Degradation of RDX and HMX in contaminated sludge and water with indigenous microbes isolated from an explosive contaminated site

Ph.D. Thesis

By

Arjun Meda

DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

AUGUST 2022

Degradation of RDX and HMX in contaminated sludge and water with indigenous microbes isolated from an explosive contaminated site

A THESIS

Submitted in partial fulfillment of the requirements for the award of the degree
of
DOCTOR OF PHILOSOPHY

by
Arjun Meda

DEPARTMENT OF BIOSCIENCES AND BIOMEDICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY INDORE
AUGUST 2022

INDIAN INSTITUTE OF TECHNOLOGY INDORE

I hereby certify that the work which is being presented in the thesis entitled "**Degradation of RDX and HMX with indigenous microbes isolated from an explosive contaminated site**" in the partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** and submitted in the **Department/School Of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from **July 2017 to August 2022** under the supervision of **Dr. Kiran Bala, Associate Professor, Biosciences and Biomedical Engineering, Indian Institute of Technology Indore** and **Dr. Pritam Sangwan, Scientist 'E', Center for Fire, Explosive and Environmental Safety, Defense Research and Development Organization (DRDO), Delhi**

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

7/08/22

signature of the student with date

Arjun Meda

This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

7/8/22

Signature of Thesis Supervisor #1 with date

Dr. Kiran Bala

07/08/22

Signature of Thesis Supervisor #2 with date

Dr. Pritam Sangwan

Arjun Meda has successfully given his/her Ph.D. Oral Examination held on.....

Signature of Thesis Supervisor #1 with date

Dr. Kiran Bala

Signature of Thesis Supervisor #2 with date

Dr. Pritam Sangwan

ACKNOWLEDGEMENTS

This thesis is a result of much hard work and dedication. This journey has been supported by so many important people without whom this work wouldn't have seen the light of the day. First and foremost, I would like to thank Lord Shiva for his blessings and for bestowing all the strength that helped me get through the journey. I want to thank my supervisors from the bottom of my heart. Dr. Kiran Bala and Dr. Pritam Sangwan, as they invested in me their valuable time and immense knowledge and gave me the pleasant opportunity to develop my career under their guidance. Everything they have done for me has been admirable. I thank them for all their support, commitment, thoughtful debates, and suggestions during my Ph.D. tenure. I am grateful for their determination and endurance that allowed me to be productive and focused during a tough period.

Besides my supervisors, I would like to thank my PSPC committee members: Dr. Mirza S. Baig and Dr. Amrendra K. Singh for their insightful comments and encouragement, but also for the hard questions which incited me to widen my research from various perspectives. I would also like to thank Prof. Amit Kumar, Head of the department, for his advice and continuous support. I appreciate the direct and indirect assistance from all the BSBE faculty members in my research work. Further, I would like to acknowledge Prof. Suhas S. Joshi, Director, Indian Institute of Technology Indore, who has been a source of inspiration for me in every manner.

I would like to express my gratitude to the University Grants Commission, Government of India, New Delhi, for funding my research. I would also like to express my gratitude to every member of the BSBE office i.e., to Mr. Arif Patel, Mr. Murphy Bhaskar Ganveer, and Mr. Gaurav Singh, who helped me with my research in some way. I would like to thank the IIT Indore's sophisticated instrumentation center (SIC) for providing instruments for measurements and sample analysis. Additionally, I would want to express my appreciation to the entire IITI community for their ongoing assistance and support.

Additionally, my sincere thanks to the Center for Fire, Explosive and Environmental Safety, DRDO for providing me an opportunity and facilities to accomplish my thesis work. A special mention to Director, Dr. Rajeev Narang for giving me an opportunity to work in the elite organization. I thank him for his constant support and encouragement throughout my research work. I would also like to thank Dr. P.K Rai, Head, EnSG, Dr. Meenakshi Gupta, AD, CFEES, and Dr. Prasun Kumar Roy Scientist 'F', Dr. Mary Celin, Scientist 'F', Dr. V.K Bind, Scientist 'E' and Dr. Shalini Anand, Scientist 'E' for their continuous support and words of encouragement that helped in the smooth running of my work. I would also like to thank the Institute of Microbial Technology (IMTECH), Chandigarh for helping in isolation and identification of microbes.

I am deeply indebted to my friends and colleagues at Algal EcoTechnology & Sustainability Group, IIT Indore (Dr. Vishal Anand, Dr. Atreyee Ghosh, Dr. Tonmoy Ghosh, Mr. Deshmukh Suchit Ashokrao, Mr. Mrinal Kashyap, Ms. Kanchan Samadhiya, Ms. Rimjhim Sangtani, Ms. Palak Saket, Mr. Rahul Chouhan, Mr. Ms. Anshul Kaushik and Dinesh Paridha). In addition, I would like to thank my friends at CFEES, DRDO (Ms. Anchita kalsi, Ms. Pallavi Bhanot, Ms. Shilpi Nagar, Ms. Jyoti Lamaba, Ms. Kirty Sharma, Ms. Shruti Kaushik, Ms. Lavi Dhiman, Ms. Pooja, and Mr. Naveen Yadav) and all the friends from around the world who have joined me along this journey inside and outside the academic world

Last but not least, my deepest love and gratitude to my father Late Sri Magan Singh Meda, and my mother Mrs. Shakuntala Meda whom I owe absolutely everything: thanks for their infinite love, support, hard work, and sacrifices. Thanks to my beloved siblings Mr. Vishal Meda, and Ms. Priya Meda for being my motivation and giving unconditional love.

Arjun Meda

DEDICATION

This thesis is dedicated to my parents and

and Lord Shiva

SYNOPSIS

Introduction

Explosives are simply defined as substances that, when subjected to chemical and thermal shock, produce a significant amount of heat, gas, radiation, and shock waves [1,2]. Due to the various advantages, they offer, explosives have garnered a lot of attention over the years. Humans have used explosives for a variety of purposes, such as cannon propellant, mining, defense, and rocket propulsion. During testing, manufacturing, transport, and use of these explosives their contamination in the environment has increased significantly. A high amount of RDX and HMX contamination in soil and water has been observed all over the world [3–7]. Nearly, 1900 mg/kg and 900 mg/kg of RDX and HMX were present in the soil at the Louisiana Army Ammunition plant [8]. It was also observed that up to 3 mg/L of HMX concentration was present in the wastewater effluent [9]. RDX and HMX contamination can be a major threat to the environment and humans. Both RDX and HMX are toxic to humans; they can cause seizures, convulsions, a decrease in body weight, neuromuscular toxicity, dilation of kidney tubules, liver lesions, etc., [10–15]. United states environmental protection agency (USEPA) has categorized RDX as a group 'C' carcinogen [16]. According to Agency for Toxic Substances and Disease Registry, USA, animal studies suggested RDX causes neurological, reproductive, and kidney damage [17]. However, HMX is classified as non-carcinogenic to humans but can cause convulsions, histopathological liver lesions, dilation of kidney tubules, mydriasis, change in body mass, etc. in mammals [15,18,19]. As both the RDX and HMX are toxic to the environment there is a need to remediate the contaminated sites. Various treatment methods have been proposed for RDX and HMX removal such as incineration, alkaline hydrolysis, adsorption, UV-radiation, oxidative treatment, reverse osmosis, chemical hydrolysis, etc [1,20]. However, these physical and chemical treatment methods have some

drawbacks such as a lot of consumption of energy, and the generation of toxic and un-disposable waste, which encourages the search for cost-efficient and eco-friendly technology. The microbial remediation technology has gained much attention in recent years. Various microbes such as *Janibacter cremeus*, *Kinneretia asachharophila*, *Arthrobacter subterraneus*, *Bacillus aryabhattai*, *Clostridium* sp., *Pelomonas aquatica*, *Paenibacillus aestuarii*, etc., are have been tested to remove RDX and HMX from the contaminated sites [21–24]. Sharma et al. 2020 studied the degradation of RDX under aerobic conditions using a microbial consortium of *Paenibacillus aestuari* and *Arthrobacter subterraneus* and observed an 80.4% reduction in RDX concentration after 12 days [25]. However, Yang et al 2021 studied the effect of HMX on the *Bacillus aryabhatti*. They observed that exposure to HMX leads to a change in the expression of 254 metabolites and the primary metabolic pathway was disoriented in the microbes [26].

However, the degradation efficiency of native microbes is not explored much. So, the goal of the current thesis was to study the degradation potential of different indigenous microbes. To achieve the goal, samples of soil and water were collected from an HMX manufacturing site in North India [27] and the identification and isolation of microorganisms were conducted by the CSIR-Institute of Microbial Technology (CSIR-IMTECH), Chandigarh. Many different microorganisms were isolated and identified from the contaminated samples. Based on some preliminary explosive degradation studies, few potential microbes like *Arthrobacter subterraneus* (MTCC No. 12883, isolate no. S2-TSB-17), *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857), *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859), and *Bacillus sonorensis* (MTCC No. 12855, isolate no. S8-TS) were selected to achieve different objectives. To optimize the degradation process RSM was used which is a uses lower-order polynomial equations for developing, improving, and optimizing a process having numerous variables influencing

the response. The degradation of RDX and HMX in water was optimized using the response surface methodology. Various factors such as initial concentration, microbial inoculum size, and the degradation time were used as an independent factor to optimize the process. Further, the RDX and HMX degradation in soil sludge was carried out within the bioreactor system. The study was planned to explore the potential of native microbes to decontaminate RDX and HMX contamination during the in-vessel composting process.

Objectives

Major objective: Degradation of RDX and HMX in contaminated sludge and water with indigenous microbes isolated from an explosive contaminated site

Minor objectives:

- **Biodegradation of RDX and possible pathway exploration.**
- **Process development for the biodegradation of HMX using response surface methodology.**
- **Bioreactor studies: Upscaled composting of actual RDX and HMX contaminated sludge.**

The current thesis is divided into six chapters. The first chapter provides an overview of RDX and HMX, their classification, fate & transport in the environment, alternative treatment strategies, and microbial remediation techniques. The design of the experiment, various procedures, and analytical strategies employed to achieve the objectives are briefly discussed in chapter 2. Furthermore, the breakdown of HMX and RDX by indigenous bacteria was observed in the aqueous phase and soil (contaminated sludge). The response surface methodology was used to optimize the experiments. The chapters 3 and 4 describe the data created during the breakdown of RDX and HMX. Further, in the follow-up chapter i.e., chapter 5, the soil sludge contaminated with RDX and HMX was

decontaminated using the in-vessel composting technology. A summary of the thesis and a conclusion are presented in the sixth chapter.

Chapter contributions:

Chapter 1 highlighted the history of explosives and their classification based on their chemical composition. Further, in the chapter, it is explained that the environmental fate of RDX and HMX is based on their physical and chemical properties. Also, various treatment technologies are available to reduce RDX and HMX contamination. But due to ecological concerns, the researchers are focusing on eco-friendly and low-cost technologies such as microbial remediation. In this chapter, it is discussed that various microbes are tested to degrade RDX and HMX under aerobic and anaerobic conditions. However, the potential of native microbes is yet to be explored.

Chapter 2 describes the experimental design for each objective. The instrumentation used and analytical methods to measure microbial growth, nitrate release, nitrite release, HMX degradation, RDX degradation, scanning electron microscopy, mass spectroscopy, and compost characterization are briefly described in this chapter. The quantitative analysis of HMX and RDX degradation was observed using HPLC. Further, the breakdown of both the explosives was co-related with the release of nitrite and nitrate, and the different metabolites formed. As the third objective is in-vessel composting of actual soil sludge, compost characterization such as C: N ratio, phosphate, potassium, total organic carbon, and total Kjeldahl nitrogen is also described in this chapter.

In **Chapter 3** two indigenous microbes, *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859), isolated from an explosive manufacturing facility in north India, have been investigated for their potential to degrade RDX in the aqueous medium. Additionally, RDX degradation has been improved in a 15-day study utilizing RSM at concentrations of 20, 40, and 60 mg/L. As shown in Figure 1 the transformation and degradation of the contaminant were shown to be

influenced by several variables, including the initial concentration of RDX, inoculum volume (2, 4, and 6 percent), and time of degradation (5, 10, and 15 days). High-performance liquid chromatography (HPLC) was used to evaluate the samples, and mass spectroscopy (MS) was used to pinpoint the intermediate products. At an initial concentration of 40 mg/L, the maximum RDX removal of 81.6 ± 1.3%and 84.7 ± 0.9% was observed on day 15 for *Bacillus toyonensis* and *Paenibacillus dendritiformis*, respectively. Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), 4-nitro-2,4-diazabutanal, bis(hydroxymethyl)nitramine, and nitrite were found to be intermediate products during the breakdown of RDX. According to the findings, both microorganisms are capable of degrading RDX in an aqueous medium and can be employed to scale up RDX degradation in explosive polluted sites. Furthermore, as HMX is also present as a co-contaminant with RDX, therefore in next chapter the HMX degradation and optimization has been performed.

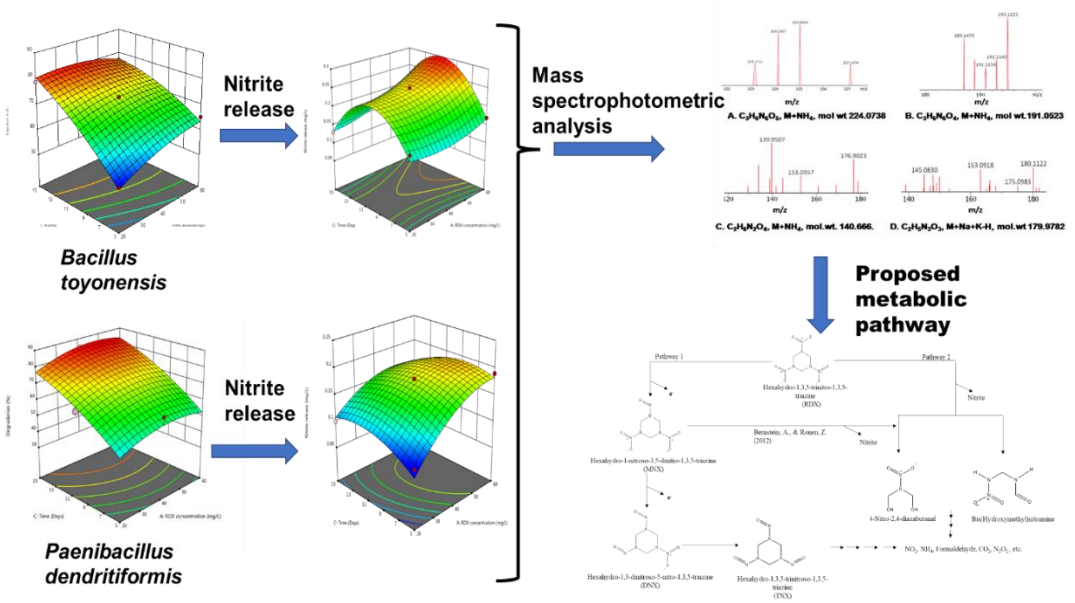


Figure 1. illustration of RDX degradation and optimization with *Bacillus toyonensis* and *Paenibacillus dendritiformis*.

Chapter 4 describes the use of response surface methodology to optimize the variable process parameters for the HMX degradation in the aqueous

phase by a locally isolated bacterial strain, *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857), from an actual HMX-contaminated site in North India. In the current investigation, the link between initial HMX concentrations (2-6 mg/L), microbial inoculum size (2-6%), and degradation time (5-15 days) was established as shown in Figure 2. Results showed that on the fifteenth day, with an initial HMX concentration of 2 mg/L and an inoculum size of 4 %, a total of 87.7 % degradation was obtained. A high regression coefficient (0.9878) further confirmed the predictability of the experimental results. The experiment's estimated concentrations of nitrite and nitrate confirm the HMX cyclic ring degradation. Findings suggest that *Bacillus toyonensis* may be able to break down HMX and be applied to microbial remediation of HMX-contaminated locations. Hence, the results obtained in Chapter 3 and 4 depicts the RDX and HMX degradation potential of native microbes in aqueous phase, therefore, in the upcoming chapter the degradation of both RDX and HMX in contaminated soil sludge has been performed.

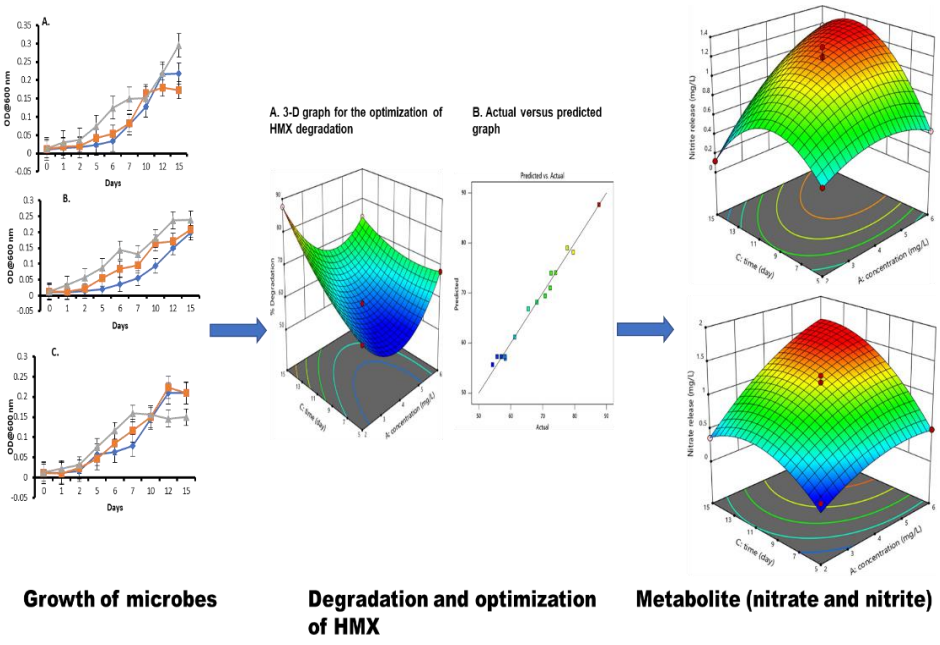


Figure 2. Illustration of different analyses performed during the optimization of the HMX degradation process.

In **Chapter 5** *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (isolate no. S8-TSB-4) were native microbes that were isolated from the same contaminated site and were used in the current study to remediate highly contaminated sludge that contained HMX and RDX. This contaminated site was located in North India. As shown in figure 3 the explosive-contaminated sludge was composted inside the vessels using garden waste and cow manure as bulking agents in 12 separate bioreactors. 78.5% degradation of HMX was observed in reactor no. 2 with *Bacillus sonorensis* having a combination of 10% sludge, 70% cow manure, and 20% garden waste on the 80th day. While researching the breakdown process two secondary metabolites, bis(hydroxymethyl)nitramine and methylenedinitramine, were observed. Similar to this, on the 80th day, RDX degradation of 91.2 % was found in reactor number 11 with a consortium of *Arthrobacter subterraneus* and *Bacillus sonorensis*. Significant nitrate and nitrite ion release were observed during the investigation. It is already known that the breakdown of RDX and HMX results in the emission of nitrite/nitrate ions. The highest nitrate (reactor no. 2) and nitrite (reactor no. 11) release measurements were made on the 50th and 70th days, respectively, and were 24.02±0.05 mg/kg and 30.65±0.99 mg/kg.

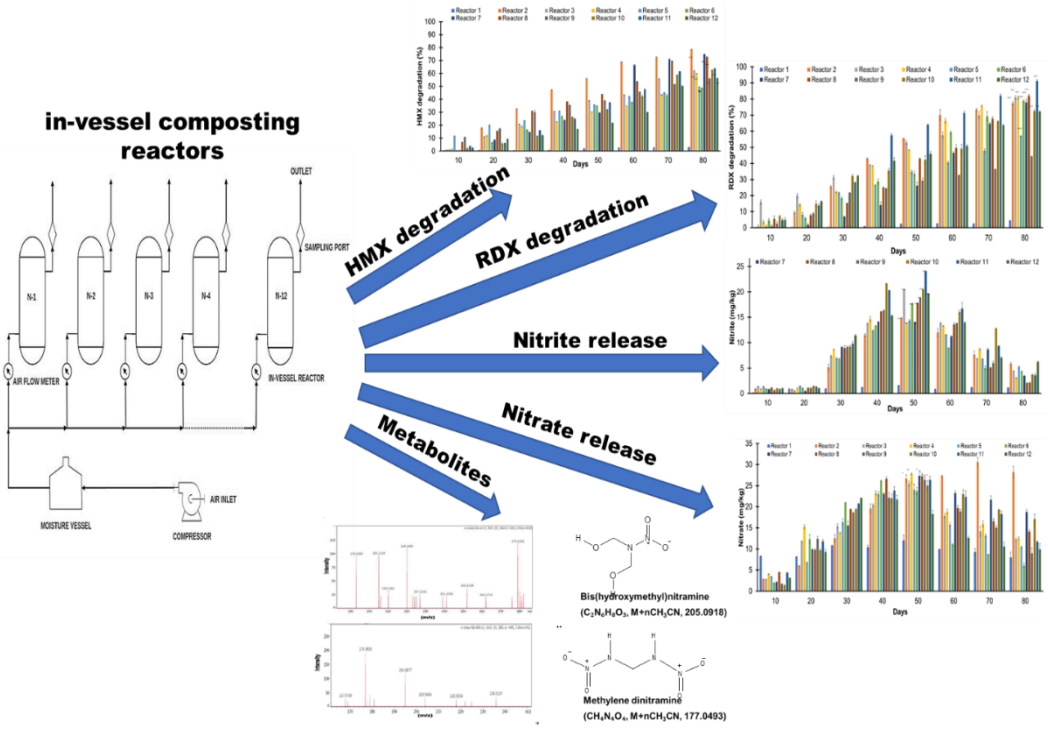


Figure 3. Illustration of in-vessel composting of HMX and RDX contaminated sludge.

Studies using scanning electron microscopy (SEM) confirmed the presence of bacteria attached to solid surfaces. No structural changes in the microbial cells were noticed due to contamination stress. The findings suggest that in-vessel composting supported by native bacterial species could be a promising technique for the treatment of explosively contaminated sludge at contaminated locations.

Chapter 6 concludes the thesis outcomes and lists the future aspects. Treatment of HMX and RDX pollution using native microorganisms isolated from the HMX manufacturing site has been investigated under various circumstances. The following outcomes were observed during the study:

- The highest levels of RDX removal were found on the 15th day for *Bacillus toyonensis* and *Paenibacillusdendritiformis*, respectively, and were 81.6 % and 84.7 %. The breakdown of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1-nitroso-1,5-dinitro-1,3,5-triazine (HNX), 4-nitro-2,4-diazabutanal, bis(hydroxymethyl)nitramine, and nitrite were discovered to produces intermediate compounds. The study's findings suggest that both microorganisms could be used to speed up the breakdown of RDX in explosive-contaminated locations and be able to do it in an aquatic environment.
- According to the results of the current study, there is a positive correlation between the initial HMX concentration, the size of the microbial inoculum, and the rate of degradation. Results showed that at an initial HMX level of 2 mg/L, 87.7 % degradation was achieved on day 15 with an inoculum size of 4 %. According to the study's findings, *Bacillus toyonensis* may be able to break down HMX and be applied to microbial remediation of HMX-contaminated locations.
- During the in-vessel composting study, in reactor number 2, the highest HMX degradation utilizing *Bacillus sonorensis* and a mixture of 10% sludge, 70% cow manure, and 20% garden waste were observed on day 80. The two secondary metabolites

bis(hydroxymethyl)nitramine and methylene dinitramine were discovered while studying the breakdown process. Similar to this, reactor number 11 with consortia of *Arthrobacter subterraneus* and *Bacillus sonorensis* has RDX degradation of 91.2 % on the 80th day. During the research, significant nitrate and nitrite ion emission was seen. On the 50^{th} and 70^{th} days, respectively, the highest nitrate (reactor no. 2) and nitrite (reactor no. 11) release values were taken; they were 24.02 mg/kg and 30.65 mg/kg. The results suggest that treating explosive contaminated sludge in polluted areas through in-vessel composting with the aid of native bacterial species may be a feasible solution.

References:

[1] S. Chatterjee, U. Deb, S. Datta, C. Walther, D.K. Gupta, Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation, Chemosphere. 184 (2017) 438–451.

[2] J.P. Agrawal, High energy materials: propellants, explosives and pyrotechnics, John Wiley & Sons, 2010.

[3] K.A. Thorn, P.G. Thorne, L.G. Cox, Alkaline hydrolysis/polymerization of 2, 4, 6-trinitrotoluene: characterization of products by 13C and 15N NMR, Environ. Sci. Technol. 38 (2004) 2224–2231.

[4] A.L. Juhasz, R. Naidu, Explosives: fate, dynamics, and ecological impact in terrestrial and marine environments, Rev. Environ. Contam. Toxicol. (2007) 163–215.

[5] J.C. Pennington, T.F. Jenkins, G. Ampleman, S. Thiboutot, J.M. Brannon, Distribution and fate of energetics on DoD test and training ranges: Interim Report 3, ENGINEER RESEARCH AND DEVELOPMENT CENTER VICKSBURG MS, 2003.

[6] P. Broomandi, M. Guney, J.R. Kim, F. Karaca, Soil contamination in areas impacted by military activities: A critical review,

Sustainability. 12 (2020) 9002.

[7] E.P. Burrows, D.H. Rosenblatt, W.R. Mitchell, D.L. Parmer, Organic Explosives and Related Compounds. Environmental and Health Considerations, (1989).

[8] B. Clark, R. Boopathy, Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana, J. Hazard. Mater. 143 (2007) 643–648.

[9] S.S. Talmage, D.M. Opresko, C.J. Maxwell, C.J.E. Welsh, F.M. Cretella, P.H. Reno, F.B. Daniel, Nitroaromatic munition compounds: environmental effects and screening values, Rev. Environ. Contam. Toxicol. (1999) 1–156.

[10] B.P. Heerspink, S. Pandey, H. Boukhalfa, D.S. Ware, O. Marina, G. Perkins, V. V Vesselinov, G. WoldeGabriel, Fate and transport of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and its degradation products in sedimentary and volcanic rocks, Los Alamos, New Mexico, Chemosphere. 182 (2017) 276–283.

[11] K.M. Dontsova, S.L. Yost, J. Šimunek, J.C. Pennington, C.W. Williford, Dissolution and transport of TNT, RDX, and Composition B in saturated soil columns, J. Environ. Qual. 35 (2006) 2043–2054.

[12] M.S. Johnson, M.J. McAtee, Characterizing potential for toxicity: Estimating risks to wildlife, in: Wildl. Toxic. Assessments Chem. Mil. Concern, Elsevier, 2015: pp. 3–11.

[13] B. Zhang, X. Pan, RDX induces aberrant expression of microRNAs in mouse brain and liver, Environ. Health Perspect. 117 (2009) 231–240.

[14] N.R. Schneider, S.L. Bradley, M.E. Andersen, Toxicology of cyclotrimethylenetrinitramine: distribution and metabolism in the rat and the miniature swine, Toxicol. Appl. Pharmacol. 39 (1977) 531–541.

[15] C.A. McFarland, M.J. Quinn Jr, M.A. Bazar, L.G. Talent, M.S.

Johnson, Toxic effects of oral hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine in the western fence lizard (Sceloporus occidentalis), Environ. Toxicol. Chem. An Int. J. 28 (2009) 1043–1050.

[16] U. Environmental Protection Agency Federal Facilities Restoration, R. Office, Fact Sheet – Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), (2021) 1–7.

[17] H. Services, Toxicological Profile for RDX, ATSDR's Toxicol. Profiles. (2002). https://doi.org/10.1201/9781420061888_ch137.

[18] D.J. Everett, I.R. Johnson, P. Hudson, M. Jones, HMX: 13 week toxicity study in rats by dietary administration, INVERESK RESEARCH INTERNATIONAL LTD MUSSELBURGH (UNITED KINGDOM), 1985.

[19] R.J. Greenough, P. McDonald, HMX: 14 Day Toxicity Study in Rats by Dietary Administration, INVERESK RESEARCH INTERNATIONAL LTD MUSSELBURGH (UNITED KINGDOM), 1985.

[20] S. Nagar, S. Anand, S. Chatterjee, C.D. Rawat, J. Lamba, P.K. Rai, A review of toxicity and biodegradation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) in the environment, Environ. Technol. Innov. 23 (2021) 101750.

[21] A. Kalsi, S.M. Celin, S. Sharma, S. Sahai, J.G. Sharma, Bioaugmentation for remediation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) contaminated soil using a clay based bioformulation, J. Hazard. Mater. 420 (2021) 126575.

[22] M.A. Khan, A. Sharma, S. Yadav, S.M. Celin, S. Sharma, A. Noureldeen, H. Darwish, Enhancing remediation of RDX-contaminated soil by introducing microbial formulation technology coupled with biostimulation, J. Environ. Chem. Eng. 9 (2021) 106019. https://doi.org/10.1016/j.jece.2021.106019.

[23] C.F. Shen, J. Hawari, G. Ampleman, S. Thiboutot, S.R. Guiot, Enhanced biodegradation and fate of hexahydro-1, 3, 5-trinitro-1, 3,

5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) in anaerobic soil slurry bioprocess, Bioremediat. J. 4 (2000) 27–39.

[24] B. Bhushan, A. Halasz, S. Thiboutot, G. Ampleman, J. Hawari, Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by Clostridium sp. EDB2, Biochem. Biophys. Res. Commun. 316 (2004) 816–821. https://doi.org/10.1016/j.bbrc.2004.02.120.

[25] K. Sharma, P. Sharma, S.M. Celin, P.K. Rai, P. Sangwan, Degradation of high energetic material hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a microbial consortium using response surface methodological approach, SN Appl. Sci. 3 (2021) 1–8. https://doi.org/10.1007/s42452-020-04021-1.

[26] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Biodegradation of octogen and hexogen by Pelomonas aquatica strain WS2-R2A-65 under aerobic condition, Environ. Technol. (United Kingdom). 43 (2022) 1003–1012. https://doi.org/10.1080/09593330.2020.1812731.

[27] A.A.C.A.S. Numbera, Method 8330B nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC), US Environ. Prot. Agency, Washington, DC. (2006).

List of publications

From thesis

1. Meda, A., Sangwan, P., & Bala, K. (2020). Optimization of process parameters for degradation of HMX with *Bacillus toyonensis* using response surface methodology. ISSN 1735-1472 International Journal of Environmental Science and Technology, Springer. DOI 10.1007/s13762-020-02783-0 **(Impact factor 3.463).**
2. Meda, A., Sangwan, P., & Bala, K. (2021). Optimization and Degradation Studies on Hexahydro-1, 3, 5-Trinitro-1, 3, 5-Triazine (RDX) with Selected Indigenous Microbes under Aerobic Conditions. Water, MDPI, 13(9), 1257, DOI 10.3390/w13091257 **(Impact factor 3.530).**
3. Meda, A., Sangwan, P., & Bala, K. (2021). In-Vessel composting of HMX and RDX contaminated sludge using microbes isolated from a contaminated site. Environmental Pollution, Elsevier, Vol. 285, 117394. DOI 10.1016/j.envpol.2021.117394 **(Impact factor 9.988).**

Conference

1. Meda, A., Sangwan, P., & Bala, K. Optimization and transformation studies on hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with selected indigenous microbes under aerobic conditions. **Best paper award** at 5^{th} international conference on Bioenergy, Environment and Sustainable technology (BEST), 2021.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
HMX	Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
USEPA	United states of Environmental protection agency
TSB	Tryptic soya broth
MSM	Minimal salt media
RSM	Response surface methodology
BBD	Box–Behnken designs
ANOVA	Analysis of variance
Cor Total	Correction total
df	Degree of freedom
MNX	Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine
DNX	Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine
ESI	Electron spray ionization
MTCC	Microbial Type Culture Collection
MEDINA	Methylenedinitramine
TKN	Total Kjeldahl Nitrogen
TOC	Total organic carbon
E.C	Electrical conductivity

ACRONYMS

HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-Mass spectroscopy
UV-vis	Ultraviolet visible spectroscopy
SEM	Scanning electron microscope

CHAPTER 1

Chapter 1

1.1 Introduction

Explosives are simply classified as compounds that store a high amount of potential energy in various forms and produce a great amount of heat, gas, radiation, and shock waves when subjected to chemical and thermal shock [1,2]. Explosives have been utilized by humans for a variety of purposes, including defense, mining, rocket propulsion, and cannon propellant. Although the usage of the explosive compound may be traced back to the 5th century AD, Arabs utilized Greek fire, which further evolved into the use of gunpowder in China and then to the development of nitro-glycerine in 1847. In addition, in 1867, Alfred Nobel discovered dynamite. However, during World Wars I and II, the necessity for a stable explosive compound led to the development of explosives like 2,4,6-trinitroglycerin, PETN (Pentaerythritol Tetranitrate), hexogen, 1,3,5-Trinitro-1,3,5-triazinane (RDX/ Royal Demolition Explosive), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, Octogen (HMX/ High Melting Explosive) [1,3].

To date, various explosives are being produced, so there was a need to classify these compounds based on chemical composition for their identification and better understanding [2,4,5]. These different explosives have been categorized into different classes based on chemical composition (Figure 1.1). However, nitrogen-based explosives, on the other hand, are the most commonly utilized explosive. Nitrogen-based explosives have one or more nitro groups linked to the cyclic ring structure [4,5]. It is divided into subcategories based on the type of connection between the nitro-group and the ring structure, such as nitroaromatics, nitramines, nitrite esters, etc. 2,4,6-trinitrotoluene (TNT), picric acid, and methyl-2,4,6-trinitrophenyl-nitramine are examples of nitroaromatic compounds in which the carbon atom of an aromatic ring is directly connected to the nitrite group [6–8].

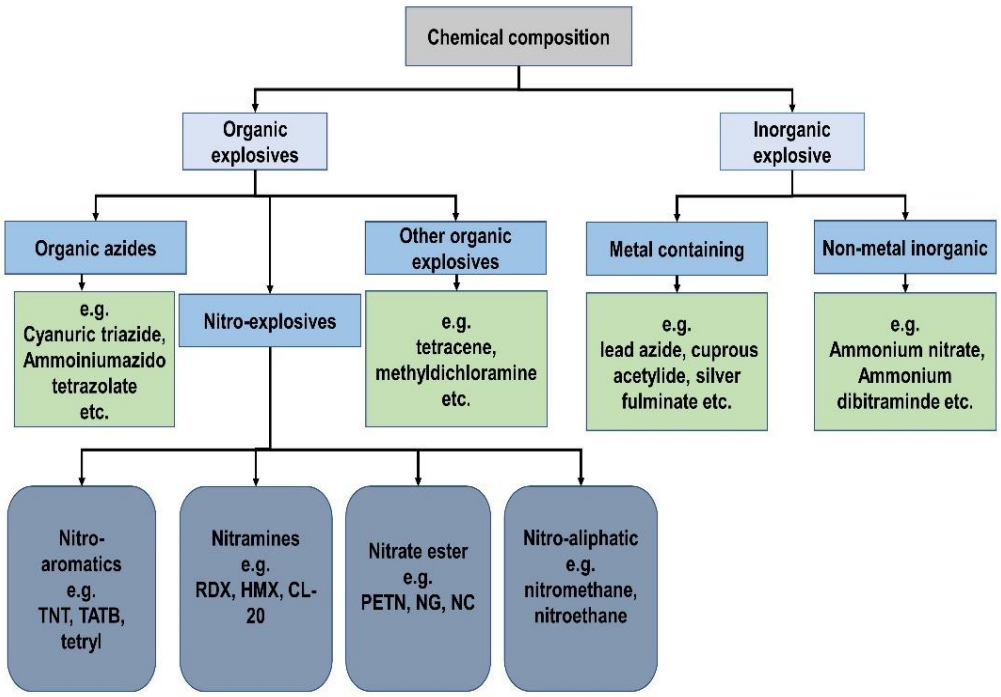


Figure 1.1 Classification of explosives based upon chemical composition.

The nitrogen atom works as an electron acceptor in this family of molecules, allowing it to self-oxidize and release a large amount of energy [9]. Nitramine compounds, such as RDX and HMX, are another type of explosive with a nitrite-nitrogen connection within alicyclic rings. Another is nitrite esters, where the nitrite is bound to the oxygen atom found in the aliphatic chain, such as PETN [4].

However, during world war I & II the production of nitramines such as RDX and HMX increased significantly. During their manufacturing, testing, transport, and use in different activities RDX and HMX causes contamination of the environment. Due to the extensive use, a high amount of RDX and HMX contamination in soil and water has been reported all over the world [10–13,14,15]. Nearly, 1900 mg/kg of RDX and 900 mg/kg of HMX were observed in the soil at the Louisiana Army Ammunition plant [15]. It was also observed that up to 3 mg/L of HMX concentration was present in the wastewater effluent [16]. The major cause of the contamination is an unexploded ordinance, which can lead to the further long-term contamination of soil and groundwater. Also, the waste produced during the manufacturing process of RDX and HMX

can lead to environmental contamination. The fate of these compounds in the environment is mainly dependent upon their physical properties of RDX and HMX.

1.2 Physical properties of RDX and HMX-

Nitramines such as RDX and HMX are the most used explosive compounds due to their high impact, lot of applications, and their stable nature. These compounds are manufactured by a process called modified Bachmann processes [14,17]. This process requires hexamine, glacial acetic acid, ammonium nitrate, and nitrator as raw materials. The only difference between RDX and HMX manufacturing is the temperature at which they are produced i.e., 75℃ and 45℃ for RDX and HMX respectively. The Physico-chemical properties of RDX and HMX are shown in table 1.1. RDX is a white, odourless, and crystalline powder having high stability and produces large amount of energy [18]. It is mostly used in munition testing, hand grenades, rocket propulsion, bomb-making, etc. Further, RDX is sparingly soluble in water (59.7 mg/L), has low vapour pressure (4.0 x 10^{-9} (HSDB 2016), octanal water partition coefficient (0.86) and Henry law constant (2.0 x10^{-11}). Therefore, it has low adsorption in soil and sediments and when released into the environment may exert harmful effects.

Another important explosive is HMX is a white crystalline solid with a high melting point and low water solubility [19]. Similar to RDX, HMX also has low soil adsorption and can travel through soil layers. Further, it is exclusively used for military purposes as plastic bonded explosives, rocket propellent, etc. According to Patterson et al. 1976 B, 1×10^5 kg/month of HMX was produced from 1969 to 1971 in the United States [20]. Kitchens et al. 1978 estimated that 61 kg/day was discharged from the AAP Holston manufacturing facility [21]. Gibbs and Popolato 1980 suggested the maximum concentration of HMX in the air to be 1.5mg/m^3 [22]. It has also low water solubility, henry constant, and vapor pressure. Due to this, HMX also has a high probability to transport deep into the soil and contaminating underground aquifers. Garg et al. 1991 suggested

the movement of HMX is mainly based on the partition coefficient, which is a function of soil organic content [23].

As both the physical properties of RDX and HMX suggest that they can travel through soil barriers and can contaminate groundwater. So, there is a need to assess the environmental risk associated with these contaminants. To assess the risk associated, fate and transport studies are required to be carried out.

1.3 Fate and transport of RDX and HMX

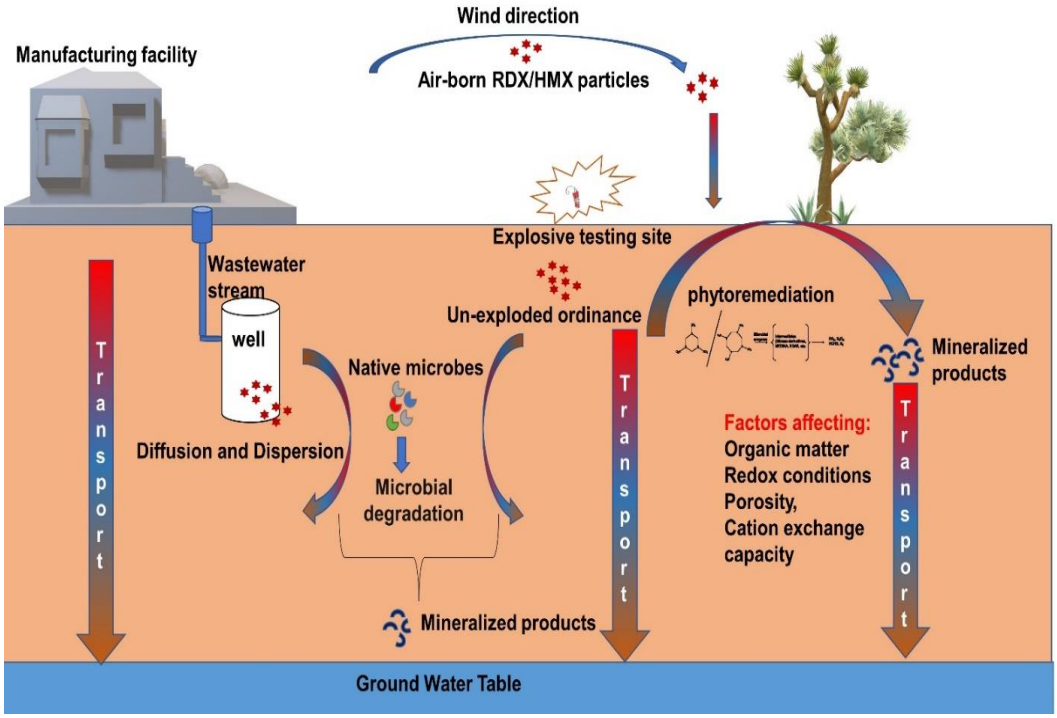


Figure 1.2 Schematic representation of fate and transport of RDX and HMX

Table 1.1 Physico-chemical properties and permissible limits of HMX and RDX

Properties		**RDX**	**HMX**
Synonyms		Cyclonite, hexogen, 1,3,5-Trinitro-1,3,5-triazinane, Cyclonite, Hexogen	Octogen, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, Octogen, Tetramethylene-tetranitramine
Molecular weight		222.12	296.16
State		White, crystalline powder	White, crystalline solid
Boiling point		276°C-280°C	280°C
Melting point		205.5°C	281°C
Solubility (Water)		59.7 mg/L	5-6 mg/L
Octanol-water partition coefficients		0.87	0.16
Henry's law constant (25 °C)		2.0×10^{-11} atm-cm cu/mole	$8.7X10^{-10}$ atm-cu m/mol
Formulations		Semtex A, Semtex H, C-4	plastic bonded explosive, rocket propellent
Analysis method		USEPA 8803 A	USEPA 8803 A
Permissible limits (USEPA)	Drinking water	0.002 mg/L	0.04 mg/L
	Resident soil	6.1 mg/kg	8300 mg/kg
	Industrial soil	28 mg/kg	49000 mg/kg

The fate and transport of RDX and HMX in the environment are depicted in figure 1.2. The understanding of the process of fate and transport of RDX and HMX requires knowing about their behaviour and mobilization in soil and water. As it is used and produced for several military purposes (including UXO), Whenever the RDX or HMX comes in contact with precipitation, they dissolve and infiltrate to the soil and reach the groundwater table [24–27]. Volatility of RDX and HMX is reported low, so they are supposed not to migrate into the atmosphere. However, the aerial transport of nitramine after post-explosion has been

studied. It was observed that the distribution is mainly based on wind direction, wind speed, and distance [28]. Various physical properties and properties of the matrices, such as the presence of cation/anion, organic content, partition coefficient, and distribution coefficient affect the transport of these compounds. The very low distribution coefficients (K_d) and partition coefficients (K_{ow}) of RDX and HMX show that they interact with organic molecules and minerals in the soil [26]. The effect of soil organic content on the distribution coefficient and adsorption was studied earlier. It was observed that the distribution coefficient of both RDX and HMX were higher in organic matter-rich soil (8.7 L/kg and 3.1 L/kg, respectively) rather than the clay-containing soil (3.4 L/kg and 1.2 L/kg, respectively). Also, during the sorption study higher amount of RDX (15%) was found in colloidal fraction rather than HMX (9%). Colloidal transport has an important role in the explosive sorption process [29]. Further, functional groups ($X\text{-}NO_2$, X = Carbon or nitrogen), cations, and anions have an important role in RDX and HMX transport in soil [30]. Sulfate and chloride ions majorly contribute to the transport of RDX and HMX compounds [31]. Also, the effect of exchangeable cations (K^+, NH^{4+}, Ca^{++}) on the sorption of RDX, HMX, and TNT has been studied earlier. It was observed that the presence of cations does not affect the adsorption of RDX and HMX in aquifer soil, however, the sorption of TNT can be increased by many folds [24]. Further, the effect of depth and the organic content/cation exchange capacity on the adsorption of RDX has been explored. It was observed that K_d of RDX at the surface was 0.36 however, at a certain depth it changed to 0.076. This shows that RDX has more tendency to bind at surface soil rather than soil present at a certain depth. It is due to the difference in the amount of organic fraction present surface and depth [32]. The interaction of RDX and HMX with soil has been demonstrated earlier. In a study, the effect of burnt soil and normal soil was studied. It was observed that more RDX and HMX were adsorbed in burnt soil due to a reduction in pores and the presence of charcoal [33]. In another study, transport of RDX and HMX was observed and compared with TNT and DNT in volcanic soil. It was observed that the adsorption of

TNT and DNT (k_f = 2.6 and 2.7 cm^3/g, respectively) was higher as compared to RDX and HMX (k_f = 1.5 and 1.8 cm^3/g, respectively). In contrast, the half-life and mobility of RDX and HMX in the soil were higher than the other two compounds[30].Further, the fate and transport of solute in the soil also depend upon different factors associated with the soil such as porosity, cation exchange capacity, organic matter, etc [34–37].

Further, the adsorption of HMX and RDX on the surface and subsurface was also evaluated using Freundlich equations. Unlike other explosives, Sorption isotherms of both RDX and HMX are liner, which shows a non-specific and non-saturated sorption process [38–40]. A Low K_d value indicates high infiltration capacity through the soil which can lead to groundwater contamination. The isotherms linearity explains that the movement of explosives is not dependent on initial concentration. However, adsorption and limited transformation lead to the long-term equilibrium of the system [41]. Furthermore, to see the transport of RDX from soil to groundwater different mathematical models were applied. To simulate the trend in the movement of RDX at the military facility, ARAMS^{TM} was applied which is the combination of three different mathematical models. It was observed that with a load capacity of 1kg/year for 10 years, the dissolution and degradation of RDX would be very slow in soil and water. Also, this model can help in predicting the spread of RDX and its concentration at different time intervals [42]. Furthermore, the transport of RDX and its degradation products has already been studied earlier in volcanic soil. In column and batch experiments with RDX, it was observed that there was no difference between the RDX and its by-products transport properties (i.e., MNX, DNX, and TNX). The observed values for K_d and retardation factor were in the range of 0-0.70 L/kg and 1.0-1.8 respectively. However, no abiotic degradation was observed within the system [43]. In another saturated column study, the transport of RDX, TNT, and comp B (mixture of RDX and TNT) was checked as compared to each other. It was observed that the mobility of RDX was higher than TNT. However, the mobility of

both the pure compound separately and in comp B was nearly similar. HYDRUS_1D was used to simulate the transport process and it was observed that sorption and dissolution of RDX was the rate-limiting step [44]. In another study, the effect of fractured and pristine soil was also evaluated on the transformation and transport of RDX and HMX. Fractured soil has a higher adsorption affinity for RDX and HMX. Also, the degree of transformation in fractured soil is high, due to the lattice defect of minerals that act as an electron donors to break the ring structure [45]. The fate and transport of RDX and HMX is an important parameter to assess the environmental risk factor associated with both compounds. As RDX and HMX can be toxic to the environment and human health. However, the challenge to assess the risk of explosives to wildlife is to know the exposure limit, toxicity information, and its interpretation.

1.4 Toxicity of RDX and HMX

RDX and HMX have a harmful effect on human health and the environment. The harmful effect of these nitramines has been studied earlier. Most likely they tend to negatively affect human beings, the environment, and wildlife near military bases and manufacturing facilities. Large surface area is being controlled for military operations, which results in overtaking of populated habitates for many wildlife species. According to a United states department of defense report 330 threatened and endangered wildlife species were covered in military installations. Apart from it, during world wars, I and II huge quantities of unexploded explosives and their by-products have been spread all over the war zones [1]. Several governmental agencies all over the world such as USEPA, ASTDR, etc. are working to establish the threshold toxicity limits for different explosives in soil and water [46].

Further united states environmental protection agency (USEPA) has categorized RDX as a group ‘C’ carcinogen [47]. Apart from this, RDX toxicity has been studied in small mammalian species such as rats and rabbits. The major observable effect was seizures in the test species. The

reason behind seizures was studied by Williams et al. 2011, that RDX has a binding affinity for the $GABA_A$ receptor which is a neuronal inhibitory receptor present in the brain. RDX blocks chloride channels present in the receptor, leading to high activity of excitatory neurons which leads to a seizure [48]. RDX is also known to change the gene expression in brain cells [49,50]. In another study to track the fate of RDX in the body, radio-labeled RDX was fed to rats. It was observed that a small amount of RDX was present in the excretory products, also the majority of radioactivity was expired which suggests the metabolism of RDX in the body [51]. The cytochrome P450 present in the liver was responsible for the metabolism of RDX under anaerobic conditions [52]. Also, metabolism under anaerobic conditions was higher than in aerobic conditions due to the competition between oxygen and RDX to bind to the enzyme. The presence of oxygen reduces the binding capacity of RDX to P450. Apart from this RDX can also get accumulated in muscles, brain, liver, and heart [46]. However, to check the toxicity of RDX in birds, amphibians, and reptile various studies have been performed. It was observed that RDX can cause a decrease in body weight, hematological disorder, neuromuscular toxicity, reduced growth rate, reduced survival, and accumulation in the brain [53–55].

However, HMX has been classified as non-carcinogenic to humans, it is reported to be toxic to some animals. It can cause convulsions, histopathological liver lesions, dilation of kidney tubules, mydriasis, change in body mass, etc. in mammals [53,56,57]. However, very few studies have focused on the toxicity of HMX to date. It has been observed that during oral feeding of HMX to rats there was the deposition of HMX in the brain, liver, and kidney [58]. HMX has been observed to affect body weight in many test animals and leads to degeneration of the liver, and an increase in eosinophils in the thymus and liver [57]. As both the RDX and HMX can be toxic to the abiotic and biotic environment there is a need to remediate the contaminated sites. Various treatment methods have been proposed for RDX and HMX removal from contaminated sites.

