)	127
4.2	Concentration of nitrate nitrogen (mg/L)observed during the aqueous phase phytoremediation of DNT and TNT by *A.microphylla* (Mean of three replicates ± SD)	128
4.3	DNT and TNT degradation by *Azolla sp*.	131
4.4	Withering study	131
4.5	Nitrate – nitrogen content of DNT and TNT contaminated soil treated with*Jatropha curcas* plant species	139
4.6	Nitrate – nitrogen content of DNT and TNT contaminated soil treated with *Ricinus communis* plant species	139
4.7	Chlorophyll a,b and total chlorophyll content of leaf of *Jatropha curcas.*	143
4.8	Chlorophyll a,b and total chlorophyll content in *Ricinus communis* plant sample.	144
4.9	Nitroreductase activity in Jatropha curcas plant leaf sample	146
4.10	Nitroreductase activity in *R. communis* plant leaf sample	147

Abbreviations

ACN	Acetonitrile
AAP	Army ammunition plant
AWWA	American water works association
BTEX	Benzene, toluene, ethylbenzene, and xylene
CFU	Colony Forming Unit
DNT	2,4-dinitrotoluene
EC	Electrical conductivity
EPA	Environmental Protection Agency
ETP	Effluent treatement plant
US EPA	U.S. Environmental Protection Agency
HMX	High Melt Explosive
HPLC	High Pressure Liquid Chromatography
MSM	Minimal Salt Medium
NA	Nutrient Agar
NEDD	n- Naphthyl ethylenediamine dihydrochloride
NRA	Nitroreductase activity
RDX	Royal Demolition Explosive
O. D.	Optical density
PDA	Photo Diode Array Detector
PPM	Parts per million
PCB	Polychlorinated biphenyl
PCR	Polymerase Chain Reaction
RDX	Royal Demolition Explosive
SVOC	Semivolatile organic compound
TNB	1,3,5-trinitrobenzene
TNT	2,4,6-trinitrotoluene
TSA	Tripticase Soya Agar
TSB	Trypticase Soya Broth