1.5 Treatment methods of RDX and HMX

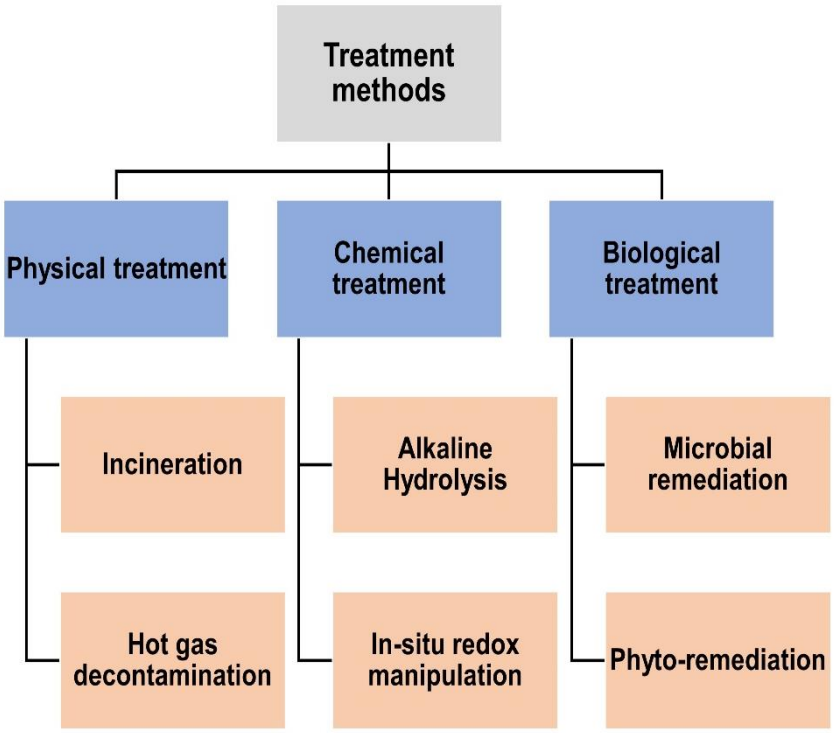


Figure 1.3 Hierarchical representation of commonly employed RDX and HMX treatment techniques.

1.5.1 Abiotic remediation of RDX and HMX

As explained in earlier sections, unplanned and unsystematic disposal and unattended explosives present in the environment can create human and wildlife toxicity concerns. It is necessary to remediate the contaminated sites. Various physical and chemical methods have been proposed and developed such as incineration, alkaline hydrolysis, adsorption, UV-radiation, oxidative treatment, reverse osmosis, chemical hydrolysis, etc [1,59]. Open burning and incineration are the most commonly employed techniques to treat RDX and HMX contamination. In open burning, brisance (heat or mechanical shock) is applied to the contaminants for its remediation, but it can lead to environmental concerns [59,60]. Another method is incineration which has two phases (i.e., incineration and condensation). Skilled workers and proper systems are required to operate the process. Even the final produced such as ash is tough to dispose off and can lead to

environmental contamination. The advanced oxidation process is another explosive treatment method used nowadays[61–63]. Bhanot et al. 2021, showed the removal of HMX from the wastewater by an oxidation process. They observed the complete removal of HMX. The whole degradation process follows the first-order reaction kinetics [64]. Another important method to remediate RDX/HMX contamination is based on the use of iron-dependent catalysts.

1.5.2 Iron-dependent remediation of RDX and HMX

The use of zerovalent iron to remove redox-sensitive pollutants from the contaminated zone has already been established. It generally utilizes Fe^0 barriers to treat contaminants by serving as an electron donor and reducing the chemical structure. During soil slurry treatment of RDX (510 mg/kg) the total removal observed was nearly 99% within 24 hrs. Understanding the Fe^0-dependent removal of RDX requires a better understanding of thermodynamic stability under different Eh and pH conditions of soil [65]. Singh et al. 1999 showed the effect of electrical conductivity (-130 to +150 mV) and pH (2-10) on the RDX degradation in soil and water. They observed that RDX(20 mg/L) was efficiently removed (95%) from the aqueous phase at Eh of -150 mV, 7 pH in 4 hrs [65]. In another study by Scott & Hoag, 2006 the effect of pH on RDX degradation in groundwater was studied with the help of radical produced by Fe (III)-EDTA activated persulphate. It was observed that an acidic environment (3pH) causes the production of radicals. However, at neutral pH, no reactions were observed due to the buffering action of the system. Apart from this, the effect of complex organic species was also explored using RDX degradation [66]. Fe^{II} catechol and thiol complexes were shown that they were efficient in removing RDX and its by-products from the solution. They have also shown that the concentration of ligands and pH of the solution has a positive impact on the degradation process followed by first-order kinetics. The ring cleavage was initiated by a single electron transfer from the complex to RDX [66]. As the shelf life of zero-valent Fe^0 is very low, modifications in the elementary treatment process are required. Gong et al 2015

developed a novel bimetallic zero-valent composite with iron (Fe) and bismuth (Bi). Bi/Fe^0 complex was observed to be effective in the treatment of RDX under anaerobic conditions. Due to the presence of Bi, the electron generation was increased from the complex which supports higher degradation efficiency for RDX degradation [67]. However, the used catalyst is difficult to dispose of after use, so there is a need to explore other disposal methods.

1.5.3 Alkaline hydrolysis of RDX and HMX

Alkaline hydrolysis is another RDX and HMX treatment method. It uses a proton deletion mechanism in the presence of various alkali under different conditions to remediate RDX or HMX from the contaminated soil and water. Hydrolysis of RDX and HMX has been performed at the 10-12 pH range and the formation of different metabolites was reported. Metabolites such as formaldehyde, nitrate, acetic acid, nitrous oxide, etc. have been the end products of the process [68]. Also, the mass balance for the process is proven to be efficient with a recovery rate of 94 and 90 % for carbon and nitrogen. This efficiency was proven to be temperature-dependent and follows pseudo-first-order kinetics [69]. However, treatment with lime RDX and HMX are known to be remediated by 74 and 57 % by the end of 21 days. In another study, the remediation of RDX at 300℃ and pH13 was observed. It was observed that the RDX was remediated completely from the wastewater with the pseudo-first-order rate constant of 16×10^{12} [70]. However, various studies have focused on the remediation of RDX and HMX [23,71–75], but all the processes took place under extreme conditions which require sophisticated instrumentation and also lead to the production of toxic intermediate products. So, the focus of various researchers has been diverted to the development of green technologies such as biological treatment processes.

1.5.4 Bioremediation of RDX and HMX

1.5.4.1 Phytoremediation of RDX and HMX

Phytoremediation is a cost-effective, environmentally friendly technique that is employed to treat various contaminants present in the soil, water, and sediments. It uses processes such as photo-stabilization, rhizo-filtration, phytodegradation, etc. to remediate the contaminants [76–82]. Many plants have been shown to remediate RDX and HMX from the ecosystem. The treatment of HMX contamination in the lagoon at the Milan ammunition plant, Tennessee has been shown to remediate by the phytoremediation process [83,84]. Various plants have been known to reduce HMX contaminations at contaminated sites such as *Myriophyllum aquaticum*, *Triticum aestivum*, *Poplar* plant, *alfalfa Medicago sativa*, *Paspalum notatum,* etc. [84–88]. The downflow constructed wetlands have been shown to remediate RDX runoff. However, phytoremediation has a limitation of toxicity to plants, the more time required, and the area to be covered. The RDX was able to get accumulated in plant tissues and different nitroso-derivatives were shown to be present during the treatment process [86]. However, the phytoremediation technique has another disadvantage, in that it requires a large surface area, bioaccumulation of the parent and intermediate products, and un-useful products (food or ornamental) [87]. So, the new eco-friendly method that is microbial degradation has gained much more attention as compared to phytoremediation.

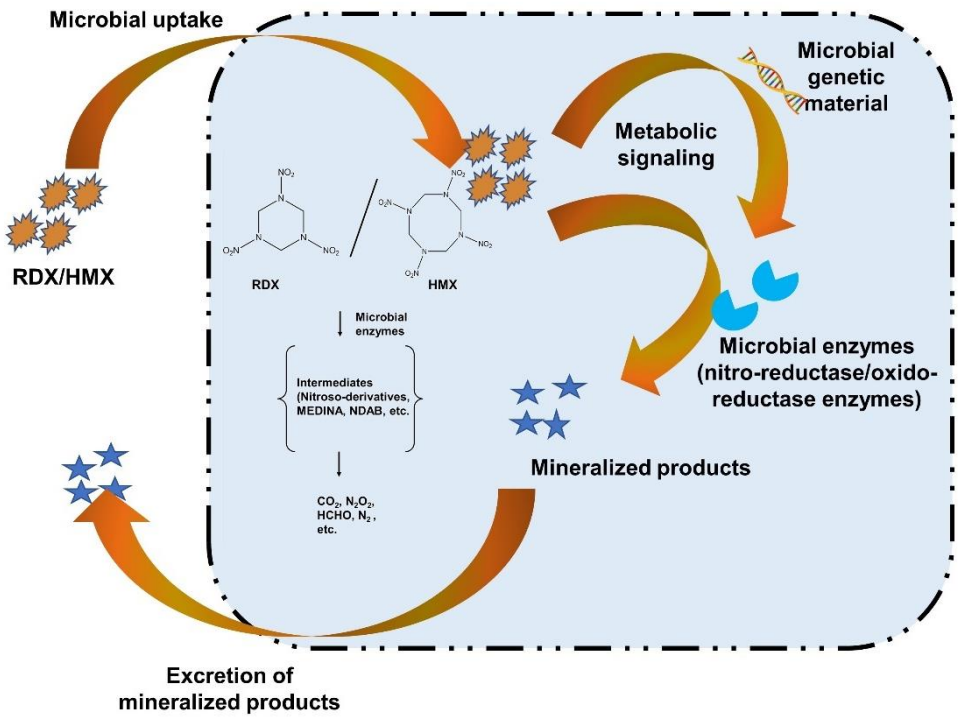


Figure 1.4 The microbial uptake and metabolism of RDX/HMX.

1.5.4.2 Microbial remediation of RDX and HMX

As shown in figure 1.4 the microbes can uptake the RDX and HMX for their metabolic growth. It has been observed that microbes can use RDX and HMX as a source of carbon and nitrogen during their growth. As microbial remediation is getting much more attention these days various researchers have tried to degrade the RDX and HMX with different microbes. Some of the recent works on microbial remediation of RDX and HMX are shown in table 1.2.

Various microbial species are known to degrade RDX and HMX under aerobic and anaerobic conditions such as *Clostridium* sp. strain EDB2, *Methylobacterium* sp. (strain BJ001), *Klebsiella pneumoniae* SCZ1, *Desulfovibrio* strain midref-29, *Bacillus toyonensis*, *Pelomonas aquatica*, *Planomicrobium flavidum*, *Bacillus aryabhattai*, etc [89–96]. Microbial community analysis has been done at the air force bombing range, where various microbes such as *Mesorhizobium* sp., *Rodococcus* sp. *Rhizomium* sp., *Williamsia* sp., etc were reported to be present. Various identified microbial species were having the potential to

degrade RDX from the contaminated site [97]. Apart from this, it was also observed that RDX and HMX readily change the soil microbial community having *Gammaproteo* bacteria and *Sphingomonadaceae* as major microbial species present. The presence of these explosives leads to reduced dehydrogenase activity and soil microbial respiration; however, organic acid and phosphotransferase system pathways were the most enriched pathways. Apart from it, lipid and carbohydrate pathways were inhibited during explosive exposure. This suggests that the presence of HMX and RDX can lead to a change in the metabolic process of the microbial community [98].

However, in earlier studies, it has been demonstrated that RDX can be degraded anaerobically by *Rhodococcus* strain. They have observed different intermediate products such as MEDINA and NDAB formed during the process [99]. *Williamsia* and *Gordonia* sp. were also able to utilize RDX as a source of nutrients for their growth and completely utilized it [100]. Sharma et al. 2020 studied the degradation of RDX under aerobic conditions using a microbial consortium of *Paenibacillus aestuari* and *Arthrobacter subterraneus* and observed an 80.4% reduction in RDX concentration after 12 days [101]. Waisner et al. 2002 studied the potential of native microbes to degrade RDX under aerobic and anaerobic conditions. They observed the maximum degradation under aerobic conditions with the consortium of microbes isolated from the soil [102]. Another microbial species i.e., *Clostridium bifermentans* can aerobically degrade RDX into different end products such as formaldehyde, CO_2, nitrous oxide, etc. through different intermediate products such as MNX and DNX [103]. Ronen et al (2008) studied the metabolism of RDX in a contaminated unsaturated vadose zone at a coastal aquifer. They observed that the soil has a majority of proteobacteria having RDX degradation potential. Furthermore, they observed that the degradation rate was higher at the surface than in deep soil. Also, RDX was observed to be mineralized and form CO_2 through the formation of 4-nitro-2,4-diazabutanal as an intermediate product [104]. In another study, the effect of RDX on cell metabolism was

observed in *Klebsiella* sp. isolated from sewage. *Klebsiella* sp. was able to remove 81.9% of RDX within 24 hrs. However, the cell structure was changed which can be due to the destruction of cell membrane/cell wall proteins. Also, RDX was able to up regulate 193 different metabolites and disrupt the galactose metabolism pathway [105].

The HMX present in the soil was degraded by an indigenous microbe i.e. *Janibacter cremeus* immobilized on calcite. They have observed that HMX was readily degraded under anoxic conditions even at a very high concentration (3000 mg/kg). Also, the formulation was efficient and have a high shelf life (180 days). The metabolites formed during the HMX degradation were nitroso-derivatives of HMX (two-electron reduction pathway), 5-hydroxy-4-nitro2,4-diazapentana (denitration step). This metabolite can ultimately convert to formaldehyde [106]. However, Yang et al 2021 studied the effect of HMX on the *Bacillus aryabhatti*. They observed degradation was 90.5% at 5 mg/L initial concentration under aerobic conditions. However, they observed that exposure to HMX leads to a change in the expression of 254 metabolites and the primary metabolic pathway was disoriented in the microbe. Also, the degradation product of HMX can participate in cell metabolism pathways such as amino acid, nucleoside sugars, and purine formation [89]. The anaerobic granules taken from the UASB reactor were observed to degrade HMX up to 99.04% in 16 days. It was observed that the adsorption of HMX in granules played an important role in the degradation process. However, the accumulation of nitrite negatively affects the degradation process. On the contrary the presence of a suitable carbon source the degradation process is known to enhance. The Major microbe participating in HMX degradation was observed to be acetogenic bacteria [107]. *Pichia sydowiorum* MCM Y-3 (Yeast) isolated from the soil is also known to thrive in HMX contaminated wastewater. It was observed that in the fixed-film bioreactor, the presence of this indigenous microbe the HMX was known to degrade up to 28-50%. Apart from this, nitrate and acetate were also co-metabolized during the process and removed up to 50-70% [108]. Further, the

degradation of RDX and HMX with municipal anaerobic sludge was studied. It was observed that microbes present in explosive contaminated soil were less efficient as compared to the microbes present in municipal anaerobic sludge. The reduction of both the compounds was through the formation of their respective nitroso-derivatives. However, when both the explosives are present RDX is the preferred electron acceptor as compared to HMX [109]. However, the environmental fate of RDX and HMX has been studied earlier. They observed that the degradation of these nitramines follows two different pathways, one is the sequential reduction pathway, and another is the enzymatic cleavage of carbon and nitrogen bond. The sequential reduction pathway produces nitroso-derivatives of the compound; however, C-N bond cleavage pathway involves ring cleavage and eventually conversion to carbon-di-oxide and nitrous oxide [110]. Further, the effect of TNT on RDX and HMX degradation has been studied. The degradation of explosives in a mixture is a tough process, as TNT can inhibit the degradation of RDX and HMX. The mechanism of TNT inhibition exerts a cytotoxic effect on the microbial community which degrades RDX and HMX. [111]. The aerobic degradation of HMX in water was observed using manure and soil. The degradation rate constant was -0.144 /day and -0.0382/day was observed for manure and the combination of manure and soil. Different nitroso-derivatives were observed during the degradation process which confirms the breakdown of HMX with the native microbes [112]. Bio-slurry degradation of RDX and HMX using anaerobic sludge was done and it was observed that 45 days was required for RDX degradation while HMX removal took 35 days more to completely mineralize. The limiting step in this process was the mass transfer from the solid to the liquid phase. Here also the degradation took place through the formation of nitroso-derivatives of individual compounds [113].

Table 1.2 Microbes degrading RDX and HMX

Microbe	Degradation product	Experimental conditions	Outcomes	Ref.
		RDX degradation		
In-situ	Nitroso derivatives	Aerobic	Degradation below detection levels (i.e., 0.1ug), waste glycerol enhances the degradation rate.	[114]
Anaerobic dehalogenating consortium	methylene dinitramine, Nitroso derivatives,	Anaerobic	Degradation below detection levels (i.e., 0.1µg), presence of co-contaminant and carbon doner helps in bioremediation of RDX.	[115]
Native micro-organisms	Mono nitroso derivative of RDX	Aerobic	90% RDX degradation, waste glycerol helps in the bioremediation process.	[116]
Janibacter cremeus	Nitroso derivatives, 5-hydroxy-4-nitro-2,4-diazapentanal	Anaerobic	73% RDX degradation, eggshell can act as bio carrier	[117]
Kinneretia asachharophila	methylenedinitramine (MEDINA) and 4-nitro-2,4-diazabutanal (NDAB)	Aerobic conditions	75% RDX degradation, water-dispersible granules help in the survival of microbes	[118]
Arthrobacter subterraneus and *Paenibacillus aestuarii*	Nitrate and nitrite	Aerobic	80 % RDX degradation, the microbial consortium has higher degradation compared to individual species	[101]
Janibacter cremeus	Trinitroso-RDX, diamino-RDX, trimino-RDX, bis-(hydroxymethyl) nitramine and methylenedintramine	Aerobic	88 % RDX degradation, Nitro reductase enzyme was found to play a major role	[119]
Pelomonas aquatica	Nitrite	Aerobic	80% RDX degradation, water-	[120]

			dispersible granules formulation enhances degradation efficiency	
Microbacterium esteraromaticum	N-methyl-N, N0 - dinitromethanediamine, and methylenedintramine,	Aerobic	60-70% RDX degradation, water dispersible granule exhibits 8% enhanced removal efficiency	[121]
Bacillus toyonensis and Paenibacillus dendritiformis	Hexahydro-1-nitroso-3,5-dinitro-1,3,5 triazine (MNX), Hexahydro-1,3-dinitroso-5- nitro-1,3,5-triazine (DNX), 4-Nitro-2,4-diazabutanal, Bis(hydroxymethyl)nitramine and nitrite	Aerobic	81-84% RDX degradation, indigenous isolates have potential to remediate RDX	[122]
Pelomonas aquatica	Nitrosoderivatives of RDX, methylenedinitramine (MEDINA), diamino derivative of RDX, etc.	Aerobic	86% degradation of RDX, Co-metabolism of RDX and HMX by indigenous microbes	[91]
		HMX degradation		
Bacillus aryabhattai		Aerobic	~90% HMX degradation Differential expression of 254 genes	[89]
Rumen fluid consortium	Nitroso-derivatives of HMX	Anaerobic	~80 % degradation The consortium performs better than individual species	[123]
Clostridium sp. strain EDB2	Nitrate, nitrous oxide, formaldehyde	Anaerobic	~ 46% degradation Released nitrite ion helps in chemotaxis of microbes during HMX degradation	[96]
Sheep rumen fluid	Methylenedinitramine (MEDINA) Nitroso-derivatives of HMX	Anaerobic	~55% degradation *Prevotella* enriched rumen fluid is capable of detoxifying HMX	[124]
Methanogens and acetogen		Anaerobic	~95% degradation	[125]

			Electron donors such as ethanol and propylene glycol can increase degradation	
microcosm culture	Nitroso derivatives (MNX, DNX and TNX)	Aerobic	Enhancing oxygen uptake by microbes increases HMX degradation with high degradation rates	[126]
M.Morganii, *P.rettgeri*, and *C.freundii*	Nitroso derivatives (MNX, DNX, and TNX)	Aerobic followed by O_2 depleted conditions	50-60% degradation RDX inhibit transformation of HMX	[127]
Methanogenic mixed culture	Nitroso derivatives (MNX, DNX, and TNX)	Anaerobic	22-53% degradation, The introduction of Hydrogen donors increases the degradation rate	[128]
Pelomonas aquatica	Nitroso derivatives, bishydroxymethyl nitramine, MEDINA, nitrosoamine, hydrazinyl derivatives	Aerobic	78% degradation Co-metabolism of HMX and RDX, degradation pathway involves single e^- transfer ring cleavage	[90]
Bacillus toyonensis	Nitrate and nitrite	Aerobic	Nearly 87 % degradation of HMX was observed, and microbial degradation efficiency was affected by initial concentration and inoculum size.	[92]

As various studies have been performed to study the degradation of RDX and HMX. Also, different metabolites present have been observed during the degradation process. Now it is important to identify and know the degradation pathway for both the RDX and HMX.

1.6 Metabolic pathways for RDX and HMX degradation

Various metabolic pathways have been proposed for the RDX and HMX under aerobic and anaerobic conditions. Figure 1.5 shows the metabolic degradation pathway for RDX in microbes. The metabolic conversion of RDX and MNX under anaerobic conditions. The conversion of both the compounds takes place via a de-nitration process which subsequently leads to the formation of nitrite, nitrate, NDAB, formaldehyde, nitrous oxide, etc. Denitration is an important pathway for aerobic degradation of RDX. Denitration process is accompanied by a ring cleavage step [129]. In another study, the RDX degradation has been shown to lead to the formation of 4-nitro-2,4-diazabutanal. The process is known to be catalyzed by cytochrome p450 2B4 present in the *Rhodococcus* sp. via two single-electron transport processes releasing the nitro group from the ring structure. Later it was discovered that the p450 enzyme was encoded by the XplA gene which helps NADPH as an electron donor [52]. This enzyme has known to catalyze an oxidative and reductive transformation of various environmental pollutants such as agrochemicals, steroids, fatty acids, and xenobiotic compounds. However, under anaerobic conditions, the denitration step occurs by single-electron transport catalyzed by c-type cytochrome [130]. Under anaerobic conditions single denitration, single hydration, and single ring cleavage steps are observed which is different compared to the aerobic process. Also, the end product formed during the process is MEDINA in contrast to NADB produced in aerobic conditions. Microbial remediation of RDX has been suggested earlier. The degradation of RDX under anaerobic conditions with the microbes present in the ovine rumen was studied. It was observed that different metabolites such as nitroso-derivatives of RDX, methylenedinitramine, and pentahydro-1,3-dinitro-1,3,5- Furthermore, the metagenomic analysis of ovine rumen showed the presence of different RDX-degrading genes such as xenA, xenB, diaA, etc. *Clostridium*, which is predominantly present in the ovine rumen does have diaA gene which

reduces RDX to a simpler form. Also, Pfam family of genes was observed to be present in the ovine rumen. The RDX degradation is regulated at the transcriptomics level in the microbial cell [131]. In another study, the degradation of RDX with municipal anaerobic sludge was studied. They observed that two different pathways were followed during the degradation. In one pathway de-nitration of RDX occurred and forms nitroso-derivatives (MNX, DNX, and TNX) which can further mineralize into hydroxylamino derivative of RDX and then to formaldehyde and methanol. However, in another pathway, the enzymatic hydrolysis of the RDX ring leads to the ring cleavage product formation such as methylenedinitramine and bis(hydroxymethyl)nitramine. Methylenedinitramine can further undergo degradation to either form formaldehyde and formic acid or nitrous acid and water [132].

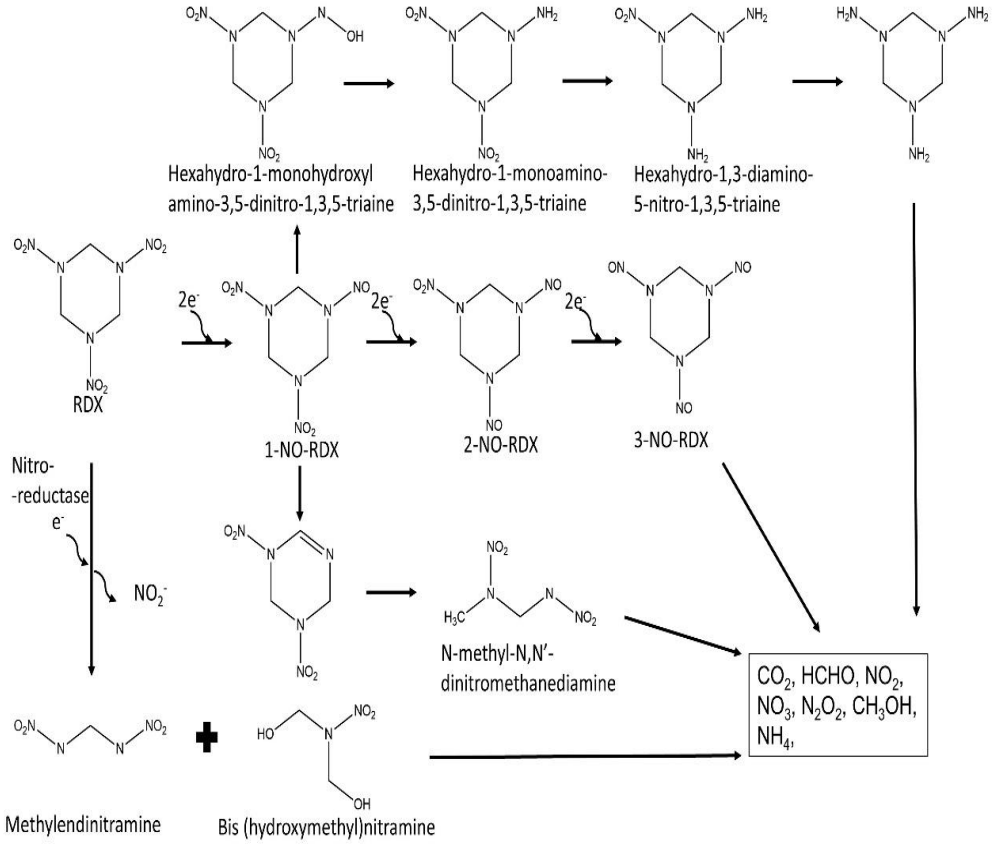


Figure 1.5 Schematic representation of RDX metabolism pathway [129-131]

The microbial community present at the contaminated site can have the potential to remediate HMX contamination. Community analysis done in Hawaii showed the presence of different phylogenetic clusters using 16s rRNA analysis. *Clostridiales* were observed to degrade HMX upto 26 % in 308 days. The mineralized products of HMX observed were nitrite, and formaldehyde, via the formation of methylenedinitramine [93]. Similar to RDX degradation, degradation of HMX can also occur through the reduction process by forming mono nitroso derivatives of HMX. HMX was degraded by a fungal species through a similar pathway but sequential dehydration of HMX did not occur and the ring cleavage started just after the mononitroso derivative formation and leads to the formation of NADB [133]. Figure 1.6 explains the degradation mechanism of HMX by microbes. Mono, di, tri- nitroso derivatives of HMX have been reported to be produced during the HMX degradation process. Recently, Nagar et al. 2020, has observed the degradation of HMX with *Pelomonas aquatica* in the aqueous phase. Products observed were mono-nitroso derivatives which were further reduced to N-nitroso-aminomethylene nitramine through ring cleavage [91]. Further, the ring cleavage occurs through single nitrite elimination which leads to the formation of methylenedinitramine, and N, N'-bishydroxymethyl-methylene dinitramine [93,130,134].

Xanthine oxidase which is metallo-flavoprotein is known to decompose HMX into simpler products. It starts the reaction with a single N-denitration step and breaks HMX into nitrite, formaldehyde, 4-nitro-2,4-diazabutanal (NDAB), ammonium, methylenedinitramine (MDNA), etc [135]. However, various studies have been done to know the metabolic degradation pathway for RDX and HMX still there is a need to further explore the degradation pathway under aerobic conditions and different kinds of metabolites formed. Also, various research gaps are yet to be filled.

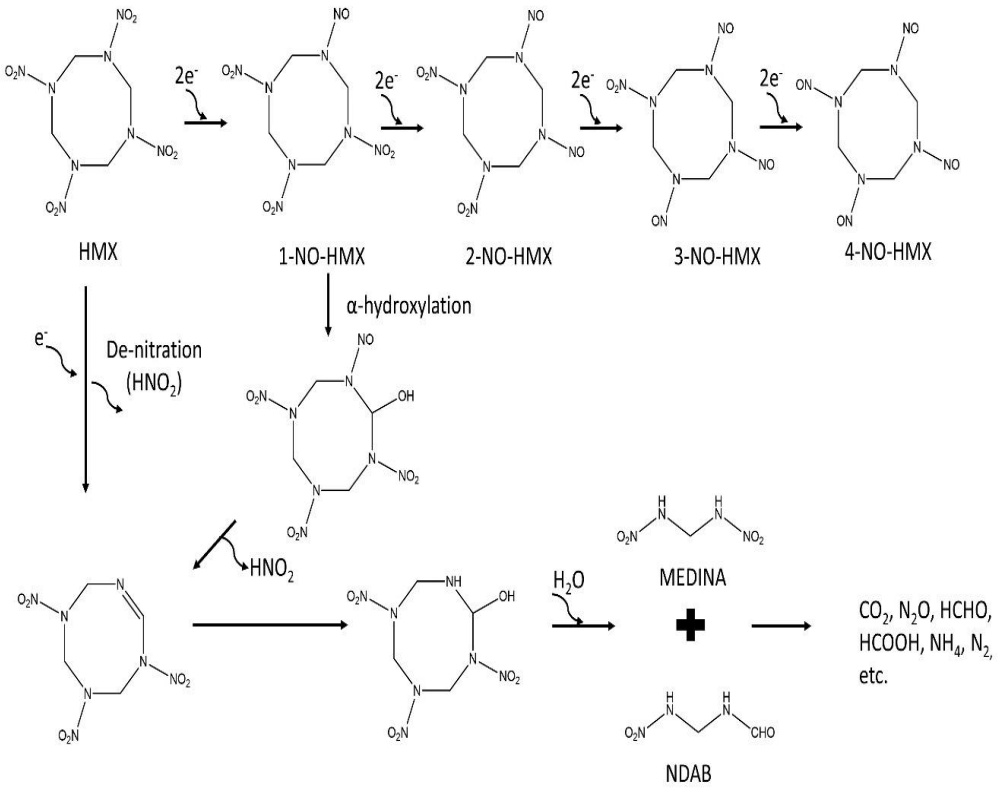


Figure 1.6 Schematic representation of HMX degradation pathway [91, 134,135]

1.7 Gaps in research and opportunities for investigation

Different research gaps that are present in the RDX/HMX degradation study are represented in Figure 1.7. Degradation of RDX and HMX depends upon various environmental factors such as the presence of oxygen, nutrient conditions, contaminated matrix (soil or water), native microorganisms, initial concentration, and degradation time. Variations in these factors could have a direct impact on the degradability rate and half-life of RDX and HMX. Further, the potential of native microbes in the removal of RDX and HMX is yet to be explored. The metagenomic analysis of the contaminated sites could help in isolating and identifying microbes with higher efficiencies. As native microbes can have higher tolerance toward high HMX and RDX concentration, they can be useful during the in-situ remediation of both compounds.

To enhance the degradation of both the compounds first step is to optimize the independent factors that affect the degradation process. Factors such as microbial biomass, initial concentration of explosive, duration of exposure, pH, and temperature mainly affect the degradation process. So, to implement the microbial degradation process at a large scale there is a need to optimize these variables. In addition, many studies have shown the degradation of RDX and HMX under anaerobic conditions. As the anaerobic degradation process could lead to incomplete degradation and the generation of harmful intermediate products. So, there is a need for understanding and conducting aerobic degradation studies which could result in complete mineralization of RDX and HMX. However, very few studies have focused on the degradation of these compounds in aerobic conditions.

Apart from this, nowadays the microbial techniques to efficiently remove the RDX and HMX at very high concentration are still a point of research interest. Various techniques have been employed such as land farming, reactor systems, incineration, catalytic treatment, etc. but none of them are proven to be cost-efficient, eco-friendly, and less toxic end-generating methods. Thus, there is need to develop efficient bioreactor techniques which should be eco-friendly to remediate the RDX and HMX. In this work, the authors have tried to build an in-vessel composting bioreactor to efficiently remove the RDX and HMX contamination. These bioreactor studies can be directly implemented at field contaminated sites. Further, as the degradation process starts, there is the formation of different intermediate metabolites, which can be toxic to humans and the environment. So, there is a need to identify different metabolites formed during the aerobic degradation of RDX and HMX. Based on different research gaps analyzed author has a hypothesis, which has different objectives.

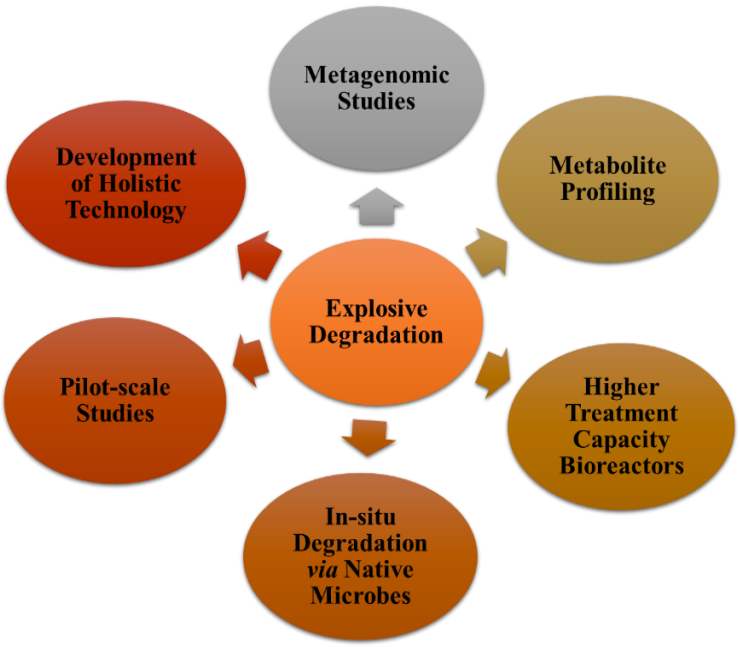


Figure 1.7 Various research gaps in the RDX and HMX bioremediation studies

1.8 Hypothesis to carry out the present study

As with the notion that the native microbes can have a high tolerance and higher degradability rate, novel and native microbes from the actual contaminated sites can be isolated and explored for RDX and HMX. Also, the degradation under aerobic conditions needs much more attention as it can be a possible tool to completely mineralize RDX and HMX, without generating toxic end products. Apart from this, another important part is the optimization of different factors associated with the degradation process. Factors such as initial concentration of RDX and HMX, degradation time, microbial biomass, species, etc. can be the rate-limiting factors during RDX and HMX degradation. So, there is a need to optimize these various factors simultaneously with the degradation process. Apart from this, the major part is the technique involved in the degradation process. Various physical processes employed are not cost-efficient, so there is a need to devise a method that will be eco-friendly as well as cost-efficient. Compositing is one of the techniques, that uses microbes as a toxic product remediator. These microbes utilize the waste products and convert them into simpler forms that can be used as a

fertilizer during plant growth. The Overall thesis theme representing objectives of the study are shown in Figure 1.8. So based upon all the above hypothesis the following objectives were selected-

1. Biodegradation of RDX and possible metabolic pathway exploration
2. Process development for the biodegradation of HMX using response surface methodology
3. Bioreactor studies: up-scaled composting of actual RDX and HMX contaminated sludge

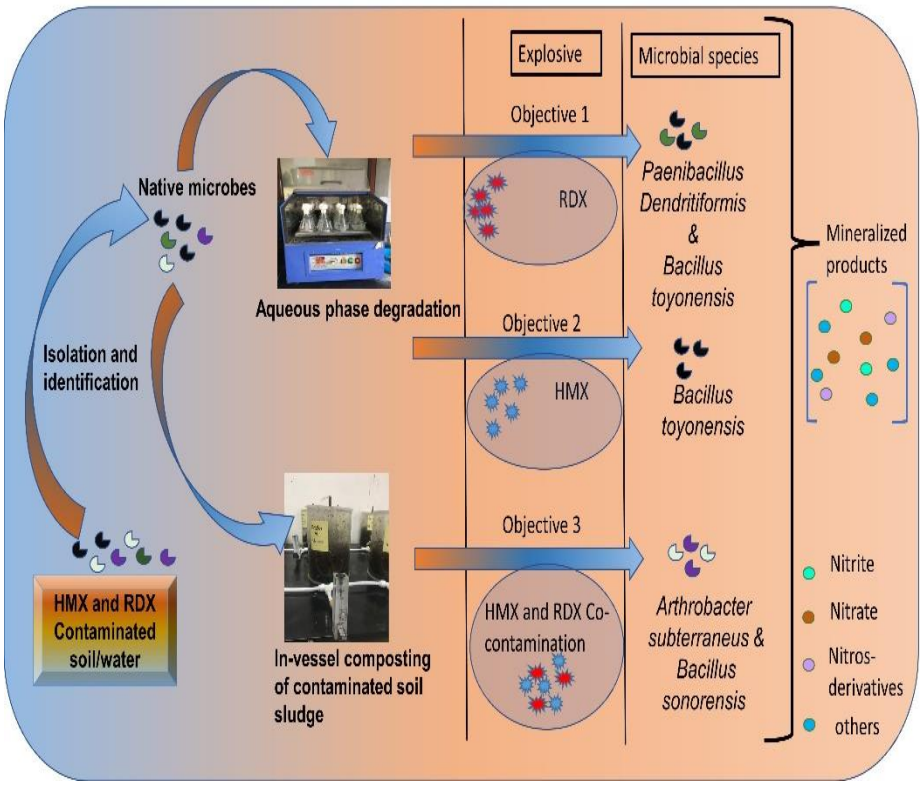


Figure 1.8 Overall thesis theme representing objectives of the study

References

[1] S. Chatterjee, U. Deb, S. Datta, C. Walther, D.K. Gupta, Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation, Chemosphere. 184 (2017) 438–451.

[2] J.P. Agrawal, High energy materials: propellants, explosives and pyrotechnics, John Wiley & Sons, (2010).

[3] B.J. Buchanan, Gunpowder, explosives and the state: a technological history, Ashgate Publishing, Ltd., (2006).

[4] F. Zapata, C. García-Ruiz, Chemical classification of explosives, Crit. Rev. Anal. Chem. 51 (2021) 656–673.

[5] T. Urbanski, S. Laverton, W. Ornaf, Chemistry and technology of explosives, pergamon press Oxford, (1964).

[6] J. Akhavan, The Chemistry of Explosives 4E, Royal Society of Chemistry,(2022).

[7] M. Marshall, Post-blast detection issues, in: Asp. Explos. Detect., Elsevier, (2009): pp. 223–243.

[8] D.H. Rosenblatt, E.P. Burrows, W.R. Mitchell, D.L. Parmer, Organic explosives and related compounds, in: Anthropog. Compd., Springer, (1991): pp. 195–234.

[9] K.-S. Ju, R.E. Parales, Nitroaromatic compounds, from synthesis to biodegradation, Microbiol. Mol. Biol. Rev. 74 (2010) 250–272.

[10] K.A. Thorn, P.G. Thorne, L.G. Cox, Alkaline hydrolysis/polymerization of 2, 4, 6-trinitrotoluene: characterization of products by 13C and 15N NMR, Environ. Sci. Technol. 38 (2004) 2224–2231.

[11] A.L. Juhasz, R. Naidu, Explosives: fate, dynamics, and ecological impact in terrestrial and marine environments, Rev.

Environ. Contam. Toxicol. (2007) 163–215.

[12] J.C. Pennington, T.F. Jenkins, G. Ampleman, S. Thiboutot, J.M. Brannon, Distribution and fate of energetics on DoD test and training ranges: Interim Report 3, ENGINEER RESEARCH AND DEVELOPMENT CENTER VICKSBURG MS, 2003.

[13] P. Broomandi, M. Guney, J.R. Kim, F. Karaca, Soil contamination in areas impacted by military activities: A critical review, Sustainability. 12 (2020) 9002.

[14] E.P. Burrows, D.H. Rosenblatt, W.R. Mitchell, D.L. Parmer, Organic Explosives and Related Compounds. Environmental and Health Considerations, (1989).

[15] B. Clark, R. Boopathy, Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana, J. Hazard. Mater. 143 (2007) 643–648.

[16] S.S. Talmage, D.M. Opresko, C.J. Maxwell, C.J.E. Welsh, F.M. Cretella, P.H. Reno, F.B. Daniel, Nitroaromatic munition compounds: environmental effects and screening values, Rev. Environ. Contam. Toxicol. (1999) 1–156.

[17] M.G. Ryon, B.C. Pal, S.S. Talmage, R.H. Ross, Database assessment of the health and environmental effects of munition production waste products, (1984).

[18] National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 87236934, Cyclotrimethylene-trinitramine. Retrieved August 7, 2022 from https://pubchem.ncbi.nlm.nih.gov/compound/Cyclotrimethylene-trinitramin

[19] National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 17596, Octogen Retrieved August 7, 2022 from

https://pubchem.ncbi.nlm.nih.gov/compound/Octogen..

[20] J. Patterson, State-of-the-art, Military Explosives and Propellants Production Industry: Volume III, Wastewater Treatment, Industrial Environmental Research Laboratory, Office of Research and …, 1976.

[21] J.F. Kitchens, W.E. Harward III, D.M. Lauter, R.S. Wentsel, R.S. Valentine, Preliminary Problem Definition Study of 48 Munitions Related Chemicals. Volume I. Explosives Related Chemicals, ATLANTIC RESEARCH CORP ALEXANDRIA VA, 1978.

[22] T.R. Gibbs, J.F. Baytos, LASL explosive property data, Univ of California Pr, 1980.

[23] R. GARG, D. GRASSO, G. HOAG, Treatment of explosives contaminated lagoon sludge, Hazard. Waste Hazard. Mater. 8 (1991) 319–340.

[24] J.M. Brannon, J.C. Pennington, Environmental fate and transport process descriptors for explosives, (2002).

[25] J.M. Brannon, T.E. Meyers, Review of fate and transport processes of explosives, US Army Engineer Waterways Experiment Station, 1997.

[26] F. Monteil-Rivera, A. Halasz, C. Groom, J.S. Zhao, S. Thiboutot, G. Ampleman, J. Hawari, Fate and transport of explosives in the environment: A chemist's view, Ecotoxicol. Explos. (2009) 5–33.

[27] J.L. Clausen, N. Korte, Fate and transport of energetics from surface soils to groundwater, in: Environ. Chem. Explos. Propellant Compd. Soils Mar. Syst. Distrib. Source Charact. Remedial Technol., ACS Publications, 2011: pp. 273–316.

[28] E.H. Denis, K.A. Morrison, S. Wharton, S. Phillips, S.C. Myers, M.P. Foxe, R.G. Ewing, Trace explosive residue detection of

HMX and RDX in post-detonation dust from an open-air environment, Talanta. 227 (2021) 122124.

[29] P. Sharma, M.A. Mayes, G. Tang, Role of soil organic carbon and colloids in sorption and transport of TNT, RDX and HMX in training range soils, Chemosphere. 92 (2013) 993–1000.

[30] G. Alavi, M. Chung, J. Lichwa, M. D'Alessio, C. Ray, The fate and transport of RDX, HMX, TNT and DNT in the volcanic soils of Hawaii: A laboratory and modeling study, J. Hazard. Mater. 185 (2011) 1600–1604.

[31] T.C. Schutt, M.K. Shukla, Predicting the Impact of Aqueous Ions on Fate and Transport of Munition Compounds in the Environment, J. Phys. Chem. A. 123 (2019) 4973–4979.

[32] H. Yamamoto, M.C. Morley, G.E. Speitel, J. Clausen, Fate and transport of high explosives in a sandy soil: Adsorption and desorption, Soil Sediment Contam. 13 (2004) 459–477.

[33] M. D'Alessio, J. Lichwa, C. Ray, Transport of explosive chemicals in fire-Affected hawaiian topsoil, J. Hazardous, Toxic, Radioact. Waste. 25 (2021) 6020006.

[34] J.C. Lynch, J.M. Brannon, K. Hatfield, J.J. Delfino, An exploratory approach to modeling explosive compound persistence and flux using dissolution kinetics, J. Contam. Hydrol. 66 (2003) 147–159.

[35] J. Šimůnek, D. Jacques, G. Langergraber, S.A. Bradford, M. Šejna, M.T. Van Genuchten, Numerical modeling of contaminant transport using HYDRUS and its specialized modules, J. Indian Inst. Sci. 93 (2013) 265–284.

[36] M.S. Dortch, B.E. Johnson, J.A. Gerald, Modeling fate and transport of munitions constituents on firing ranges, Soil Sediment Contam. An Int. J. 22 (2013) 667–688.

[37] R. Ravikrishna, K.T. Valsaraj, C.B. Price, J.M. Brannon, C.A.

Hayes, S.L. Yost, Vapor-phase transport of explosives from buried sources in soils, J. Air Waste Manage. Assoc. 54 (2004) 1525–1533.

[38] D.C. Leggett, Sorption of military explosive contaminants on bentonite drilling muds, (1985).

[39] F. Monteil-Rivera, C. Groom, J. Hawari, Sorption and degradation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine in soil, Environ. Sci. Technol. 37 (2003) 3878–3884.

[40] T.W. Sheremata, A. Halasz, L. Paquet, S. Thiboutot, G. Ampleman, J. Hawari, The fate of the cyclic nitramine explosive RDX in natural soil, Environ. Sci. Technol. 35 (2001) 1037–1040.

[41] J. Singh, S.D. Comfort, L.S. Hundal, P.J. Shea, Long-term RDX sorption and fate in soil, Wiley Online Library, 1998.

[42] M.S. Dortch, S. Fant, J.A. Gerald, Modeling fate of RDX at demolition area 2 of the Massachusetts military reservation, Soil Sediment Contam. 16 (2007) 617–635.

[43] B.P. Heerspink, S. Pandey, H. Boukhalfa, D.S. Ware, O. Marina, G. Perkins, V. V Vesselinov, G. WoldeGabriel, Fate and transport of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and its degradation products in sedimentary and volcanic rocks, Los Alamos, New Mexico, Chemosphere. 182 (2017) 276–283.

[44] K.M. Dontsova, S.L. Yost, J. Šimunek, J.C. Pennington, C.W. Williford, Dissolution and Transport of TNT, RDX, and Composition B in Saturated Soil Columns, J. Environ. Qual. 35 (2006) 2043–2054.

[45] T.A. Douglas, M.E. Walsh, C.J. McGrath, C.A. Weiss, Investigating the fate of nitroaromatic (TNT) and nitramine (RDX and HMX) explosives in fractured and pristine soils, J. Environ. Qual. 38 (2009) 2285–2294.

[46] M.S. Johnson, M.J. McAtee, Characterizing potential for toxicity: Estimating risks to wildlife, in: Wildl. Toxic. Assessments Chem. Mil. Concern, Elsevier, 2015: pp. 3–11.

[47] U. Environmental Protection Agency Federal Facilities Restoration, R. Office, Fact Sheet – Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), (2021) 1–7.

[48] L.R. Williams, V. Aroniadou-Anderjaska, F. Qashu, H. Finne, V. Pidoplichko, D.I. Bannon, M.F.M. Braga, RDX binds to the GABAA receptor–convulsant site and blocks GABAa receptor–mediated currents in the amygdala: a mechanism for RDX-induced seizures, Environ. Health Perspect. 119 (2011) 357–363.

[49] K.A. Gust, M. Pirooznia, M.J. Quinn Jr, M.S. Johnson, L. Escalon, K.J. Indest, X. Guan, J. Clarke, Y. Deng, P. Gong, Neurotoxicogenomic investigations to assess mechanisms of action of the munitions constituents RDX and 2, 6-DNT in Northern bobwhite (Colinus virginianus), Toxicol. Sci. 110 (2009) 168–180.

[50] B. Zhang, X. Pan, RDX induces aberrant expression of microRNAs in mouse brain and liver, Environ. Health Perspect. 117 (2009) 231–240.

[51] N.R. Schneider, S.L. Bradley, M.E. Andersen, Toxicology of cyclotrimethylenetrinitramine: distribution and metabolism in the rat and the miniature swine, Toxicol. Appl. Pharmacol. 39 (1977) 531–541.

[52] B. Bhushan, S. Trott, J.C. Spain, A. Halasz, L. Paquet, J. Hawari, Biotransformation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by a rabbit liver cytochrome P450: insight into the mechanism of RDX biodegradation by Rhodococcus sp. strain DN22, Appl. Environ. Microbiol. 69 (2003) 1347–1351.

[53] C.A. McFarland, M.J. Quinn Jr, M.A. Bazar, L.G. Talent, M.S.

Johnson, Toxic effects of oral hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine in the western fence lizard (Sceloporus occidentalis), Environ. Toxicol. Chem. An Int. J. 28 (2009) 1043–1050.

[54] M.S. Johnson, M.J. Quinn, M.A. Bazar, K.A. Gust, B.L. Escalon, E.J. Perkins, Subacute toxicity of oral 2, 6-dinitrotoluene and 1, 3, 5-trinitro-1, 3, 5-triazine (RDX) exposure to the northern bobwhite (Colinus virginianus), Environ. Toxicol. Chem. An Int. J. 26 (2007) 1481–1487.

[55] M.J. Quinn Jr, M.A. Bazar, C.A. McFarland, E.J. Perkins, K.A. Gust, M.S. Johnson, Sublethal effects of subacute exposure to RDX (1, 3, 5-trinitro-1, 3, 5-triazine) in the northern bobwhite (Colinus virginianus), Environ. Toxicol. Chem. An Int. J. 28 (2009) 1266–1270.

[56] D.J. Everett, I.R. Johnson, P. Hudson, M. Jones, HMX: 13 week toxicity study in rats by dietary administration, INVERESK RESEARCH INTERNATIONAL LTD MUSSELBURGH (UNITED KINGDOM), 1985.

[57] R.J. Greenough, P. McDonald, HMX: 14 Day Toxicity Study in Rats by Dietary Administration, INVERESK RESEARCH INTERNATIONAL LTD MUSSELBURGH (UNITED KINGDOM), 1985.

[58] A.B. Wilson, Determination of the acute and subchronic mammalian toxicity of HMX, US Army Med. Res. Dev. Command. Ft. Detrick, Frederick, MD. (1985).

[59] S. Nagar, S. Anand, S. Chatterjee, C.D. Rawat, J. Lamba, P.K. Rai, A review of toxicity and biodegradation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) in the environment, Environ. Technol. Innov. 23 (2021) 101750.

[60] E. National Academies of Sciences and Medicine, Exploring Medical and Public Health Preparedness for a Nuclear Incident: Proceedings of a Workshop, National Academies Press, 2019.

[61] J. Zhang, J. Gu, Y. Han, W. Li, Z. Gan, J. Gu, Supercritical water oxidation vs supercritical water gasification: which process is better for explosive wastewater treatment?, Ind. Eng. Chem. Res. 54 (2015) 1251–1260.

[62] R. Alnaizy, A. Akgerman, Oxidative treatment of high explosives contaminated wastewater, Water Res. 33 (1999) 2021–2030.

[63] J.D. Rodgers, N.J. Bunce, Treatment methods for the remediation of nitroaromatic explosives, Water Res. 35 (2001) 2101–2111.

[64] P. Bhanot, S.M. Celin, A. Kalsi, S.K. Singh, S.K. Sahai, P. Sharma, Treatment of high explosive HMX (octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine) production effluent by advanced oxidation processes, Int. J. Environ. Sci. Technol. 19 (2022) 1775–1784.

[65] J. Singh, S.D. Comfort, P.J. Shea, Iron-mediated remediation of RDX-contaminated water and soil under controlled Eh/pH, Environ. Sci. Technol. 33 (1999) 1488–1494.

[66] S. Waisner, G.E. Hoag, Fe (III)-EDTA-activated persulfate destruction of explosives and pH dependence of chemistry, in: Proc. 5 Th Int. Conf. Remediat. Chlorinated Recalcitrant Compd. (Monterey, CA) Columbus, OH, Battelle Press, 2006.

[67] J. Gong, C.-S. Lee, Y.-Y. Chang, Y.-S. Chang, Novel self-assembled bimetallic structure of Bi/Fe0: The oxidative and reductive degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX), J. Hazard. Mater. 286 (2015) 107–117.

[68] V.K. Balakrishnan, A. Halasz, J. Hawari, Alkaline hydrolysis of the cyclic nitramine explosives RDX, HMX, and CL-20: new insights into degradation pathways obtained by the observation of novel intermediates, Environ. Sci. Technol. 37 (2003) 1838–1843.

[69] H.M. Heilmann, U. Wiesmann, M.K. Stenstrom, Kinetics of the alkaline hydrolysis of high explosives RDX and HMX in aqueous solution and adsorbed to activated carbon, Environ. Sci. Technol. 30 (1996) 1485–1492.

[70] J.L. Davis, M.C. Brooks, S.L. Larson, C.C. Nestler, D.R. Felt, Lime treatment of explosives-contaminated soil from munitions plants and firing ranges, Taylor & Francis, 2006.

[71] S.-Y. Oh, D.-S. Shin, Remediation of explosive-contaminated soils: alkaline hydrolysis and subcritical water degradation, Soil Sediment Contam. An Int. J. 24 (2015) 157–171.

[72] C.E. VanEngelen, Alkaline hydrolysis of explosives, (2010).

[73] R.L. Bishop, D.M. Harradine, R.L. Flesner, S.A. Larson, D.A. Bell, Safe operating temperatures for pressurized alkaline hydrolysis of HMX-based explosives, Ind. Eng. Chem. Res. 39 (2000) 1215–1220.

[74] L.K. Sviatenko, L. Gorb, F.C. Hill, D. Leszczynska, M.K. Shukla, S.I. Okovytyy, D. Hovorun, J. Leszczynski, In silico alkaline hydrolysis of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine: Density functional theory investigation, Environ. Sci. Technol. 50 (2016) 10039–10046.

[75] K.-D. Zoh, M.K. Stenstrom, Application of a membrane bioreactor for treating explosives process wastewater, Water Res. 36 (2002) 1018–1024.

[76] C.A. Groom, A. Halasz, L. Paquet, N. Morris, L. Olivier, C. Dubois, J. Hawari, Accumulation of HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) in indigenous and agricultural plants grown in HMX-contaminated anti-tank firing-range soil, Environ. Sci. Technol. 36 (2002) 112–118.

[77] K. Panz, K. Miksch, Phytoremediation of explosives (TNT, RDX, HMX) by wild-type and transgenic plants, J. Environ.

Manage. 113 (2012) 85–92.

[78] L. S.L., J. R.P., E. L., P. D., Classification of explosives transformation products in plant tissue, Environ. Toxicol. Chem. 18 (1999) 1270–1276. http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L29233316.

[79] J.L. Ramos, M.M. González-Pérez, A. Caballero, P. Van Dillewijn, Bioremediation of polynitrated aromatic compounds: Plants and microbes put up a fight, Curr. Opin. Biotechnol. 16 (2005) 275–281.

[80] N.K. Hannink, S.J. Rosser, N.C. Bruce, Phytoremediation of explosives, CRC. Crit. Rev. Plant Sci. 21 (2002) 511–538.

[81] J.D. Kiiskila, P. Das, D. Sarkar, R. Datta, Phytoremediation of Explosive-Contaminated Soils, Curr. Pollut. Reports. 1 (2015) 23–34.

[82] B. Van Aken, Transgenic plants for enhanced phytoremediation of toxic explosives, Curr. Opin. Biotechnol. 20 (2009) 231–236.

[83] F.J. Sikora, R.A. Almond, L.L. Behrends, J.J. Hoagland, D.A. Kelly, Demonstration Results of Phytoremediation of Explosives-Contaminated Groundwater Using Constructed Wetlands At The Milan Army Ammunition Plant, Milan, Tennessee Volume IV., TENNESSEE VALLEY AUTHORITY MUSCLE SHOALS AL, 1998.

[84] L. Behrends, F. Sikora, D. Kelly, S. Coonrod, B. Rogers, Demonstration Plan for Phytoremediation of Explosive-Contaminated Groundwater in Constructed Wetlands at Milan Army Ammunition Plant Milan Tennessee. Volume 2., TENNESSEE VALLEY AUTHORITY MUSCLE SHOALS AL, 1996.

[85] V.F. Medina, R. Rivera, S.L. Larson, S.C. McCutcheon,

Phytoreactors show promise in treating munitions contaminants, Soil Groundw. Cleanup, February/March Issue. (1998) 19–24.

[86] D. Low, K. Tan, T. Anderson, G.P. Cobb, J. Liu, W.A. Jackson, Treatment of RDX using down-flow constructed wetland mesocosms, Ecol. Eng. 32 (2008) 72–80.

[87] S.M. Via, Phytoremediation of explosives, in: Phytoremediation, Springer, 2020: pp. 261–284.

[88] A. Lee, B. Kim, J. Park, B. Bae, Removal of RDX using Lab-scale Plug Flow Constructed Wetlands Planted with Miscanthus sacchariflorus (Maxim.) Benth, J. Soil Groundw. Environ. 20 (2015) 85–94.

[89] X. Yang, J. long Lai, J. Li, Y. Zhang, X. gang Luo, M. wei Han, Y. bing Zhu, S. ping Zhao, Biodegradation and physiological response mechanism of Bacillus aryabhattai to cyclotetramethylenete-tranitramine (HMX) contamination, J. Environ. Manage. 288 (2021) 112247.

[90] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Aerobic biodegradation of HMX by Planomicrobium flavidum, 3 Biotech. 8 (2018) 1–7.

[91] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Biodegradation of octogen and hexogen by Pelomonas aquatica strain WS2-R2A-65 under aerobic condition, Environ. Technol. (United Kingdom). 43 (2022) 1003–1012.

[92] A. Meda, P. Sangwan, K. Bala, Optimization of process parameters for degradation of HMX with Bacillus toyonensis using response surface methodology, Int. J. Environ. Sci. Technol. 17 (2020) 4601–4610.

[93] J.-S. Zhao, D. Manno, J. Hawari, Abundance and diversity of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX)-metabolizing bacteria in UXO-contaminated marine sediments,

FEMS Microbiol. Ecol. 59 (2007) 706–717.

[94] J.-S. Zhao, L. Paquet, A. Halasz, D. Manno, J. Hawari, Metabolism of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine by Clostridium bifermentans strain HAW-1 and several other H2-producing fermentative anaerobic bacteria, FEMS Microbiol. Lett. 237 (2004) 65–72.

[95] B. Van Aken, J.M. Yoon, J.L. Schnoor, Biodegradation of nitro-substituted explosives 2, 4, 6-trinitrotoluene, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine, and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5-tetrazocine by a phytosymbiotic Methylobacterium sp. associated with poplar tissues (Populus d, Appl. Environ. Microbiol. 70 (2004) 508–517.

[96] B. Bhushan, A. Halasz, S. Thiboutot, G. Ampleman, J. Hawari, Chemotaxis-mediated biodegradation of cyclic nitramine explosives RDX, HMX, and CL-20 by Clostridium sp. EDB2, Biochem. Biophys. Res. Commun. 316 (2004) 816–821.

[97] P. Andeer, D.A. Stahl, L. Lillis, S.E. Strand, Identification of microbial populations assimilating nitrogen from RDX in munitions contaminated military training range soils by high sensitivity stable isotope probing, Environ. Sci. Technol. 47 (2013) 10356–10363.

[98] X. Yang, J. Lai, Y. Zhang, X. Luo, M. Han, S. Zhao, Microbial community structure and metabolome profiling characteristics of soil contaminated by TNT, RDX, and HMX, Environ. Pollut. 285 (2021) 117478.

[99] M.E. Fuller, N. Perreault, J. Hawari, Microaerophilic degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by three Rhodococcus strains, Lett. Appl. Microbiol. 51 (2010) 313–318.

[100] K.T. Thompson, F.H. Crocker, H.L. Fredrickson, Mineralization of the cyclic nitramine explosive hexahydro-1, 3, 5-trinitro-1, 3,

5-triazine by Gordonia and Williamsia spp, Appl. Environ. Microbiol. 71 (2005) 8265–8272.

[101] K. Sharma, P. Sharma, S.M. Celin, P.K. Rai, P. Sangwan, Degradation of high energetic material hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a microbial consortium using response surface methodological approach, SN Appl. Sci. 3 (2021) 1–8.

[102] S. Waisner, L. Hansen, H. Fredrickson, C. Nestler, M. Zappi, S. Banerji, R. Bajpai, Biodegradation of RDX within soil-water slurries using a combination of differing redox incubation conditions, J. Hazard. Mater. 95 (2002) 91–106.

[103] J.-S. Zhao, L. Paquet, A. Halasz, J. Hawari, Metabolism of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine through initial reduction to hexahydro-1-nitroso-3, 5-dinitro-1, 3, 5-triazine followed by denitration in Clostridium bifermentans HAW-1, Appl. Microbiol. Biotechnol. 63 (2003) 187–193.

[104] Z. Ronen, Y. Yanovich, R. Goldin, E. Adar, Metabolism of the explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) in a contaminated vadose zone, Chemosphere. 73 (2008) 1492–1498.

[105] J. Li, X. Yang, J.-L. Lai, Y. Zhang, X.-G. Luo, S.-P. Zhao, Y.-B. Zhu, Characteristics of RDX degradation and the mechanism of the RDX exposure response in a Klebsiella sp. strain, Biochem. Eng. J. 176 (2021) 108174.

[106] A. Kalsi, S.M. Celin, S. Sharma, S. Sahai, J.G. Sharma, Bioaugmentation for remediation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) contaminated soil using a clay based bioformulation, J. Hazard. Mater. 420 (2021) 126575.

[107] C. An, Y. He, G. Huang, Y. Liu, Performance of mesophilic anaerobic granules for removal of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) from aqueous solution, J.

Hazard. Mater. 179 (2010) 526–532.

[108] S.P. Kanekar, P.P. Kanekar, S.S. Sarnaik, N.P. Gujrathi, P.N. Shede, M.R. Kedargol, K.F. Reardon, Bioremediation of nitroexplosive wastewater by an yeast isolate Pichia sydowiorum MCM Y-3 in fixed film bioreactor, J. Ind. Microbiol. Biotechnol. 36 (2009) 253–260.

[109] C.F. Shen, J. Hawari, G. Ampleman, S. Thiboutot, S.R. Guiot, Enhanced biodegradation and fate of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) in anaerobic soil slurry bioprocess, Bioremediat. J. 4 (2000) 27–39.

[110] C.A. Groom, S. Beaudet, A. Halasz, L. Paquet, J. Hawari, Detection of the cyclic nitramine explosives hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazine (HMX) and their degradation products in soil environments, J. Chromatogr. A. 909 (2001) 53–60.

[111] S.S.-B. Moshe, Z. Ronen, O. Dahan, N. Weisbrod, L. Groisman, E. Adar, R. Nativ, Sequential biodegradation of TNT, RDX and HMX in a mixture, Environ. Pollut. 157 (2009) 2231–2238.

[112] V.R. Harkins, T. Mollhagen, C. Heintz, K. Rainwater, Aerobic biodegradation of high explosives, phase I-HMX, Bioremediat. J. 3 (1999) 285–290.

[113] S.R. Guiot, C.F. Shen, L. Paquet, J. Breton, J. Hawari, S. Thiboutot, G. Ampleman, Pilot-scale anaerobic bioslurry remediation of RDX and HMX contaminated soils, Situ On-Site Bioremediation. 5 (1999) 15–20.

[114] L.B. Jugnia, D. Manno, S. Dodard, C.W. Greer, M. Hendry, Manipulating redox conditions to enhance in situ bioremediation of RDX in groundwater at a contaminated site, Sci. Total Environ. 676 (2019) 368–377.

https://doi.org/10.1016/j.scitotenv.2019.04.045.

[115] M.M. Lorah, E. Vogler, F.E. Gebhardt, D. Graves, J.F. Grabowski, Enhanced bioremediation of RDX and Co-Contaminants perchlorate and nitrate using an anaerobic dehalogenating consortium in a fractured rock aquifer, Chemosphere. 294 (2022) 133674.

[116] L.B. Jugnia, D. Manno, K. Drouin, M. Hendry, In situ pilot test for bioremediation of energetic compound-contaminated soil at a former military demolition range site, Environ. Sci. Pollut. Res. 25 (2018) 19436–19445.

[117] A. Kalsi, S.M. Celin, P. Bhanot, S. Sahai, J.G. Sharma, A novel egg shell-based bio formulation for remediation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) contaminated soil, J. Hazard. Mater. 401 (2021) 123346.

[118] S. Yadav, A. Sharma, M.A. Khan, R. Sharma, M. Celin, A. Malik, S. Sharma, Enhancing hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) remediation through water-dispersible Microbacterium esteraromaticum granules, J. Environ. Manage. 264 (2020) 110446.

[119] A. Kalsi, S.M. Celin, J.G. Sharma, Aerobic biodegradation of high explosive hexahydro-1,3,5- trinitro-1,3,5-triazine by Janibacter cremeus isolated from contaminated soil, Biotechnol. Lett. 42 (2020) 2299–2307.

[120] M.A. Khan, S. Yadav, R. Sharma, M. Dalela, S.M. Celin, A. Sharma, S. Sharma, Augmentation of stimulated Pelomonas aquatica dispersible granules enhances remediation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) contaminated soil, Environ. Technol. Innov. 17 (2020) 100594.

[121] M.A. Khan, A. Sharma, S. Yadav, S.M. Celin, S. Sharma, A. Noureldeen, H. Darwish, Enhancing remediation of RDX-contaminated soil by introducing microbial formulation

technology coupled with biostimulation, J. Environ. Chem. Eng. 9 (2021) 106019.

[122] A. Meda, P. Sangwan, K. Bala, Optimization and Degradation Studies on Hexahydro-1, 3, 5-Trinitro-1, 3, 5-Triazine (RDX) with Selected Indigenous Microbes under Aerobic Conditions, Water. 13 (2021) 1257.

[123] H.L. Eaton, L.D. Murty, J.M. Duringer, M.A. Craig, Ruminal bioremediation of the high energy melting explosive (HMX) by sheep microorganisms, FEMS Microbiol. Lett. 350 (2014) 34–41.

[124] S. Perumbakkam, A.M. Craig, Anaerobic transformation of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) by ovine rumen microorganisms, Res. Microbiol. 163 (2012) 567–575.

[125] N.R. Adrian, C.M. Arnett, Anaerobic biotransformation of explosives in aquifer slurries amended with ethanol and propylene glycol, Chemosphere. 66 (2007) 1849–1856.

[126] J. Won, R.C. Borden, Impact of glycerin and lignosulfonate on biodegradation of high explosives in soil, J. Contam. Hydrol. 194 (2016) 1–9.

[127] C.L. Kitts, D.P. Cunningham, P.J. Unkefer, Degrading Species of the Family Enterobacteriaceae from Nitramine Explosive-Contaminated Soil, Appl. Environ. Microbiol. 60 (1994) 4608–4711. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC202029/pdf/aem00029-0402.pdf.

[128] N.R. Adrian, C.M. Arnett, R.F. Hickey, Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen, Water Res. 37 (2003) 3499–3507.

[129] E.L. Rylott, A. Lorenz, N.C. Bruce, Biodegradation and

biotransformation of explosives, Curr. Opin. Biotechnol. 22 (2011) 434–440.

[130] H.L. Eaton, J.M. Duringer, L.D. Murty, A.M. Craig, Anaerobic bioremediation of RDX by ovine whole rumen fluid and pure culture isolates, Appl. Microbiol. Biotechnol. 97 (2013) 3699–3710.

[131] R.W. Li, J.G. Giarrizzo, S. Wu, W. Li, J.M. Duringer, A.M. Craig, Metagenomic insights into the RDX-degrading potential of the ovine rumen microbiome, PLoS One. 9 (2014) e110505.

[132] J. Hawari, S. Beaudet, A. Halasz, 374 S Thiboutot, G. Ampleman, Microbial degradation of explosives: biotransformation versus mineralization, Appl. Microbiol. Biotechnol. 54 (2000) 605–618.

[133] D. Fournier, A. Halasz, J. Spain, R.J. Spanggord, J.C. Bottaro, J. Hawari, Biodegradation of the hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine ring cleavage product 4-nitro-2, 4-diazabutanal by Phanerochaete chrysosporium, Appl. Environ. Microbiol. 70 (2004) 1123–1128.

[134] D. Kalderis, A.L. Juhasz, R. Boopathy, S. Comfort, Soils contaminated with explosives: environmental fate and evaluation of state-of-the-art remediation processes (IUPAC technical report), Pure Appl. Chem. 83 (2011) 1407–1484.

[135] B. Bhushan, L. Paquet, A. Halasz, J.C. Spain, J. Hawari, Mechanism of xanthine oxidase catalyzed biotransformation of HMX under anaerobic conditions, Biochem. Biophys. Res. Commun. 306 (2003) 509–515.

CHAPTER 2

Chapter 2

Material and Methods

2.1 Experimental Plan

This chapter involves various methodologies used to achieve different objectives discussed in the introduction section. Figure 2.1 depicts a schematic overview of the experimental design and analytical methodologies. In brief, the aim was to use native bacteria to clean up RDX and HMX contamination because they can tolerate higher explosive concentrations. Microbes studied were isolated from soil and water samples that had been collected from the HMX manufacturing facility in North India. The CSIR-Institute of Microbial Technology (CSIR-IMTECH), Chandigarh supported with the identification and isolation of microbes. The tainted samples yielded a variety of microorganisms. However, *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857), *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) *Arthrobacter subterraneus* (MTCC No. 12883, isolate no. S2-TSB-17) and *Bacillus sonorensis* (MTCC no. 12855 isolate no. S8-TSB-4) were used to complete different objectives as they were found potential degraders in preliminary studies. As shown in Figure 2.1, isolated microorganisms were employed to decompose RDX and HMX in aqueous and soil-sludge environments. The *Bacillus toyonensis* and *Paenibacillus dendritiformis* were used to study the aqueous phase degradation of RDX and HMX. However, the *Arthrobacter subterraneus* and *Bacillus sonorensis* were used for the degradation of RDX and HMX in soil sludge.

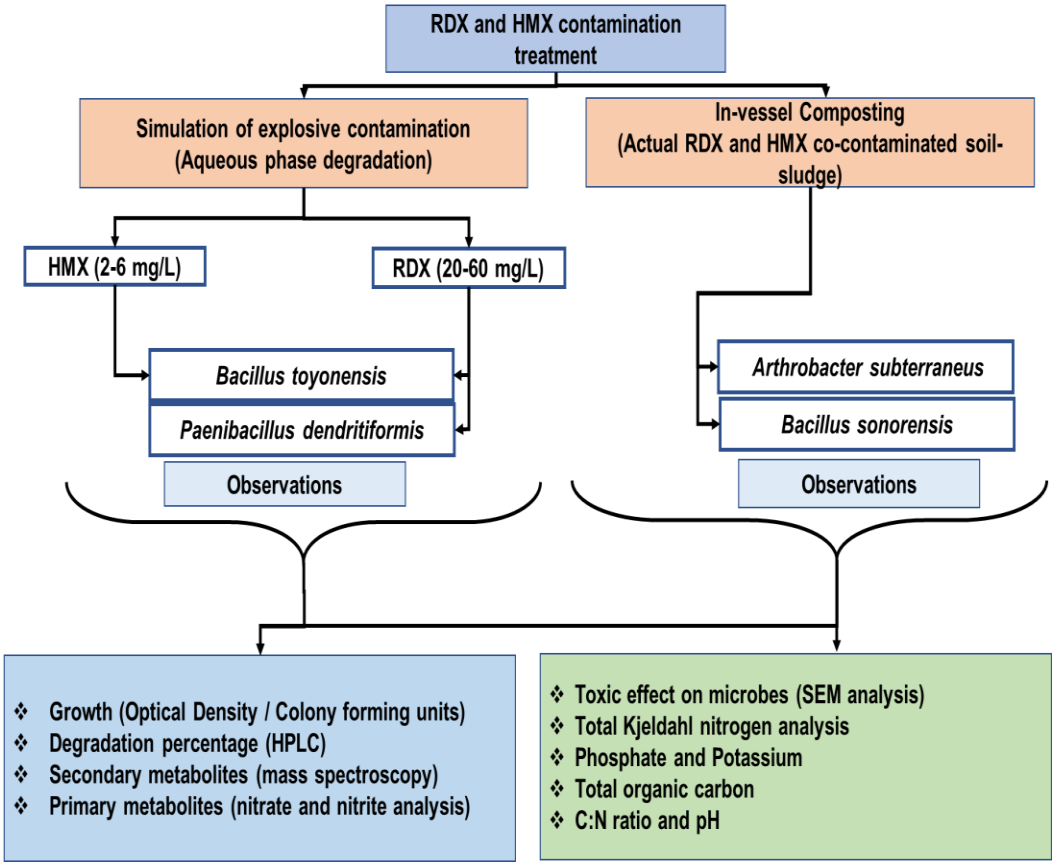


Figure 2.1 Schematic representation of experimental framework for RDX and HMX degradation with native microorganisms.

2.2 Microbial growth

As discussed earlier microbial identification and isolation was done by CSIR-IMTECH, Chandigarh, from contaminated samples and obtained in lyophilized form for further research. Various microbial species were isolated and identified out of which, *Bacillus toyonensis*, *Paenibacillus dendritiformis*, *Arthrobacter subterraneus*, and *Bacillus sonorensis* were revived in tryptic soya broth (TSB, HIMEDIA, LQ508) and preserved on agar slants at 4 °C before use. The composition of TSB is shown in Table 2.1. Afterward, the microbes were sub-cultured in a minimum salt medium (MSM) (Table 2.2) [1] for three generations to acclimatize them to nutrient-limited conditions. During the entire duration of the experiment, microorganisms were cultivated at 32±2 °C in shaking flasks (120 rpm) to

reach an optical density of 1.2 (1 mL ≈10^8 cells/mL). These adapted and revived microbes were further used for the aqueous phase and composting studies.

Table 2.1 Composition of tryptic soya broth (TSB) medium

Compound	**g/1000 mL**
Casein peptone	17.0
Soya peptone	3.0
Sodium chloride	5.0
Potassium phosphate dibasic	2.48
Dextrose	2.48

Table 2.2 Composition of minimal salt medium [1]

Compound	**g/1000 mL**
Potassium phosphate dibasic (K_2HPO_4)	1.74
Potassium phosphate monobasic (KH_2PO_4)	1.44
Magnesium sulfate ($MgSO_4.7H_2O$)	0.06
Glycerol	0.92
Glucose	0.90
Succinic acid	0.58
Ammonium chloride	0.05
Trace elements [$CaCl_2$ (2.7 mg), H_3BO_3 (6.18 mg), $CuSO_4.5H_2O$ (0.625 mg), $MnCl_2.4H_2O$ (3.95 mg), $ZnSO_4.7H_2O$ (1.43 mg), $FeSO_4$ (69.5 mg), $CoCL_2.6H_2O$ (1.78 mg) in 250 ml of distilled water]	1 mL

2.3 Aqueous phase degradation study

In the aqueous phase study, degradation of HMX and RDX was observed with previously revived microbes. For the degradation study, the MSM medium was spiked with the specific concentration of RDX or HMX. To get the desired concentration (2, 4, and 6 mg/L for HMX and 20, 40, and 60 mg/L for RDX), MSM was spiked with 1000 mg/L RDX/HMX stock solutions, prepared in acetonitrile. Following spiking, the solutions were left open under aseptic conditions in laminar airflow chamber for 18 hours to allow the solvent (acetonitrile) to evaporate [2]. Afterward, the microorganisms were inoculated into the spiked medium and incubated at

32±3 °C in an orbital shaker at 120 rpm. *Bacillus toyonensis* was used for both HMX as well as RDX degradation while *Paenibacillus dendritiformis* was used only for RDX. Both the RDX and HMX degradation experiments were carried out in 250 mL Erlenmeyer flasks. Each combination, which included MSM medium, contaminant, and microbial culture, had a total volume of 100 mL. The growth of microbes was monitored as optical density (O.D) at 600 nm using UV–visible spectrophotometer (Perkin Elmer, Model Lambda 650S) [3,4].

2.3.1 Optimization of RDX/HMX degradation

To optimize the degradation process, experiments were designed, and the data were analyzed using the DESIGN EXPERT ® VERSION 12 (Stat-Ease®, Minneapolis). It's a statistical tool that uses lower-order polynomial equations to develop, improve, and optimize a process with multiple variables impacting the result. The whole study had been optimized using response surface methodology (RSM). RSM reduces the number of alternative outcomes, which saves time and costs during testing [6-9]. The Box–Behnken design (BBD) model was used in this study which is a multivariate mathematical model for 3-level factorial design. Relationships between several independent and dependent parameters were explored during microbial degradation to better understand their inter-relationships [5]. The factors which were considered during the study were the initial concentration of RDX or HMX, inoculum size, and the time of degradation. Different combinations (total 17) obtained from the response surface methodology were operated during the study in triplicates. Further to statistically validate the data Box Behnken design was used as a second-order polynomial model [3,6]. The number of experimental set up is defined as,

$$N = k (k - 1) + C_0 \quad (1)$$

where 'C_0' is the central point and 'k' is the number of factors selected during the study. Microbial culture of 1.2 O.D (1 mL = 10^8 cells) was used

as an inoculant in the explosive spiked medium. Afterward, the flasks were incubated in an orbital shaker at a temperature of 32±3 ℃ at 120 rpm. The entire experiment was performed under aerobic conditions and sampling was done at specific time intervals (5, 10, and 15 days) to analyze % degradation of HMX/RDX, nitrite and nitrate production, and metabolites. Cotton plugs were used to ensure aerobic biodegradation conditions in each flask. As it aids in the optimization of process parameters, the RSM results can be used to scale up the degrading process.

2.4 Composting

Various ways to remediate RDX and HMX contamination in soil have been proposed and tried by different researchers, but only a few studies have concentrated on soil-sludge decontamination. To operate, traditional technologies like incineration necessitates complex instruments and a lot of energy. As a result, these processes are inefficient and produce waste like ash, which is difficult to dispose-of. Composting, a green technology that is eco-friendly, cost-effective, and requires no fuel or energy during the process, is receiving a lot of acceptance as a solution. During the procedure, there is a chance that contaminants will co-metabolize with the bulking agents. Bulking agents, which are also waste materials with high organic content and are readily available, can be used to improve the product's quality during composting. So, to remediate contaminated sludge (HMX and RDX) in-vessel composting study was designed.

2.4.1 In-vessel composter design

For the composting of the sludge, 3 L composting bioreactors constructed of acrylic and molded into a cylindrical shape were designed and manufactured. The entire experimental setup is depicted in figure 2.2. Twelve different reactors were used during the study with different combinations of explosive sludge, microbes, and cow manure. Also, these reactors were connected to air compressors through air flow meters. The

airflow was kept at 50 mL/min. Each day, the airflow was maintained for 8 hours. Each vessel's moisture was maintained at 60 ±10 % by adding autoclaved water to compost weekly and it was checked using a hygrometer (Hanna HI9565, US). The temperature in the reactors was 32±3 ℃, and the compost was rotated once a week to maintain aerobic conditions [10].

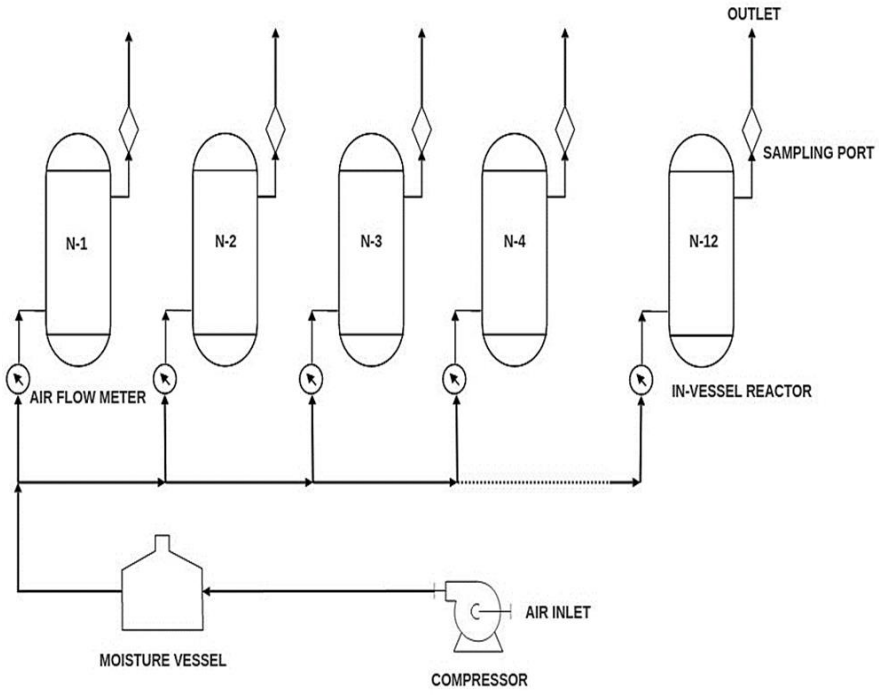


Figure 2.2 Schematic diagram for in-vessel composting reactors

2.4.2 Composting materials

Explosive contaminated sludge and soil samples were collected from an HMX manufacturing facility in north India. Large particles were hand-picked from sludge that had been dried in the air. Cow manure was gathered from cattle shed in Delhi, air-dried, and pre-composted in the open air to remove any volatile compounds. Garden debris was collected from the laboratory's garden which consisted primarily of grass trimmings, *Azadirachta indica* leaves, and a little number of other leaves [10].

2.5 Analytical methods

2.5.1 HPLC analysis

To observe the reduction in the concentration of RDX and HMX during the study; samples were extracted using EPA 8330B standard methods [2]. In aqueous phase extraction, the contaminated water samples were extracted using acetonitrile. Briefly, 5 mL of the experimentally treated sample was taken and mixed with 5 mL of HPLC grade acetonitrile. The solution was vortexed properly and centrifuged at 10000×g for 10 min. The supernatant was filtered through a 0.45 µm Teflon membrane filter. The filtrate was further analyzed using HPLC.

The solid-phase extraction of explosives from the compost samples, which was done as per the standard protocol described by USEPA 8330 B [2]. Briefly, the samples collected were air-dried. Afterward, they were properly homogenized and passed through a mesh (2 mm) to remove larger particles. 2 g of sample was taken in an amber-colored sonication vial and mixed with 10 mL of HPLC grade acetonitrile. The suspension was mixed properly and ultrasonicated for 18 hrs at 18 ℃. Then, the samples were allowed to settle down for 30 min. 5 mL of the resulting supernatant was taken from the tube and mixed with 5 ml of calcium chloride (5 g/L). The solution was filtered through a 0.45 µm Teflon membrane filter and analyzed using HPLC.

The filtrate obtained from the extraction process was further subjected to HPLC as per standard method USEPA 8330B [2]. The filtrate obtained was fed into the autosampler section of the HPLC (Flexer, Perkin Elmer, Waltham, MA, USA). The HPLC was equipped with a C-18 reverse-phase column (3µm, 150 mm x 4.6 mm) and a photodiode array (PDA) detector (254 nm). The mobile phase was an acetonitrile and water (50:50) mixture. The sample run time was 10 min, and the injection volume was 50-100 µL. Peaks were identified and quantified using the retention time and UV profile of standard compounds.

2.5.2 Nitrite analysis

As it is important to observe the release of nitrite during the degradation process. The nitrite estimation was done by a colorimetric method [11]. Briefly, 1 ml of the collected sample was centrifuged and used for nitrite analysis. 600 µL of the sample was initially mixed with 150 µl of sulphanilamide (5g sulphanilamide mixed in 26 mL HCl and volume adjusted to 500 mL). After 5 min incubation, 150 µl of N-(1-naphthyl)ethylenediamine dihydrochloride (0.5 g per 500 mL distilled water) was added and the solution was further incubated for 20 min. After the incubation, 2.1 mL of distilled water was added to the reaction mixture and its absorbance was recorded at 540 nm (Perkin Elmer, Model Lambda 650S, Waltham, MA, USA). The calibration curve was plotted using sodium nitrite (1.23 gm/L sodium nitrite = 250 ug/ml available nitrite).

Additionally, either during RDX or HMX degradation nitrite may tend to convert into nitrate or nitrate may be directly released as a degradation product. So, its estimation also signifies the degradation process.

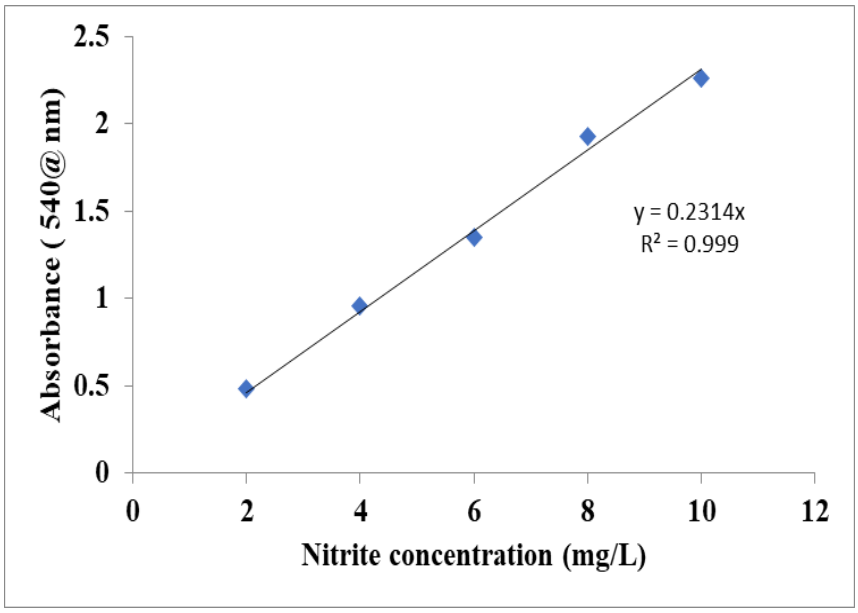


Figure 2.3 Standard curve for nitrite.

2.5.3 Nitrate analysis

Nitrate was estimated using the spectrophotometric method [12]. Briefly, 1 mL of centrifuged sample supernatant was taken in a clean tube. Then 5 mL of 1 N HCl was added to the sample and incubated for 5 min. The absorbance of sample was taken at 220 nm. However, to reduce the interference due to organic matter the absorbance at 220 nm is reduced by absorbance at 275 nm. The calibration was done using potassium nitrate as a standard (0.721 g/L potassium nitrate = 100 µg/mL available nitrate).

Further to identify different secondary metabolites formed during the degradation process mass spectroscopy was performed for the different samples at different intervals of time.

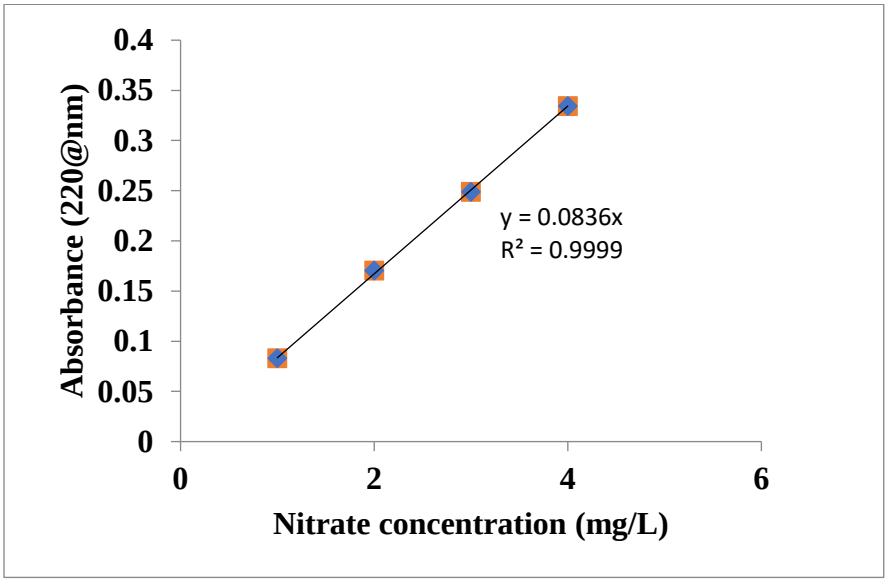


Figure 2.4 Standard curve for nitrate

2.5.4 Mass spectroscopic analysis

The microTOF-Q (Bruker Daltonics, Billerica, Massachusetts, USA) MS system was used to perform the studies, which used atmospheric pressure chemical ionization in the positive ion (ES+) mode [3,6]. The sample preparation method was similar to that described for the HPLC sample preparation method in section 2.5.1. However, the filter used was 0.22 µm

Teflon filter. To separate RDX or HMX and their degradation products, the C-18 column was employed. The sample run time was 5 minutes and the flow rate was 1 mL/min. 0.1% formic acid, 50% acetonitrile, and 49.9% triple distilled water made up the solvent system. The acquired peaks were analyzed using the system software and metabolites already described in the literature.

2.5.5 Scanning electron microscopy (SEM) analysis

The effect of RDX and HMX contamination on microbial cells was observed through scanning electron microscopy (SEM) analysis of samples. The samples for SEM were prepared in different steps such as fixation, dehydration, and post-fixation [13]. In brief, a small amount of sample (0.1 g) was taken and incubated overnight with 3% glutaraldehyde. After the incubation, samples were pelleted using a centrifuge (10000×g, 10 min) and washed 2-3 times with triple distilled water. After washing, the samples were dehydrated using ethanol. Initially, 35% of ethanol solution was mixed with pelleted sample, vortexed, and incubated for 5 min. Then the sample was centrifuged at 10000×g for 10 min and the pellet was collected. The dehydration process was carried out with a series of ethanol concentrations i.e., 35%, 50%, 75%, 95%, and 100%. Further, the pelleted sample was postfixed with hexamethyldisilazane (HMDS) and incubated for 30 min. It was spread onto a glass slide using the drop cast method and allowed to dry. The slide was placed in a sputterer to coat the sample with gold film and was analyzed using SEM (Supra55 Zeiss, Oberkochen, Germany). The instrument was operated at 10–12 mA current under vacuum with a voltage of 5 kV to increase the image quality.

The SEM images of isolated microbes are shown in figure 2.5 (A, B, C, and, D)

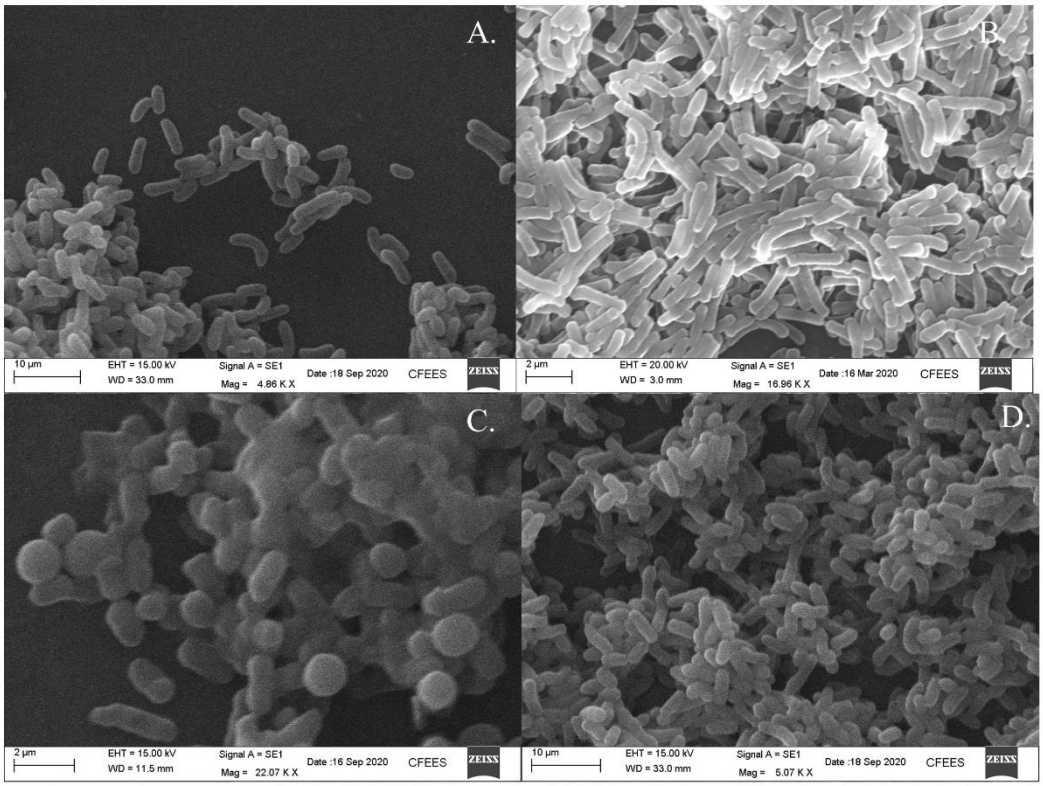


Figure 2.5 Scanning electron microscopic (SEM) images of microbes.

A. *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) B. *Arthrobacter subterraneus* (MTCC No. 12883, isolate no. S2-TSB-17) C. *Bacillus sonorensis* (MTCC no. 12855 isolate no. S8-TSB-4) D. *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857)

2.5.6. Total Kjeldahl nitrogen analysis (TKN)

As the nitrogen content is indicative of the quality of the compost, nitrogen analysis was done using a nitrogen analyzer (Pelican Equipment, KelPlusEmVa, Chennai, India) as per manufacturers' protocol. Briefly, 0.1 g of sample was taken in the tubes and mixed with 10 ml of concentrated H_2SO_4 and 3 g mixture of potassium sulfate and cupric sulfate (10:1). Afterward, the tubes were placed on the heater with the manifolds on so that the fumes are not released into the atmosphere. The distilled boric acid was titrated with 0.1 N HCl.

$$\text{TKN } (\%) = \frac{14.01 \times \text{N} \times (\text{T.V} - \text{B.V}) \times 100}{\text{S.wt} \times 1000} \qquad (2)$$

Where T.V is titrated volume of sample, B.V is titrated volume of blank, N is the normality of acid and S.wt is sample weight.

2.5.7 Total organic carbon analysis (TOC)

Total organic carbon (TOC) present in the compost is another important parameter for monitoring the composting process. The analysis was done as per Walkley and Black method [14]. Briefly, 1 g sample was put in a muffle furnace at 550°C for 8 hrs in a dried and pre-weighed crucible. The crucible was allowed to cool down (in a vacuum desiccator) post-treatment and weighed. Afterward, the TOC of the sample was calculated as follows:

$$Ash\% = \frac{\text{Wt. of sample after ignition} \times 100}{\text{Wt of sample before ignition}} \quad (3)$$

$$\text{organic carbon} = (100 - \text{Ash}\%)/1.724 \quad (4)$$

2.5.8. Compost characterization

The other parameters essential for the characterization of compost such as phosphate, potassium, moisture, and C: N ratio [12]. In brief, the phosphate and potassium estimation were carried out by Hach Spectrophotometric methods. Initially, the compost extract was prepared using Mehlich 2 extractant. 2 gm of soil was mixed with 20 mL of Mehlich extractant. Then after 5 min of shaking, the mixture was filtered and subjected to further analysis. The potassium analysis was done using the turbidimetric method (8049 methods). Further, the phosphate analysis was done using the phosver3 method (8048). Another major parameter is temperature and moisture which were measured using a hygrometer instrument (Hanna HI9565, United States). The C:N ratio was calculated based on the TOC and TKN ratio of the compost.

2.6 Conclusions

The instrumentation, analytical approach, and experimental plan used in the current thesis are described in this chapter. The study's main focus is on the utilization of native microbes in the treatment of RDX and HMX in water and soil. As a result, these methodologies are used in the next chapters to provide fascinating findings.

References

[1] A.M. Cook, R. Hütter, s-Triazines as Nitrogen Sources for Bacteria, J. Agric. Food Chem. 29 (1981) 1135–1143.

[2] A.A.C.A.S. Numbera, Method 8330B nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC), US Environ. Prot. Agency, Washington, DC. (2006).

[3] A. Meda, P. Sangwan, K. Bala, Optimization of process parameters for degradation of HMX with Bacillus toyonensis using response surface methodology, Int. J. Environ. Sci. Technol. 17 (2020) 4601–4610.

[4] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Aerobic biodegradation of HMX by Planomicrobium flavidum, 3 Biotech. 8 (2018) 1–7.

[5] M. Mäkelä, Experimental design and response surface methodology in energy applications: A tutorial review, Energy Convers. Manag. 151 (2017) 630–640.

[6] Meda, A., Sangwan, P., & Bala, K. (2021). Optimization and Degradation Studies on Hexahydro-1, 3, 5-Trinitro-1, 3, 5-Triazine (RDX) with Selected Indigenous Microbes under Aerobic Conditions. Water, 13(9), 1257.

[7] A. Meda, P. Sangwan, K. Bala, In-vessel composting of HMX and RDX contaminated sludge using microbes isolated from contaminated site, Environ. Pollut. 285 (2021) 117394.

[8] G.S. Nyanhongo, A. Erlacher, M. Schroeder, G.M. Gübitz,

Enzymatic immobilization of 2,4,6-trinitrotoluene (TNT) biodegradation products onto model humic substances, Enzyme Microb. Technol. 39 (2006) 1197–1204.

[9] A. APhA, WPCF (1985) Standard methods for the examination of water and wastewater, Am. Public Heal. Assoc. Washington, DC. 1 (1985) 134.

[10] M.D. Murtey, P. Ramasamy, Life science sample preparations for scanning electron microscopy, Acta Microsc. 30 (2021) 80–91.

[11] A. Walkley, I.A. Black, An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method, Soil Sci. 37 (1934) 29–38.

[12] Soil Kit Manual Npk-1, Hach company, 2001.

CHAPTER 3

Chapter 3

Biodegradation of RDX and possible metabolic pathway exploration

3.1 Introduction

Explosives are energetic nitrogen-based compounds with high potential energy. This category includes RDX, also known as hexahydro-1,3,5-trinitro-1,3,5-triazine, which is primarily employed for military purposes. Manufacturing, testing, training, demilitarisation, open burning, and waste discharge are all examples of military activities that have contaminated the soil and groundwater in the nearby region [1,2]. Furthermore, it has been established that RDX has a reasonably stable ring structure and the presence of electron-withdrawing nitro groups makes it less resistant to degradation in nature [3]. Since RDX has a low soil adsorption coefficient, it has a high risk of contaminating groundwater near military sites, testing facilities, and combat zones [1,4]. Since RDX is relatively mobile in the soil and degrades slowly, it poses serious environmental concerns [5]. As RDX is water-soluble (60 mg/L at 25 °C), it may get mixed into underground reservoirs and migrate to remote locations, posing a risk to the environment and human health. The USEPA has proposed a lifetime drinking standard of 2 µg/L for RDX [6].

RDX has been proven to be a harmful substance in previous research, and it is designated as a group 'C' human carcinogen by the USEPA [7]. It can produce seizures, convulsions, nausea, vomiting, and other symptoms [8–10]. It has the potential to harm the neurological system and the liver. In a variety of species, RDX can easily pass through the blood-brain barrier, change the expression of several brain genes, and cause dramatic seizure-like responses [11–14]. As a result, RDX polluted locations must be remedied to preserve human health and ecosystems. Thermal decomposition [15,16], photolysis [17], and catalyst treatment [18] are the most common methods for RDX clean

up. Traditional methods are not cost-effective and environment friendly because they necessitate complex instrumentation and produce additional by-products, such as ash, which is difficult to dispose of. The other option, microbial remediation, is earning a lot of attention these days. It is environmentally friendly, cost-effective, and considerably simpler to establish and carry out. RDX breakdown has been observed in a variety of microorganisms. *Klebsiella pneumonia*, a bacteria isolated from anaerobic sludge, can degrade RDX chains into methanol, CO_2, formaldehyde, and nitrous oxide by forming intermediates like methylene di-nitramine [19]. RDX is reported to be aerobically degraded by *Phanerochaete chrysosporium*, which produces 4-nitro-2,4-diazabutanal (NDAB) as an intermediate product, which can be mineralized entirely into CO_2 and N_2O [20,21]. *Clostridium bifermentans* may break down RDX aerobically into formaldehyde, methanol, and CO_2 via the intermediate products hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) [22]. Even though many studies have been conducted to better understand the RDX degradation process, the majority of them have focused on anaerobic degradation. As a result, this work was aimed to investigate the aerobic degradation of RDX and optimize the parameters that can influence the degradation process. Response surface methodology (RSM) was used to optimize the process [23–26]. RSM has also been applied by some previous researchers to enhance dye and explosive/pollutant removal from the medium [27–30]. Hence, this study was designed with the primary goal of determining the RDX degrading potential of two microbes, *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859), which were isolated from an actual explosive contaminated site and have yet to be investigated for optimization. The interaction of RDX degradation with independent factors (starting RDX concentration, inoculum volume, and time) was also investigated. For RDX degradation investigation, identification of metabolites, and understanding of the mechanism, mass spectroscopy (LC-MS/MS) was used. Figure 3.1 shows a schematic presentation of

highlighted section from the overall thesis theme addressed in the present chapter.

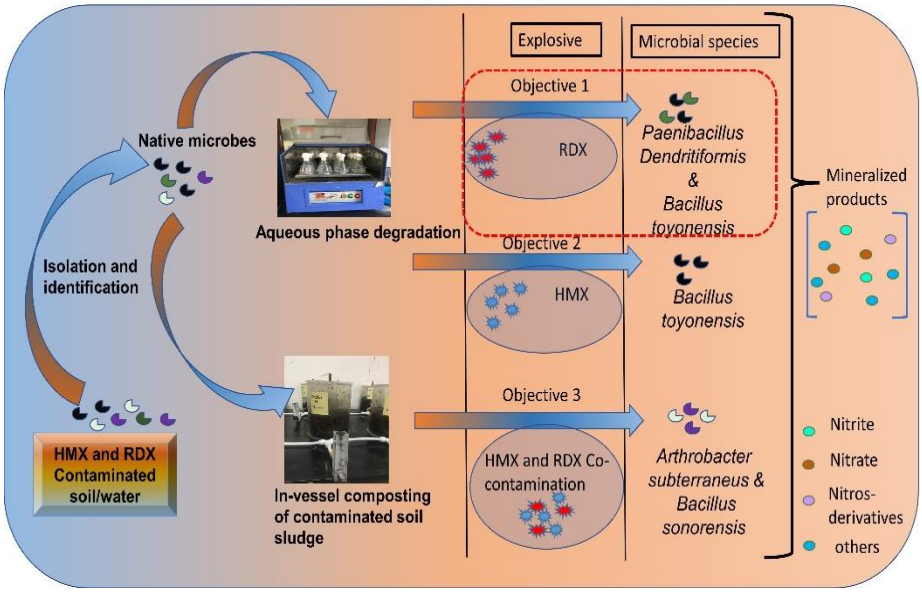


Figure 3.1 Schematic representation of RDX degradation study

3.2 Materials and methods

3.2.1 Chemicals

RDX with a purity of higher than 99.9% was taken from an explosive manufacturing plant in north India. Other chemicals utilized were of analytical grade and came from standard suppliers.

3.2.2 Microbial culture

Standard procedures were used to prepare microbial cultures as described in section 2.2 (Chapter 2). Briefly, aqueous phase degradation of RDX was done using *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859). Lyophilized microbes obtained from IMTECH, Chandigarh were first revived in TSB and then sub-cultured in MSM [31]. Further, an RDX degradation study was performed as described in section 2.3 [32–34]. RDX spiked MSM was inoculated with the microorganisms and cultured in an orbital shaker at 120 rpm at 32±3°C.

3.2.3 Experimental setup

As described in section 2.3.1, different combinations, are made with 3 independent variables i.e., the initial RDX concentration (20–60 mg/L),

time duration (5–15 days), and inoculum volume (2–6%) were observed (Table 3.1).

Table 3.1 Different levels and ranges used during the experiment

Parameters	**Upper limit (+1)**	**Lower limit (-1)**
Concentration RDX (mg/L)	60	20
Inoculum volume (%)	6	2
Time of degradation (days)	15	5

Table 3.2 Different runs for optimization of parameters for RDX degradation

RDX concentration (mg/L)	**Inoculation volume (%)**	**Time (Days)**
20	2	10
20	4	5
20	4	15
20	6	10
40	2	5
40	2	15
40	4	10
40	4	10
40	4	10
40	4	10
40	4	10
40	6	5
40	6	15
60	2	10
60	4	5
60	4	15
60	6	10

During the experiment, total 17 combinations for *Bacillus toyonensis* (isolate No. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate No.

S10-TSA-3) were created, as shown in table 3.2. All combinations were created with Box Behnken Design (DESIGN-EXPERT® VERSION 12 program, Stat-Ease, Minneapolis, MN, USA). The flasks were incubated at 32±3 °C at 120 rpm for 15 days and samples were collected at specific intervals (5, 10, and 15 days) and used to analyze different parameters.

3.2.4. Analysis

Reduction in the concentration was analyzed using HPLC as described in section 2.4. Nitrite was analyzed as discussed in section 2.5.4. Briefly, 600 µl of the sample was mixed with 150 µl of sulphanilamide and then incubated for 5 min. Afterward, 150 µl of NEDD was added to the mixture and again incubated for 20 min. The absorbance was recorded at 540 nm (Perkin Elmer, Model Lambda 650S, Waltham, MA, USA). Mass spectroscopic (MS) analysis was also performed to analyze the metabolites formed during the degradation process. The MS was done as described in section 2.5.2. The acquired peaks were analyzed using the system software and metabolites already described in the literature.

3.3. Results and discussion

3.3.1 Degradation of RDX

Degradation of RDX during the experiment can be observed in Figure 3.2 (A & B) with *Bacillus toyonensis* (isolate no. WS4-TSB-3). The Maximum RDX degradation achieved with this microbe was 81.7±1.3% with 40 mg/L initial RDX concentration and 6% inoculum volume on 15th day. This was followed by 78.7±1.1 and 77.01±0.8% RDX degradation at 20 mg/L and 60 mg/L, respectively with 4% inoculum volume on 15th day. Minimum degradation was 74.2±0.3% achieved at 40 mg/L initial RDX concentration and 2% inoculum volume. Figure 3.2.A shows the interactive effect of initial RDX concentration and time on RDX degradation. It was observed that with increase in time, there was increase in RDX removal. However, at higher RDX concentration (60 mg/L) removal efficiency was much lower, which can be due to toxic effect of RDX on *Bacillus toyonensis* (isolate no. WS4-TSB-3). Figure 3.2.B shows the interactive effect of inoculum

volume and RDX concentration on RDX degradation. It was observed that *Bacillus toyonensis* (isolate no. WS4-TSB-3) showed increased removal of RDX with increase in inoculum volume. The whole set of data for RDX degradation with *Bacillus toyonensis* (isolate no. WS4-TSB-3) was subjected to two-way analysis of variance (ANOVA) as shown in Table 3.3. The p-value (0.0002), F- value (23.6) and R^2 (0.9) of the model shows that the data was significant and best suited for the quadratic model. Figure 3.3 presents data between actual versus predicted value, which shows that there was very less dispersion of data between experimentally obtained and predicted values by the model. Also, very low standard deviation (2.2) was observed for the model, which confirms the suitability of the model. All the parameters were fitted for second order polynomial equation as follows-

$$Y = 73.07 + 1.94\ A + 0.9663\ B + 10.43\ C + 1.88\ AB - 3.32\ AC + 1.07\ BC - 2.02\ A^2 - 4.32\ B^2 - 2.43\ C^2 \quad (1)$$

Similarly, in figure 3.4 (A & B), it was observed that with *Paenibacillus dendritiformis* (isolate no. S10-TSA-3) in media, there was also a reduction in RDX concentration with time. With the increase in inoculum volume, increased degradation of RDX was observed. At the end of 15th day, maximum (84.7±0.9%) RDX degradation was observed at 40 mg/L initial concentration with 6% inoculum volume, which was followed by 78.1±1.1% at 20 mg/L concentration and 2 % inoculum volume. The maximum degradation achieved was nearly 1.2 times higher than the minimum degradation (71.7±1.1) observed at 20 mg/L concentration with 4 % inoculum volume on 15th day.

However, degradation in the control (unmodified media) due to abiotic factors was 0.8 and 1.1% for *Bacillus toyonensis* (isolate no. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate no. S10-TSA-3) respectively, which is negligible as compared to test samples. RDX degradation with *Planomicrobium flavidum, Rhodococcus* strain, *Phanerochaete chrysosporium*, *Clostridium bifermentans*, *Paenibacillus aestuarii and Arthrobacter subterraneus* having

degradation efficiency of more than 80% was observed by other researchers in 30-40 days [27,35–37].

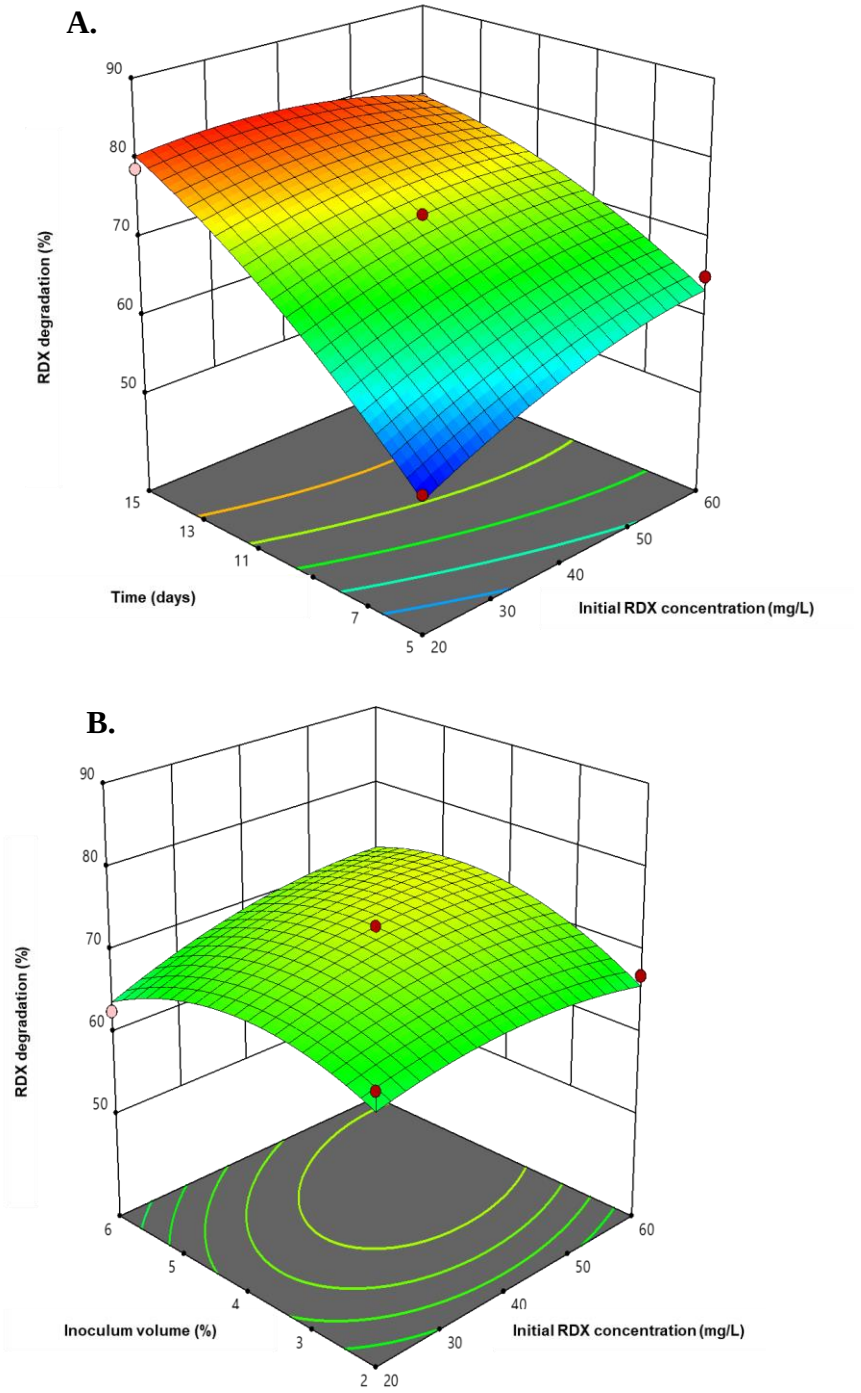


Figure 3.2 (A) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and time (days) with *Bacillus toyonensis*. (B) 3-D model plot for the degradation of RDX under

varying initial concentration (mg/L) and inoculum volume (%) with *Bacillus toyonensis*.

Table 3.3 ANOVA of Quadratic model for percent degradation of RDX with *Bacillus toyonensis*

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	1103.52	9	122.61	23.64	0.0002	Significant
A-RDX concentration	30.11	1	30.11	5.81	0.0468	
B-Inoculum volume	7.47	1	7.47	1.44	0.2692	
C-Time	870.49	1	870.49	167.83	< 0.0001	
AB	14.18	1	14.18	2.73	0.1423	
AC	44.02	1	44.02	8.49	0.0226	
BC	4.62	1	4.62	0.8912	0.3766	
A^2	17.14	1	17.14	3.30	0.1119	
B^2	78.58	1	78.58	15.15	0.0060	
C^2	24.86	1	24.86	4.79	0.0647	
Residual	36.31	7	5.19			
Lack of Fit	36.31	3	12.10			
Pure Error	0.0000	4	0.0000			
Cor Total	1139.83	16				

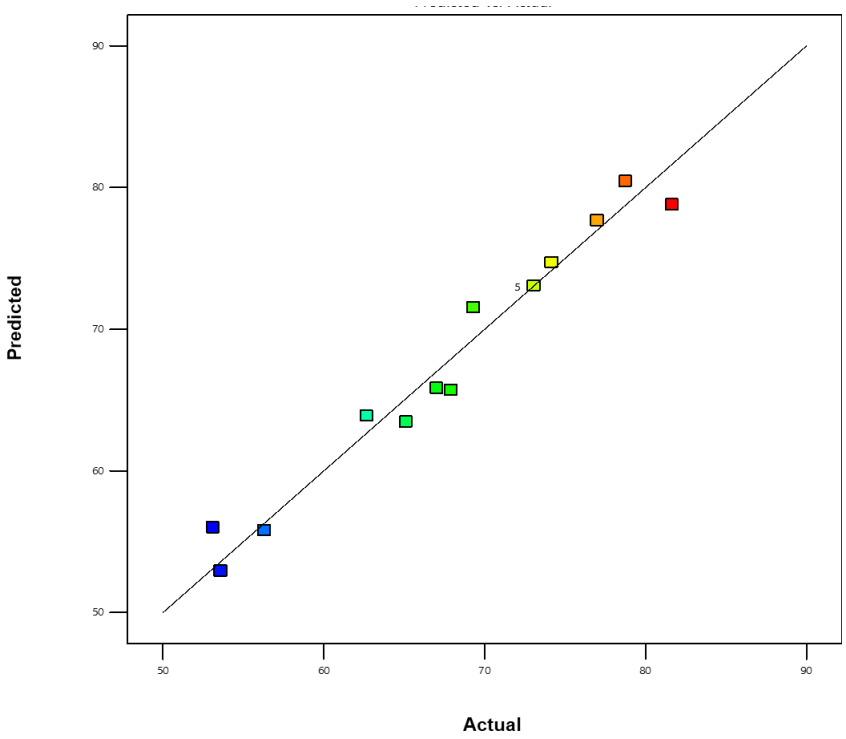


Figure 3.3 Actual versus predicted graph for the RDX degradation with *Bacillus toyonensis*.

Figure 3.4.A shows the 3-D plot for interaction of initial RDX concentration and time during the RDX degradation. It was observed that with the increase of both the variables, there was increase in RDX degradation. Even, increase in initial RDX concentration does not have negative impact on the degradation efficiency of microbes. This observation implies that *Paenibacillus dendritiformis* (isolate no. S10-TSA-3) can survive and performs better at higher concentration of RDX (60 mg/L). Similarly, Figure 3.4.B shows the effect of inoculum volume on RDX degradation. It was observed that there was higher degradation of RDX with 6 % inoculum volume. To validate the model, two-way ANOVA was performed, and it was observed that the model was statistically significant (Table 3.4). Obtained p-value (0.0003), F-value (21.6) and R^2 (0.9) were significant and shows that the model best suited for quadratic model.

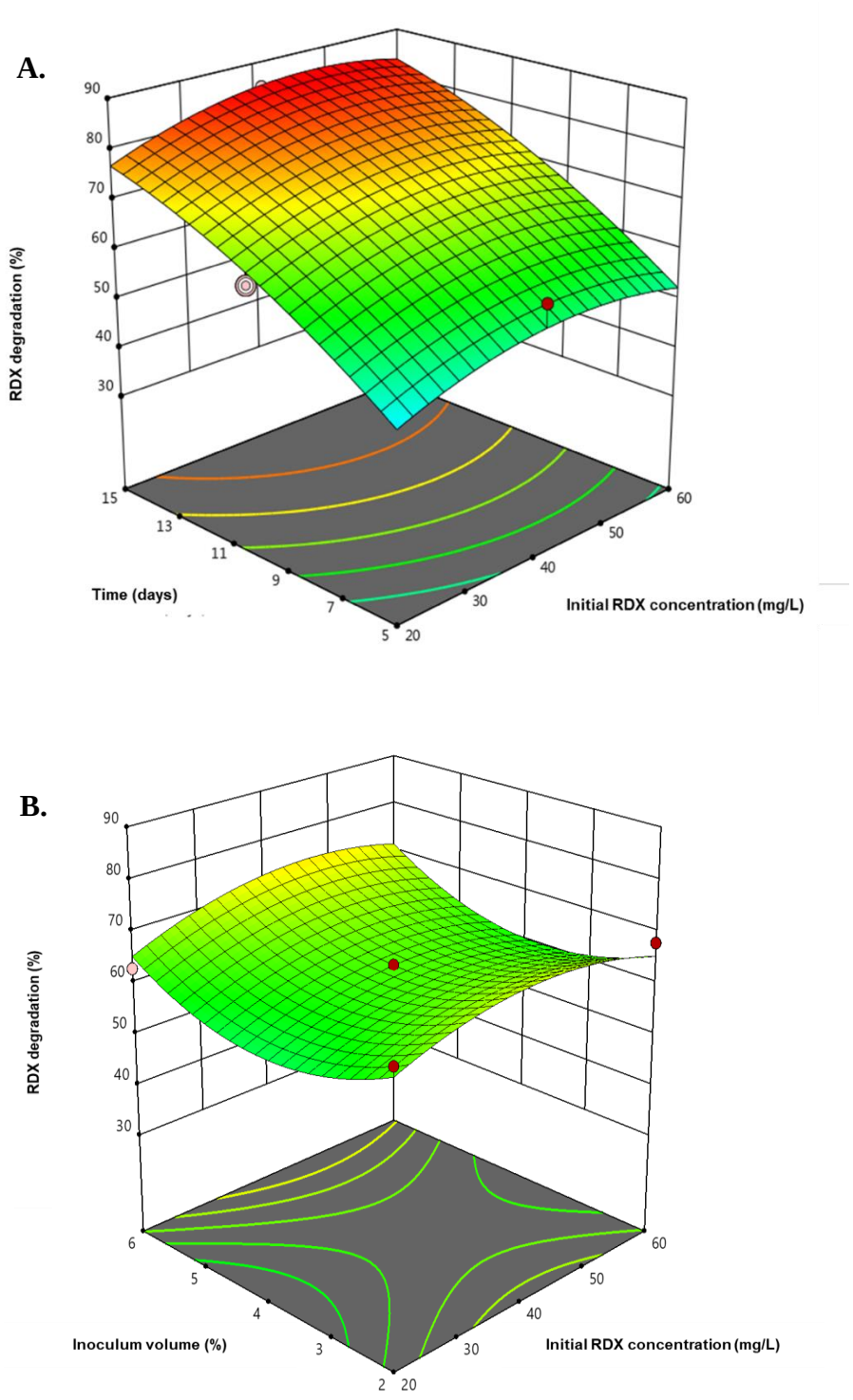


Figure 3.4 (A) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and time (days) with *Paenibacillus dendritiformis.* (B) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and inoculum volume (%) with *Paenibacillus dendritiformis.*

Figure 3.5 shows the difference between actual and predicted values, which shows that there was very less variation between experimentally obtained values and values predicted by the model for RDX degradation. All parameters were fitted for second order polynomial equation as follows-

$$Y = 63.74 + 2.02\,A + 1.99\,B + 15.94\,C + 0.9850\,AB + 0.3925\,AC - 0.7525\,BC - 5.01\,A2 + 7.73\,B2 - 3.42\,C2 \quad (2)$$

Similar results for ANOVA were obtained in earlier studies by other authors also. Mohanty & Jena, (2018) obtained similar results during the optimization of butachlor remediation with *Enterobacter cloacae* [38]. Sharma et al. (2021) observed a similar two-way ANOVA results during the remediation of RDX in aqueous phase with the consortium of microbes [27].

3.3.2. Release of nitrite during RDX degradation

It is well established that nitrite ions are released during the degradation of RDX. Ring cleavage of RDX starts with the denitration-hydration step, with the formation of NADB and formaldehyde resulting into the release of nitrite ion [36,39]. Similar observations were made during this study. As RDX degraded, there was change in the nitrite concentration in the medium with both the microbes in their respective combinations. Figure 3.6.A shows the change in nitrite concentration for *Bacillus toyonensis* (isolate no. WS4-TSB-3) with respect to RDX concentration and time. Maximum concentration of nitrite release (0.3±0.01 mg/L) with *Bacillus toyonensis* was observed on 10^{th} day with 60 mg/L concentration and 6% inoculum volume, which was followed by 0.3±0.01 mg/L at 60 mg/L RDX concentration and 4% inoculum volume. Similarly, Figure 3.6.B shows the nitrite release during the RDX degradation with *Paenibacillus dendritiformis* (isolate no. S10-TSA-3).

Table 3.4 ANOVA of Quadratic model for percent degradation of RDX with *Paenibacillus dendritiformis*.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	2489.85	9	276.65	21.68	0.0003	Signif-icant
A-RDX concentration	32.76	1	32.76	2.57	0.1531	
B-Inoculum volume	31.72	1	31.72	2.49	0.1588	
C-Time	2032.03	1	2032.03	159.28	< 0.0001	
AB	3.88	1	3.88	0.3042	0.5984	
AC	0.6162	1	0.6162	0.0483	0.8323	
BC	2.27	1	2.27	0.1775	0.6861	
A^2	105.79	1	105.79	8.29	0.0237	
B^2	251.75	1	251.75	19.73	0.0030	
C^2	49.25	1	49.25	3.86	0.0902	
Residual	89.30	7	12.76			
Lack of Fit	89.30	3	29.77			
Pure Error	0.0000	4	0.0000			
Cor Total	2579.15	16				

Maximum nitrite release was observed on 10^{th} day, which was 0.2±0.01 mg/L at 60 mg/L RDX concentration and 2% inoculum volume. It was observed, that with increase in RDX concentration there was increase in the release of nitrite. Also, with the degradation of RDX, there was increase in nitrite concentration till 10^{th} day, and afterward it started decreasing. Decrease in the nitrite concentration can be due to its utilization by microbes or conversion into nitrate [40,41].

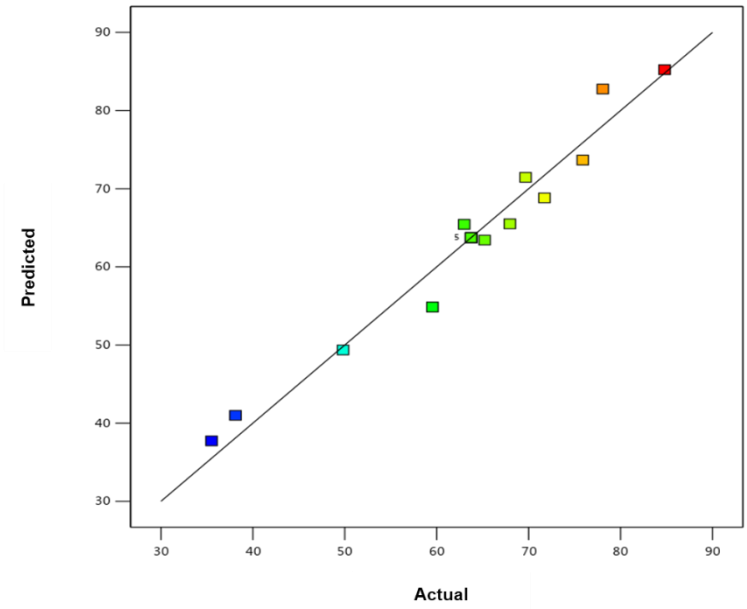


Figure 3.5 Actual versus predicted graph for the RDX degradation with *Paenibacillus dendritiformis*.

To validate, the data was subjected to two-way ANOVA. For nitrite release it was observed that microbes, *Bacillus toyonensis* (isolate no. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate no. S10-TSA-3) has significant p-value (<0.0001 & 0.0009), F-value (34.5 and 14.9) and R^2 (0.9 and 0.9 respectively) which statistically validate the model for both the microbes (Table 3.5). Correction total (sum of square) for both the microbes shows that the model has high reproducibility and less variation around the mean. Statistically similar results were obtained by Chaudhary et al. (2019) during their optimization of tannery wastewater remediation with *Aspergillus fumigates* [42]. Garg et al. (2015) found similar ANOVA results during the optimization of decolorization of different dyes with *Pseudomonas* strain [43].

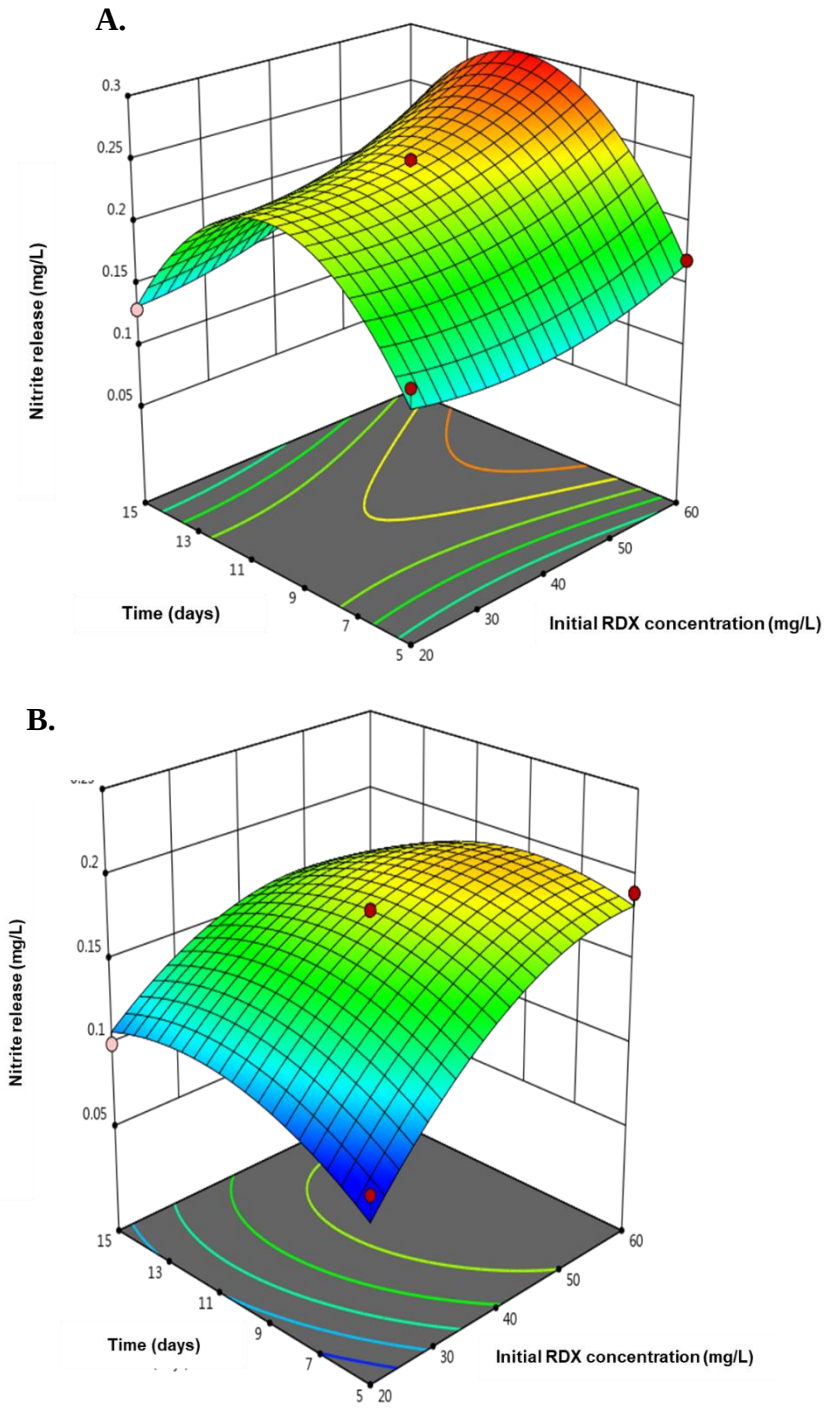


Figure 3.6 (A) Response surface plot (3-D plot) showing the interactive effect of RDX concentration (mg/L) and time (days) on nitrite release with *Bacillus toyonensis.* (B) Response surface plot (3-D plot) showing interactive effect of RDX concentration (mg/L) and time (days) on nitrite release with *Paenibacillus dendritiformis*.

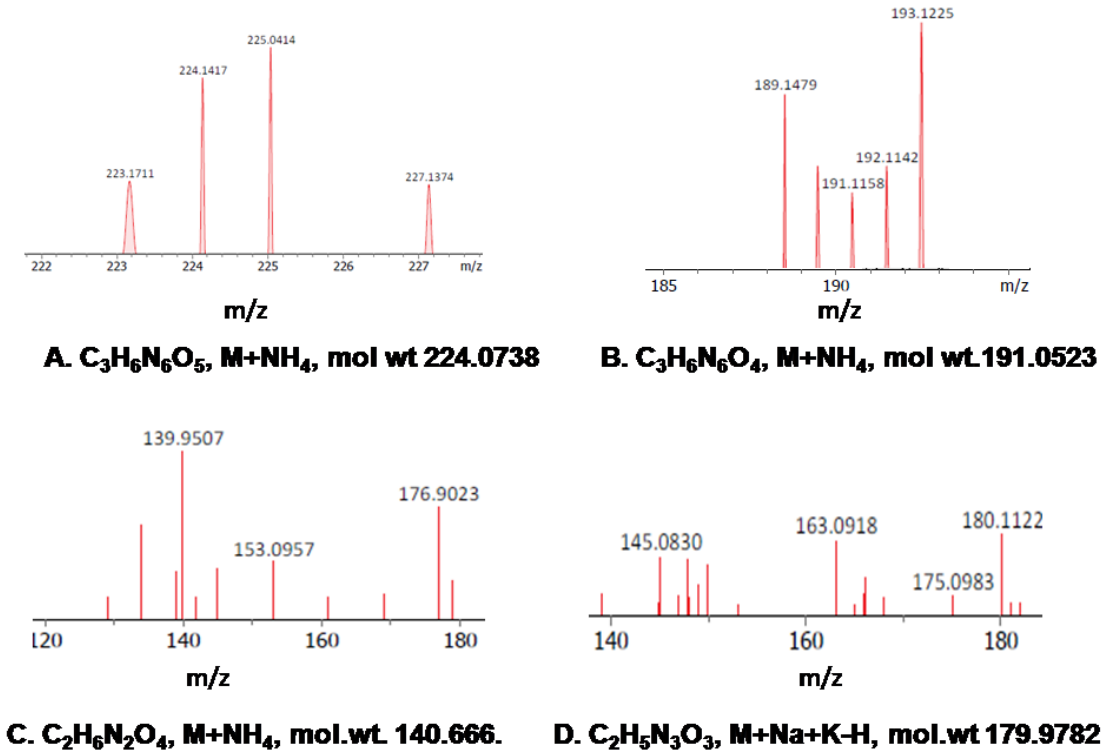
Table 3.5 ANOVA of Quadratic model for the release of nitrite during RDX degradation.

Factor	***Bacillus toyonensis***	***Paenibacillus dendritiformis***
p-value	< 0.0001	0.0009
F-value	34.52	14.88
R^2	0.97	0.95
Cor-total	0.0624	0.0236
Std. dev	0.0140	0.0130

3.3.3 Degradation pathway

It is already known that during the microbial degradation process, the cyclic structure of RDX tends to break into intermediate products. To understand and elucidate the RDX degradation pathway for both the microbes, mass spectroscopy of the samples was done at different intervals of time i.e., 5^{th} day and 10^{th} day in the combination having highest degradation. Positive ESI (Electron spray ionization) revealed the presence of different metabolites in the samples. The peaks obtained were at m/z values of 224.07 and 191.05 on 5^{th} day samples and 140.66 and 179.97 on 10^{th} day samples (Figure 3.7. A, B, C and D). To identify the metabolites, molecular weight (m/z ratio) of the obtained peaks were compared with the metabolites reported in earlier studies [37,39,44–47]. The peaks 224.07 and 191.05, were identified as Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, $C_3H_6N_6O_5$, $M+NH_4$, mol. wt. 224.07 Da) and Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, $C_3H_6N_6O_4$, $M+NH_4$, mol. wt. 191.05 Da), respectively. The presence of MNX and DNX during the study suggests that the RDX degradation occurred by single nitrite elimination pathway in which, the transfer of a single electron to nitramino group leads to the RDX ring cleavage. Further, on 10^{th} day the peaks observed were 140.66 and 179.97 (m/z) which were identified as Bis(hydroxymethyl)nitramine ($C_2H_6N_2O_4$, $M+NH_4$, mol. wt. 140.66 Da) and 4-Nitro-2,4-diazabutanal ($C_2H_5N_3O_3$, M+Na+K-H, mol. wt. 179.97 Da) respectively. Subsequent studies have

shown that both 4-nitro-2,4-diazabutanal and Bis(hydoxymethyl)nitramine are the de-nitration ring cleavage products of RDX. Also, earlier studies have shown that, MNX can get transformed into 4-nitro-2,4-diazabutanal [48]. Further, Halasz & Hawari (2011) showed that DNX, 4-nitro-2,4-diazabutanal and Bis(hydoxymethyl)nitramine can undergo degradation and forms CO_2, and nitrous oxide, formaldehyde and ammonia as end products [49]. It was observed that the metabolites identified for both the microbes were similar. This shows that both the microbes follow the same degradation pathway. Based on the findings mentioned above the RDX degradation pathway was proposed which is shown in Figure 3.8.



A. $C_3H_6N_6O_5$, M+NH_4, mol wt 224.0738 **B. $C_3H_6N_6O_4$, M+NH_4, mol wt.191.0523**

C. $C_2H_6N_2O_4$, M+NH_4, mol.wt. 140.666. **D. $C_2H_5N_3O_3$, M+Na+K-H, mol.wt 179.9782**

Figure 3.7 Mass Spectra of metabolites formed during RDX degradation. A. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, $C_3H_6N_6O_5$, M+NH_4, mol. wt. 224.07), B. Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, $C_3H_6N_6O_4$, M+NH_4, mol. wt. 191.05), C. Bis(hydroxymethyl)nitramine ($C_2H_6N_2O_4$, M+NH_4, mol. wt. 140.66) and D. 4-Nitro-2,4-diazabutanal ($C_2H_5N_3O_3$, M+Na+K-H, mol. wt. 179.97)

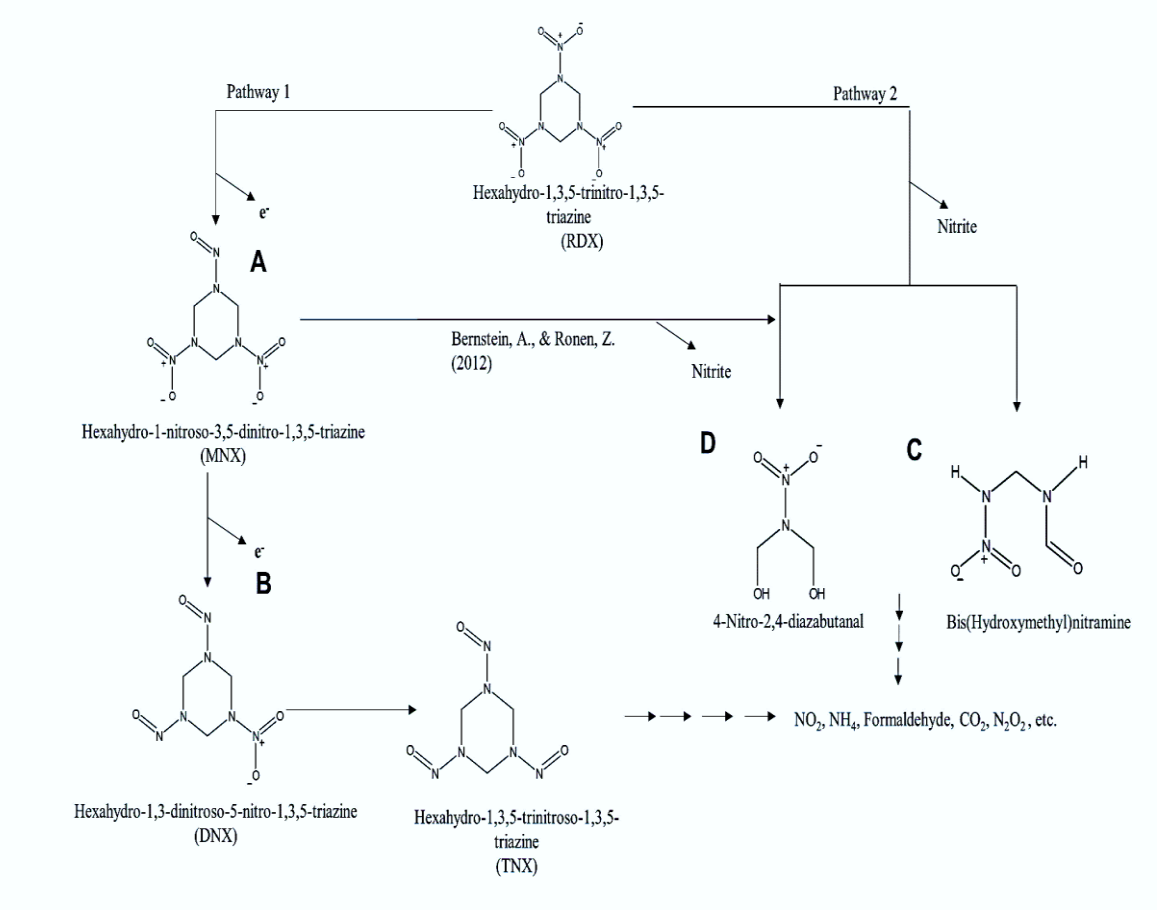


Figure 3.8 Proposed pathway for biodegradation of RDX with *Bacillus toyonensis* and *Paenibacillus dendritiformis*

3.4 Conclusions

Two microbial species obtained from an explosive polluted location were used in this work to investigate the decomposition of RDX. Both species were discovered to be capable of removing RDX from contaminated water. At a dose of 40 mg/L, the maximal degradation recorded with *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) was 81.6±1.3%, and with *Paenibacillusden dritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) was 84.7±0.9%.

The three-dimensional graph depicted the optimization of process parameters for RDX degradation as well as the interaction between the independent variables. Each variable has a direct and beneficial impact on RDX degradation, as shown in these graphs. The model for RDX breakdown obtained with both microorganisms demonstrated great

repeatability and statistical significance. MNX, DNX, 4-Nitro-2,4-diazabutanal, and bis(hydroxymethyl)nitramine were found as intermediary metabolites during the breakdown of RDX. These metabolites can be mineralized into CO_2, NH_4, formaldehyde, and nitrous oxide after a further breakdown.

The study concludes that *Bacillus toyonensis* and *Paenibacillus dendritiformis* were both have good potential of eliminating RDX. Also, Chapter 3 concludes that the bacteria recovered from the polluted site are capable of decomposing explosives like RDX from the aqueous phase.

References

[1] T.W. Sheremata, A. Halasz, L. Paquet, S. Thiboutot, G. Ampleman, J. Hawari, The fate of the cyclic nitramine explosive RDX in natural soil, Environ. Sci. Technol. 35 (2001) 1037–1040.

[2] S. Chatterjee, U. Deb, S. Datta, C. Walther, D.K. Gupta, Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation, Chemosphere. 184 (2017) 438–451.

[3] E. Sisco, M. Najarro, C. Bridge, R. Aranda IV, Quantifying the degradation of TNT and RDX in a saline environment with and without UV-exposure, Forensic Sci. Int. 251 (2015) 124–131.

[4] W.A. Tucker, G.J. Murphy, E.D. Arenberg, Adsorption of RDX to soil with low organic carbon: Laboratory results, field observations, remedial implications, Soil Sediment Contam. 11 (2002) 809–826.

[5] M. Lapointe, R. Martel, E. Diaz, A conceptual model of fate and transport processes for RDX deposited to surface soils of North American active demolition sites, J. Environ. Qual. 46 (2017) 1444–1454.

[6] United States Environmental Protection Agency, 2006 Edition of the Drinking Water Standards and Health Advisories, Environ. Prot. (2006) 18.

http://water.epa.gov/action/advisories/drinking/upload/dwstandards2009.pdf.

[7] U. Environmental Protection Agency Federal Facilities Restoration, R. Office, Fact Sheet – Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), (2021) 1–7.

[8] Services, U.S. Department Of Health And Human Service, Public Health, Agency for Toxic Substances and Disease Registry, P.H. for T.S. and D.R. Service, Toxicological Profile for RDX, 2002. https://doi.org/10.1201/9781420061888_ch137.

[9] USEPA, Handbook on the Management of Munitions Response Actions, (2005) 315. http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P100304J.txt.

[10] Hazardous Substance Data Bank (HSDB) (2016). https://www.nlm.nih.gov/databases/download/hsdb.html (accessed August 3, 2022).

[11] K.A. Gust, M.S. Wilbanks, X. Guan, M. Pirooznia, T. Habib, L. Yoo, H. Wintz, C.D. Vulpe, E.J. Perkins, Investigations of transcript expression in fathead minnow (Pimephales promelas) brain tissue reveal toxicological impacts of RDX exposure, Aquat. Toxicol. 101 (2011) 135–145.

[12] M.J. Quinn Jr, M.A. Bazar, C.A. McFarland, E.J. Perkins, K.A. Gust, M.S. Johnson, Sublethal effects of subacute exposure to RDX (1, 3, 5-trinitro-1, 3, 5-triazine) in the northern bobwhite (Colinus virginianus), Environ. Toxicol. Chem. An Int. J. 28 (2009) 1266–1270.

[13] L. Williams, K. Wong, A. Stewart, … C.S.-… and P.P.C., undefined, Behavioral and physiological effects of RDX on adult zebrafish, Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology (2011)155(1), 33-38.

[14] B. Zhang, X. Pan, RDX induces aberrant expression of microRNAs in mouse brain and liver, Environ. Health Perspect. 117 (2009) 231–240.

[15] W. Jung, B.W. Nyande, S.H. Lee, W.Y. Jeong, J. Su Park, I.L. Moon, M. Oh, CFD simulation for demilitarization of RDX in a

rotary kiln by thermal decomposition, J Eng Sci and Tech (2017)1662–1676.

[16] Bulusu, S., & Jr., R. A Review of the Thermal Decomposition Pathways in RDX, HMX and Other Closely Related Cyclic Nitramines. Defence Science Journal (2013) 46(5), 347-360.

[17] Mahbub, P., Nesterenko, P.N. Application of photo degradation for remediation of cyclic nitramine and nitroaromatic explosives. RSC Adv (2016).

[18] Wildman, M.J., Alvarez, P.J.J. RDX degradation using an integrated Fe(0)-microbial treatment approach. Water Sci. Technol (2001) 43, 25–33.

[19] J.S. Zhao, A. Halasz, L. Paquet, C. Beaulieu, J. Hawari, Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by Klebsiella pneumoniae strain SCZ-1 isolated from an anaerobic sludge, Appl. Environ. Microbiol. 68 (2002) 5336–5341.

[20] J. Stahl, B. Aken, M. Cameron, S.A.-B. journal, undefined 2001, Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) Biodegradation in Liquid and Solid-State Matrices by Phanerochaete chrysosporium, Taylor Fr. 5 (2001) 13–25.

[21] D. Fournier, A. Halasz, J. Spain, R.J. Spanggord, J.C. Bottaro, J. Hawari, Biodegradation of the Hexahydro-1,3,5-Trinitro-1,3,5-Triazine Ring Cleavage Product 4-Nitro-2,4-Diazabutanal by Phanerochaete chrysosporium, Appl. Environ. Microbiol. 70 (2004) 1123–1128.

[22] J.S. Zhao, L. Paquet, A. Halasz, J. Hawari, Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in Clostridium bifermentans HAW-1, Appl. Microbiol. Biotechnol. 63 (2003) 187–193.

[23] M. Mäkelä, Experimental design and response surface methodology in energy applications: A tutorial review, Energy Convers. Manag. 151 (2017) 630–640.

[24] M.A. Bezerra, R.E. Santelli, E.P. Oliveira, L.S. Villar, L.A. Escaleira, Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta. 76 (2008) 965–977.

[25] R.H. Myers, D.C. Montgomery, C.M. Anderson-Cook, Response surface methodology: process and product optimization using designed experiments, John Wiley & Sons, (2016).

[26] N.R. Draper, J.A. John, Response-surface designs for quantitative and qualitative variables, Technometrics. 30 (1988) 423–428.

[27] K. Sharma, P. Sharma, S.M. Celin, P.K. Rai, P. Sangwan, Degradation of high energetic material hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a microbial consortium using response surface methodological approach, SN Appl. Sci. 3 (2021) 1–8.

[28] A. Meda, P. Sangwan, K. Bala, Optimization of process parameters for degradation of HMX with Bacillus toyonensis using response surface methodology, Int. J. Environ. Sci. Technol. 17 (2020) 4601–4610.

[29] P. Sangwan, S.M. Celin, L. Hooda, Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using Acinetobacter noscomialis, Eur. J. Adv. Eng. Technol. 2 (2015) 51–56.

[30] B. Kiran, A. Kaushik, C.P. Kaushik, Response surface methodological approach for optimizing removal of Cr (VI) from aqueous solution using immobilized cyanobacterium, Chem. Eng. J. 126 (2007) 147–153.

[31] A.M. Cook, R. Hütter, s-Triazines as Nitrogen Sources for Bacteria, J. Agric. Food Chem. 29 (1981) 1135–1143.

[32] J. Lamba, S. Anand, J. Dutta, S. Chatterjee, S. Nagar, S.M. Celin, P.K. Rai, Study on aerobic degradation of 2,4,6-trinitrotoluene (TNT) using Pseudarthrobacter chlorophenolicus collected from the contaminated site, Environ. Monit. Assess. 193 (2021).

[33] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Aerobic biodegradation of HMX by Planomicrobium flavidum, 3 Biotech. 8 (2018) 1–7.

[34] United states of environmental protection agency, Method 8330B nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC), US Environ. Prot. Agency, Washington, DC. (2006).

[35] H.A. Mercimek, S. Dincer, G. Guzeldag, A. Ozsavli, F. Matyar, Aerobic Biodegradation of 2,4,6-Trinitrotoluene (TNT) by Bacillus cereus İsolated from Contaminated Soil, Microb. Ecol. 66 (2013) 512–521.

[36] M.E. Fuller, N. Perreault, J. Hawari, Microaerophilic degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by three Rhodococcus strains, Lett. Appl. Microbiol. 51 (2010) 313–318.

[37] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Biodegradation of octogen and hexogen by Pelomonas aquatica strain WS2-R2A-65 under aerobic condition, Environ. Technol. (United Kingdom). 43 (2022) 1003–1012.

[38] S.S. Mohanty, H.M. Jena, Process optimization of butachlor bioremediation by Enterobacter cloacae using Plackett Burman design and response surface methodology, Process Saf. Environ. Prot. 119 (2018) 198–206.

[39] D. Fournier, A. Halasz, J. Spain, P. Fiurasek, J. Hawari, Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with Rhodococcus sp. strain DN22, Appl. Environ. Microbiol. 68 (2002) 166–172.

[40] R. Singh, P. Soni, P. Kumar, S. Purohit, A. Singh, Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria, World J. Microbiol. Biotechnol. 25 (2009) 269–275.

[41] K.-D. Zoh, M.K. Stenstrom, Fenton oxidation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX), Water Res. 36 (2002)

1331–1341.

[42] P. Chaudhary, V. Beniwal, R. Kaur, R. Kumar, A. Kumar, V. Chhokar, Efficacy of Aspergillus fumigatus MCC 1175 for bioremediation of Tannery Wastewater, CLEAN–Soil, Air, Water. 47 (2019) 1900131.

[43] S.K. Garg, M. Tripathi, N. Lal, Response surface methodology for optimization of process variable for reactive orange 4 dye discoloration by Pseudomonas putida SKG-1 strain and bioreactor trial for its possible use in large-scale bioremediation, Desalin. Water Treat. 54 (2015) 3122–3133.

[44] Indest KJ, Jung CM, Chen HP, Hancock D, Florizone C, Eltis LD, Crocker FH. Functional characterization of pGKT2, a 182-kilobase plasmid containing the xplAB genes, which are involved in the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by Gordonia sp. strain KTR9. Appl Environ Microbiol. (2010) 76(19):6329-37.

[45] Bhatt, M., Zhao, J.S., Halasz, A., Hawari, J. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment. J. Ind. Microbiol. Biotechnol. 33, 850–858.

[46] Sheremata, T.W., Hawari, J. Mineralization of RDX by the white rot fungus Phanerochaete chrysosporium to carbon dioxide and nitrous oxide. Environ. Sci. Technol (2000) 34, 3384–3388.

[47] K.T. Thompson, F.H. Crocker, H.L. Fredrickson, Mineralization of the cyclic nitramine explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by Gordonia and Williamsia spp, Appl. Environ. Microbiol. 71 (2005) 8265–8272.

[48] A. Bernstein, Z. Ronen, Biodegradation of the explosives TNT, RDX and HMX, in: Microb. Degrad. Xenobiotics, Springer, (2012): pp. 135–176.

[49] Halasz, A., Hawari, J. Degradation routes of RDX in various redox systems, in: ACS Symposium Series. American Chemical Society, (2011) pp. 441–462.

CHAPTER 4

Chapter 4

Process development for the biodegradation of HMX using response surface methodology

4.1 Introduction

HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine, Octogen) is extensively utilized in military applications. It may get into the environment during the manufacturing, transportation, usage, and demilitarization operations. In previous studies, HMX is shown to be toxic to mammals [1,2]. Convulsions, nausea, and loss of consciousness have been reported in mammalian species exposed to HMX [3]. In numerous animal studies, HMX has been shown to impact the liver and central nervous system, accumulate in the kidney, liver, brain, and heart, and even induce mortality and histopathological abnormalities [4]. However, the United States Environmental Protection Agency (USEPA, 1998) has categorized HMX as non-carcinogenic [5]. Due to low adsorption and high mobility in the soil, HMX can lead to contamination of groundwater [6,7]. It can limit soil microbial diversity, resulting in lower natural degradation capability of such contaminants, affecting the soil profile and suitability for farming in locations near military bases [8]. High level of HMX contamination has been observed all over the world [9]. USEPA has set a lifetime safe drinking water limit of 0.4 mg/L for HMX [10].

Various approaches have been proposed for treating HMX contamination of soil and water. Iron-dependent depletion [11], catalytic transformation [12], incineration, and oxidation are general ways for HMX treatment [13]. However, conventional treatment processes are not cost-efficient, as they waste a lot of energy, and produce toxic end products such as non-reusable catalysts, effluents, and ash as residual waste [14,15]. Nowadays, many researchers have focused on microbial

degradation process, as an environmentally benign and cost-effective method [16–20]. Under aerobic conditions, Nagar et al. (2018) investigated the breakdown of HMX by *Planomicrobium flavidum* [21]. *Phanerochaete chrysosporium* was found to degrade HMX (600 nmol) within 25 days of incubation [22]. As, many of the microbes have been shown to remove HMX, but to the best of our knowledge, there have been no literature on the breakdown of HMX by *Bacillus toyonensis*. Furthermore, as the degradation of HMX is species-specific and must be optimized to scale up the process.

Response surface methodology (RSM) can be used to optimize various process parameters. It is a statistical tool for designing, improving, and optimizing a process with several variables influencing the response that employs lower-order polynomial equations. RSM minimizes the overall number of possible possibilities, saving time and resources during testing [23–26]. The Box–Behnken design (BBD) model, which is a multivariate mathematical model for 3-level factorial design, was employed in this work. In earlier studies, RSM has been used to study the removal of chromium ions with the help of a cyanobacterial species [27]. Sangwan et al. (2015) used RSM to investigate the effect of pH, concentration, and degradation time on the removal of TNT [20].

The objective of this work is to optimize the process parameters involved in the degradation of HMX using *Bacillus toyonensis*, which was isolated from an actual HMX-contaminated site. To the best of our knowledge, this is the first investigation of a novel bacterial strain i.e., *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) to investigate HMX degradability. The experiment was designed, and the data were analyzed using the DESIGN-EXPERT ® VERSION 12 program (Stat-Ease®, Minneapolis). During the microbial degradation of HMX, relationships between numerous independent and dependent factors were investigated to better understand the interaction influence. Figure 4.1 shows a schematic presentation of highlighted section from the overall thesis theme addressed in the present chapter.

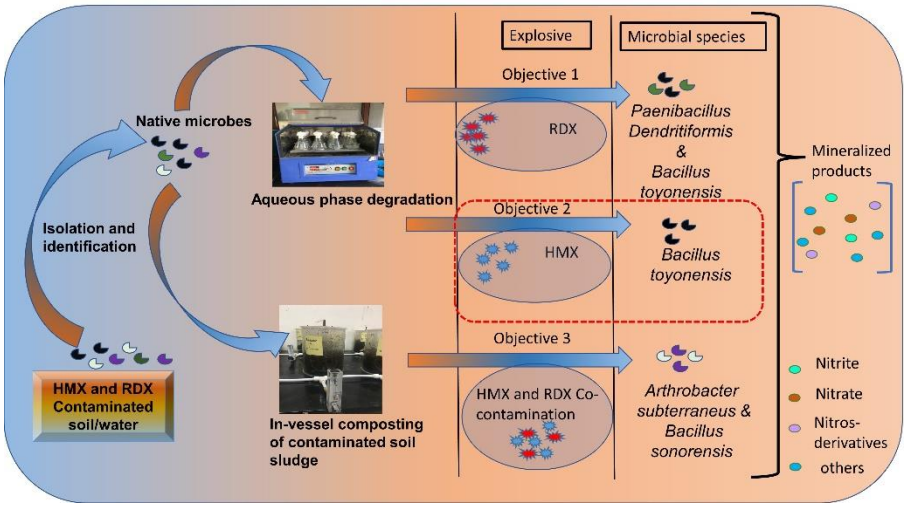


Figure 4.1 Schematic representation of HMX degradation study in overall thesis theme.

4.2 Materials and methods

4.2.1 Chemicals

Commercial grade HMX was obtained from the HMX manufacturing facility in North India with a purity of greater than 99.5%. Chemicals for high-performance liquid chromatography (HPLC) were purchased from Sigma-Aldrich, while other analytical grade chemicals were purchased from other established suppliers.

4.2.2 Microbial culture

The microbes were cultured as explained earlier in section 2.2, chapter 2 [26]. Briefly, *Bacillus toyonensis* was revived in tryptic soya broth (TSB). Further, before cultivating microbes into modified minimal salts medium (MSM) containing HMX, microbes were sub-cultured for three generations in MSM.

The degradation study was performed as per discussed in section 2.3 (Chapter 2). Briefly, MSM was spiked with stock solution (1000 mg/L) and the desired concentration of HMX i.e., 2, 4, and 6 mg/L were obtained. Flasks were kept open for 18 hours in an aseptic laminar airflow chamber to evaporate the acetonitrile and avoid any interaction with the medium composition [21,28]. Bacterial growth was measured

at regular intervals by monitoring the absorbance at 600 nm (Perkin Elmer, Model Lambda 650S).

4.2.3 Experimental setup

The experimental setup was prepared as discussed in section 2.3.1. In brief, HMX concentration (2–6 mg/L), degradation time (5–15 days), and inoculum size (2–6%) were used as the variables during the study (table 4.1). A total of 17 runs (table 4.2) were operated, which were prepared using Box–Behnken design (DESIGN EXPERT ® VERSION 12, Stat-Ease®, Minneapolis). Flasks were inoculated and incubated in an orbital shaker at 32±3 ℃ and 120 rpm. The entire study was carried out in an aerobic environment. Samples were taken at specified intervals of time (5 days), to assess optical density (O.D), nitrite release, nitrate content, and HMX degradation.

Table 4.1 Different levels and ranges used during the experiment

Parameters	**Upper limit (+1)**	**Lower limit (-1)**
Concentration HMX (mg/L)	6	2
Inoculum volume (%)	6	2
Time of degradation (days)	15	5

4.2.4 Analysis

Samples were analyzed using HPLC for HMX degradation according to standard procedure 8330A, mentioned in section 2.5.1 [28].

Further, nitrate and nitrite in the samples were evaluated using a Hach spectrophotometer (DR1900) by cadmium reduction method. Employing NitraVer 5_8192 LR powder pillow and NitriVer 3_8507 DR900 powder pillow provided by Hach (Colorado, United States) nitrate, and nitrite release were observed at a wavelength of 270 nm and 540 nm, respectively.

Table 4.2 Different runs for optimization of parameters for HMX degradation

RDX concentration (mg/L)	Inoculation volume (%)	Time (Days)
60	4	5
20	4	15
20	4	5
40	2	5
40	6	15
40	4	10
40	2	15
60	4	15
60	6	10
40	4	10
40	4	10
40	6	5
40	4	10
20	2	10
60	2	10
20	6	10
40	4	10

4.3. Results and discussion

4.3.1 Growth of *Bacillus toyonensis* in MSM spiked with HMX

Growth of microbes was monitored by measuring optical density at different time intervals. Figure 4.2 shows growth of *Bacillus toyonensis* at varying HMX concentration and inoculum size with different intervals of time. It is clear from Figure 4.2 (A, B, C) that initially microbes were in lag phase till 2nd day for all the combinations. After 2 days, microbes attained exponential phase showing acclimatization to the presence of HMX and may be started utilizing it as a nitrogen source. *Bacillus toyonensis* at concentrations, 2 and 4mg/L showed continuous growth till 15th day while at 6mg/L microbes attained stationary phase after 7th day. Maximum optical density (0.239) was observed at 2 mg/L HMX concentration with 6% of inoculum size on 15th day, which is nearly double as compared to the lowest growth observed in 6 mg/L HMX concentration with 2% inoculum on 15th day. Higher growth of

microbes can be correlated with the degradation of HMX whereas lower growth can be explained by the toxic effect of HMX at 6 mg/L concentration. It was also observed that the initial inoculum size tends to affect the growth of *Bacillus toyonensis* in the medium. When concentration of HMX was kept constant with varying initial inoculum size, there was a gradual change in the optical density on 15^{th} day and nearly 20% higher growth of microbes was observed with 6% inoculum size (0.239) as compared to 2% (0.198). The higher growth of microbes can be correlated with higher degradation of HMX in the medium, which shows their interdependency on each other.

4.3.2 Degradation of HMX

Different responses for the different runs observed during the HMX degradation are shown in Table 4.3. Degradation of HMX during the experiment is shown in Figure 4.3. A maximum 87.7% reduction was achieved at 2 mg/L initial HMX concentration with 4% inoculum size on 15^{th} day. It was followed by 79.5% and 74.1% respectively with 6 mg/l and 4 mg/l initial concentration of HMX on 15^{th} day. In Figure 4.3, the 3-D model plot shows the interactive effect of different selected variables on HMX removal. Range of responses can be estimated and maximized by analyzing the plot and tracking the efficiency of optimum value for variable.

From Figure 4.3 it was observed that, when time factor was kept constant (15^{th} day) change in HMX removal can be observed with changing cell inoculum size. It was also observed that on increasing the initial inoculum size, there was an increase in percent degradation of HMX. Degradation of 65.5 and 79.5% was observed with 2 and 6% inoculation size, respectively on 15^{th} day at 4 mg/L initial HMX concentration.

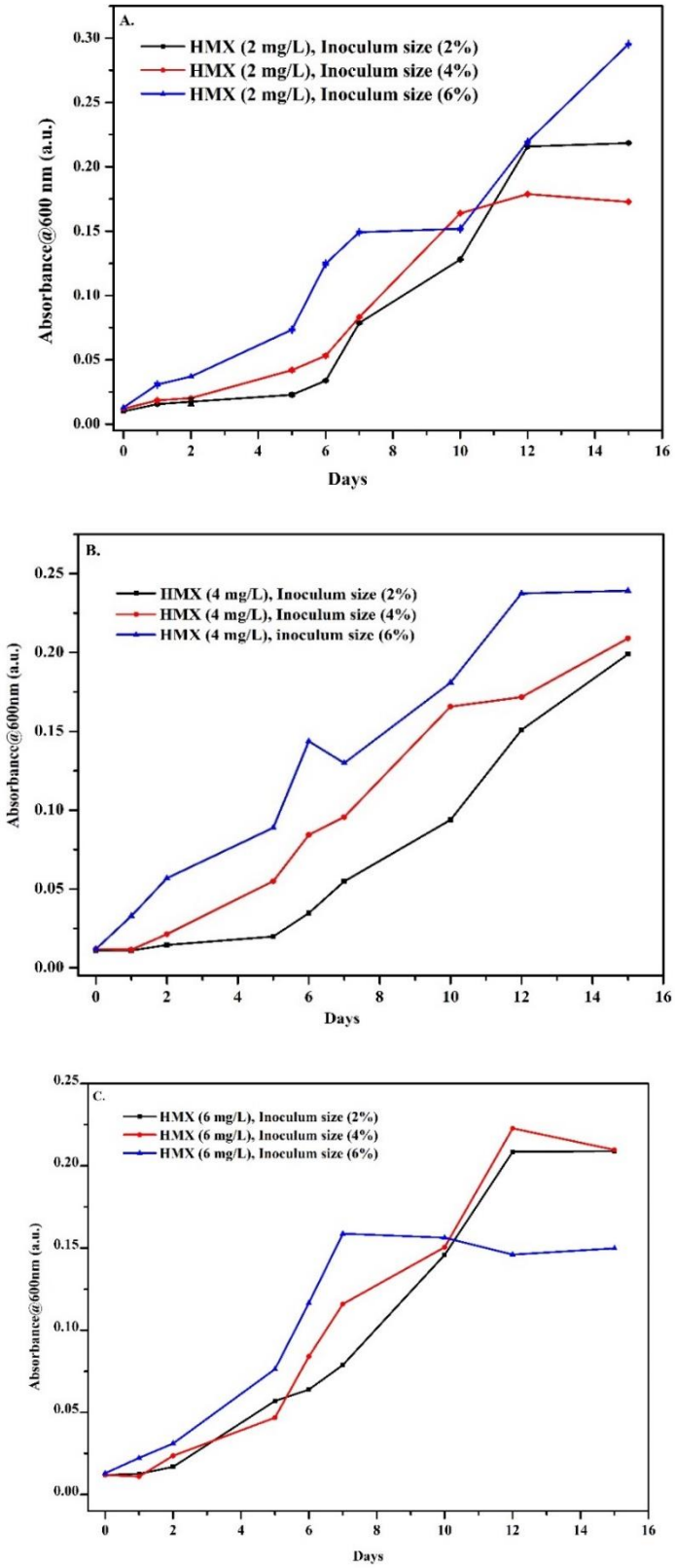


Figure 4.2 Growth of *Bacillus toyonensis* with varying inoculum size (2–6%) at initial HMX concentration of (A) 2 mg/L, (B) 4 mg/L, and (C) 6 mg/L.

Similarly, Figure 4.4 shows increase in HMX removal with the increase in time period at constant microbial inoculum size. Degradation of 58.2 and 79.5% was observed on 5^{th} and 15^{th} day respectively with 4 mg/L concentration and 6% volume of microbial suspension. Similarly, Figure 4.5 shows the effect of microbial inoculum size and time. It was observed that both the factors positively affect the % degradation of HMX. These observations made from figures 4.3, 4.4 and 4.5 supports the fact that on varying time as well as initial volume of suspension, there was effect on HMX removal efficiency i.e., with increasing time of exposure and initial microbial inoculum, there was increase in the removal of HMX with *Bacillus toyonensis.* In a similar study by An et al. (2010), 96.42% reduction in the concentration of HMX with the use of mesophillic anaerobic microbial granules is reported [29]. Similarly, under denitrifying conditions Singh et al. (2009) showed that *Pseudomonas* species can efficiently remove HMX from the medium [30].

Table 4.4 shows the analysis of variance for the obtained experimental data. The P-value (<0.0001), R^2 value (0.9878) and F-value (62.97) for degradation of HMX also suggested that the obtained data was accurate and significant. Similar findings have also been reported in previous studies during the optimization of different process parameters [31]. Adjusted R^2 (0.9721) explains total variation in degradation of HMX with different variables and shows its agreement with predicted R^2. Figure 4.6 shows the predicted versus actual values plot, which explains the dispersion of data for % removal of HMX. Variations between the predicted values were measured by functions used for the model and actual values determined by actual experimentation [32]. Small value of standard deviation for the model (1.65) explains a better relationship between different variables and response.

Table 4.3. Different runs for optimization of parameters for HMX degradation and responses

A: concentration (mg/L)	B: volume of microbial suspension (%)	C: time (day)	Degradation (%)	Nitrite release (mg/L)	Nitrate release (mg/L)
4	4	10	57.0±1.89	1.2±0.14	1.1±0.15
4	2	5	54.3±3.19	0.58±0.05	0.2±0.02
4	4	10	58.2±1.89	1.01±0.14	1.3±0.15
6	2	10	70.7±2.61	1.23±0.13	1.3±0.23
2	2	10	72.3±2.60	0.43±0.18	0.35±0.01
4	6	15	79.5±3.76	0.73±0.25	1.15±0.16
4	4	10	55.7±1.89	1.3±0.14	1.2±0.17
6	6	10	72.6±3.53	1.06±0.12	1.4±0.22
2	4	5	61.2±1.47	0.4±0.143	0.2±0.22
6	4	15	74.0±7.39	1.18±0.22	1.51±0.31
4	6	5	58.2±5.30	0.38±0.034	0.34±0.03
6	4	5	68.1±7.70	0.44±0.14	0.49±0.04
2	4	15	87.6±4.46	0.12±0.01	0.36±0.02
4	4	10	57.2±1.89	1.35±0.14	1.2±0.15
4	4	10	58.2±1.89	1.0±0.18	1.1±0.15
2	6	10	77.6±1.75	0.73±0.14	0.43±0.08
4	2	15	65.5±8.47	0.68±0.13	1.11±0.28

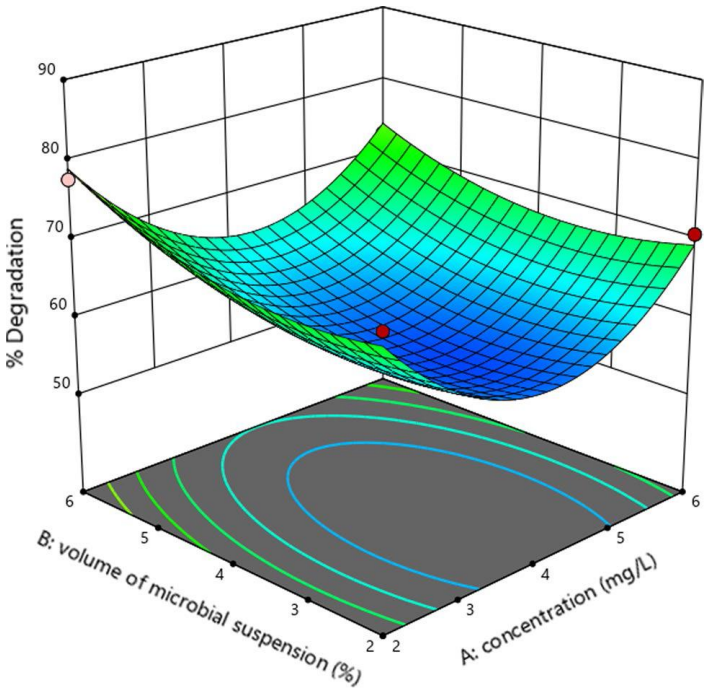


Figure 4.3 Percent degradation of HMX with varying initial HMX concentration (mg/L) and microbial suspension volume (%)

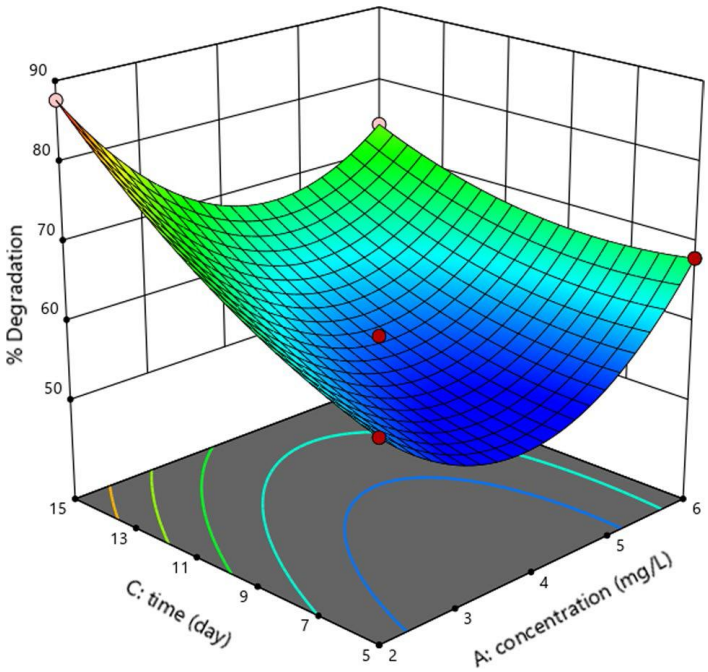


Figure 4.4 Percent degradation of HMX with varying initial HMX concentration (mg/L) and time (days)

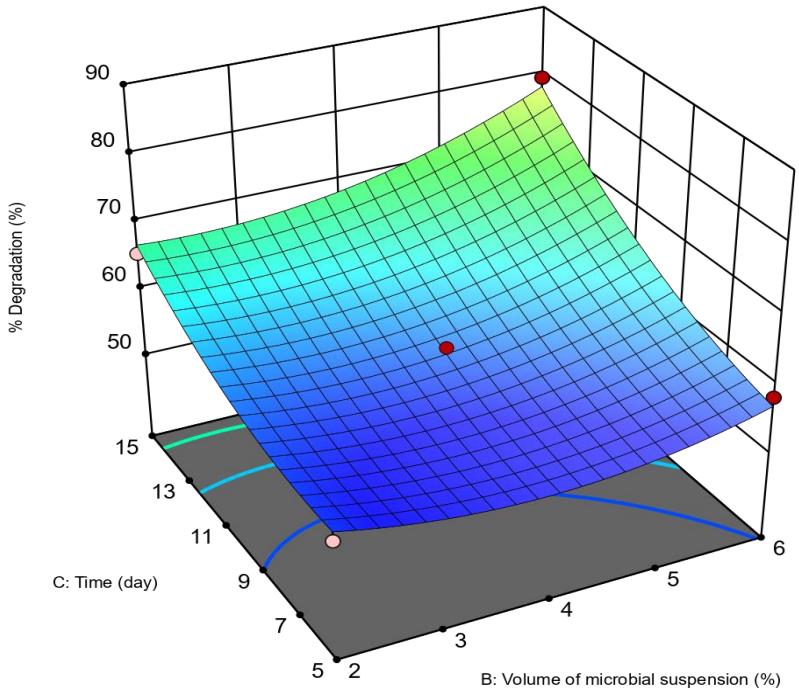


Figure 4.5 Percent degradation of HMX with varying microbial suspension volume (%) and time (days)

All the parameters were fitted for second order polynomial equation as follows-

$$Y = 56.63 - 1.67A + 3.14B + 8.09\,C - 0.8646AB - 5.13AC + 2.52BC + 12.55A^2 + 4.18B^2 + 3.61C^2 \quad (1)$$

Where, Y is % degradation, A, B and C are coded values for initial concentration of HMX, volume of inoculum size and time of degradation, respectively.

Table 4.4. ANOVA of quadratic model for % degradation of HMX

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1545.03	9	171.67	62.97	< 0.0001	Significant
A-concentration	22.27	1	22.27	8.17	0.0244	
B-volume of microbial suspension	78.91	1	78.91	28.94	0.0010	
C-time	523.62	1	523.62	192.05	< 0.0001	
AB	2.99	1	2.99	1.10	0.3298	
AC	105.35	1	105.35	38.64	0.0004	
BC	25.42	1	25.42	9.32	0.0185	
A^2	628.02	1	628.02	230.34	< 0.0001	
B^2	62.24	1	62.24	22.83	0.0020	
C^2	45.07	1	45.07	16.53	0.0048	
Residual	19.08	7	2.73			
Lack of Fit	14.78	3	4.93	4.58	0.0877	not significant
Pure Error	4.30	4	1.07			
Cor Total	1564.12	16				

df degree of freedom

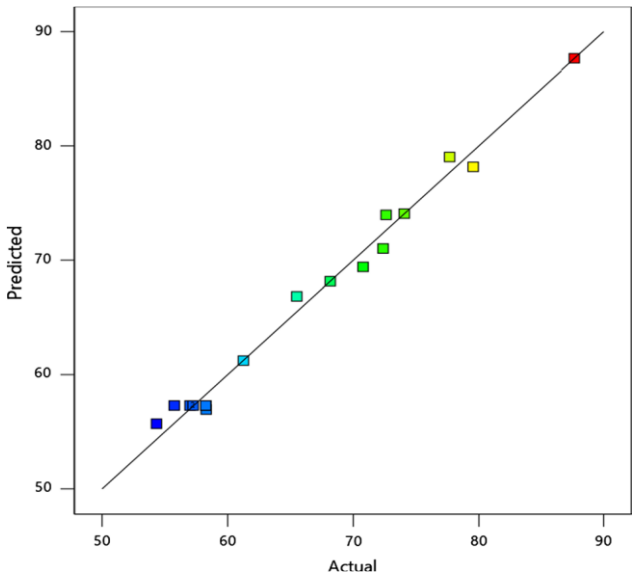


Figure 4.6 Actual versus predicted graph for % HMX degradation

4.3.3 Release of nitrite in different treatments

Increased nitrite ion levels in the medium can be further correlated with degradation of HMX. It is well confirmed in literature that during HMX degradation, nitrite is released as a by-product and get converted into methylenedinitramine (MEDINA) [21,33,34]. Previous studies has also shown than microbes utilize HMX as a carbon and nitrogen source and produce various products such as formaldehyde, CO_2, 4-Nitro-2,4-diazabutanal and nitrous oxide (N_2O) [22,35,36].

Figure 4.7 shows that at constant inoculum size (4%), concentration of nitrite in solution increased with increase of initial HMX concentration from 5^{th} day (0.38 mg/L) to 10^{th} day (1.3 mg/L) and then decreased down to 0.73 mg/L at 15^{th} day which may be due to its transformation into nitrate [37] or utilization of nitrite by microbes. Nitrite release was found maximum at 4 mg/L (1.3 mg/L) and minimum at 2mg/L (0.43 mg/L) concentration, which shows the effect of initial concentration of HMX on the nitrite release. Similarly, in figure 4.8 and figure 4.9 it was observed that initial HMX concentration, microbial suspension and time positively affects removal of nitrite. It has been already established that during the degradation of HMX, there is hydrolytic and hydroxylation of the rings, which leads to the sequential release of nitrite [21,22,36]. During HMX degradation, nitrite concentration found to be varying with different independent variables in the current study.

Fitting the experimentally obtained data in Box-Behnken design indicated that quadratic model was significantly applicable to show the relationship between different independent variables to their response. Analysis of variance is shown in Table 4.5, which shows the statistical significance of variables and their interactions with response in terms of high regression values (R^2 = 0.9566) (Table 4.6), F-value (17.15) and low p-value (0.0006). P value < 0.05 for A, C, AC, A^2, C^2 concludes them as significant model terms.

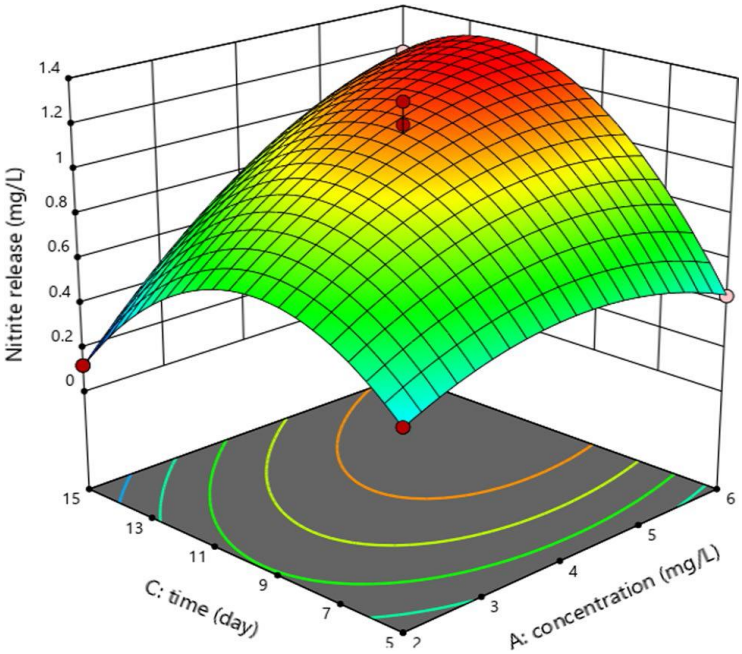


Figure 4.7 Nitrite release (mg/L) with varying initial HMX concentration(mg/L) and time (days)

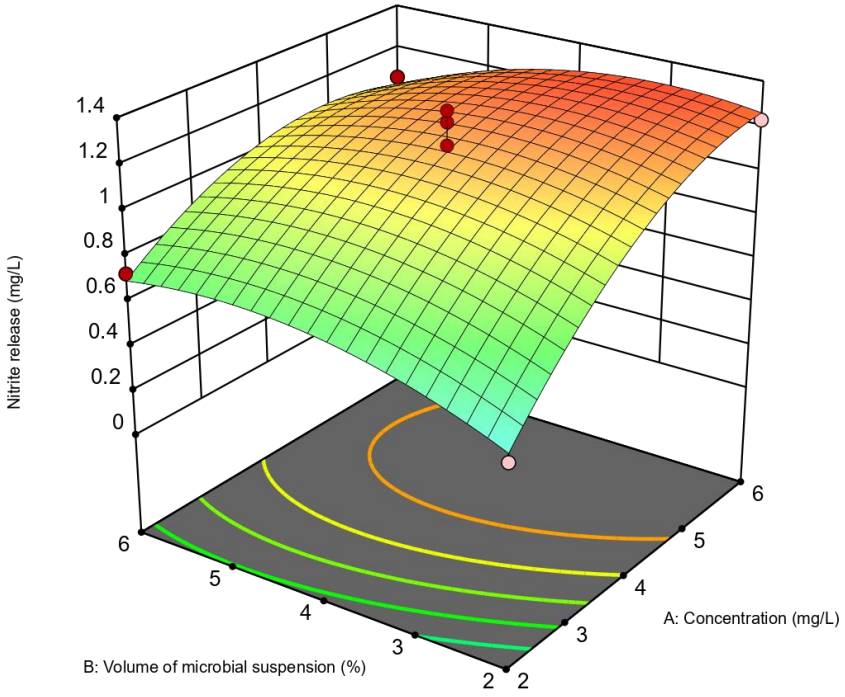


Figure 4.8 Nitrite release (mg/L) with varying initial HMX concentration(mg/L) and varying microbial suspension volume (%)

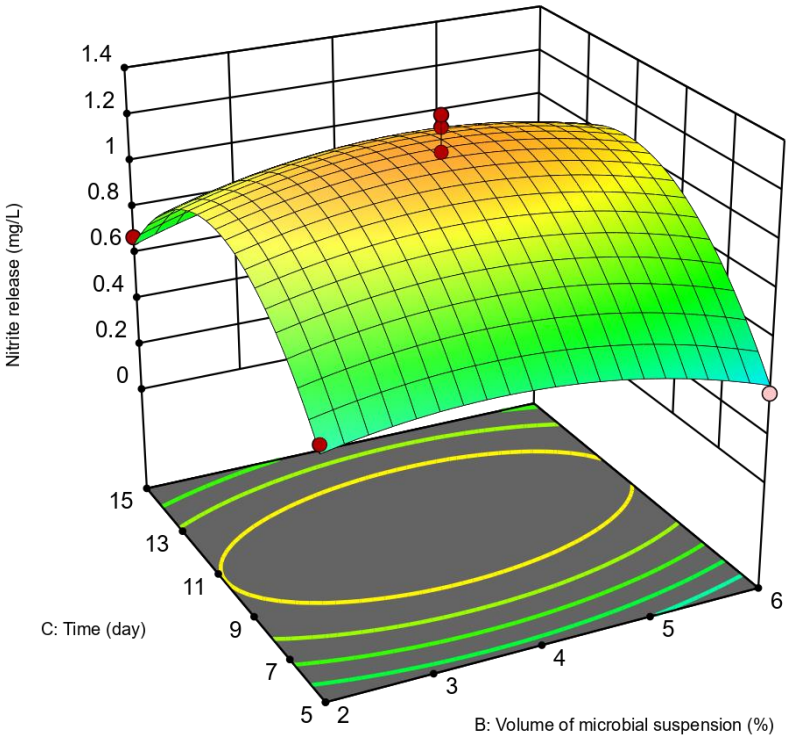


Figure 4.9 Nitrite release (mg/L) with varying microbial suspension volume (%) and time (days)

These model terms have positive relationship with nitrite production during HMX degradation, while other terms showed negative impact but not significant. Correction total (sum of squares = 2.28) obtained from the model showed high reproducibility of the data and less variation around mean of observations whereas residual value (0.0988) showed the variations, which were still unexplained from the model. From this statistically significant data, it was observed that model can be applied for the optimization of nitrite release during HMX degradation. Similar results were obtained by earlier researchers during the optimisation of different process parameters [38]. All the parameters were fitted for second order polynomial equation as follows:

$$Y = 1.16 + 0.2787\,A - 0.0025B + 0.1137C - 0.1175AB - 0.2550AC + 0.0625\,BC - 0.1785\,A^2 - 0.1210B^2 - 0.4485C^2 \quad (2)$$

Where, Y is % nitrite release; A, B and C are coded values for initial concentration of HMX, volume of microbial suspension and time of degradation, respectively.

Table 4.5 ANOVA for Quadratic model for nitrite release

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.18	9	0.2420	17.15	0.0006	significant
A-concentration	0.6216	1	0.6216	44.04	0.0003	
B-volume of microbial suspension	0.0000	1	0.0000	0.0035	0.9542	
C-time	0.1035	1	0.1035	7.33	0.0303	
AB	0.0552	1	0.0552	3.91	0.0884	
AC	0.2601	1	0.2601	18.43	0.0036	
BC	0.0156	1	0.0156	1.11	0.3277	
A^2	0.1342	1	0.1342	9.50	0.0177	
B^2	0.0616	1	0.0616	4.37	0.0750	
C^2	0.8470	1	0.8470	60.00	0.0001	
Residual	0.0988	7	0.0141			
Lack of Fit	0.0099	3	0.0033	0.1489	0.9252	not significant
Pure Error	0.0889	4	0.0222			
Cor Total	2.28	16				

df degree of freedom

Table 4.6 Fit statistics of the model

Model	R^2	Adjusted R^2	Predicted R^2	Mean	Std. deviation	CV %
% Degradation of HMX	0.9878	0.9721	0.8445	66.40	1.65	2.49
Nitrite release	0.9566	0.9008	0.8693	0.8100	0.1188	14.67
Nitrate release	0.9739	0.9402	0.6950	0.8671	0.1154	13.31

4.3.4 Conversion of nitrite to nitrate

During the process of HMX degradation, nitrite gets converted into nitrate with help of different microbial enzymes through oxidation process [4,14,30]. Effect of different variables (inoculum size, exposure time and initial HMX concentration) on nitrate production during HMX transformation is shown with model graphs. In Figure 4.10, it is showed that at constant volume of inoculum size with increasing time of degradation and increased concentration of HMX, there is an increase in nitrate release. Nitrate concentration increased from 0.34 to 1.15 mg/L from 5^{th} to 15^{th} day at 4 mg/L HMX concentration with 6% inoculum size. The maximum nitrate was observed at 15^{th} day (1.51 mg/L) with 6 mg/L HMX initial concentration. Similarly, in figure 4.11 and 4.12 shows the positive impact of inoculum size, initial concentration and time on the nitrite formation in the medium. With the increase in either inoculum size or initial concentration, there was an increase in nitrate concentration with respect to time.

The significance of variables and their interaction with response at different probability levels is explained by ANOVA. Table 4.7 shows that after fitting the data with the model, a quadratic model with significant F-value (28.97) and P-value (0.0001) was obtained. The terms of the model are explained on the basis of P-value, which should be less than 0.05 for the model. The significant model terms having less P-value (0.05) are A, C, AC, A^2 and C^2, which have positive effect with the variable-variable interaction. Correction total (sum of squares = 3.57) explains that the variation around the mean was less and the model was significant for the response. So, ANOVA explains the significance of model, which can be applied to optimize nitrate release during the HMX degradation process. All the parameters were fitted for second order polynomial equation as follows-

$$Y= 1.15 + 0.4200A + 0.0450B + 0.3625C + 0.0050AB + 0.2150AC - 0.0250BC - 0.1700A^2 - 0.1100B^2 - 0.3400C^2 \quad (3)$$

Where, Y is nitrate release; A, B and C are coded values for initial concentration of HMX, volume of microbial suspension and time of degradation, respectively.

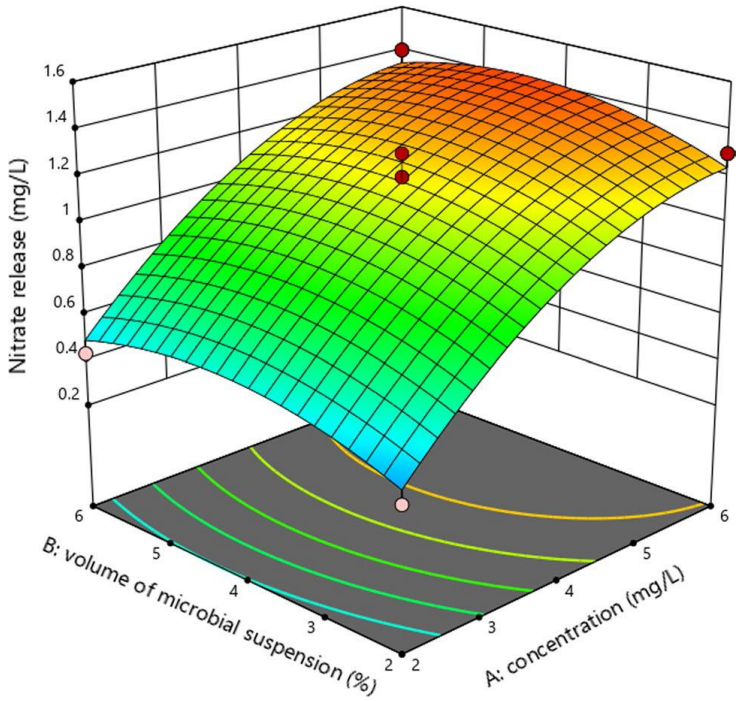


Figure 4.10 Nitrate release (mg/L) with varying initial HMX concentration (mg/L) and microbial suspension volume (%)

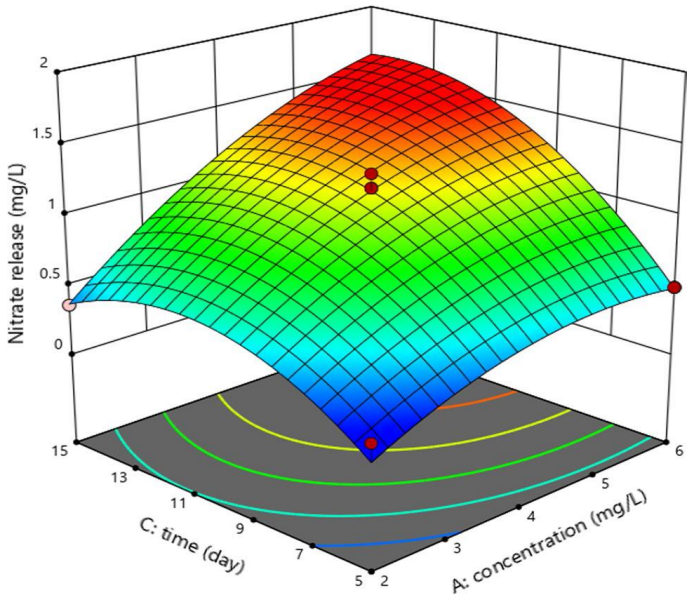


Figure 4.11 Nitrate release (mg/L) with varying initial HMX concentration (mg/L) and time (days)

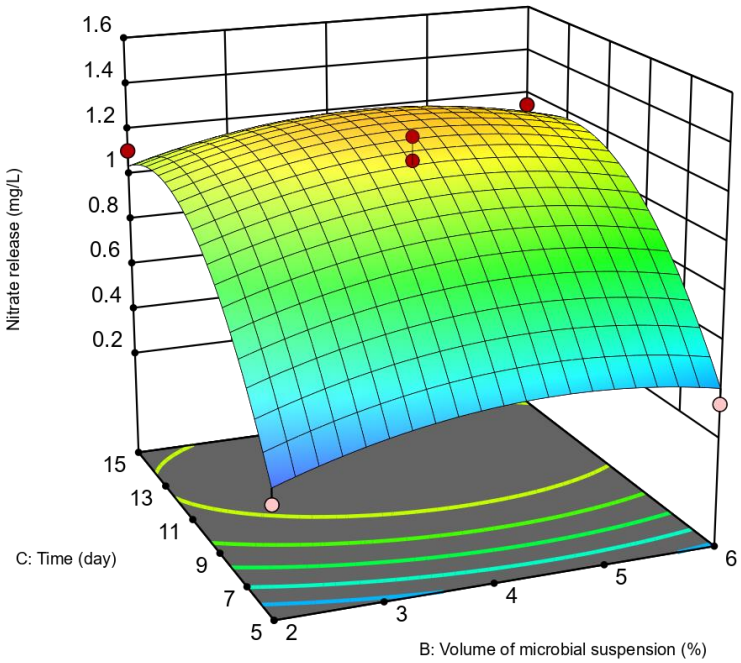


Figure 4.12 Nitrate release (mg/L) with varying microbial suspension volume (%) and time (days)

Table 4.7. ANOVA for Quadratic model for nitrate release

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.47	9	0.3860	28.97	0.0001	significant
A-concentration	1.41	1	1.41	105.93	< 0.0001	
B-volume of microbial suspension	0.0162	1	0.0162	1.22	0.3066	
C-time	1.05	1	1.05	78.91	< 0.0001	
AB	0.0001	1	0.0001	0.0075	0.9334	
AC	0.1849	1	0.1849	13.88	0.0074	
BC	0.0025	1	0.0025	0.1877	0.6779	
A^2	0.1441	1	0.1441	10.82	0.0133	
B^2	0.0658	1	0.0658	4.94	0.0617	
C^2	0.5306	1	0.5306	39.83	0.0004	
Residual	0.0932	7	0.0133			
Lack of Fit	0.0652	3	0.0217	3.11	0.1510	not significant
Pure Error	0.0280	4	0.0070			
Cor Total	3.57	16				

df degree of freedom

4.4. Conclusions

In this study Process development for the biodegradation of HMX using response surface methodology was carried out. RSM was used to optimize HMX degradation process parameters like degradation time, inoculum size, and initial HMX concentration with outputs such as percent degradation, nitrite, and nitrate release. It aided in the optimization of broad experimental domains during the HMX degradation process by *Bacillus toyonensis*. It was discovered that the initial HMX concentration, time, and inoculum size are the variables that had the most impact on HMX degradation, nitrite release, and nitrate conversion. On the 15^{th} day, the maximum (87.7%) breakdown of HMX was achieved at 2 mg/L concentration with a 4% volume of microbial inoculum size. ANOVA using various significant values (e.g., p-value, F-value, R^2, etc.) revealed the model's significance and also explained the effect of each variable on the various responses.

The study's findings reveal that *Bacillus toyonensis* was able to survive and withstand high HMX concentrations and found to be effective at removing HMX from the aqueous phase. As a result, it's critical to see if *Bacillus toyonensis* can break down RDX. The model obtained for each response can be utilized to optimize the degradation process and product release. *Bacillus toyonensis*, which was isolated from an actual HMX-contaminated location, appears to be a viable candidate for HMX degradation. HMX is a higher homolog of RDX, so RDX is always present as an impurity during the manufacturing of HMX. A substantial amount of wastewater is created during the manufacture of these compounds, which contains RDX and HMX residues. Hence, the results obtained in Chapter 3 and 4 depicts the RDX and HMX degradation potential of native microbes in aqueous phase, therefore, in the upcoming chapter the degradation of both RDX and HMX present in contaminated soil sludge has been studied by performing in-vessel study.

References

[1] J.A. Cuthbert, K.J. D'Arcy-Burt, S.M. Carr, HMX: Acute Toxicity Tests in Laboratory Animals., Inveresk Research International LTD Musselburgh (Scotland), 1985.

[2] R.J. Greenough, P. McDonald, HMX: 14 Day Toxicity Study in Rats by Dietary Administration, INVERESK RESEARCH INTERNATIONAL LTD MUSSELBURGH (UNITED KINGDOM), 1985.

[3] A.B. Wilson, Determination of the acute and subchronic mammalian toxicity of HMX, US Army Med. Res. Dev. Command. Ft. Detrick, Frederick, MD. (1985).

[4] A. Berry, S.E. Radford, R.E. Williams, D.A. Rathbone, P.C.E. Moody, N.S. Scrutton, N.C. Bruce, Degradation of explosives by nitrate ester reductases, in: Biochem. Soc. Symp., Portland Press, 2001: pp. 143–153.

[5] I.R.I.S.D. USEPA, Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetr... (HMX) (CASRN 2691-41-0) | IRIS | US EPA, (2018) 1–10. https://cfpub.epa.gov/ncea/iris/iris_documents/documents/subst/0311_summary.pdf.

[6] G.R. Lotufo, M. Nipper, R.S. Carr, J.M. Conder, Fate and toxicity of explosives in sediments, Ecotoxicol. Explos. CRC Press. Boca Rat. (2009) 117–134.

[7] W. Zheng, J. Lichwa, M. D'Alessio, C. Ray, Fate and transport of TNT, RDX, and HMX in streambed sediments: Implications for riverbank filtration, Chemosphere. 76 (2009) 1167–1177.

[8] P. Gong, J. Hawari, S. Thiboutot, G. Ampleman, G.I. Sunahara, Toxicity of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) to soil microbes, Bull. Environ. Contam. Toxicol. 69 (2002) 97–103.

[9] B. Clark, R. Boopathy, Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana, J. Hazard. Mater. 143 (2007) 643–648.

[10] United States Environmental Protection Agency, 2006 Edition

of the Drinking Water Standards and Health Advisories, Environ. Prot. (2006) 18. http://water.epa.gov/action/advisories/drinking/upload/dwstandards2009.pdf.

[11] J.S. Kim, P.J. Shea, J.E. Yang, J.-E. Kim, Halide salts accelerate degradation of high explosives by zerovalent iron, Environ. Pollut. 147 (2007) 634–641.

[12] M.E. Fuller, C.E. Schaefer, J.M. Lowey, Degradation of explosives-related compounds using nickel catalysts, Chemosphere. 67 (2007) 419–427.

[13] J. Pichtel, Distribution and fate of military explosives and propellants in soil: a review, Appl. Environ. Soil Sci. 2012 (2012).

[14] K.-D. Zoh, M.K. Stenstrom, Fenton oxidation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX), Water Res. 36 (2002) 1331–1341.

[15] S. Chatterjee, U. Deb, S. Datta, C. Walther, D.K. Gupta, Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation, Chemosphere. 184 (2017) 438–451.

[16] V.R. Harkins, T. Mollhagen, C. Heintz, K. Rainwater, Aerobic biodegradation of high explosives, phase I-HMX, Bioremediat. J. 3 (1999) 285–290.

[17] J. Hawari, S. Beaudet, A. Halasz, 374 S Thiboutot, G. Ampleman, Microbial degradation of explosives: biotransformation versus mineralization, Appl. Microbiol. Biotechnol. 54 (2000) 605–618.

[18] N.R. Adrian, C.M. Arnett, Anaerobic biotransformation of explosives in aquifer slurries amended with ethanol and propylene glycol, Chemosphere. 66 (2007) 1849–1856. https://doi.org/10.1016/j.chemosphere.2006.08.042.

[19] T. Kalafut, M.E. Wales, V.K. Rastogi, R.P. Naumova, S.K. Zaripova, J.R. Wild, Biotransformation patterns of 2, 4, 6-

trinitrotoluene by aerobic bacteria, Curr. Microbiol. 36 (1998) 45–54.

[20] P. Sangwan, S.M. Celin, L. Hooda, Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using Acinetobacter noscomialis, Eur. J. Adv. Eng. Technol. 2 (2015) 51–56.

[21] S. Nagar, A.K. Shaw, S. Anand, S.M. Celin, P.K. Rai, Aerobic biodegradation of HMX by Planomicrobium flavidum, 3 Biotech. 8 (2018) 1–7.

[22] D. Fournier, A. Halasz, J. Spain, R.J. Spanggord, J.C. Bottaro, J. Hawari, Biodegradation of the hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine ring cleavage product 4-nitro-2, 4-diazabutanal by Phanerochaete chrysosporium, Appl. Environ. Microbiol. 70 (2004) 1123–1128.

[23] M. Mäkelä, Experimental design and response surface methodology in energy applications: A tutorial review, Energy Convers. Manag. 151 (2017) 630–640. https://doi.org/10.1016/j.enconman.2017.09.021.

[24] M.A. Bezerra, R.E. Santelli, E.P. Oliveira, L.S. Villar, L.A. Escaleira, Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta. 76 (2008) 965–977.

[25] R.H. Myers, D.C. Montgomery, C.M. Anderson-Cook, Response surface methodology: process and product optimization using designed experiments, John Wiley & Sons, 2016.

[26] N.R. Draper, J.A. John, Response-surface designs for quantitative and qualitative variables, Technometrics. 30 (1988) 423–428.

[27] B. Kiran, A. Kaushik, C.P. Kaushik, Response surface methodological approach for optimizing removal of Cr (VI) from aqueous solution using immobilized cyanobacterium, Chem. Eng. J. 126 (2007) 147–153.

[28] Method 8330B nitroaromatics, nitramines, and nitrate esters by

high performance liquid chromatography (HPLC), US Environ. Prot. Agency, Washington, DC. (2006).

[29] C. An, Y. He, G. Huang, Y. Liu, Performance of mesophilic anaerobic granules for removal of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) from aqueous solution, J. Hazard. Mater. 179 (2010) 526–532.

[30] R. Singh, P. Soni, P. Kumar, S. Purohit, A. Singh, Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria, World J. Microbiol. Biotechnol. 25 (2009) 269–275.

[31] S.K. Garg, M. Tripathi, N. Lal, Response surface methodology for optimization of process variable for reactive orange 4 dye discoloration by Pseudomonas putida SKG-1 strain and bioreactor trial for its possible use in large-scale bioremediation, Desalin. Water Treat. 54 (2015) 3122–3133.

[32] S.S. Mohanty, H.M. Jena, Process optimization of butachlor bioremediation by Enterobacter cloacae using Plackett Burman design and response surface methodology, Process Saf. Environ. Prot. 119 (2018) 198–206.

[33] B. Bhushan, L. Paquet, A. Halasz, J.C. Spain, J. Hawari, Mechanism of xanthine oxidase catalyzed biotransformation of HMX under anaerobic conditions, Biochem. Biophys. Res. Commun. 306 (2003) 509–515.

[34] J.-S. Zhao, D. Manno, J. Hawari, Abundance and diversity of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX)-metabolizing bacteria in UXO-contaminated marine sediments, FEMS Microbiol. Ecol. 59 (2007) 706–717.

[35] K.T. Thompson, F.H. Crocker, H.L. Fredrickson, Mineralization of the cyclic nitramine explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by Gordonia and Williamsia spp, Appl. Environ. Microbiol. 71 (2005) 8265–8272.

[36] F.H. Crocker, K.J. Indest, H.L. Fredrickson, Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20, Appl. Microbiol. Biotechnol. 73 (2006) 274–290.

[37] K.-D. Zoh, M.K. Stenstrom, Application of a membrane bioreactor for treating explosives process wastewater, Water Res. 36 (2002) 1018–1024.

[38] P. Chaudhary, V. Beniwal, R. Kaur, R. Kumar, A. Kumar, V. Chhokar, Efficacy of Aspergillus fumigatus MCC 1175 for bioremediation of Tannery Wastewater, CLEAN–Soil, Air, Water. 47 (2019) 1900131.

CHAPTER 5

Chapter 5

Bioreactor studies: up-scaled composting of actual RDX and HMX contaminated sludge

5.1 Introduction

The nitrogen-containing energetic chemicals hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are mostly employed for military objectives. HMX is made by nitrating hexamine with nitric acid and ammonium nitrate in the presence of acetic acid as a catalyst. Since HMX is a higher analog of RDX, RDX is always present as an impurity during HMX production. A substantial amount of wastewater containing RDX and HMX residues is released during the manufacture of these explosives. After completing the process of neutralization, this wastewater is typically discharged into unlined lagoons or pits, resulting in the formation of polluted sludge sediments [1,2]. Prior to final disposal at dumping sites, this sludge is not treated any further generally. Because RDX and HMX have a low soil adsorption coefficient, they may contaminate the groundwater table [3,4]. These contaminants pose a significant concern to humans, other species, and the environment due to their slow biological decay and toxic nature [5,6]. HMX exposure can cause liver, central nervous system, and kidney malfunction [7,8]. The US Environmental Protection Agency (USEPA), on the other hand, has categorized RDX as a group 'C human carcinogen [5,9]. Seizures, convulsions, vomiting, and nausea are all possible side effects that may occur due to RDX exposure [5,10].

Different researchers have developed and tested several techniques to remediate RDX and HMX contamination in water and soil, but only a few studies have concentrated on sludge decontamination. Incineration [11,12], composting [13,14], and aqueous thermal decomposition are some of the procedures that entail explosive sludge treatment [12]. To operate, traditional technologies such as incineration require complex sensors and a lot of energy. As a result, these processes are inefficient

and produce wastes such as ash and other toxic emissions, which are difficult to dispose off [15]. Composting, a green technology that is eco-friendly, cost-effective, and requires no fuel or energy during the process, is receiving a lot of attention as a solution to these concerns. During the process, there is also the possibility of contaminant co-metabolism with the bulking agents. Bulking agents, which are also waste materials with high organic content and are readily available, can be used to improve the product's quality after composting. The Louisiana Army Ammunition Plant composted explosive-contaminated sediments, removing 98% of total explosives during the process [14]. HMX and RDX have half-lives of 42 days and 30 days, respectively. Emery and Faessler (1997) described the development of a composting approach to remediate explosive pollution at the Umatilla Army Depot Activity (UMDA) [2]. They discovered that the composting method was inexpensive as well as effective. Clark and Boopathy (2007) investigated land-farming for explosive polluted soil and observed the efficient removal TNT and RDX. [16]. Composting procedures for the treatment of oily sludge, municipal garbage, and polycyclic aromatic hydrocarbons have also been established and are well understood [17–19]. Various studies have looked into microbial remediation of explosives in soil and water [20,21], and it appears to be a viable approach for treating explosive-contaminated sludge as well.

So, this study was designed with an objective of determining the potential of two unexplored native microbes, *Arthrobacter subterraneus* (MTCC no. 12883, isolate no. S5-TSB-17) and *Bacillus sonorensis* (MTCC no. 12855, isolate no. S8-TSB-4) isolated from an explosive contaminated site, to decontaminate actually RDX and HMX contaminated sludge using an in-vessel composting technique in an 80-days experiment. The decrease in concentration of contaminants, the mechanism of degradation, and the half-life of both chemicals during the composting process were investigated. Figure 5.1 shows the bioreactor study for RDX and HMX removal from soil sludge in overall thesis theme addressed in the present chapter.

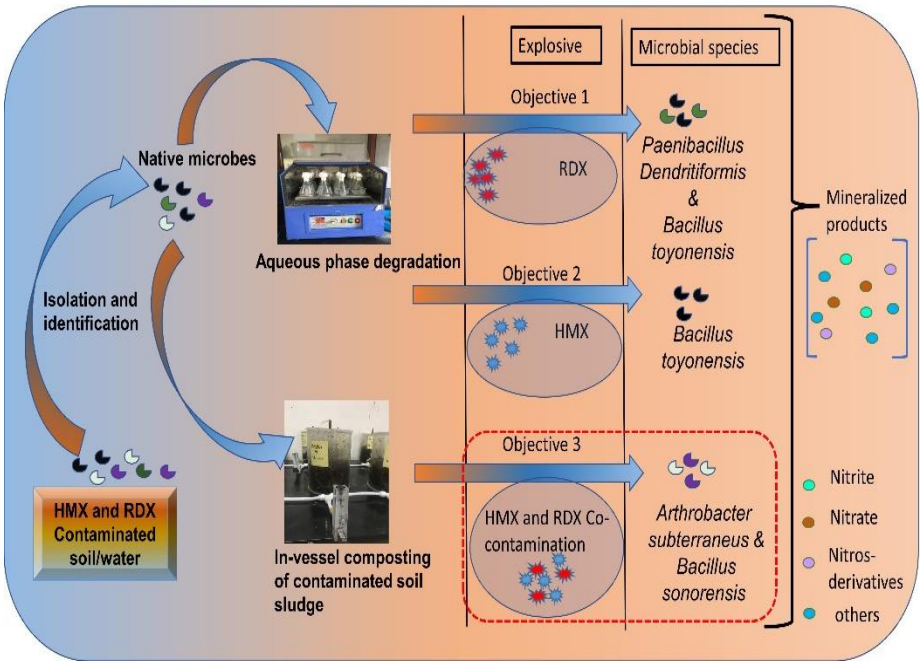


Figure 5.1 Schematic representation of bioreactor study for RDX and HMX removal from soil sludge

5.2 Materials and methods

5.2.1 Composting materials and chemicals

The actually contaminated sludge samples were collected from the contaminated location and subjected to initial physio-chemical analysis (table 5.1) using standard methods, as stated above in section 2.4.2 (chapter 2). Cow dung was obtained from cattle shed in Delhi and air-dried before using for composting. The laboratory collected garden waste (grass trimmings, *Azadirachta indica* leaves, and other leaves) were used as bulking agents during composting.

5.2.2 In-vessel reactor design

The in-vessel bioreactors were designed and fabricated as discussed in section 2.4.1. In brief, a total of 12 acrylic bioreactors were manufactured with a 3 L capacity. To maintain aerobic conditions, each reactor was connected to an air compressor through a moisture vessel and airflow meters. The airflow maintained was 50 mL/ min for 8 hrs each day (80 days). Also, the moisture was maintained at 60±10% using autoclaved water. The temperature of the reactors was maintained at

32±3 °C and the compost was turned weekly to maintain aerobic conditions.

5.2.3 Microbial culture

As explained earlier in chapter 2 sections 2.1 and 2.2 soil and water samples were collected and sent to the Chandigarh-based Institute of Microbial Technology (IMTECH). *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (MTCC no. 12855 isolate no. S8-TSB-4) were chosen for this study after preliminary screening of the numerous microbial species isolated and identified from sludge. The bacteria were also revived as detailed in section 2.2. Microbes were revived in TSB at 32±2 °C and 120 rpm in a shaking flask. The bacteria were cultivated until their optical density (OD) reached 1.5 OD (1 mL = 10^{12} cells/mL). Following that, 10 mL of microbial suspension was used to seed reactors 2 to 9. A consortium of microbes under study was employed in reactors 10–12, which was generated using 10 mL broth of each species. As indicated in table 5.2, many combinations with variable compositions of contaminated sludge, cow dung, and garden waste were made with both bacteria. The dry weight of all composting ingredients was calculated. For 80 days, the entire experiment was carried out under aerobic conditions.

5.2.4 Analysis

The HPLC analysis of RDX and HMX was done as mentioned in section 2.5. Mass spectroscopic analysis (microTOF-Q, Bruker Daltonics, Billerica, Massachusetts, USA) was done (Section 2.5.2) to identify metabolites formed during RDX and HMX degradation. The analysis was done using positive electron spray ionization (+ve ESI). C-18 column was used to separate RDX and HMX degradation products. The mobile phase was 0.1% formic acid, 50% acetonitrile, and 49.9% triple distilled water with a flow rate of 1 mL/min and 5 min run time.

Nitrite and nitrate analysis was done as mentioned in sections 2.5.4 and section 2.4.5 respectively. The absorbance of nitrate and nitrite was taken at 220 nm and 540 nm respectively, using a UV-

spectrophotometer (PerkinElmer, Model Lambda 650S, USA). Further, for the characterization of compost initial and final content of potassium and phosphate was estimated. The analysis was done as mentioned in section 2.4.8. As the carbon to nitrogen ratio is an important parameter that helps in plant growth. The was calculated based upon TKN and TOC ratio as mentioned in sections 2.4.6 and 2.4.7. Other parameters such as temperature and moisture were measured using a hygrometer (Hanna HI9565, US).

SEM analysis was carried out for the compost samples to observe the changes in microbial structure. SEM analysis was performed as mentioned in section 2.5.3. In brief, a pinch of the compost sample (0.1 g) was incubated overnight with 1 mL of 3% glutaraldehyde. Then the samples were washed and dehydrated with distilled water and ethanol solution (35, 50, 75, 95, and 100%). After dehydration, the sample was post fixed with hexamethyldisilazane (HMDS) for 30 min. Using the drop cast method the sample was spread on a glass slide, which was further coated with gold film using a sputterer. The slides were analyzed using SEM (Supra55 Zeiss, Oberkochen, Germany) operated at 10-12mA (5kV) under a vacuum.

5. 3 Result and discussion

5.3.1 Change in physico-chemical properties of compost

Physico-chemical properties of the actual explosive contaminated sludge obtained from manufacturing site are shown in table 5.1. It was observed that there was high concentration of HMX (95594.4 ± 2.3 mg/kg) and RDX (9638.4 ± 5.9 mg/kg) in the sludge. The moisture, nitrate, phosphate, potassium and pH of the sludge were 51.13±2.0%, 6.25±0.1 mg/kg, 29.9±0.4 mg/kg, 22.1±0.4 mg/kg and 3.50±0.02, respectively. Table 5.2. shows different combinations of contaminated sludge, cow manure and garden waste with both the microbes used in the study.

Table 5.1 Initial physico-chemical parameters of explosives contaminated sludge

Parameters	**Explosive contaminated sludge**
pH	3.50 ± 0.02
E.C	0.5 ± 0.1mS
Moisture	51.13 ± 2.0 %
Nitrate	6.25 ± 0.1 mg/kg
Phosphate	29.9 ± 0.4 mg/ kg
Potassium	22.1 ± 0.4 mg/kg
C:N ratio	25.6 ± 3.7
HMX concentration	95594.4 ± 2.3 mg/kg
RDX concentration	9638.4 ± 5.9 mg/kg

Different physico-chemical parameters were studied to assess the properties of compost, which are given in table 5.3. The initial pH of sludge was acidic having a value of 3.5±0.02, whereas after mixing with the bulking agents it got increased towards alkalinity with a value from 6.86 to 8.49 for different combinations. During the process of composting, pH got inclined towards neutrality ranging from 6.96 to 7.23 for all the combinations. The initial phosphate and potassium concentration in the compost were in the range of 20.1±0.1 to 27.2±0.1 mg/kg and 15.2±0.2 to 20.3±0.4 mg/kg, respectively. The phosphate and potassium concentrations were decreased on 80^{th} day in each combination. Decrease in phosphate and potassium concentration can be due to their up-take by microbes for growth and metabolism. The initial C:N ratio was in the range from 23.3 to 39.8 for different in-vessel reactors. Whereas, at the end of 80 days the final C:N ratio of the compost in each combination was in the range of 20.5 to 35.9. The initial Total Kjeldahl Nitrogen (TKN) values for different combinations were 0.7 to 1.3%, which were increased at the end of 80^{th} day from 0.8 to

1.5%. Increase in TKN shows the release of nitrogen containing compounds into the compost, which are accessible to microbes.

Table 5.2. Different combinations of explosive sludge, cow dung, garden waste, and microbes during the composting process

In-vessel reactor no.	**Sludge (%)**	**Cow manure (%)**	**Garden waste (%)**	**Microbes**
1	100 (control)	0	0	No microbes inoculated
2	10	70	20	*Bacillus sonorensis (B.S.)*
3	20	60	20	*Bacillus sonorensis (B.S.)*
4	30	50	20	*Bacillus sonorensis (B.S.)*
5	50	30	20	*Bacillus sonorensis (B.S.)*
6	10	70	20	*Arthrobacter subterraneus (A.S)*
7	20	60	20	*Arthrobacter subterraneus (A.S)*
8	30	50	20	*Arthrobacter subterraneus (A.S)*
9	50	30	20	*Arthrobacter subterraneus (A.S)*
10	10	70	20	*Arthrobacter subterraneus and Bacillus sonorensis (A.S. + B.S.)*
11	20	60	20	*Arthrobacter subterraneus* and *Bacillus sonorensis (A.S. + B.S.)*
12	30	50	20	*Arthrobacter subterraneus* and *Bacillus sonorensis (A.S. + B.S.)*

However, TOC of different reactors increased from 22.8 - 30.7% (initial day) to 21.89 - 39.28% (final day). This can be due to the release of carbon metabolites during the microbial growth. The whole experiment was performed with three replicates

Table 5.3. Initial and final physico-chemical characterization of different waste combinations during in-vessel composting (n=3)

In-vessel Reactor no.	pH		Phosphate (mg/kg)		Potassium (mg/kg)		TKN (%)		TOC (%)		C:N ratio	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
1	3.50±0.02	3.53±0.02	29.9±0.4	12.34±0.2	22.1±0.4	11.34±0.4	0.9±0.1	0.9±0.1	22.8±0.2	21.89±0.1	25.62±3.7	24.63±4.0
2	7.42±0.02	6.96±0.02	21.3±0.4	10.0±0.2	19.8±0.2	15.25±0.4	0.9±0.1	1.3±0.2	30.7±0.2	39.28±0.4	34.51±5.1	30.91±6.3
3	6.86±0.04	7.02±0.02	22.4±0.2	6.77±0.2	17.3±0.4	15.23±0.2	0.7±0.0	1.2±0.1	27.9±0.1	38.94±0.2	39.85±0.2	32.66±3.6
4	7.30±0.02	7.15±0.01	25.7±0.5	7.43±0.1	16.9±0.4	11.67±0.2	0.8±0.1	1.2±0.2	27.8±0.2	25.40±0.5	35.26±5.8	21.82±5.6
5	7.43±0.04	7.10±0.02	26.2±0.2	3.55±0.4	16.2±0.1	13.27±0.2	0.7±0.1	0.8±0.0	25.5±0.1	28.73±0.5	37.16±7.3	35.91±0.7
6	7.26±0.04	7.08±0.01	22.9±0.4	8.45±0.2	18.5±0.2	9.35±0.1	1.2±0.2	1.5±0.1	27.3±0.4	31.33±0.4	23.35±5.1	20.96±1.6
7	7.52±0.02	7.11±0.02	23.6±0.1	9.27±0.1	17.9±0.4	15.22±0.1	0.9±0.1	1.2±0.2	25.9±0.1	30.52±0.5	29.12±4.4	26.10±5.6
8	7.39±0.02	7.23±0.02	25.5±0.2	10.59±0.1	17.1±0.1	12.34±0.4	0.8±0.1	1.3±0.2	26.4±0.1	37.27±0.8	33.50±5.7	29.29±5.7
9	7.62±0.04	7.07±0.01	27.2±0.1	10.19±0.2	15.2±0.2	10.56±0.2	0.8±0.1	0.9±0.1	24.7±0.4	24.09±0.4	31.31±5.0	27.13±4.7
10	7.60±0.02	7.12±0.02	20.1±0.1	8.96±0.2	20.3±0.4	8.97±0.4	1.3±0.2	1.5±0.1	30.4±0.4	30.69±0.5	23.91±3.4	20.53±1.5
11	8.49±0.04	7.08±0.04	24.2±0.2	9.06±0.4	18.6±0.2	16.34±0.1	0.9±0.1	1.3±0.1	28.1±0.2	35.54±0.1	31.58±4.6	27.50±3.1
12	8.14±0.02	7.01±0.01	25.9±0.1	11.22±0.1	15.2±0.2	15.23±0.1	0.8±0.1	1.1±0.1	27.3±0.2	33.49±0.7	34.63±5.7	30.74±4.5

5.3.2 Degradation of HMX and RDX during in-vessel composting

Degradation pattern of HMX and RDX during the in-vessel composting of sludge over a time period of 80 days is given in Figure 5.3 and 5.5, respectively.

5.3.2.1 Degradation of HMX

Figure 5.3 revealed that with the increase in time, there was significant reduction in HMX concentration for each composting reactor as compared to the control. However, in each reactor the rate of degradation with time was different. The reactors inoculated with *Bacillus sonorensis* (reactor 2-5) showed that with increase of sludge concentration, there was decrease in HMX removal efficiency. The degradation on 80^{th} day in reactors 2, 3, 4 and 5 were 78.5±0.12%, 62.01±0.08%, 59.9±0.06% and 49.6±0.06%, respectively. However, for reactor 6 to 9 which were inoculated with *Arthrobacter subterraneus* the maximum degradation observed was 74.7±0.17% in reactor no. 7 with 20% explosive sludge and 80% bulking agents. Further, the reactors inoculated with consortium of microbes (reactor 10, 11 and 12) showed low HMX degradation values (62.7±0.03, 63.8±0.14 and 56.1±0.23%, respectively) on 80^{th} day. This may be due to the fact that the microbes did not interacted positively with each other to remove significant amount of HMX in the compost. Maximum HMX degradation (78.5±0.12%) was observed with *Bacillus sonorensis* (isolate no. S8-TSB-4) in combination having 10% sludge, 70% cow manure and 20% garden waste in reactor no. 2. It was followed by 74.7±0.17% HMX reduction with microbe, *Arthrobacter subterraneus* (isolate no. S2-TSB-17) with combination of 20% sludge, 60% cow manure and 20% garden waste in reactor no. 7. The minimum degradation observed was 48.5±0.37% with isolate no. S2-TSB-17 having 10% sludge, 70% cow manure and 20% grass cuttings in reactor no. 6, which was approx. 37% lesser than the maximum degradation. In other composters, degradation was in the range of 48.5 to 78.5%. HMX removal was higher at lower

sludge concentration which may be due to lower toxicity at lower concentrations

However, as the microbe, *Bacillus sonorensis* performs better at lower HMX concentration (10% sludge), *Arthrobacter subterraneus* works better at 20 and 30% sludge concentration. Further for the consortium of microbes, the degradation at 10 and 20% sludge concentrations were significant as compared to 30% sludge. Earlier studies shows that cyclic ring of nitramines (HMX & RDX) is less stable in nature and got breakdown in other intermediate products by different mechanisms. Single nitrite ion (NO_2^-) elimination is one of the routes. The reduction in concentration of RDX and HMX was observed with formation of nitrite ions. During in-vessel composting, microbes utilized nitrogen and carbon present in the explosive sludge as a source of nutrition for their growth. The nitro-reductase enzymes are known to break complex ring structure into simpler secondary metabolites before complete mineralization.

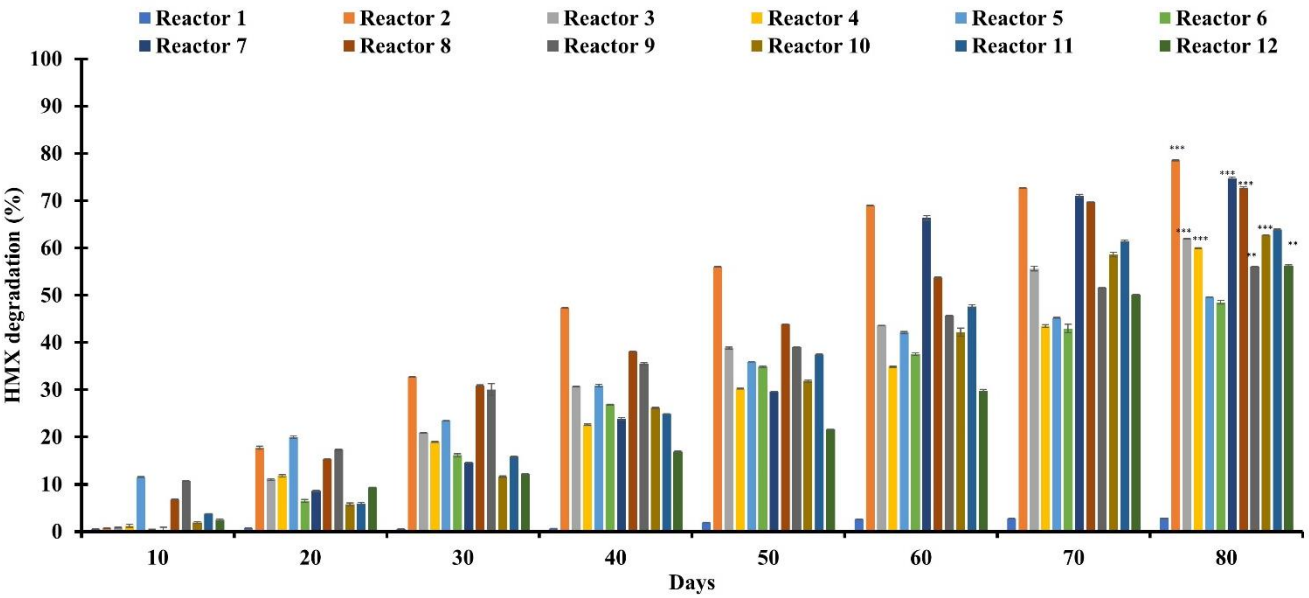


Figure 5.2 Degradation of HMX in different combinations of contaminated sludge. The asterisk sign shows a significant level of the p-value (*** indicates p-value ≤ 0.0005 and ** indicates p-value ≤ 0.005 on the 80th day).

It was observed that the degradation of HMX/RDX and microbial growth for individual microbes at 50% sludge concentrations were very low may be due to more toxicity at higher concentration. Williams et al. (1992) performed the composting of soil contaminated with explosives under thermophilic and mesophilic conditions. They observed that the RDX and HMX were decreased below the detection level from the initial concentration, 17900 mg/kg and 2390 mg/kg in the compost in 153 days with the help of indigenous microbes present in the compost [22]. US Army Environmental Center (1993), has done windrow composting of explosive contaminated soil under aerated conditions and observed 76.6% removal of HMX from the initial concentration (199.5 mg/kg) [23]. Authors obtained similar results during the study. Further, there was negligible degradation of 2.8% in HMX concentration in control which may be due to photolysis of the contaminants. It also showed that the microbes played an important role in degradation of contaminants during the study. ANOVA (single factor) was performed to check the significance of degradation efficiency between reactors on 80^{th} day. The ANOVA was performed between different reactors and the reactor having least degradation (reactor 6). A p-value less than 0.0001 was observed for reactor 2, 3, 4, 7, 8, 10 and 11 and a p-value less than 0.01 was observed for reactor 9 and 12 indicating significant degradation of HMX in these reactors. The combination with maximum degradation was further evaluated for half-life of HMX during the composting process. Pseudo first order kinetic model was applied which is represented in Eq. (1).

$$\ln A = - kt + \ln A_0 \qquad (1)$$

Where, A is concentration after incubation, A_0 is the initial concentration of HMX, $t_{1/2}$ is the time for 50% HMX degradation and k is the degradation rate. The observed rate of degradation (k) and half-life ($t_{1/2}$=0.693/K) of the selected combination was 0.0223 day^{-1} and 31 days, respectively with the correlation coefficient (R^2) of 0.99 (figure 5.4). The high half-life of HMX shows, it is recalcitrant in nature. Garg

et al. (1991) have done a sludge composting study and found that the half-life of HMX was nearly 42 days [12].

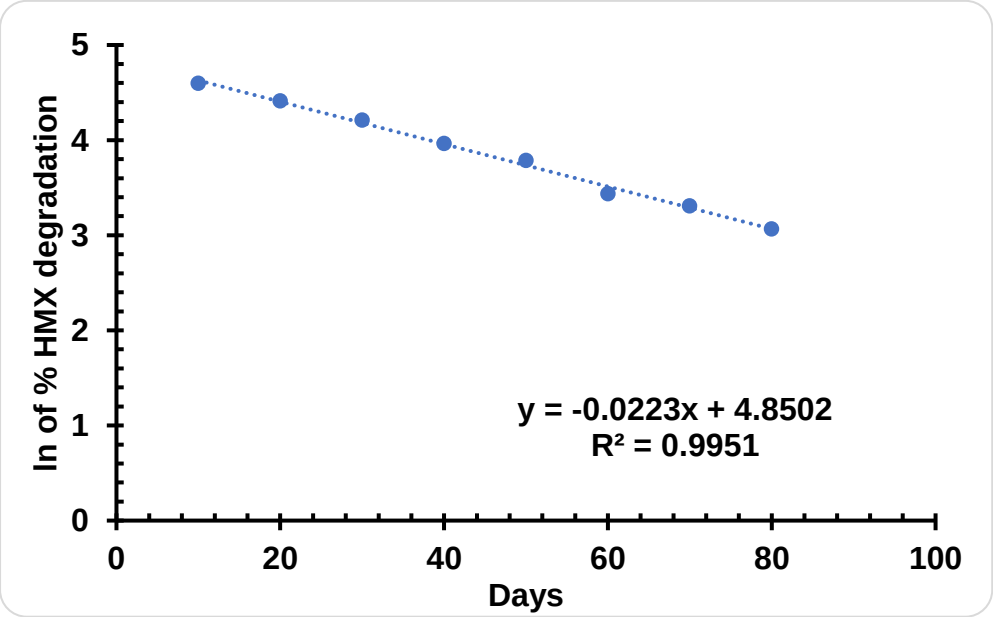


Figure 5.3 The degradation kinetics for HMX degradation in reactor no. 2

5.3.2.2 Degradation of RDX

RDX was present in the sludge as a co-contaminant of HMX and its degradation was also evaluated during the composting process as shown in figure 5.5. It was observed that both microbes were well adapted to utilize RDX during composting process. The reactor number 2, 3, 4 and 5 inoculated with *Bacillus sonorensis* showed a degradation of 77.36±0.95%, 81.09±1.02%, 80.60±0.79% and 56±0.17%, respectively at a concentration of 10, 20, 30 and 50% sludge concentration. Maximum degradation achieved with *Bacillus sonorensis* was 81.09±1.02%, in reactor number 3 with 20% explosive sludge concentration. Similarly, the reactor number 6, 7, 8 and 9 seeded with *Arthrobacter subterraneus* showed a maximum degradation of 81.96 ± 0.77% in reactor no. 8 which was 54.56% higher than the minimum degradation in reactor no. 9. The maximum reduction observed in RDX concentration was 91.2±0.80% in reactor no. 11 having 20% sludge, 60% cow manure, 20% garden waste in presence of both the microbes. The presence of both microbes has positive impact on the removal of

RDX. During RDX degradation, both the microbes performed very well at 10, 20 and 30% sludge concentration whereas, 50% sludge concentration tends to decrease the degradation efficiency, which can be due to high toxic effect of RDX on microbes. The degradation achieved was more with RDX in comparison with HMX. It may be due to the high concentration of HMX in contaminated sludge. The concentration of HMX was nearly 9-10 times higher as compared to the RDX concentration in explosive sludge (Table 5.1). It is well proven that quantity and concentration of the contaminant plays an important role in microbial degradation. So RDX degradation observed was more as compared to HMX.

.

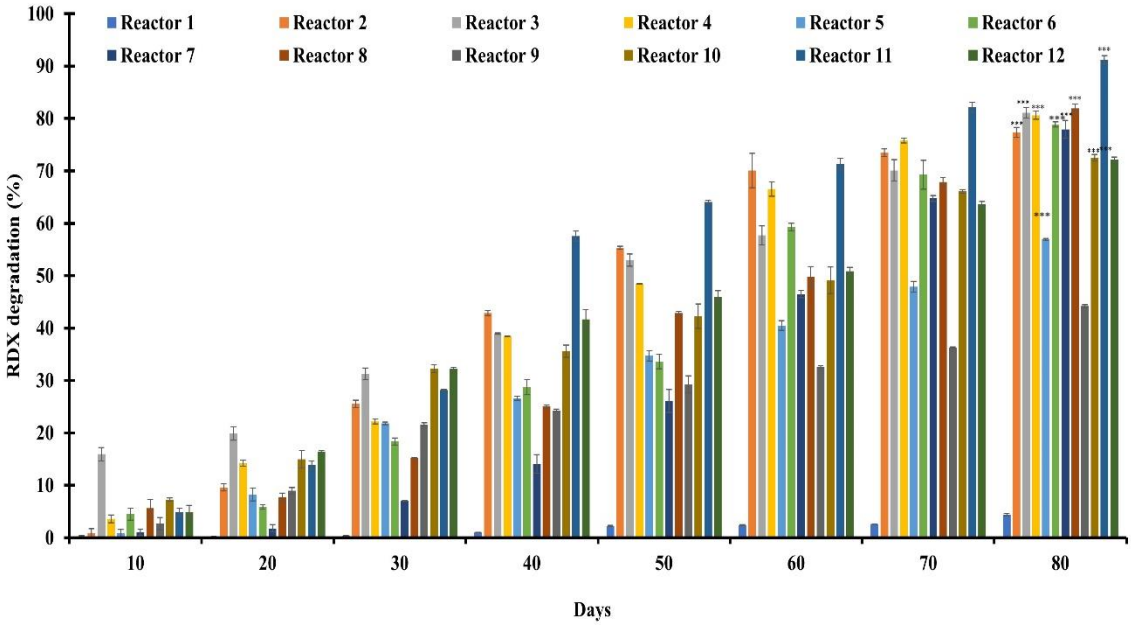


Figure 5.4 RDX degradation in different combinations of contaminated sludge. The asterisk sign shows a significant level of the p-value (*** indicates p-value ≤ 0.0005 and ** indicates p-value ≤ 0.005 on the 80th day).

Apart from this, based on the equation 1, half-life for RDX degradation was calculated for the combination having maximum degradation. The rate constant (k) for the RDX in the combination having 20% sludge, 60% cow manure, 20% garden waste with both the microbes was found

0.0327 day^{-1} with a half-life ($t_{1/2}$) of 21 days with the correlation coefficient of 0.95 figure 5.6. Further, negligible degradation of 4.3% RDX was observed in control which showed that the indigenous microbes present in the sludge were unable to uptake and mineralize the RDX and were not effective. ANOVA single factor was performed and significant RDX degradation was achieved for reactor no. 2, 3, 4, 5, 6, 8, 10, 11 and 12 with a p-value of less than 0.0001 and reactor no. 7 with a p-value less than 0.01.

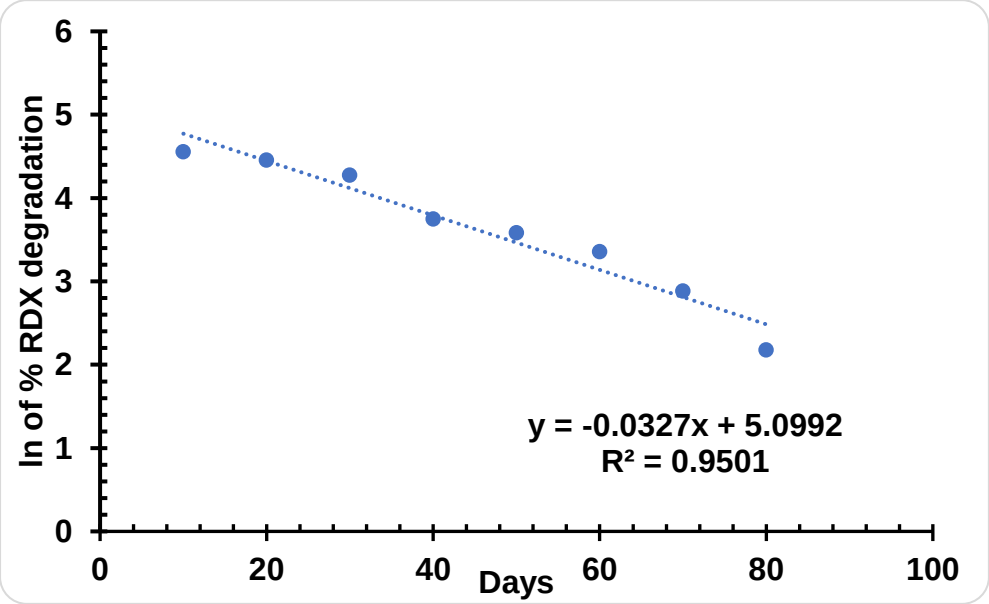


Figure 5.5 The degradation kinetics for RDX degradation in reactor no. 11

The findings of the work were in line with the previous studies done by the US Army Environmental division in 1993, where windrow composting of soil contaminated with explosives was carried out. Also, Griest et al. (1991) studied static pile composting for the explosive contaminated soil and found up to 97% reduction in the RDX concentrations [24]. Similar degradation efficiency was observed for *Pseudomonas fluorescens*, *Rhodococcus* sp., *Gordonia* sp. and *Willamsia* sp. during the RDX degradation in soil [25,26].

Some intermediate products peaks were also observed while quantifying the concentration of remaining explosives using HPLC. The samples showing those peaks were chosen and subjected to mass

spectroscopic analysis using LC-MS/MS to elucidate the HMX degradation intermediates formed during in-vessel composting of explosive sludge due to cleavage of HMX and RDX ring. Under the Positive electron spray ionization (ESI) mode, the presence of intermediate molecular ions were identified having the major $[M+H]^+$ peaks at ~205.09 Da and 177.04 Da *m/z* ratio (Figure 5.7). Different molecular ion fragments identified, indicate the presence of two different intermediate products which were Bis(hydroxymethyl)nitramine ($C_2N_6H_8O_3$, M+nCH_3CN) [27] and Methylene dinitramine ($CH_4N_4O_4$, M+nCH_3CN)[28] having m/z 205.09 and 177.04, respectively. The microbes may utilized HMX and RDX as a source of nitrogen during their growth and metabolic activities. With the help of nitro-reductase enzyme microbes tends to break the HMX ring structure into nitroso-derivatives of HMX, Bis(hydroxymethyl)nitramine, CO_2, nitrous oxide, formaldehyde, nitrate and nitrite [27–29]. The sequential hydroxylation and de-nitration of the ring may lead to the breakdown of HMX ring structure.

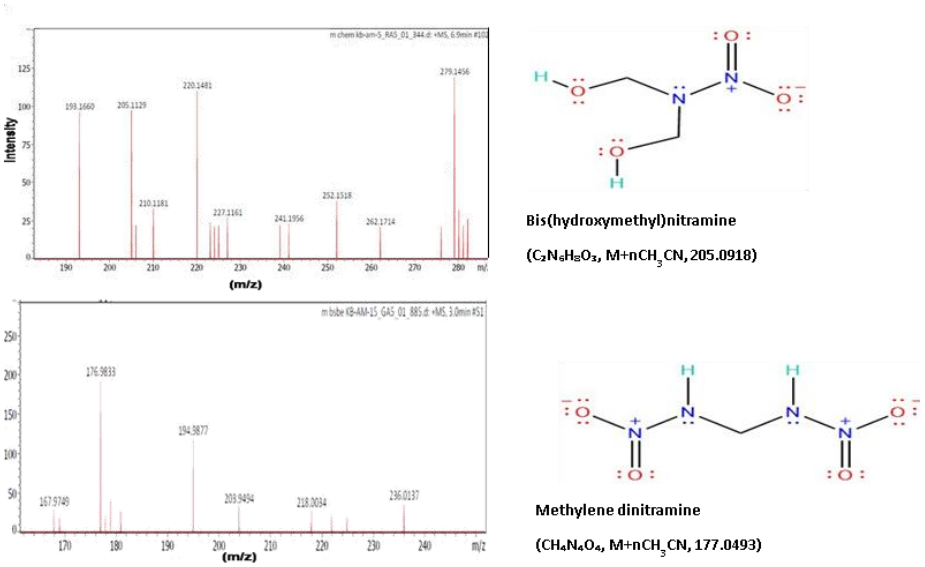


Figure 5.6 Mass spectrometric analysis of HMX degradation samples of reactor 2 on 50th day.

The Scanning Electron Microscope (SEM) imaging of the microbes was carried out at high magnification and the attachment and presence of microbes with substrate was confirmed (figure 5.8). SEM image of isolate S2-TSB-17 and isolate no. S8-TSB-4) with explosive treatment and without explosive treatment (figure 5.8) also illustrated the isolates to be rod-shaped cells and affirmed that there was no deformation in their shape in presence of explosives. Both the microbes were not adversely affected by the toxic environment of HMX and RDX. Good growth of both microbes showed that they could easily tolerate high concentration of both the contaminants during the composting process and were able to maintain their originality at the same time.

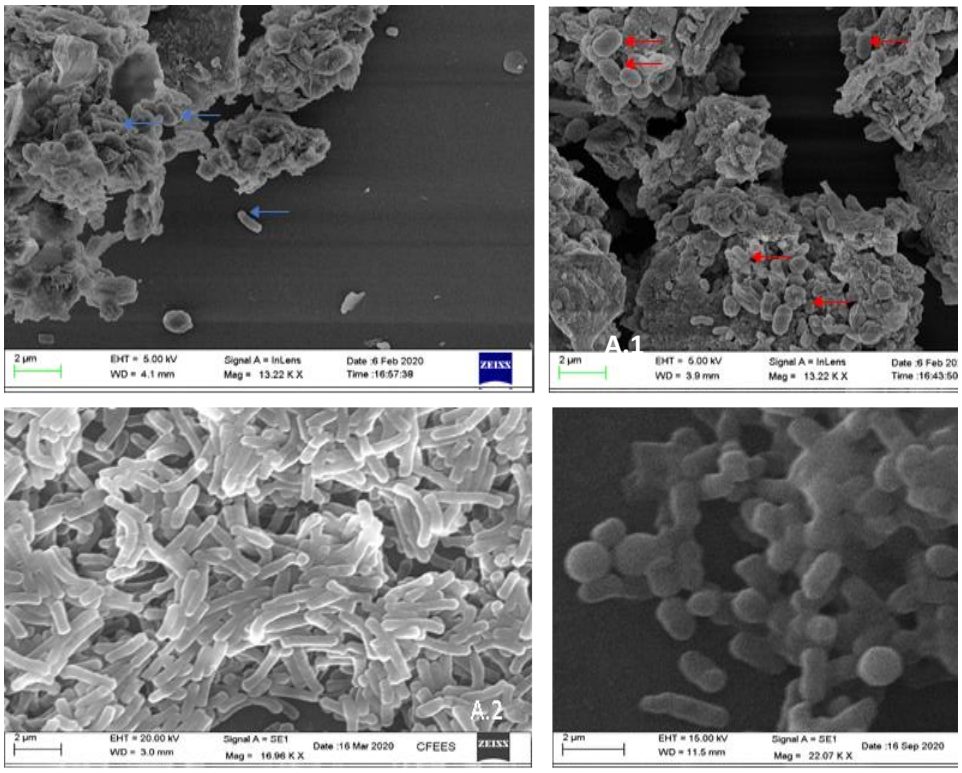


Figure 5.7 Scanning electron micrograph of microbes, *Arthrobacter subterraneus (*isolate no. S2-TSB-17) with (A.1) and without treatment (A.2) and *Bacillus sonorensis* (isolate no. S8-TSB-4) with (B.1) and without treatment (B.2) on 10^{th} day in reactor 2 and 6, respectively

5.3.3. Release of nitrite and nitrate during the composting

Nitrite is the by-product of HMX and RDX degradation. So, nitrite and nitrates were estimated to understand the degradation mechanism of contaminants. The changes in concentration of nitrite and nitrate were also observed during the in-vessel composting of explosive sludge over 80 days' time period which is mentioned in figure 5.9 and figure 5.10 respectively. During the composting of sludge, the concentration of nitrite increased till 50^{th} day and after that a decrease was observed. The increasing nitrite concentration can be co-related with the degradation of RDX and HMX during the composting process. However, further decrease in nitrite concentration may be due to uptake by microbes or conversion into nitrate. It is due to the fact that nitrite ions are less stable under aerobic conditions and readily get converted into nitrate. The maximum nitrite release observed was 24.02±0.05 mg/L in reactor no. 11 with *Bacillus sonorensis* and *Arthrobacter subterraneus* having 20% sludge concentration on 50^{th} day. This was followed by reactor no. 10 having 10% sludge with *Bacillus sonorensis* and *Arthrobacter subterraneus,* and reactor no. 3 with 20% sludge having *Bacillus sonorensis* on 50^{th} day i.e., 20.48±0.07 mg/L and 20.41±0.05 mg/L, respectively.

The change in nitrate concentration observed during the composting process is shown in figure 5.10. *Bacillus sonorensis* in reactor no. 2 with 10% sludge, 70% cow manure and 20% grass cuttings, showed accumulation of nitrate ions till 80^{th} day and maximum nitrate concentration was 30.65±0.9 mg/L on 70^{th} day. Whereas, in other combinations the nitrate concentration increased till 40 to 50 days and then it started to decline. As from the earlier work it has already been established that during RDX and HMX degradation, there is a release of nitrite and nitrate ions [30–32], ANOVA showed significant p-values for the reactors having maximum nitrite (reactor 11) and nitrate (reactor 2) released on 50^{th} and 70^{th} day.

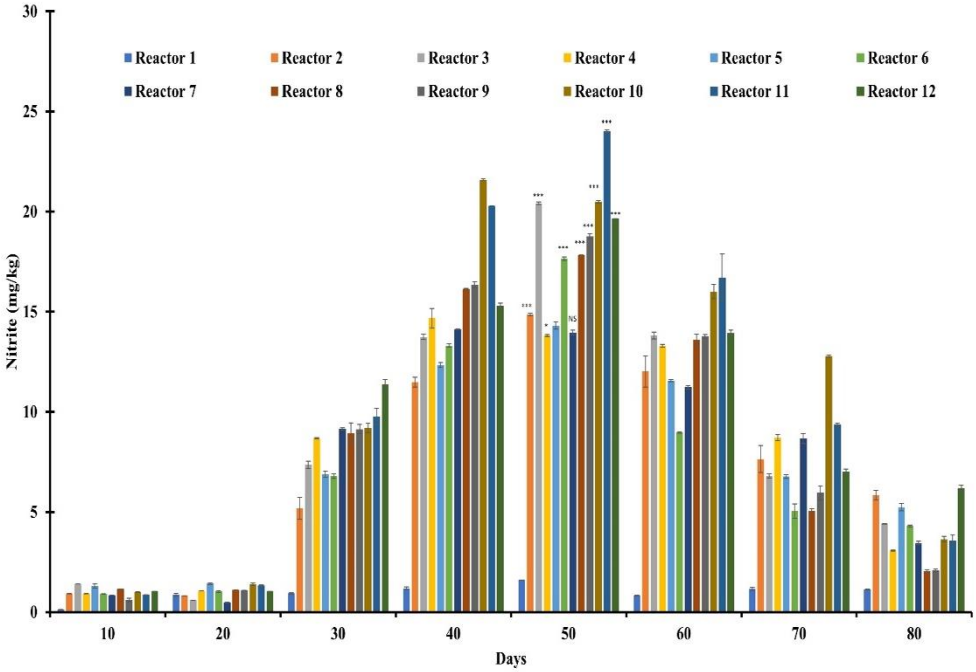


Figure 5.8 Release of nitrite during the RDX and HMX degradation in different combinations of contaminated sludge. The asterisk sign shows the significance level of the p-value (*** indicates p-value ≤ 0.0005 and ** indicates p-value ≤ 0.005 and NS indicates non-significant values on the 50th day).

The ring cleavage of HMX and RDX follows two different steps which involves de-nitration and hydrolytic cleavage. It has been found that during the degradation of mono-nitroso derivatives of the explosives, nitrite and nitrate are released [29,33].

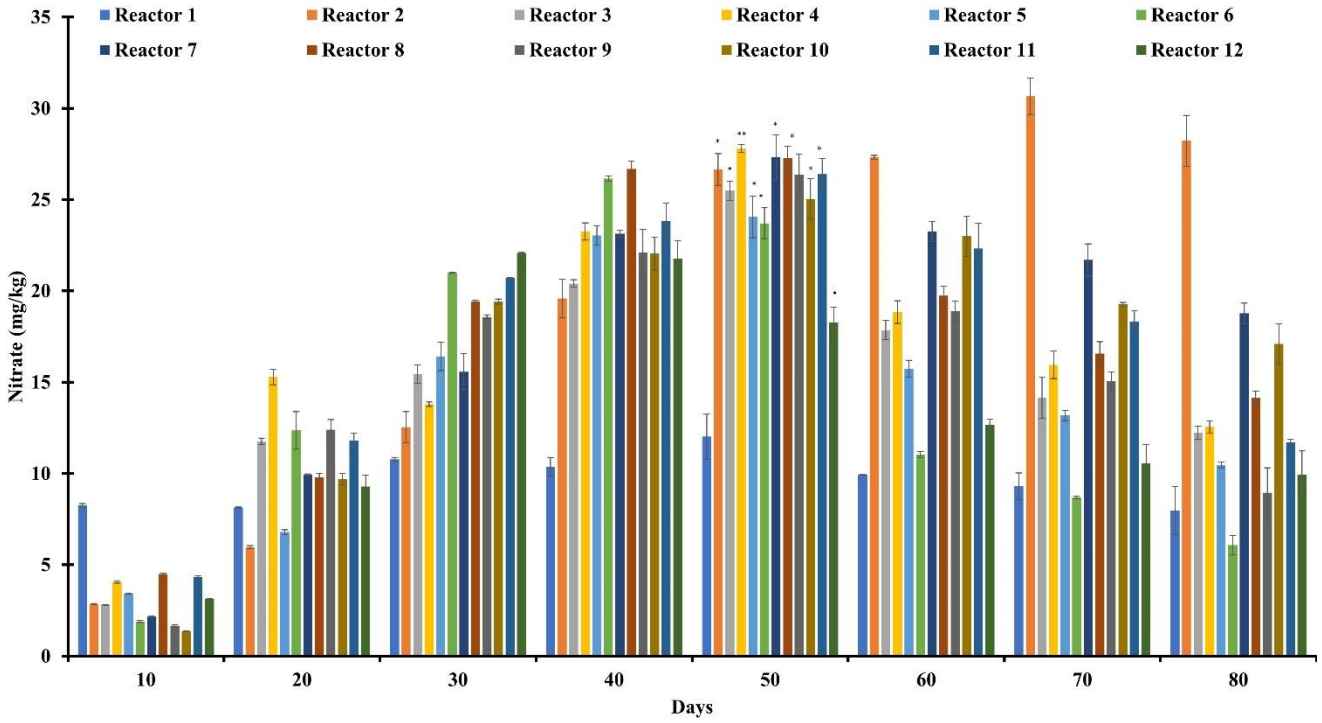


Figure 5.9 Release of nitrate during the RDX and HMX degradation in different combinations of contaminated sludge. The asterisk sign shows the significance level of the p-value (*** indicates p-value ≤ 0.0005 and ** indicates p-value ≤ 0.005 and NS indicates non-significant values on the 50th day).

5.4 Conclusions

The explosive contaminated sludge was obtained from an HMX production facility in North India for this study. In this sludge, there were high levels of HMX and RDX residues. Microbes *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (isolate number. S8-TSB-4) were isolated from the same contaminated location and used in an 80-day experiment under aerobic conditions to degradeHMX and RDX contaminated sludge through In-vessel composting. In reactor no. 2, *Bacillus sonorensis* with 10% sludge, 70% cow dung, and 20% garden waste showed the maximum (78.5%) HMX degradation. Further, RDX breakdown was found highest (91.2 %) in reactor no. 11 seeded with a consortium of microorganisms consisting

of 20% sludge, 60% cow manure, and 20% garden waste. In the combinations with the highest degradations, the half-lives of HMX and RDX were found to be 33 days ($k = 0.223day^{-1}$) and 21 days ($k = 0.0327day^{-1}$), respectively. In the treatment of explosive contaminated sludge, one-way ANOVA revealed that in-vessel composting is efficient in removing RDX and HMX. Bis(hydroxymethyl)nitramine and methylene dinitramine were discovered to be the HMX degradation intermediate products during degradation. Analyzing the release of nitrite and nitrate during the composting process confirmed the HMX and RDX breakdown. According to the findings, both indigenous bacteria have potential to degrade and compost RDX and HMX polluted sludge and can be efficiently used for this purpose. Also, this is the first study on use of these bacteria for an in-vessel composting investigation of explosive contaminated sludge. This research could be expanded to include a pilot-scale composting procedure for decontaminating sludge in real-world contaminated areas. Furthermore, the precise mechanism and metabolic pathway for RDX and HMX aerobic degradation have to be thoroughly investigated.

Objective 1, 2, and 3 concludes that the native microbes [*Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857), *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) *Arthrobacter subterraneus* (MTCC No. 12883, isolate no. S2-TSB-17) and *Bacillus sonorensis* (MTCC no. 12855 isolate no. S8-TSB-4)] have high RDX and HMX removal efficiencies. They can tolerate high explosives concentrations which do not have any toxic effect on them. Further during the degradation of RDX and HMX, nitrite, nitrate, and many intermediate degradation products were released as primary by-products, which suggests structural breakdown of both the compounds

References

[1] K.A. Gust, M.S. Wilbanks, X. Guan, M. Pirooznia, T. Habib, L. Yoo, H. Wintz, C.D. Vulpe, E.J. Perkins, Investigations of transcript expression in fathead minnow (Pimephales promelas) brain tissue reveal toxicological impacts of RDX exposure, Aquat. Toxicol. 101 (2011) 135–145.

[2] Emery, D.D., Faessler, P.C. First production-level bioremediation of explosives-contaminated soil in the United States. Ann. N. Y. Acad. Sci. (1997) 829, 326–340.

[3] W. Zheng, J. Lichwa, M. D'Alessio, C. Ray, Fate and transport of TNT, RDX, and HMX in streambed sediments: Implications for riverbank filtration, Chemosphere. 76 (2009) 1167–1177.

[4] T.W. Sheremata, A. Halasz, L. Paquet, S. Thiboutot, G. Ampleman, J. Hawari, The fate of the cyclic nitramine explosive RDX in natural soil, Environ. Sci. Technol. 35 (2001) 1037–1040.

[5] U.S.D.O.H.A.H. SERVICES, P.H. for T.S. and D.R. Service, Toxicological Profile for RDX, 2002.

[6] M. Lapointe, R. Martel, E. Diaz, A conceptual model of fate and transport processes for RDX deposited to surface soils of North American active demolition sites, J. Environ. Qual. 46 (2017) 1444–1454.

[7] Williams, M.A. (Biologist), Reddy, G., Quinn, M.J., Johnson, M.S. (Toxicologist). Wildlife Toxicity Assessments for Chemicals of Military Concern, Wildlife Toxicity Assessments for Chemicals of Military Concern (2015).

[8] J.A. Cuthbert, K.J. D'Arcy-Burt, S.M. Carr, HMX: Acute Toxicity Tests in Laboratory Animals., Inveresk Research International LTD Musselburgh (Scotland), 1985.

[9] U. Environmental Protection Agency Federal Facilities

Restoration, R. Office, Fact Sheet – Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), (2021) 1–7.

[10] USEPA, Handbook on the Management of Munitions Response Actions, (2005) 315. http://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P100304J.txt.

[11] N.H.A. Van Ham, Recycling and disposal of munitions and explosives, Waste Manag. 17 (1998) 147–150.

[12] R. GARG, D. GRASSO, G. HOAG, Treatment of explosives contaminated lagoon sludge, Hazard. Waste Hazard. Mater. 8 (1991) 319–340.

[13] R.C. Doyle, J.D. Isbister, G.L. Anspach, J.F. Kitchens, Composting explosives/organics contaminated soils, (1986). https://apps.dtic.mil/sti/citations/ADA169994 (accessed August 3, 2022).

[14] R. Williams, P. Ziegenfuss, P. Marks, Installation Restoration Program. Environmental Technology Development Task Order-8. Field Demonstration-Composting of Explosives-Contaminated Sediments at, (1988). https://apps.dtic.mil/sti/citations/ADA202383 (accessed August 3, 2022).

[15] S. Chatterjee, U. Deb, S. Datta, C. Walther, D.K. Gupta, Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation, Chemosphere. 184 (2017) 438–451.

[16] B. Clark, R. Boopathy, Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana, J. Hazard. Mater. 143 (2007) 643–648.

[17] Koolivand, A., Godini, K., Saeedi, R., Abtahi, H., Ghamari, F. Oily sludge biodegradation using a new two-phase composting

method: Kinetics studies and effect of aeration rate and mode. Process Biochem (2019) 79, 127–134.

[18] M. Vasudevan, K. Karthika, S. Gowthaman, K. Karthick, P. Balaganesh, S.M. Suneeeth Kumar, N. Natarajan, Aerobic in-vessel co-composting of dewatered sewage sludge with mixed municipal wastes under subhumid and semiarid atmospheric conditions, Taylor Fr. 43 (2021) 3403–3414.

[19] B. Antizar-Ladislao, J. Lopez-Real, A. Beck, J.M. Lopez-Real, A.J. Beck, Bioremediation of polycyclic aromatic hydrocarbon (PAH)-contaminated waste using composting approaches, Taylor Fr. 34 (2004) 249–289.

[20] A. Meda, P. Sangwan, K. Bala, Optimization of process parameters for degradation of HMX with Bacillus toyonensis using response surface methodology, Int. J. Environ. Sci. Technol. 17 (2020) 4601–4610.

[21] P. Sangwan, S.M. Celin, L. Hooda, Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using Acinetobacter noscomialis, Eur. J. Adv. Eng. Technol. 2 (2015) 51–56.

[22] R.T. Williams, P.S. Ziegenfuss, W.E. Sisk, Composting of explosives and propellant contaminated soils under thermophilic and mesophilic conditions, J. Ind. Microbiol. 9 (1992) 137–144.

[23] US Army Environmental Center, 1993. Windrow Composting of ExplosivesContaminatedSoil at Umatilla Army Depot Activity Hermiston, Oregon., (1933). http://erwiki.net/images/0/05/1993-AEC_Technology_Applicatons_Analysis.pdf.

[24] W.H. Griest, R.L. Tyndall, A.J. Stewart, J.E. Caton, A.A. Vass, C. -H Ho, W.M. Caldwell, Chemical characterization and toxicological testing of windrow composts from explosives-contaminated sediments, Environ. Toxicol. Chem. 14 (1995)

51–59.

[25] K.T. Thompson, F.H. Crocker, H.L. Fredrickson, Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro- 1,3,5-triazine by Gordonia and Williamsia spp., Appl. Environ. Microbiol. 71 (2005) 8265–8272.

[26] Lorenz, A., Rylott, E.L., Strand, S.E., Bruce, N.C. Towards engineering degradation of the explosive pollutant hexahydro-1,3,5-trinitro-1,3,5-triazine in the rhizosphere. FEMS Microbiol. Lett (2013) 340, 49–54.

[27] D. Fournier, A. Halasz, J. Spain, P. Fiurasek, J. Hawari, Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with Rhodococcus sp. strain DN22, Appl. Environ. Microbiol. 68 (2002) 166–172.

[28] J. Hawari, A. Halasz, S. Beaudet, L. Paquet, G. Ampleman, S. Thiboutot, Biotransformation routes of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine by municipal anaerobic sludge, Environ. Sci. Technol. 35 (2001) 70–75.

[29] S. Zhang, H.N. Nguyen, T.N. Truong, Theoretical study of mechanisms, thermodynamics, and kinetics of the decomposition of gas-phase α-HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), J. Phys. Chem. A. 107 (2003) 2981–2989.

[30] D. Fournier, A. Halasz, J. Spain, R.J. Spanggord, J.C. Bottaro, J. Hawari, Biodegradation of the Hexahydro-1,3,5-Trinitro-1,3,5-Triazine Ring Cleavage Product 4-Nitro-2,4-Diazabutanal by Phanerochaete chrysosporium, Appl. Environ. Microbiol. 70 (2004) 1123–1128.

[31] R. Singh, P. Soni, P. Kumar, S. Purohit, A. Singh, Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria, World J. Microbiol. Biotechnol. 25 (2009) 269–275.

[32] B. Bhushan, A. Halasz, J.H.-J. of Applied, undefined 2006, Effect of iron(III), humic acids and anthraquinone-2,6-disulfonate on biodegradation of cyclic nitramines by Clostridium sp. EDB2, Wiley Online Libr. 100 (2006) 555–563.

[33] Zoh, K.D., Stenstrom, M.K. Fenton oxidation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Water Res (2002) 36, 1331–1341.

CHAPTER 6

Chapter 6

Conclusions and scope for the future work

The nitrogen-containing energetic compounds such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), are mainly used for military purposes. Both are manufactured by the modified Bachmann process through nitration of hexamine. Since HMX is a higher analog of RDX, so RDX is always present as an impurity during the manufacture of HMX. A significant amount of wastewater is produced during the production of these substances, which contains RDX and HMX residues. Both RDX and HMX have a negative impact on the environment and humans, there is a need to remediate the contaminated sites. Nowadays, the microbial remediation method has gained much attention due to its eco-friendly and cost-effective nature. Various microbes have been applied to treat RDX and HMX contamination. However, the potential of native microbes to remediate HMX and RDX is yet to be explored.

In the present work, as explained two native microbes isolated from an HMX manufacturing contaminated site have been explored to treat HMX and RDX contamination under different conditions. In Chapter 3 the RDX degradation has been studied using the *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) and *Bacillus toyonenesis* (isolate No. WS4-TSB-3, MTCC No. 12857). Further, As HMX is also present as a co-contaminant during RDX contamination, the degradation of HMX with *Bacillus toyonensis* has been explored in Chapter 4. As it was observed that microbes isolated from the polluted sites were capable of decomposing RDX and HMX in the aqueous phase. So, it is also crucial to look into the breakdown of RDX and HMX in soil/sludge. Therefore, in Chapter 5 the objective was to clean up RDX and HMX-affected actual soil sludge that had been obtained from the same polluted site.

Chapter 3: Biodegradation of RDX and possible pathway exploration

In this study, RDX degradation was observed using *Bacillus toyonensis* and *Paenibacillus dendritiformis*. It was observed that both the microbes have high RDX removal efficiency under aerobic conditions. The maximum degradation observed was 81% and 84% with *Bacillus toyonensis* and *Paenibacillus dendritiformis* respectively at the end of 15^{th} day. High degradation efficiency for both the microbes shows that both the microbes have a high potential to reduce RDX contamination. The RDX degradation process was optimized using the response surface methodology with process variables RDX initial concentration (20-60 mg/L), inoculum volume (2-6%), and degradation time (5-15 days). It was observed that inoculum volume and degradation time have a positive impact on the degradation of RDX for both microbial species. However, with *Bacillus toyonensis*, an increase in the initial concentration of RDX leads to decreased degradation efficiency. The ANOVA for the degradation of RDX shows significant p-value (0.0002 & 0.0003), F-value (23.64 & 21.6), R^2 value (0.9 & 0.9), respectively for *Bacillus toyonensis* and *Paenibacillus dendritiformis*.

The metabolites formed during the RDX degradation were observed. Different peaks were observed which corresponds to the Hexahydro-1-nitroso,3,5-dinitroso-1,3,5-triazine (MNX, $C_3H_6N_6O_5$, M+NH4, mol. wt. 224.07 Da), Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, $C_3H_6N_4$, M+NH_4, mol. Wt. 191.05 Da), Bis(hydroxymethyl)nitramine ($C_2H_6N_2O_4$, M+NH_4, mol. wt. 140.66 Da) and 4-Nitro-2,4-diazabutanal ($C_2H_5N_3O_3$, M+Na+K-H, mol. wt. 179.97 Da) respectively. Based on the identified peaks the degradation pathway was proposed. However, review of literature shows that all known metabolites are destined to be mineralized into simpler forms (i.e., CO_2, methanol, nitrous oxide, formaldehyde, etc.). According to the study's findings, the ability to degrade RDX was demonstrated by both *Bacillus toyonensis* and *Paenibacillus dendritiformis*.

Chapter 4: Process development for the biodegradation of HMX using response surface methodology

The degradation of HMX was optimized with the native microbial species i.e., *Bacillus toyonenesis* isolated from the actual contaminated site. It was observed that *Bacillus toyonenesis* has the high potential to efficiently remove HMX from contaminated sites. The highest removal of HMX (87%) was observed at 2 mg/L initial HMX concentration. It was followed by 79% and 74% at 6 and 4 mg/L HMX concentration respectively. The degradation process was optimized using response surface methodology (RSM) with variables such as initial HMX concentration (2-6 mg/L), inoculum volume (2-6 %), and degradation time (5-15 days). It was observed that with an increase in inoculum size there was an increase in the HMX degradation. At the end of 15^{th} day, degradation at 4 mg/L HMX concentration was 65% and 79% at 2 and 6 % inoculum volume, respectively. Similarly, an increase in degradation time has a positive impact on HMX degradation. With 4 mg/L concentration and 6 % volume of microbial suspension, degradation of 58 and 79 % was achieved on 5^{th} and 15^{th} days, respectively. However, the microbes have high degradation efficiency at lower concentrations of HMX. The experimental data obtained were subjected to analysis of variance (ANOVA) and significant p-value (<0.0001), R^2 value (0.9878), and F-value (62.97) were obtained for the model. Also, the dispersion of data between experimentally obtained values and model predicted values were observed in actual versus predicted graphs obtained from the RSM model. The model's small standard deviation (1.65) explains the association between multiple factors and responses. This shows that the model obtained for each response can be utilized to optimize the degradation process and product release. *Bacillus toyonensis*, which was isolated from an actual HMX-contaminated location, appears to be a viable candidate for HMX degradation

Thus Chapters 3 and 4 conclude that contaminants like RDX and HMX can be broken down in the aqueous phase by the microorganisms found at the contaminated sites. In addition, the RDX and HMX breakdown in soil was the next target. So, the study's next objective was to remediate soil sludge that was contaminated with both RDX and HMX.

Chapter 5: Bioreactor studies: Upscaled composting of actual RDX and HMX contaminated sludge

In this study, HMX and RDX contaminated sludge collected from the HMX manufacturing facility, in North India was subjected to microbial degradation. The in-vessel composting of HMX and RDX contaminated sludge was carried out using native microbial species i.e., *Arthrobacter subterraneus* and *Bacillus sonorensis*. The composting was performed under controlled conditions (moisture, temperature, airflow, etc.) for 80 days in the laboratory. It was observed that maximum HMX degradation (78%) was with *Bacillus sonorensis* having 10% sludge, 70% cow manure, and 20% garden waste in reactor no. 2. Furthermore, RDX breakdown was highest (91%) in reactor seeded with a consortium of microorganisms and 20% sludge, 60% cow manure, and 20% garden waste. In the combinations with the highest degradations (reactor 2 and 11), the half-lives of HMX and RDX were found to be 33 days ($k = 0.223\ day^{-1}$) and 21 days ($k = 0.0327\ day^{-1}$), respectively. Also, One-way ANOVA results showed that the in-vessel composting process produced substantial outcomes in the treatment of explosive polluted sludge. HMX degradation intermediate compounds were found to be bis(hydroxymethyl)nitramine and methylene dinitramine. The breakdown of HMX and RDX was also confirmed by analyzing the release of nitrite and nitrate during the composting process. The findings showed that RDX and HMX-polluted sludge can be successfully digested by the native bacteria i.e., *Arthrobacter subterraneus* and *Bacillus sonorensis*. Additionally, this is the first

time these bacteria have been utilized through in-vessel composting study for the decomposition of HMX and RDX contaminated sludge.

Overall conclusions:

In this thesis, response surface methodology was used to optimize the variable process parameters for the degradation of HMX and RDX in the aqueous phase by an indigenously isolated bacterial strains *Bacillus toyonensis* and *Paenibacillus dendritiformis* from a real HMX-contaminated location in North India. The current investigation reported a positive association between different initial HMX and RDX concentrations, microbial inoculum size, and degradation time. High HMX and RDX degradation were observed during the study. The experimental data's predictability was further supported by the high regression coefficient values. Estimated concentrations of nitrite and nitrate during the experiments showed that HMX and RDX are being broken down and degraded during the process. Further, Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), 4-nitro-2,4-diazabutanal, Bis(hydroxymethyl)nitramine, and nitrite were found to be intermediate products during the breakdown of RDX. The results of the study indicate that both microorganisms may be able to break down RDX in an aqueous environment and may be employed to accelerate RDX breakdown on explosive-contaminated sites.

In continuation *Arthrobacter subterraneus* and *Bacillus sonorensis* were used to remediate highly contaminated sludge that contained HMX and RDX. The explosive-contaminated sludge was composted inside the bioreactor, using garden waste and cow manure as bulking agents. It was observed that both RDX and HMX were reduced significantly on the 80th day. During the degradation of HMX, two secondary metabolites i.e., bis(hydroxymethyl)nitramine and methylene dinitramine were found. It was also observed that significant amount of nitrate and nitrite ion were released during the study. Also, no structural changes/deformities were reported in the microbial cells as a result of contamination stress in SEM studies.

Future Prospects

The composting process has shown a positive response towards the degradation of RDX and HMX in sludge with isolated microbes. In-vessel composting assisted with native bacterial species can be a potential, economical & green technology for the treatment of explosive contaminated sludge at the contaminated sites. Some of the important aspects are also yet to be explored such as the formation of different metabolites, the role of microbial enzymes, explosive degrading genes, the effect of compost composition, etc. Also, the effective microbial formulations using indigenous microbes are yet to be tested. The degradation mechanisms need a detailed and in-depth understanding for enhancing the rate of microbial degradation process under aerobic conditions. Further, large-scale bioreactor systems can be a possible solution to treat a large amount of wastewater generated at the manufacturing site. Pilot-scale biological treatment plants are needed to be optimized according to the amount of waste generated at the contaminated sites. The pilot scale in-situ biological treatment of explosive contamination can be a cost-efficient and eco-friendly approach.

water

MDPI

Article

Optimization and Degradation Studies on Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) with Selected Indigenous Microbes under Aerobic Conditions

Arjun Meda [1,2], Pritam Sangwan [1,*] and Kiran Bala [2]

[1] Centre for Fire, Explosive and Environment Safety, Defence Research & Development Organization, New Delhi 110054, India; phd1701171016@iiti.ac.in
[2] Department of Biosciences and Biomedical Engineering, Indian Institute of Technology, Indore 453552, India; kiranb@iiti.ac.in
* Correspondence: pritam@cfees.drdo.in

Abstract: Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) a nitramine explosive, which has contaminated various military sites during its use, storage and manufacturing worldwide. As RDX is a recalcitrant, less soluble and toxic to human beings and other organisms, it is essential to remediate the contaminated sites. In the current investigation, authors have explored the potential of two indigenous microbes i.e., *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) isolated from an explosive manufacturing facility in north India, for the degradation of RDX in aqueous medium. Furthermore, RDX degradation has been optimized using response surface methodology (RSM) in a 15 days experiment at concentration of 20, 40, and 60 mg/L. It was found that various factors such as initial concentration of RDX, inoculum volume (2, 4 and 6%) and time (5, 10 and 15 days) had impact on transformation and degradation of contaminant. Samples were analyzed using high performance liquid chromatography (HPLC) and intermediate products were identified using LC-MS/MS. Maximum RDX removal of 81.6 ± 1.3 and 84.7 ± 0.9% for *Bacillus toyonensis* (isolate No. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3), respectively, was observed on 15th day at 40 mg/L initial concentration. During the degradation Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), 4-Nitro-2,4-diazabutanal, Bis(hydroxymethyl)nitramine and nitrite were identified as intermediate products. The findings of the investigation suggest that both the microbes have the potential to degrade RDX in the aqueous medium and can be used for up-scaling the degradation of RDX on explosive contaminated sites.

Keywords: RDX; degradation; *Bacillus toyonensis*; *Paenibacillus dendritiformis*; response surface methodology; contamination

Citation: Meda, A.; Sangwan, P.; Bala, K. Optimization and Degradation Studies on Hexahydro-1,3,5-Trinitro-1,3,5-Triazine (RDX) with Selected Indigenous Microbes under Aerobic Conditions. *Water* **2021**, *13*, 1257. https://doi.org/10.3390/w13091257

Academic Editor: Fernando António Leal Pacheco

Received: 23 March 2021
Accepted: 27 April 2021
Published: 30 April 2021

1. Introduction

Explosives are nitrogen-based energetic compounds, which have high potential energy. Royal Demolition Explosive (RDX), or hexahydro-1,3,5-trinitro-1,3,5-triazine, belongs to this category and is generally used for military purposes. Military activities including manufacturing, testing, training, demilitarization, open burning, and waste discharge have resulted in extensive contamination of soil and groundwater of surroundings [1,2]. Furthermore, it is already studied that RDX has a relatively stable ring structure and electron withdrawing nitro groups makes it less susceptible to degradation in nature [3]. Due to low soil adsorption coefficient of RDX, there is high possibility that it may contaminate ground water near the military bases, testing facilities, and war zones [1,4]. As, RDX is comparatively mobile in the soil and has low rates of degradation in soil, it presents distinct problems for bioremediation [5]. RDX is also known to be water soluble 60 mg/L at 25 °C, and therefore, it may get mixed into groundwater aquifers and can travel to distant places,

which may affect environment and human health. USEPA has suggested a lifetime dinking standard for RDX as 2 μg/L [6].

Earlier studies have shown that, RDX is a toxic material, and, according to USEPA, it is classified as group 'C' human carcinogen [7]. Its exposure may cause seizures, convulsion, nausea, vomiting, etc. [8–10]. It can affect the nervous system and damage the liver. RDX can readily cross the blood-brain barrier, alter the expression of multiple brain genes, and evoke pronounced seizure-like responses in a wide range of species [11–14]. Thus, the remediation of RDX contaminated sites is important for the protection of human health and ecosystems. The conventional approaches for the remediation of RDX are thermal decomposition [15,16], photolysis [17], and treatment with catalyst [18]. The Conventional methods are not cost effective as they require sophisticated instrumentation and also generate other by-products, such as ash, which is difficult to get rid of. The other method, which is gaining much more attention these days, is microbial remediation. It is eco-friendly, cost-efficient, and much easier to implement and perform. The degradation of RDX has already been reported with many microbes. *Klebsiella pneumonia*, isolated from anaerobic sludge, can break down RDX chains into methanol, CO_2, formaldehyde, and nitrous oxide through the formation of intermediate such as methylene di-nitramine [19]. *Phanerochaete chrysosporium* is known to aerobically degrade RDX and produce 4-Nitro-2,4-diazabutanal (NDAB) as an intermediate product, which can be completely mineralized into CO_2 and N_2O [20,21]. *Clostridium bifermentans* can aerobically degrade RDX into formaldehyde, methanol and CO_2 through the formation of intermediate products Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) [22]. Although many studies have been performed to understand the RDX degradation pathway, the majority of them have focused on its anaerobic degradation. So, in this investigation, authors have tried to study the aerobic degradation of RDX and optimize the parameters, which can influence the process of degradation. Optimization of the process was done by response surface methodology (RSM), which uses lower order polynomial equation to predict a model based on the interaction of different variables during the process [23–26]. Some earlier researchers have also used RSM to optimize the dye and explosive/pollutants removal from the medium [27–30].

So, this study was planned with the major objective to explore the RDX degrading potential of microbes i.e., *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) which were isolated from an actual explosive contaminated site and are unexplored yet for optimization and degradation study in the RDX. The inter-relationship between the RDX degradation and independent variables (initial RDX concentration, inoculum volume and time) were also explored. Mass spectroscopy (LC-MS/MS) was used for RDX degradation analysis, identification of the metabolites, and understanding of mechanism.

2. Materials and Methods

2.1. Chemicals

RDX was taken from an explosive manufacturing facility in north India with a purity of greater than 99.9%. High Performance Liquid Chromatography (HPLC) grade solvents were purchased from Sigma-Aldrich. Other chemicals used were also of analytical grade and purchased from standard manufacturers.

2.2. Microbial Culture

Microbial cultures were prepared using standard methods. In brief, soil and water samples were collected as per standard protocols from an actual explosive contaminated site in north India for isolation of microbes. Microbes were isolated and identified from samples by Institute of Microbial Technology (IMTECH), Council of Scientific and Industrial Research, (CSIR), Chandigarh, India and provided in lyophilized form for further research work. Lyophilized microbes, *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) were revived

in tryptic soya broth (TSB) (HIMEDIA, LQ508). Cultures were maintained on slants at a temperature of 4 °C prior to use. Then microbes were sub-cultured for three generations in minimal salt media (MSM) [31], which was deficient in nitrogen sources to make them more tolerant and adaptive to RDX stress before culturing into modified MSM containing RDX. The MSM was spiked with the desired concentration of RDX, prepared in acetonitrile. After spiking, the solutions were left open for 18 h in a laminar air flow chamber, so that solvent (acetonitrile)evaporates and does not interfere with media composition [32–34]. The spiked media was then inoculated with the microbes and grown at a temperature of 32 ± 3 °C in orbital shaker at 120 rpm. The experiment was performed in Erlenmeyer flasks of 250 mL capacity. The total volume of 100 mL was used in each combination, which consisted of MSM media, contaminant, and microbial culture.

2.3. Experimental Setup

Total 17 combinations were set during the experiment as shown in Table 1 for *Bacillus toyonensis* (isolate No. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3) each. Initial concentration of RDX (20–60 mg/L), time period (5–15 days) and inoculum volume (2–6%) of each combination are mentioned in Table 1. All these combinations were designed using DESIGN-EXPERT® VERSION 12 software (Stat-Ease®, Minneapolis, MN, USA) with RSM. Box Behnken Design (BBD) was used as a second order polynomial model for designing the experiment and to statistically validate the data. Number of experimental sets required for BBD was defined by,

$$N = k\,(k - 1) + C_o \quad (1)$$

where, 'C_o' is the central point and 'k' is the number of factors. Microbial cultures (3rd generation) of *Bacillus toyonensis* (isolate No. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3) having optical density (OD) 1.2 ± 0.2 corresponding to $\approx 10^8$ cells/mL were separately used to inoculate the freshly prepared MSM, spiked with varying concentrations of RDX. After inoculation as per the combinations given in Table 1, flasks were incubated in an orbital shaker at a temperature of 32 ± 3 °C and a rotation of 120 rpm. The whole experiment was performed under aerobic conditions. Samples were withdrawn at a fix interval of time (5 days) to analyze the nitrite, metabolites and reduction in concentration of RDX. To ensure the aerobic biodegradation conditions in each flask, cotton plugs were fitted so that air diffusion can take place in and out during the shaking of flasks in orbital shaker (120 rpm).

Table 1. Different runs for optimization of parameters for RDX degradation.

RDX Concentration (mg/L)	Inoculation Volume (%)	Time (Days)
20	2	10
20	4	5
20	4	15
20	6	10
40	2	5
40	2	15
40	4	10
40	4	10
40	4	10
40	4	10
40	4	10
40	6	5
40	6	15
60	2	10
60	4	5
60	4	15
60	6	10

2.4. Analysis

Analysis of samples for degradation of RDX was carried out using High Performance Liquid Chromatography (HPLC) as per standard method USEPA 8330A [34]. In brief, 5 mL of treated sample was withdrawn and mixed with 5 mL acetonitrile. After centrifugation, supernatant was filtered through 0.45 µm Teflon filter. This filtrate was used for analysis of RDX concentration and fed in HPLC (Flexer, Perkin Elmer, Waltham, MA, USA) equipped with photo diode array (PDA) detector. C18 reverse phase column (3 µm, 150 mm × 4.6 mm) was used as stationary phase whereas, acetonitrile: water (50:50) mixture was used as mobile phase with a flow rate of 1 mL/min. The mobile phase was prepared with triple distilled water and acetonitrile. The injection volume was 10 µL. Retention time and UV-profile of the standard compound were used for the identification and quantification of peak.

Nitrite in the samples was analyzed by the method described earlier [34]. In brief, 1 mL of sample was collected at regular interval and centrifuged. Supernatant was used to analyze nitrite concentration. Sample (600 µL) was mixed with 150 µL sulfanilamide. After incubation of 5 min, 150 µL of *N*-(1-naphthyl) ethylenediamine dihydrochloride solution was added and incubated for 20 min at room temperature. Afterwards, 2.1 mL of distilled water was added and analyzed by taking absorbance at 540 nm on UV–Visible spectrophotometer (Perkin Elmer, Model Lambda 650S, Waltham, MA, USA).

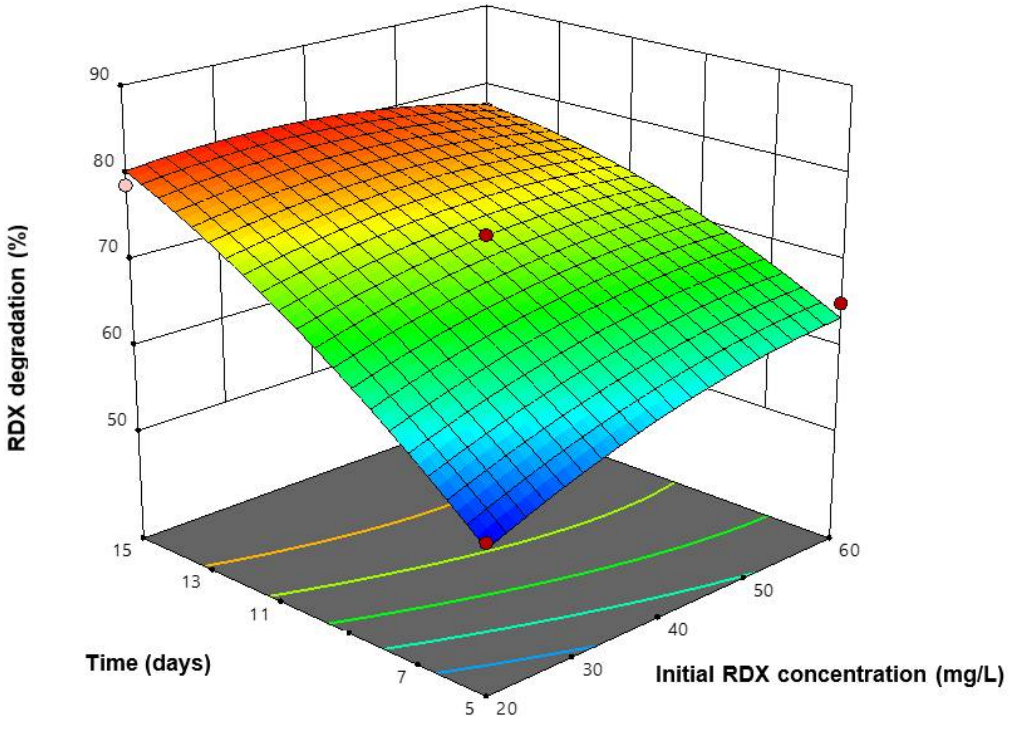
Mass spectrometric (MS) analyses were performed on microTOF-Q (Bruker Daltonics, Billerica, Massachusetts, USA) MS system using atmospheric pressure chemical ionization in the positive ion (ES+) mode. C18 column was used to separate RDX and degradation products. The flow rate was 1 mL/min for 5 min. The solvent system consisted of 0.1% formic acid, 50% acetonitrile and 49.9% triple distilled water. The obtained peaks were interpreted based on the metabolites previously reported in the literature and the system software.

3. Results and Discussion

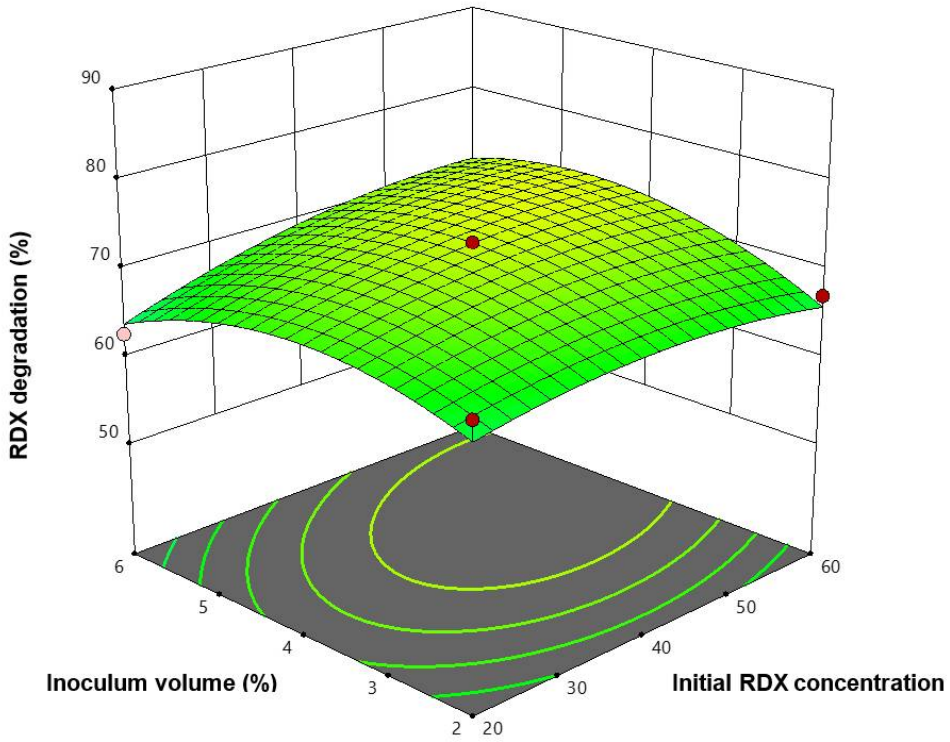
3.1. Degradation of RDX

Degradation of RDX during the experiment can be observed in Figure 1A,B with *Bacillus toyonensis* (isolate No. WS4-TSB-3). The Maximum RDX degradation achieved with this microbe was 81.7 ± 1.3% with 40 mg/L initial RDX concentration and 6% inoculum volume on 15th day. This was followed by 78.7 ± 1.1 and 77.01 ± 0.8% RDX degradation at 20 mg/L and 60 mg/L, respectively with 4% inoculum volume on 15th day. Minimum degradation was 74.2 ± 0.3% achieved at 40 mg/L initial RDX concentration and 2% inoculum volume. Figure 1A shows the interactive effect of initial RDX concentration and time on RDX degradation. It was observed that, with increase in time, there was increase in RDX removal. However, at higher RDX concentration (60 mg/L) removal efficiency was much lower, which can be due to toxic effect of RDX on *Bacillus toyonensis* (isolate No. WS4-TSB-3). Figure 1B shows the interactive effect of inoculum volume and RDX concentration on RDX degradation. It was observed that *Bacillus toyonensis* (isolate No. WS4-TSB-3) showed increased removal of RDX with increase in inoculum volume. The whole set of data for RDX degradation with *Bacillus toyonensis* (isolate No. WS4-TSB-3) was subjected to two-way analysis of variance (ANOVA) as shown in Table 2. The *p*-value (0.0002), F-value (23.6) and R^2 (0.9) of the model shows that the data was significant and best suited for the quadratic model. Figure 2 presents data between actual versus predicted value, which shows that there was less dispersion of data between experimentally obtained and predicted values by the model. Low standard deviation (2.2) was observed for the model, which confirms the suitability of the model. All the parameters were fitted for second order polynomial equation as follows:

$$Y = 73.07 + 1.94\ A + 0.9663\ B + 10.43\ C + 1.88\ AB - 3.32\ AC + 1.07\ BC - 2.02\ A^2 - 4.32\ B^2 - 2.43\ C^2 \quad (2)$$



(**A**)



(**B**)

Figure 1. (**A**) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and time (days) with *Bacillus toyonensis*. (**B**) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and inoculum volume (%) with *Bacillus toyonensis*.

Table 2. ANOVA of Quadratic model for percent degradation of RDX with *Bacillus toyonensis*.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	1103.52	9	122.61	23.64	0.0002	Significant
A-RDX concentration	30.11	1	30.11	5.81	0.0468	
B-Inoculation volume	7.47	1	7.47	1.44	0.2692	
C-Time	870.49	1	870.49	167.83	<0.0001	
AB	14.18	1	14.18	2.73	0.1423	
AC	44.02	1	44.02	8.49	0.0226	
BC	4.62	1	4.62	0.8912	0.3766	
A^2	17.14	1	17.14	3.30	0.1119	
B^2	78.58	1	78.58	15.15	0.0060	
C^2	24.86	1	24.86	4.79	0.0647	
Residual	36.31	7	5.19			
Lack of Fit	36.31	3	12.10			
Pure Error	0.0000	4	0.0000			
Cor Total	1139.83	16				

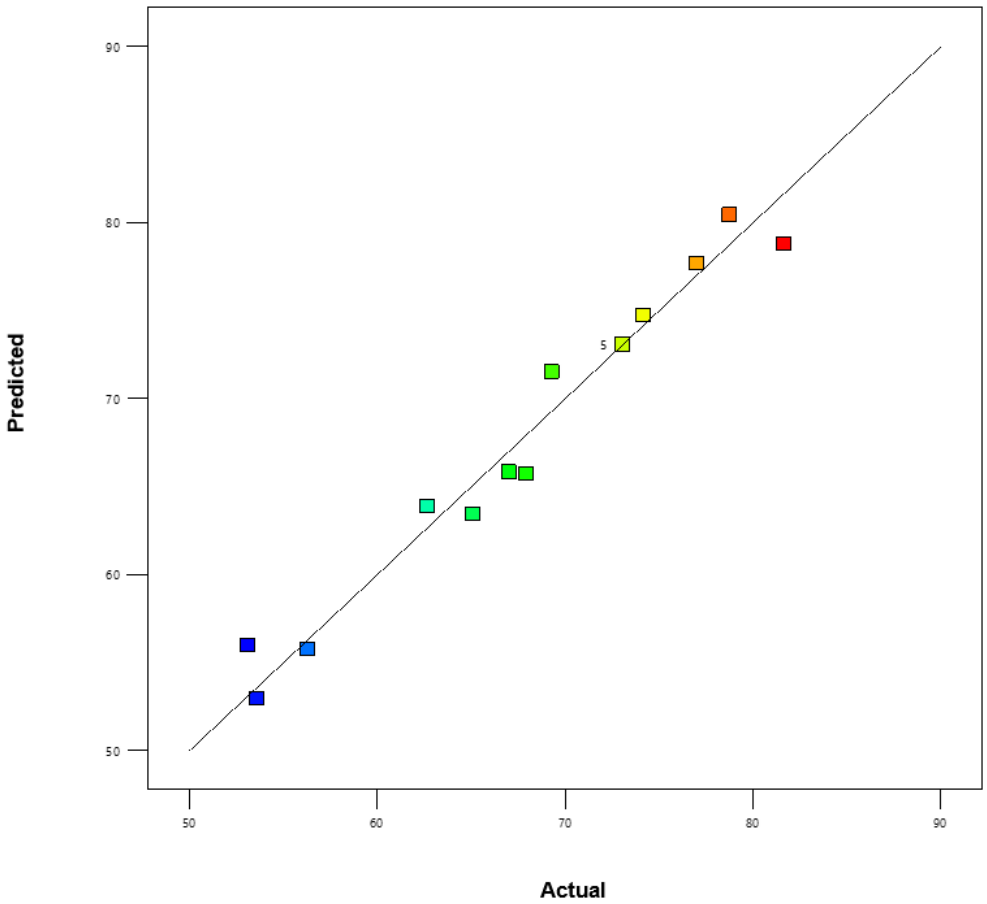


Figure 2. Actual verses predicted graph for the RDX degradation with *Bacillus toyonensis*.

Similarly, in Figure 3A,B a reduction in RDX concentration with time was observed with *Paenibacillus dendritiformis* (isolate No. S10-TSA-3). With the increase in inoculum volume, a increase in degradation of RDX was observed. At the end of 15th day, maximum (84.7 ± 0.9%) RDX degradation was observed at 40 mg/L initial concentration with 6% inoculum volume, which was followed by 78.1 ± 1.1% at 20 mg/L concentration and 2% inoculum volume. The maximum degradation achieved was nearly 1.2 times higher than the minimum degradation (71.7 ± 1.1) observed at 20 mg/L concentration with 4% inoculum volume on 15th day. However, degradation in the control due to abiotic factors was 0.8% and 1.1% for *Bacillus toyonensis* (isolate No. WS4-TSB-3) and *Paenibacillus*

dendritiformis (isolate No. S10-TSA-3), respectively, which is negligible compared to test samples. Other researchers observed RDX degradation of more than 80% with other species of microbes like *Planomicrobium flavidum*, *Rhodococcus* strain, *Phanerochaete chrysosporium*, *Clostridium bifermentans*, *Paenibacillus aestuarii and Arthrobacter subterraneus* [27,35–37].

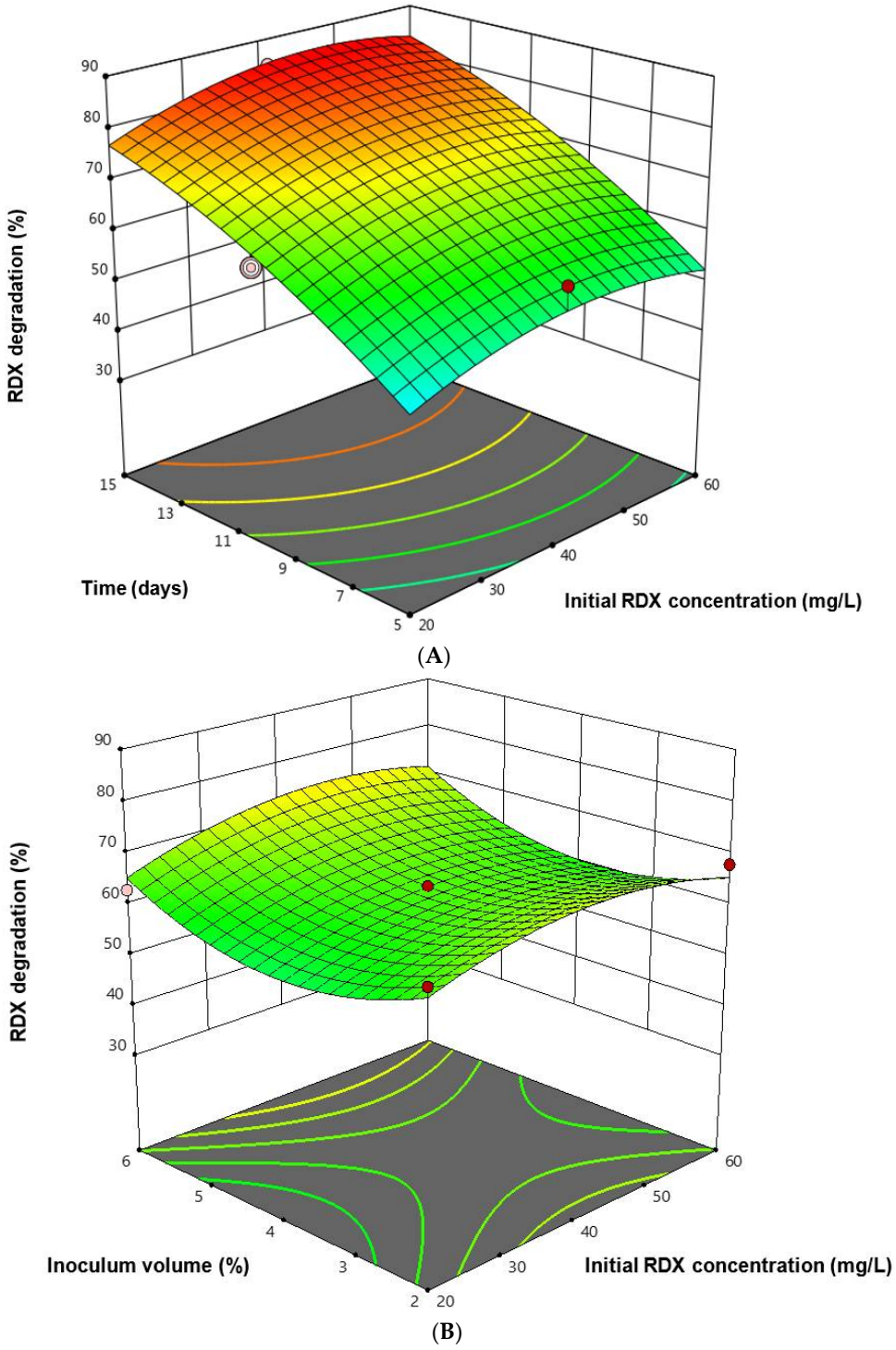


Figure 3. (**A**) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and time (days) with *Paenibacillus dendritiformis*. (**B**) 3-D model plot for the degradation of RDX under varying initial concentration (mg/L) and inoculum volume (%) with *Paenibacillus dendritiformis*.

Figure 3A shows the 3-D plot for interaction of initial RDX concentration and time during the RDX degradation. It was observed that with the increase in both the variables,

there was increase in RDX degradation. Even, increase in initial RDX concentration does not have negative impact on the degradation efficiency of microbes. This observation implies that *Paenibacillus dendritiformis* (isolate No. S10-TSA-3) can survive and performs better at higher concentration of RDX (60 mg/L) also. Similarly, Figure 3B shows the effect of inoculum volume on RDX degradation. It was observed that there was higher degradation of RDX with 6% inoculum volume. To validate the model, two-way ANOVA was performed, and it was observed that the model was statistically significant (Table 3). Obtained *p*-value (0.0003), F-value (21.6) and R^2 (0.9) were significant and shows that the model best suited for quadratic model. Figure 4 shows the difference between actual and predicted values. It is evident that there was less variation between experimentally obtained values and values predicted by the model for RDX degradation. All parameters were fitted for second order polynomial equation as follows:

$$Y = 63.74 + 2.02\ A + 1.99\ B + 15.94\ C + 0.9850\ AB + 0.3925\ AC - 0.7525\ BC - 5.01\ A^2 + 7.73\ B^2 - 3.42\ C^2 \quad (3)$$

Table 3. ANOVA of Quadratic model for percent degradation of RDX with *Paenibacillus dendritiformis*.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value	
Model	2489.85	9	276.65	21.68	0.0003	Significant
A-RDX concentration	32.76	1	32.76	2.57	0.1531	
B-Inoculation volume	31.72	1	31.72	2.49	0.1588	
C-Time	2032.03	1	2032.03	159.28	<0.0001	
AB	3.88	1	3.88	0.3042	0.5984	
AC	0.6162	1	0.6162	0.0483	0.8323	
BC	2.27	1	2.27	0.1775	0.6861	
A^2	105.79	1	105.79	8.29	0.0237	
B^2	251.75	1	251.75	19.73	0.0030	
C^2	49.25	1	49.25	3.86	0.0902	
Residual	89.30	7	12.76			
Lack of Fit	89.30	3	29.77			
Pure Error	0.0000	4	0.0000			
Cor Total	2579.15	16				

Similar results for ANOVA were also obtained in earlier studies by other authors. Mohanty and Jena, (2018) obtained similar results during the optimization of butachlor remediation with *Enterobacter cloacae* [38]. Sharma et al. (2021) observed a similar two-way ANOVA results during the remediation of RDX in aqueous phase with the consortium of microbes [27].

3.2. Release of Nitrite during RDX Degradation

It is well established that nitrite ions are released during the degradation of RDX. Ring cleavage of RDX starts with the denitration-hydration step, with the formation of NADB and formaldehyde resulting into the release of nitrite ion [36,39]. Similar observations were made during this study. As RDX degraded, there was change in the nitrite concentration in the medium with both the microbes in their respective combinations. Figure 5A shows the change in nitrite concentration for *Bacillus toyonensis* (isolate No. WS4-TSB-3) with respect to RDX concentration and time. Maximum concentration of nitrite release (0.3 ± 0.01 mg/L) with *Bacillus toyonensis* was observed on 10th day with 60 mg/L concentration and 6% inoculum volume, which was followed by 0.3 ± 0.01 mg/L at 60 mg/L RDX concentration and 4% inoculum volume. Similarly, Figure 5B shows the nitrite release during the RDX degradation with *Paenibacillus dendritiformis* (isolate No. S10-TSA-3). Maximum nitrite release was observed on 10th day, which was 0.2 ± 0.01 mg/L at 60 mg/L RDX concentration and 2% inoculum volume. It was observed, that with an increase in RDX concentration there was an increase in the release of nitrite. Also, with the degradation of RDX, there was an increase in nitrite concentration until the 10th day, and afterward,

it started decreasing. Decrease in the nitrite concentration can be due to its utilization by microbes or conversion into nitrate [40,41].

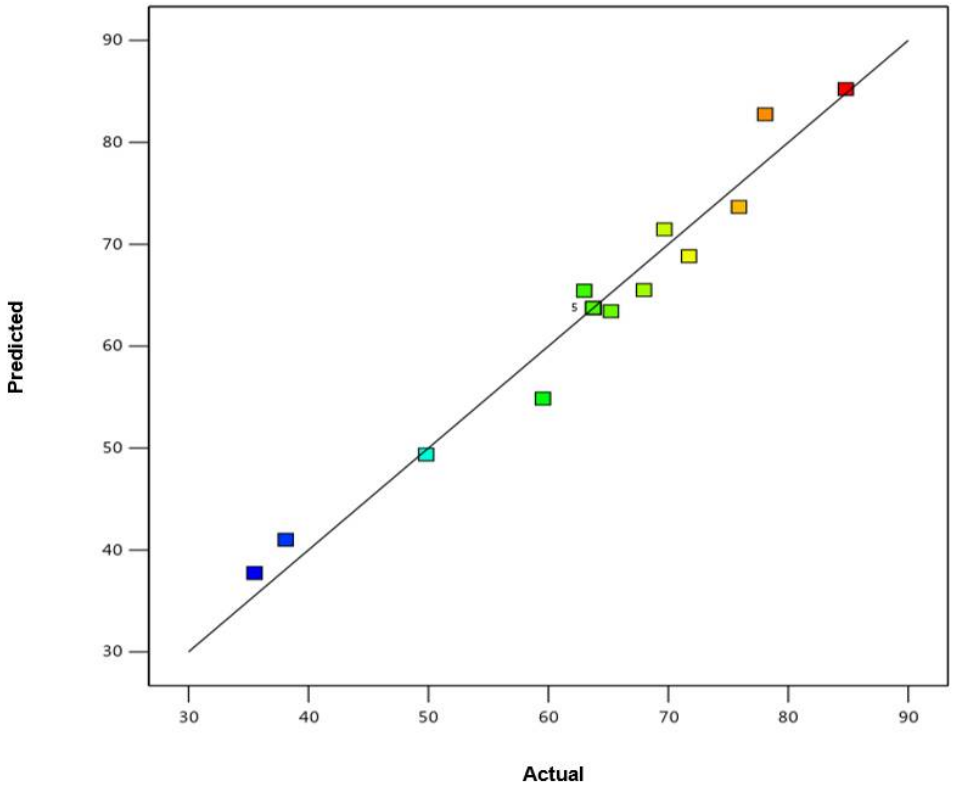


Figure 4. Actual verses predicted graph for the RDX degradation with *Paenibacillus dendritiformis*.

To validate, the data was subjected to two-way ANOVA. For nitrite release it was observed that microbes, *Bacillus toyonensis* (isolate No. WS4-TSB-3) and *Paenibacillus dendritiformis* (isolate No. S10-TSA-3) has significant *p*-value (<0.0001 and 0.0009), F-value (34.5 and 14.9) and R^2 (0.9 and 0.9 respectively) which statistically validated the model for both the microbes (Table 4). Correction total (sum of square) for both the microbes shows that the model has high reproducibility and less variation around the mean. Statistically similar results were obtained by Chaudhary et al. (2019) during their optimization of tannery wastewater remediation with *Aspergillus fumigates* [42]. Garg et al. (2015) found similar ANOVA results during the optimization of decolorization of different dyes with *Pseudomonas* strain [43].

Table 4. ANOVA of Quadratic model for the release of nitrite during RDX degradation.

Factor	*Bacillus toyonensis*	*Paenibacillus dendritiformis*
p-value	<0.0001	0.0009
F-value	34.52	14.88
R^2	0.97	0.95
Cor-total	0.0624	0.0236
Std.dev	0.0140	0.0130

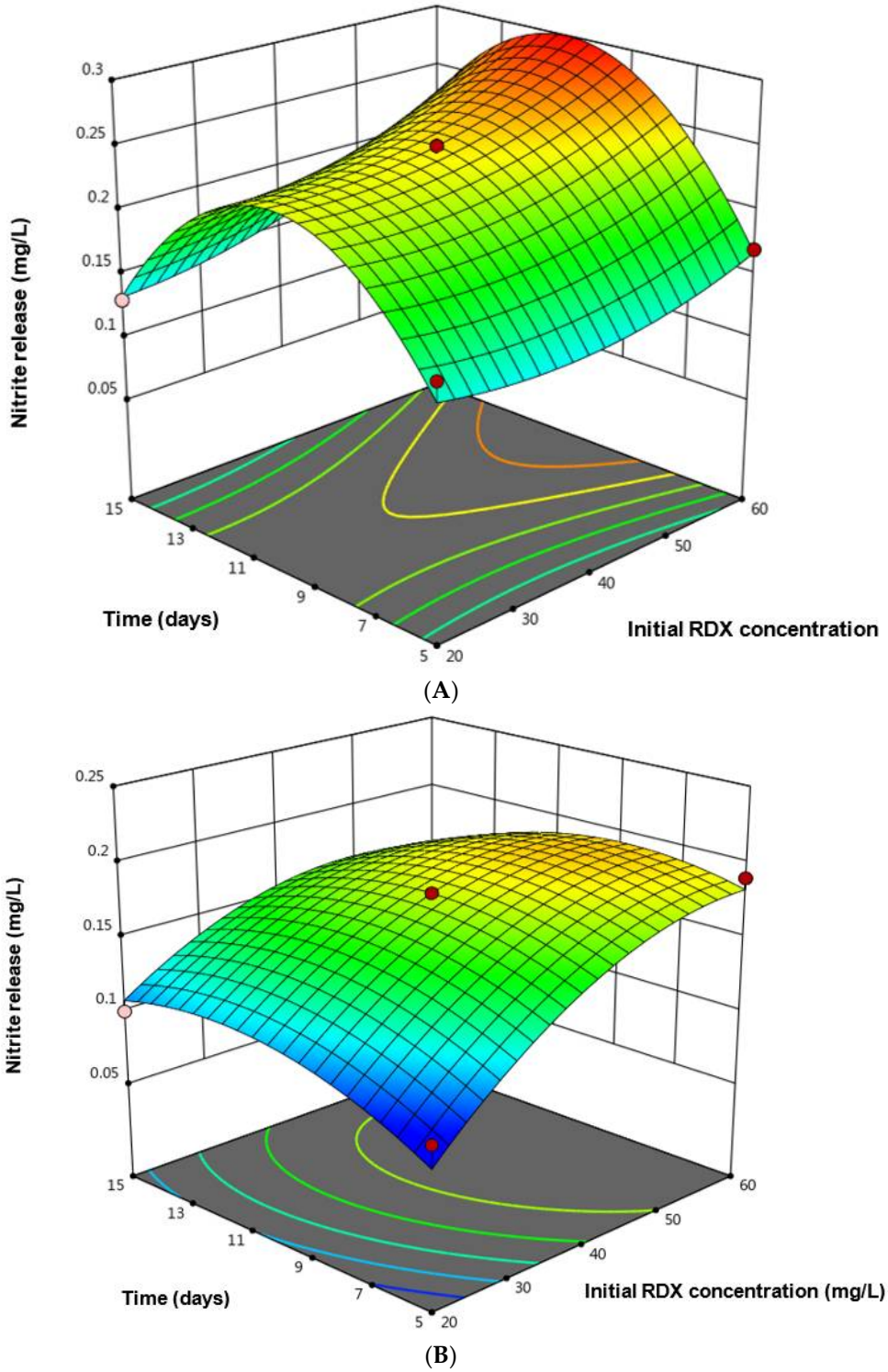


Figure 5. (**A**) Response surface plot (3-D plot) showing interactive effect of RDX concentration (mg/L) and time (days) on nitrite release with *Bacillus toyonensis*. (**B**) Response surface plot (3-D plot) showing interactive effect of RDX concentration (mg/L) and time (days) on nitrite release with *Paenibacillus dendritiformis*.

3.3. Degradation Pathway

It is already known that during the microbial degradation process, the cyclic structure of RDX tends to break into intermediate products. To understand and elucidate the RDX degradation pathway for both the microbes, mass spectroscopy of the samples was done at different intervals of time i.e., 5th day and 10th day in the combination having

highest degradation. Positive ESI (Electron spray ionization) revealed the presence of different metabolites in the samples. The peaks obtained were at m/z values of 224.07 and 191.05 on 5th day samples and 140.66 and 179.97 on 10th day samples (Figure 6A–D). To identify the metabolites, molecular weight (m/z ratio) of the obtained peaks were compared with the metabolites reported in earlier studies [37,39,44–47]. The peaks 224.07 and 191.05, were identified as Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, $C_3H_6N_6O_5$, M+NH_4, mol. wt. 224.07 Da) and Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, $C_3H_6N_6O_4$, M+NH_4, mol. wt. 191.05 Da), respectively. The presence of MNX and DNX during the study suggests that the RDX degradation occurred by single nitrite elimination pathway in which, the transfer of a single electron to nitramino group leads to the RDX ring cleavage. Furthermore, on the 10th day, the peaks observed were 140.66 and 179.97 (m/z), which were identified as Bis(hydroxymethyl)nitramine ($C_2H_6N_2O_4$, M+NH_4, mol. wt. 140.66 Da) and 4-Nitro-2,4-diazabutanal ($C_2H_5N_3O_3$, M+Na+K-H, mol. wt. 179.97 Da) respectively. Subsequent studies have shown that both 4-nitro-2,4-diazabutanal and Bis(hydoxymethyl)nitramine are the de-nitration ring cleavage products of RDX. Also, earlier studies have shown that, MNX can be transformed into 4-nitro-2,4-diazabutanal [48]. Further, Halasz and Hawari (2011) showed that DNX, 4-nitro-2,4-diazabutanal and Bis(hydoxymethyl)nitramine can undergo further degradation and form CO_2, nitrous oxide, formaldehyde, and ammonia as end products [49]. It was observed that the metabolites identified for both the microbes were similar. This shows that both the microbes follow the same degradation pathway. Based on the findings mentioned above, the RDX degradation pathway was proposed, which is shown in Figure 7.

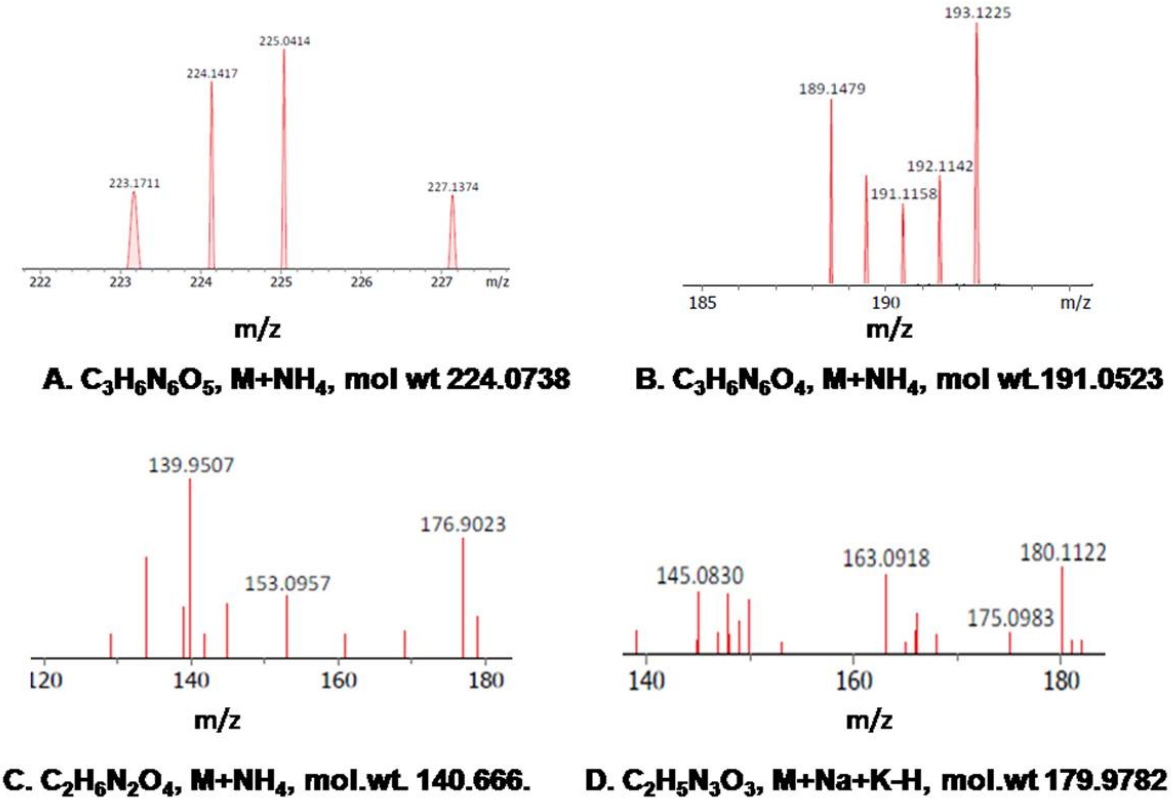


Figure 6. Mass Spectra of metabolites formed during RDX degradation. (**A**) Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX, $C_3H_6N_6O_5$, M+NH_4, mol. wt. 224.07), (**B**) Hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX, $C_3H_6N_6O_4$, M+NH_4, mol. wt. 191.05), (**C**) Bis(hydroxymethyl)nitramine ($C_2H_6N_2O_4$, M+NH_4, mol. wt. 140.66) and (**D**) 4-Nitro-2,4-diazabutanal ($C_2H_5N_3O_3$, M+Na+K-H, mol. wt. 179.97).

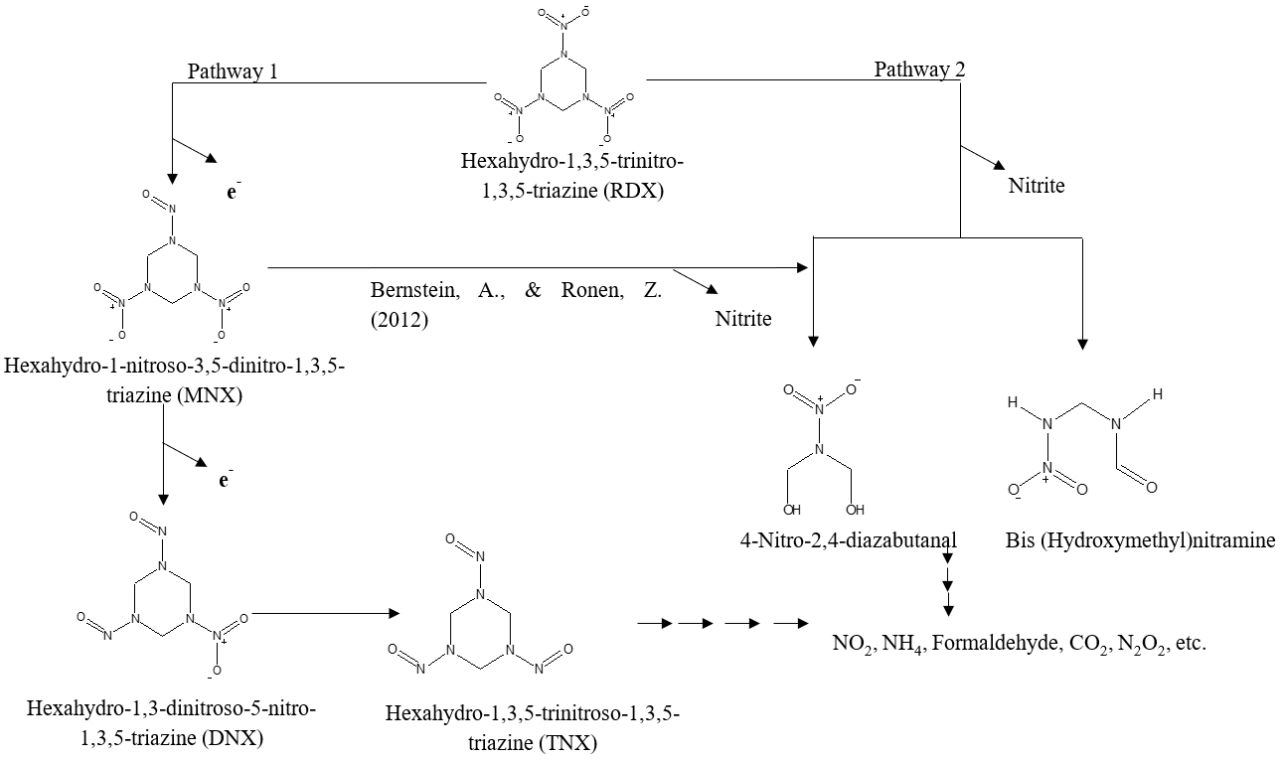


Figure 7. Proposed pathways for the biodegradation of RDX with *Bacillus toyonensis* and *Paenibacillus dendritiformis*.

4. Conclusions

In this study, the degradation of RDX was studied using two microbial species isolated from an explosive contaminated site. It was found that both the species can efficiently remove RDX from the contaminated water. The maximum degradation observed was 81.6 ± 1.3 with *Bacillus toyonensis* (isolate No. WS4-TSB-3, MTCC No. 12857) and 84.7 ± 0.9% with *Paenibacillus dendritiformis* (isolate No. S10-TSA-3, MTCC No. 12859) at the end of the 15th day at a concentration of 40 mg/L. The 3-D plot showed the optimization of process parameters for RDX degradation and the interaction between the independent variables. These plots showed that each variable has a direct, and positive, impact on the RDX degradation. The model obtained for RDX degradation with both the microbes showed that it has high reproducibility and is statistically significant. During the RDX degradation, MNX, DNX, 4-Nitro-2,4-diazabutanal, and Bis(hydroxymethyl)nitramine were identified as the intermediate metabolites. These metabolites on further degradation can be mineralized into CO_2, NH_4, formaldehyde and nitrous oxide. Further investigations related to the enzymes involved in RDX degradation are yet to be investigated. Pilot scale studies are required to be conducted for its field scale demonstration.

Author Contributions: A.M. designed, performed the experiments and used the models and wrote the draft of manuscript. P.S. and K.B. supervised with planning, designing, analyses, interpretation of data and reviewing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Funging agency, University Grant Commission (UGC), New Delhi.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All relevant data is included in the manuscript.

Acknowledgments: The authors acknowledge funding support in the form of fellowship provided to Arjun Meda by University Grant Commission (UGC), New Delhi. Funding organization has not played any role in study design, decision to publish or preparation of the manuscript. Authors are thankful to Director, Centre for Fire Explosive and Environmental Safety (CFEES), Defence Research and Development Organisation (DRDO), New Delhi and Director, Indian Institute of Technology Indore for encouraging research and providing necessary facilities. Authors acknowledge IMTECH, Chandigarh for Isolation and identification of microbial species.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sheremata, T.W.; Halasz, A.; Paquet, L.; Thiboutot, S.; Ampleman, G.; Hawari, J. The fate of the cyclic nitramine explosive RDX in natural soil. *Environ. Sci. Technol.* **2001**, *35*, 1037–1040. [CrossRef]
2. Chatterjee, S.; Deb, U.; Datta, S.; Walther, C.; Gupta, D.K. Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation. *Chemosphere* **2017**, *184*, 438–451. [CrossRef]
3. Sisco, E.; Najarro, M.; Bridge, C.; Aranda, I.V.R. Quantifying the degradation of TNT and RDX in a saline environment with and without UV-exposure. *Forensic Sci. Int.* **2015**, *251*, 124–131. [CrossRef] [PubMed]
4. Tucker, W.A.; Murphy, G.J.; Arenberg, E.D. Adsorption of RDX to soil with low organic carbon: Laboratory results, field observations, remedial implications. *Soil Sediment Contam.* **2002**, *11*, 809–826. [CrossRef]
5. Lapointe, M.C.; Martel, R.; Diaz, E. A conceptual model of fate and transport processes for RDX deposited to surface soils of North American active demolition sites. *J. Environ. Qual.* **2017**, *46*, 1444–1454. [CrossRef] [PubMed]
6. Environmental Protection Agency (EPA). *Office of Drinking Water Health Advisories, Drinking Water Health Advisory Table*; EPA 822-F-18–001; Environmental Protection Agency: Washington, DC, USA, 2018.
7. Environmental Protection Agency (EPA). *Office of Land and Emergency (5106P)*; EPA 505-F-17–008; Environmental Protection Agency: Washington, DC, USA, 2017.
8. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for RDX. 2012. Available online: www.atsdr.cdc.gov/toxprofiles/tp78.pdf (accessed on 5 February 2021).
9. Environmental Protection Agency (EPA). *Handbook on the Management of Munitions Response Actions*; EPA 505-B-01–001; Environmental Protection Agency: Washington, DC, USA, 2005. Available online: Nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P100304J.txt (accessed on 5 February 2021).
10. Hazardous Substance Data Bank (HSDB). 2016. Available online: https://www.nlm.nih.gov/databases/download/hsdb.html (accessed on 5 February 2021).
11. Gust, K.A.; Wilbanks, M.S.; Guan, X.; Pirooznia, M.; Habib, T.; Yoo, L.; Wintz, H.; Vulpe, C.D.; Perkins, E.J. Investigations of transcript expression in fathead minnow (*Pimephales promelas*) brain tissue reveal toxicological impacts of RDX exposure. *Aquat. Toxicol.* **2011**, *101*, 135–145. [CrossRef] [PubMed]
12. Quinn, M.J., Jr.; Bazar, M.A.; McFarland, C.A.; Perkins, E.J.; Gust, K.A.; Johnson, M.S. Sub lethal effects of sub-acute exposure to RDX (1,3,5-trinitro-1,3,5-triazine) in the northern bob white (*Colinus virginianus*). *Environ. Toxicol. Chem. Int. J.* **2009**, *28*, 1266–1270. [CrossRef]
13. Williams, L.R.; Wong, K.; Stewart, A.; Suciu, C.; Gaikwad, S.; Wu, N.; DiLeo, J.; Grossman, L.; Cachat, J.; Hart, P.; et al. Behavioral and physiological effects of RDX on adult zebrafish. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2012**, *155*, 33–38. [CrossRef]
14. Zhang, B.; Pan, X. RDX induces aberrant expression of microRNAs in mouse brain and liver. *Environ. Health Perspect.* **2009**, *117*, 231–240. [CrossRef]
15. Lee, S.H.; Jeong, W.Y.; Nyande, B.W.; Park, J.S.; Moon, I.; Oh, M. CFD simulation for demilitarization of RDX in a rotary kiln by thermal decomposition. *J. Eng. Sci. Technol.* **2017**, *12*, 1662–1676.
16. Bulusu, S.; Behrens, R., Jr. A Review of the Thermal Decomposition Pathways in RDX, HMX and Other Closely Related Cyclic Nitramines. *Def. Sci. J.* **1996**, *46*, 347. [CrossRef]
17. Mahbub, P.; Nesterenko, P.N. Application of photo degradation for remediation of cyclic nitramine and nitroaromatic explosives. *RSC Adv.* **2016**, *6*, 77603–77621. [CrossRef]
18. Wildman, M.J.; Alvarez, P.J. RDX degradation using an integrated Fe(0)-microbial treatment approach. *Water Sci. Technol.* **2001**, *43*, 25–33. [CrossRef]
19. Zhao, J.S.; Halasz, A.; Paquet, L.; Beaulieu, C.; Hawari, J. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine and its mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine by *Klebsiella pneumoniae* strain SCZ-1 isolated from an anaerobic sludge. *Appl. Environ. Microbiol.* **2002**, *68*, 5336–5341. [CrossRef]
20. Stahl, J.D.; Aken, B.V.; Cameron, M.D.; Aust, S.D. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) biodegradation in liquid and solid-state matrices by *Phanerochaete Chrysosporium*. *Bioremed. J.* **2001**, *5*, 13–25. [CrossRef]

21. Fournier, D.; Halasz, A.; Spain, J.; Spanggord, R.J.; Bottaro, J.C.; Hawari, J. Biodegradation of the hexahydro-1,3,5-trinitro-1,3,5-triazine ring cleavage product 4-nitro-2,4-diazabutanal by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **2004**, *70*, 1123–1128. [CrossRef] [PubMed]
22. Zhao, J.S.; Paquet, L.; Halasz, A.; Hawari, J. Metabolism of hexahydro-1,3,5-trinitro-1,3,5-triazine through initial reduction to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine followed by denitration in *Clostridium bifermentans HAW-1*. *Appl. Microbiol. Biotechnol.* **2003**, *63*, 187–193. [CrossRef]
23. Makela, M. Experimental design and response surface methodology in energy applications: A tutorial review. *Energy Convers. Manag.* **2017**, *151*, 630–640. [CrossRef]
24. Bezerra, M.A.; Santelli, R.E.; Oliveira, E.P.; Villar, L.S.; Escaleira, L.A. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta* **2008**, *76*, 965–977. [CrossRef]
25. Myers, R.H.; Montgomery, D.C.; Anderson-Cook, C.M. *Response Surface Methodology*; John Wiley & Sons: Hoboken, NJ, USA, 2002.
26. Draper, N.R.; John, J.A. Response-surface designs for quantitative and qualitative variables. *Technometrics* **1998**, *30*, 423–428. [CrossRef]
27. Sharma, K.; Sharma, P.; Celin, S.M.; Rai, P.K.; Sangwan, P. Degradation of high energetic material hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by a microbial consortium using response surface methodological approach. *SN Appl. Sci.* **2021**, *3*, 1–8. [CrossRef]
28. Meda, A.; Sangwan, P.; Bala, K. Optimization of process parameters for degradation of HMX with *Bacillus toyonensis* using response surface methodology. *Int. J. Environ. Sci. Technol.* **2020**, *17*, 4601–4610. [CrossRef]
29. Sangwan, P.; Mary, C.S.; Hooda, L. Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using *Acinetobacter noscomialis*. *Eur. J. Adv. Eng. Technol.* **2015**, *2*, 51–56.
30. Kiran, B.; Kaushik, A.; Kaushik, C.P. Response surface methodological approach for optimizing removal of Cr (VI) from aqueous solution using immobilized cyanobacterium. *Chem. Eng. J.* **2007**, *126*, 147–153. [CrossRef]
31. Cook, A.M.; Huetter, R. s-Triazines as nitrogen sources for bacteria. *J. Agric. Food Chem.* **1981**, *29*, 1135–1143. [CrossRef]
32. Lamba, J.; Anand, S.; Dutta, J.; Chatterjee, S.; Nagar, S.; Celin, S.M.; Rai, P.K. Study on aerobic degradation of 2,4,6-trinitrotoluene (TNT) using *Pseudarthrobacter chlorophenolicus* collected from the contaminated site. *Environ. Monit. Assess.* **2021**, *193*, 1–11. [CrossRef] [PubMed]
33. Nagar, S.; Shaw, A.K.; Anand, S.; Celin, S.M.; Rai, P.K. Aerobic biodegradation of HMX by *Planomicrobium flavidum*. *3 Biotech* **2018**, *3*, 455. [CrossRef] [PubMed]
34. Environmental Protection Agency (EPA). *Validated Method 8330A: Nitroaromatics, Nitramines, and Nitrate Esters by High Performance Liquid Chromatography (HPLC)*; Environmental Protection Agency: Washington, DC, USA, 2007.
35. Mercimek, H.A.; Dincer, S.; Guzeldag, G.; Ozsavli, A.; Matyar, F. Aerobic biodegradation of 2,4,6-trinitrotoluene (TNT) by Bacillus cereus isolated from contaminated soil. *Microb. Ecol.* **2013**, *66*, 512–521. [CrossRef]
36. Fuller, M.E.; Perreault, N.; Hawari, J. Microaerophilic degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by three *Rhodococcus* strains. *Lett. Appl. Microbiol.* **2010**, *51*, 313–318. [CrossRef] [PubMed]
37. Nagar, S.; Shaw, A.K.; Anand, S.; Celin, S.M.; Rai, P.K. Biodegradation of octogen and hexogen by *Pelomonas aquatica* strain WS2-R2A-65 under aerobic condition. *Environ. Technol.* **2020**, 1–10. [CrossRef]
38. Mohanty, S.S.; Jena, H.M. Process optimization of butachlor bioremediation by *Enterobacter cloacae* using Plackett Burman design and response surface methodology. *Process Saf. Environ. Prot.* **2018**, *119*, 198–206. [CrossRef]
39. Fournier, D.; Halasz, A.; Spain, J.; Fiurasek, P.; Hawari, J. Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with *Rhodococcus* sp. strain DN22. *Appl. Environ. Microbiol.* **2002**, *68*, 166–172. [CrossRef] [PubMed]
40. Singh, R.; Soni, P.; Kumar, P.; Purohit, S.; Singh, A. Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria. *World J. Microbiol. Biotechnol.* **2009**, *25*, 269–275. [CrossRef]
41. Zoh, K.D.; Stenstrom, M.K. Fenton oxidation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). *Water Res.* **2002**, *36*, 1331–1341. [CrossRef]
42. Chaudhary, P.; Beniwal, V.; Kaur, R.; Kumar, R.; Kumar, A.; Chhokar, V. Efficacy of Aspergillus fumigatus MCC 1175 for bioremediation of Tannery Wastewater. *Clean Soil Air Water* **2019**, *47*, 1900131. [CrossRef]
43. Garg, S.K.; Tripathi, M.; Lal, N. Response surface methodology for optimization of process variable for reactive orange 4 dye discoloration by *Pseudomonas putida* SKG-1 strain and bioreactor trial for its possible use in large-scale bioremediation. *Desalin. Water Treat.* **2015**, *54*, 3122–3133. [CrossRef]
44. Indest, K.J.; Jung, C.M.; Chen, H.P.; Hancock, D.; Florizone, C.; Eltis, L.D.; Crocker, F.H. Functional characterization of pGKT2, a 182-kilobase plasmid containing the xpl A B gene, which are involved in the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* sp. strain KTR9. *Appl. Environ. Microbiol.* **2010**, *76*, 6329–6337. [CrossRef] [PubMed]
45. Bhatt, M.; Zhao, J.S.; Halasz, A.; Hawari, J. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 850. [CrossRef] [PubMed]
46. Sheremata, T.W.; Hawari, J. Mineralization of RDX by the white rot fungus *Phanerochaete chrysosporium* to carbon dioxide and nitrous oxide. *Environ. Sci. Technol.* **2000**, *34*, 3384–3388. [CrossRef]

47. Thompson, K.T.; Crocker, F.H.; Fredrickson, H.L. Mineralization of the cyclic nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine by *Gordonia* and *Williamsia* spp. *Appl. Environ. Microbiol.* **2005**, *71*, 8265–8272. [CrossRef]
48. Bernstein, A.; Ronen, Z. Biodegradation of the explosives TNT, RDX and HMX. In *Microbial Degradation of Xenobiotics*; Springer: Berlin/Heidelberg, Germany, 2012; pp. 135–176.
49. Halasz, A.; Hawari, J. Degradation routes of RDX in various redox systems. In *Aquatic Redox Chemistry, American Chemical Society*; ACS Publications: Washington, DC, USA, 2011; pp. 441–462.

International Journal of Environmental Science and Technology (2020) 17:4601–4610
https://doi.org/10.1007/s13762-020-02783-0

ORIGINAL PAPER

Optimization of process parameters for degradation of HMX with *Bacillus toyonensis* using response surface methodology

A. Meda[1] · **P. Sangwan**[2] · **K. Bala**[1]

Received: 9 September 2019 / Revised: 22 May 2020 / Accepted: 26 May 2020 / Published online: 9 June 2020

Abstract

Contamination of soil and water with explosive compounds like octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX or commonly known as high melting explosives) is increasing day by day due to their extensive use all over the world. High level of contamination has been found near military sites, explosive manufacturing facilities, war-lands, mines and exercise ranges. Remediation of such contaminants is necessary as they may have adverse impact on biotic as well as on abiotic environment. Present study was carried out with an objective to optimize the variable process parameters for the degradation of HMX in aqueous phase by indigenously isolated bacterial strain, *Bacillus toyonensis* from an actual HMX contaminated site in North India using response surface methodology. The relationship among varying initial concentrations of HMX, microbial inoculum size and degradation time was revealed in the current study. Results showed that 87.7% degradation was achieved at 2 mg/L initial HMX concentration with inoculum size of 4% on 15th day. High regression coefficient value (0.9878) further supported predictability of experimental data. Nitrite and nitrate concentrations estimated during the experiment indicate breakdown and degradation process of HMX. Findings of this study concluded that *Bacillus toyonensis* can be a potential microorganism to degrade HMX and can be used for microbial remediation of HMX contaminated sites.

Keywords Explosive · HMX · *Bacillus toyonensis* · Microbial remediation · Response surface methodology

Introduction

Octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX, high melting explosive) is a heterocyclic nitramine, which is commonly used for military applications. It can enter the environment during the process of manufacturing, transport, usage and demilitarization. It is an important part of military applications and is classified as non-carcinogenic by US Environmental Protection agency (1998). However, researchers revealed its toxicity effects on laboratory animals (Cuthbert et al. 1985; Greenhough and McDonald 1985). Further exposure of HMX in different mammalian species can cause convulsions, nausea and loss of consciousness (Wilson 1985). Many animal studies have indicated that HMX affects liver and central nervous system and can get accumulated in kidney, liver, brain and heart, even can cause mortality and histopathalogical lesions in animals (Johnson and Reddy 2015). Fate and transport of explosives have been described by earlier researchers in detail (Lotufo et al. 2009). HMX has shown low adsorption and high mobility in soil, which can lead to ground water contamination (Zheng et al. 2009). It can lead to reduction in microbial diversity of soil resulting in reduced natural degradation potential of such contaminants (Gong et al. 2002), thus can also adversely affect the soil profile and suitability for farming in the areas near military bases. US Environmental Protection Agency (USEPA 2018) has recommended the lifetime safe drinking water standards for HMX as 0.4 mg/L.

Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) is known to be less stable than Tri nitro toluene (Hawari et al. 2000). Harkins et al. (1999) treated HMX in water

Editorial responsibility: Jing Chen.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13762-020-02783-0) contains supplementary material, which is available to authorized users.

✉ P. Sangwan
pritam@cfees.drdo.in

[1] Biofuels Research Laboratory, Discipline of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore, Indore, India

[2] Centre for Fire, Explosive and Environment Safety, Defence Research and Development Organization, New Delhi, India

under aerobic conditions utilizing livestock manure and indigenously isolated microbes and revealed formation of five nitroso derivatives. *Phanerochaete chrysosporium* is found to degrade HMX (600 nmol) within 25 days of incubation (Fournier et al. 2004). Different methods have been proposed to treat HMX contamination of soil and water. Common treatment methods employed for the removal of HMX include iron-dependent depletion (Kim et al. 2007), catalytic transformation (Fuller et al. 2007), incineration and oxidation (Pichtel 2012). However conventional treatment techniques are not cost-effective, consume large amount of energy and also give rise to harmful end products like non- reusable catalysts, effluents and ash as residual waste (Zoh and Stenstrom 2002; Chatterjee et al. 2017). Microbial degradation of explosives, an eco-friendly and cost-effective approach, has been studied earlier by many researchers (Kalafut et al. 1998; Adrian and Arnett 2004; Sangwan et al. 2015). Nagar et al. (2018) studied the degradation of HMX by *planomicrobium flavidum* under aerobic conditions. Similarly Kalafut et al. (1998) studied the degradation of TNT by three different microbial species (*Pseudomonas aeruginosa*, *Bacillus* sp., and *Staphylococcus* sp.) but there are no reports on degradation of HMX using *Bacillus toyonensis* till date to the best of our knowledge. Additionally, the process of degradation is species-specific and needs to be optimized at various steps to upscale it.

Optimization of different process parameters can be done using response surface methodology (RSM). RSM is a statistical tool, which uses lower-order polynomial equations for developing, improving and optimizing a process having numerous variables influencing the response. RSM reduces the total number of combinations, which saves time and resources during the experimentation (Makela et al. 2017; Bezerra et al. 2008; Myers et al. 2002; Draper and John 1988). In the current study, Box–Behnken design (BBD) model was used, which is a multivariate mathematical model for 3-level factorial design. This model has also been used by researchers to optimize the removal of chromium metal ions, using cyanobacterium species isolated from metal contaminated site (Kiran et al. 2007). Sangwan et al. (2015) studied degradation relation of TNT with pH, concentration of contaminant and degradation time as process variables using RSM.

Current study was taken up with an objective to optimize process parameters involved in the degradation of HMX with *Bacillus toyonensis,* which was isolated from an actual HMX contaminated site. The degradation potential of HMX with *Bacillus toyonensis* has not yet been reported. This is the first study of this new bacterial isolate to explore the HMX degradability to the best of our knowledge. DESIGN-EXPERT® VERSION 12 software (Stat-Ease®, Minneapolis) was used to design the experiment and analyze the data. Relationships between different independent and dependent variables were explored during the microbial degradation of HMX to understand the interactive influence.

Materials and methods

Chemicals

Commercial grade HMX was obtained from an explosive manufacturing facility in North India with a purity of greater than 99.5%. High performance liquid chromatography (HPLC) grade chemicals were purchased from Sigma-Aldrich, and other analytical grade chemicals were also purchased from standard manufacturers.

Microbial culture

The water samples were collected from an explosive manufacturing facility in North India to isolate the indigenenous, more tolerant and better adapted microbes. Sampling was carried out by the standard protocol using grab sampling methodology. Samples were stored in dark at a temperature of 4 °C (USEPA 2006). Isolation and identification of bacterial isolate *Bacillus toyonensis* (MTCC No. 12857) was carried out by Institute of Microbial Technology (IMTECH), Chandigarh, India. It was obtained in lyophilized form for further research work. Lyophilized *Bacillus toyonensis* was revived in tryptic soya broth (TSB), and composition of Media is shown in Table 1. Cultures were maintained on slants at a temperature of 4 °C prior to use. Microbes were sub-cultured for three generations in minimal salt media (MSM), which was deficient in nitrogen source to make them more tolerant and adaptive to HMX before culturing into modified MSM containing HMX. The detailed composition of MSM is given in Table 2. Due to low solubility in water, a stock solution of (1000 mg/L) HMX was prepared by dissolving it in acetonitrile. The serial dilutions were made initially with acetonitrile till 10 mg/L, afterward the solution was diluted with MSM media. MSM was spiked with desired concentration of HMX from stock solution (1000 mg/L) and left open for 18 h in aseptic conditions of laminar air flow chamber to get it (acetonitrile) evaporated and avoiding any interference with media composition (Nagar et al. 2018; USEPA 2007).

Table 1 Composition of tryptic soya broth (TSB) (MacFaddin 1985)

Compound	g/1000 mL
Casein peptone	17.0 g
Soya peptone	3.0 g
Sodium chloride	5.0 g
Potassium phosphate dibasic	2.48 g
Dextrose	2.48 g

Table 2 Composition of minimal salt media (Cook and Huetter 1981)

Compound	g/1000 mL
Potassium phosphate dibasic (K_2HPO_4)	1.74 g
Potassium phosphate monobasic (KH_2PO_4)	1.44 g
Magnesium sulfate ($MgSO_4{\cdot}7H_2O$)	0.06 g
Glycerol	0.92 g
Glucose	0.90 g
Succinic acid	0.58 g
Ammonium chloride	0.05 g
Trace elements [$CaCl_2$ (2.7 mg), H_2BO_4 (6.18 mg), $CuSO_4{\cdot}5H_2O$ (0.625 mg), $MnCl_2{\cdot}4H_2O$ (3.95 mg), $ZnSO_4{\cdot}7H_2O$ (1.43 mg), $FeSO_4$ (69.5 mg), $CoCL_2{\cdot}6H_2O$ (1.78 mg) in 250 ml of distilled water]	1 mL

Growth was monitored as optical density (O.D) at 600 nm using UV–Visible spectrophotometer (Perkin Elmer, Model Lambda 650S) at fixed intervals of time.

Experimental setup

Total 17 combinations were operated during experiment as shown in Table 3. Each combinations have different concentrations of HMX (2–6 mg/L), time period (5–15 days) and inoculum size (2–6%). All these combinations were designed using RSM and were prepared in triplicates. Box Behnken Design (BBD) was used as a second-order polynomial model for designing the experiment and to statistically validate the data. Number of experiments required for BBD is defined by

$$N = k(k-1) + C_o$$

where C_o is central point and k is number of factors. Table 3 shows the selected factors, which affect the process of degradation such as time, size of inoculation and initial concentration of HMX from the whole range of each variable. Third-generation microbial culture of *Bacillus toyonensis* having OD 1.2 ± 0.2 corresponding to $\approx 10^8$ cells/mL was inoculated in the freshly prepared MSM spiked with varying concentrations of HMX. After inoculation as per the combinations given in Table 3, flasks were incubated in orbital shaker at a temperature of 32 ± 3 °C and speed 120 rpm. The whole process was performed under aerobic conditions. Samples were withdrawn at a fixed interval of time (5 days) to check OD (optical density), nitrate, nitrite concentration and degradation of HMX.

Analysis

Analysis of samples for degradation of HMX was carried out as per standard method 8330A (USEPA 2007) using high-performance liquid chromatography (HPLC). 5 mL

Table 3 Different runs for optimization of parameters for HMX degradation and responses

A: concentration (mg/L)	B: volume of microbial suspension (%)	C: time (day)	Degradation (%)	Nitrite release (mg/L)	Nitrate release (mg/L)
4	4	10	57.0 ± 1.89	1.2 ± 0.14	1.1 ± 0.15
4	2	5	54.3 ± 3.19	0.58 ± 0.05	0.2 ± 0.02
4	4	10	58.2 ± 1.89	1.01 ± 0.14	1.3 ± 0.15
6	2	10	70.7 ± 2.61	1.23 ± 0.13	1.3 ± 0.23
2	2	10	72.3 ± 2.60	0.43 ± 0.18	0.35 ± 0.01
4	6	15	79.5 ± 3.76	0.73 ± 0.25	1.15 ± 0.16
4	4	10	55.7 ± 1.89	1.3 ± 0.14	1.2 ± 0.17
6	6	10	72.6 ± 3.53	1.06 ± 0.12	1.4 ± 0.22
2	4	5	61.2 ± 1.47	0.4 ± 0.143	0.2 ± 0.22
6	4	15	74.0 ± 7.39	1.18 ± 0.22	1.51 ± 0.31
4	6	5	58.2 ± 5.30	0.38 ± 0.034	0.34 ± 0.03
6	4	5	68.1 ± 7.70	0.44 ± 0.14	0.49 ± 0.04
2	4	15	87.6 ± 4.46	0.12 ± 0.01	0.36 ± 0.02
4	4	10	57.2 ± 1.89	1.35 ± 0.14	1.2 ± 0.15
4	4	10	58.2 ± 1.89	1.0 ± 0.18	1.1 ± 0.15
2	6	10	77.6 ± 1.75	0.73 ± 0.14	0.43 ± 0.08
4	2	15	65.5 ± 8.47	0.68 ± 0.13	1.11 ± 0.28

of sample was withdrawn and mixed with 5 mL acetonitrile, centrifuged at 10,000 rpm for 15 min and supernatant obtained was filtered through 0.45 µm Teflon filter. This filtrate was fed in HPLC (Flexer, Perkin Elmer) equipped with photodiode array (PDA) detector and injection volume was 10 µl. C-18 reverse phase column (3 µm, 150×4.6 mm) was used as stationary phase, and acetonitrile: water (50:50) mixture was used as mobile phase with a flow rate of 1 mL/min. The mobile phase was prepared with triple distilled water from Millipore, and HPLC grade acetonitrile was used in this study. Retention time and UV-profile of the standard compounds were used for the identification of peak. Nitrate and nitrite in the samples were analyzed using Hach spectrophotometer (DR1900) at a wavelength of 270 nm and 540 nm using NitraVer 5_8192 LR powder pillow and NitriVer 3_8507 DR900 powder pillow, respectively, by cadmium reduction method.

Results and discussion

Growth of *Bacillus toyonensis* in MSM spiked with HMX

Growth of microbes was monitored by measuring optical density at different time intervals. Figure 1 shows O.D. of *Bacillus toyonensis* at varying HMX concentration and inoculum size with different intervals of time. It is clear from Fig. 1a–c that initially microbes were in lag phase till 2nd day for all the combinations. After 2 days, microbes attained exponential phase showing acclimatization to the presence of HMX and may be started utilizing it as a nitrogen source. *Bacillus toyonensis* at concentrations, 2 and 4 mg/L showed continuous growth till 15th day while at 6 mg/L microbes attained stationary phase after 7th day. Maximum optical density (0.239) was observed at 2 mg/L HMX concentration with 6% of inoculum size on 15th day, which is nearly double as compared to the lowest growth observed in 6 mg/L HMX concentration with 2% inoculum on 15th day. Higher growth of microbes can be correlated with the degradation of HMX, whereas lower growth can be explained by the toxic effect of HMX at 6 mg/L concentration. It was also observed that the initial inoculum size tends to affect the growth of *Bacillus toyonensis* in the medium. When concentration of HMX was kept constant with varying initial inoculum size, there was a gradual change in the optical density on 15th day and nearly 20% higher growth of microbes was observed with 6% inoculum size (0.239) as compared to 2% (0.198). The higher growth of microbes can be correlated with higher degradation of HMX in the medium, which shows their interdependency on each other.

Degradation of HMX

Degradation in the concentration of HMX during the experiment is shown in Fig. 2. A maximum 87.7% reduction was achieved at 2 mg/L initial HMX concentration with 4% inoculum size on 15th day. It was followed by 79.5% and 74.1%, respectively, with 6 mg/l and 4 mg/l initial concentration of HMX on 15th day. In Fig. 2, the 3-D model plot shows the interactive effect of different selected variables on HMX removal. Range of responses can be estimated and maximized by analyzing the plot and tracking the efficiency of optimum value for variable. From Fig. 2, it was observed that, when time factor was kept constant (15th day) change in HMX removal can be observed with changing cell inoculum size. It was also observed that on increasing the initial inoculum size, there was an increase in percent degradation of HMX. Degradation of 65.5 and 79.5% was observed with 2 and 6% inoculation size, respectively, on 15th day at 4 mg/L initial HMX concentration. Similarly, Fig. 3 shows increase in HMX removal with the increase in time period at constant microbial inoculum size. Degradation of 58.2 and 79.5% was observed on 5th and 15th day, respectively, with 4 mg/L concentration and 6% volume of microbial suspension. These observations made from both Figs. 2 and 3 support the fact that on varying time as well as initial volume of suspension, there was effect on HMX removal efficiency, i.e., with increasing time of exposure and initial microbial inoculum, there was increase in the removal of HMX with *Bacillus toyonensis.* In a similar study by An et al. (2010), 96.42% reduction in the concentration of HMX with the use of mesophillic anaerobic microbial granules is reported. Similarly, under denitrifying conditions Singh et al. (2009) showed that *Pseudomonas* species can efficiently remove HMX from the medium.

Table 4 shows the analysis of variance for the obtained experimental data. The P value (<0.0001), R^2 value (0.9878) and F-value (62.97) for degradation of HMX also suggested that the obtained data were accurate and significant. Similar findings have also been reported in previous studies during the optimization of different process parameters (Garg et al. 2015). Adjusted R^2 (0.9721) explains total variation in degradation of HMX with different variables and shows its agreement with predicted R^2. Figure 4 shows the predicted versus actual values plot, which explains the dispersion of data for % removal of HMX. Variations between the predicted values were measured by functions used for the model and actual values determined by actual experimentation (Mohanty and Jena 2018). Small value of standard deviation for the model (1.65) explains a better relationship between different variables and response. All the parameters were fitted for second-order polynomial equation as follows-

$$Y = 56.63 - 1.67A + 3.14B + 8.09C - 0.8646AB - 5.13AC + 2.52BC + 12.55A^2 + 4.18B^2 + 3.61C^2 \quad (1)$$

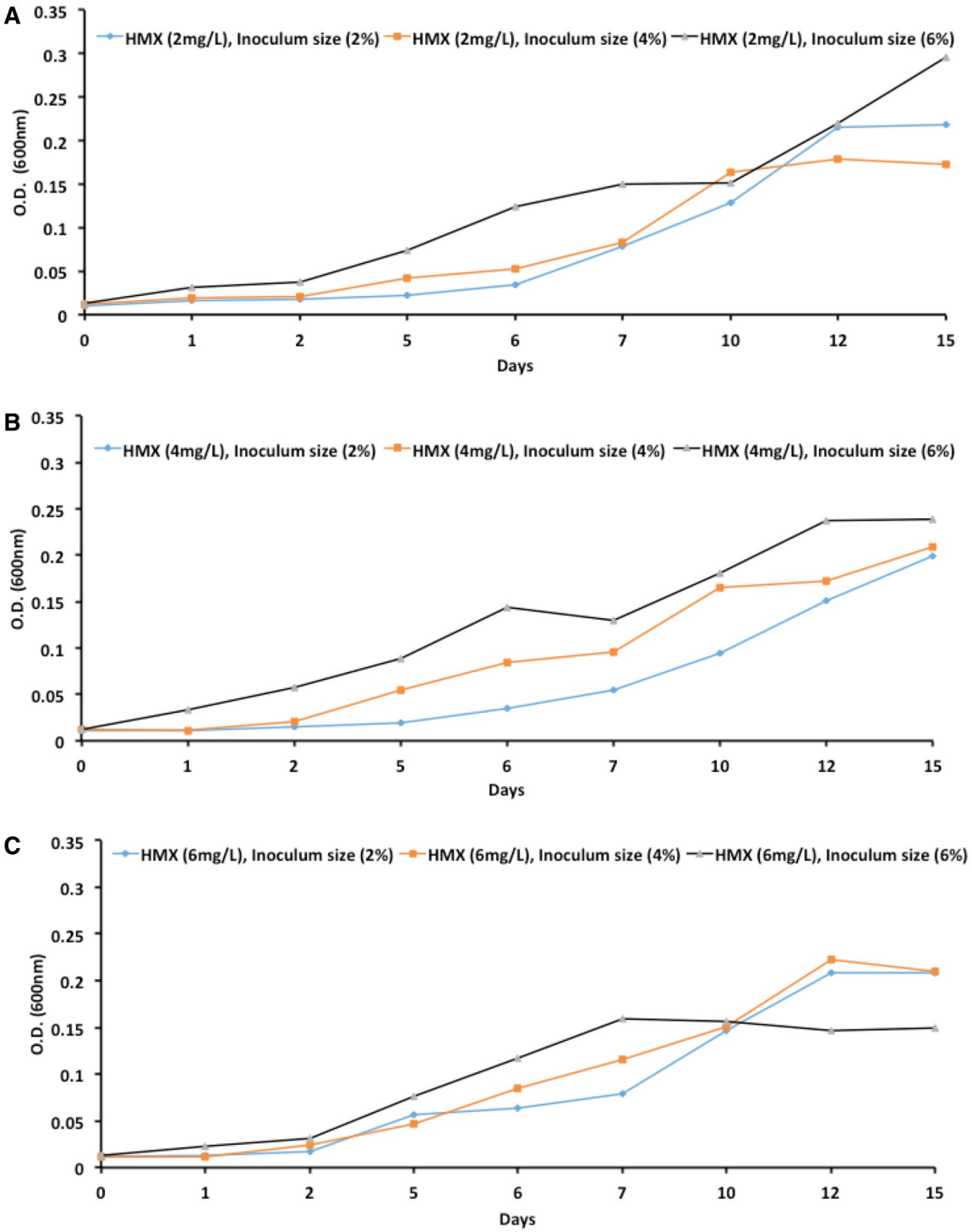


Fig. 1 Growth of *Bacillus toyonensis* with varying inoculum size (2–6%) at initial HMX concentration of 2 mg/L (**a**), 4 mg/L (**b**), 6 mg/L (**c**)

where Y is % degradation, A, B and C are coded values for initial concentration of HMX, volume of inoculum size and time of degradation, respectively.

Release of nitrite in different treatments

Increased nitrite ion levels in the medium can be further correlated with degradation of HMX. It is well confirmed in the

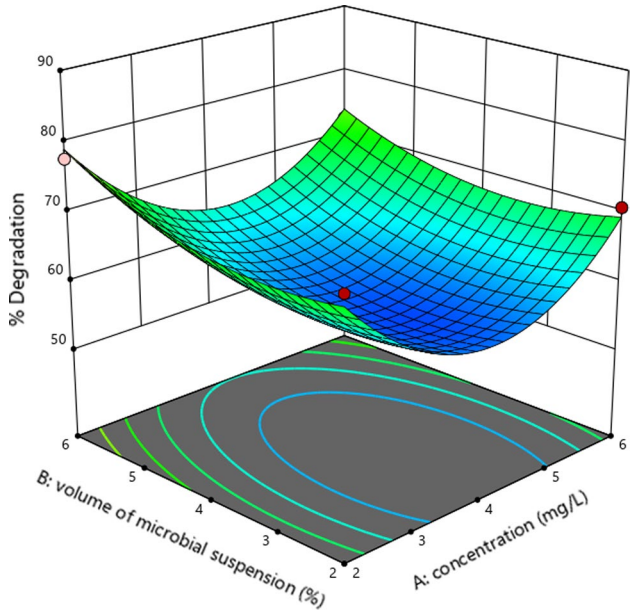


Fig. 2 Percent degradation of HMX with varying initial HMX concentration (mg/L) and microbial suspension volume (%)

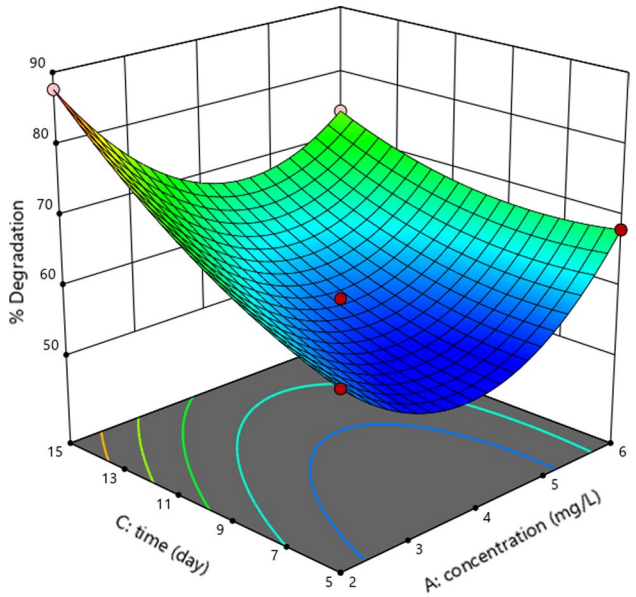


Fig. 3 Percent degradation of HMX with varying initial HMX concentration (mg/L) and time (days)

literature that during HMX degradation, nitrite is released as a by-product (Nagar et al. 2018; Bhushan et al. 2003) and can breakdown into methylenedinitramine (MEDINA) (Nagar et al. 2018; Zhao et al. 2007). Previous studies have also shown than microbes utilize HMX as a carbon and nitrogen source and produce various products such as formaldehyde, CO_2, 4-nitro-2,4-diazabutanal and nitrous oxide (N_2O) (Bernstein 2012; Fournier et al. 2004; Thompson et al. 2005).

Figure 5 shows that at constant inoculum size (4%), concentration of nitrite in solution increased with increase in initial HMX concentration from 5th day (0.38 mg/L) to 10th day (1.3 mg/L) and then decreased down to 0.73 mg/L at 15th day which may be due to its transformation into nitrate (Zoh and Stenstrom 2002) or utilization of nitrite by microbes. Nitrite release was found maximum at 4 mg/L (1.3 mg/L) and minimum at 2 mg/L (0.43 mg/L) concentration, which shows the effect of initial concentration of HMX on the nitrite release. It has been already established that during the degradation of HMX, there is hydrolytic and hydroxylation of the rings, which leads to the sequential release of nitrite (Nagar et al. 2018; Fournier et al. 2004; Crocker et al. 2006). During HMX degradation, nitrite concentration found to be varying with different independent variables in the current study.

Fitting the experimentally obtained data in Box–Behnken design indicated that quadratic model was significantly applicable to show the relationship between different independent variables to their response. Analysis of variance is shown in Table 5, which shows the statistical significance of variables and their interactions with response in terms of high regression values ($R^2 = 0.9566$) (Table 6), F-value (17.15) and low P-value (0.0006). P value < 0.05 for A, C, AC, A^2, C^2 concludes them as significant model terms. These model terms have positive relationship with nitrite production during HMX degradation, while other terms showed negative impact but not significant. Correction total (sum of squares = 2.28) obtained from the model showed high reproducibility of the data and less variation around mean of observations whereas residual value (0.0988) showed the variations, which were still unexplained from the model. From this statistically significant data, it was observed that model can be applied for the optimization of nitrite release during HMX degradation. Similar results were obtained by earlier researchers during the optimization of different process parameters (Chaudhary et al. 2019). All the parameters were fitted for second-order polynomial equation as follows

$$\begin{aligned} Y = {} & 1.16 + 0.2787A - 0.0025B + 0.1137C \\ & - 0.1175AB + 0.2550AC + 0.0625BC \\ & - 0.1785A^2 - 0.1210B^2 - 0.4485C^2 \end{aligned} \quad (2)$$

where Y is % nitrite release; A, B and C are coded values for initial concentration of HMX, volume of microbial suspension and time of degradation, respectively.

Conversion of nitrite to nitrate

During the process of HMX degradation, nitrite gets converted into nitrate with help of different microbial enzymes through oxidation process (Zoh and Stenstrom 2002;

Table 4 ANOVA of quadratic model for % degradation of HMX

Source	Sum of squares	Df	Mean square	*F*-value	*P*-value	
Model	1545.03	9	171.67	62.97	< 0.0001	Significant
A-concentration	22.27	1	22.27	8.17	0.0244	
B-volume of microbial suspension	78.91	1	78.91	28.94	0.0010	
C-time	523.62	1	523.62	192.05	< 0.0001	
AB	2.99	1	2.99	1.10	0.3298	
AC	105.35	1	105.35	38.64	0.0004	
BC	25.42	1	25.42	9.32	0.0185	
A^2	628.02	1	628.02	230.34	< 0.0001	
B^2	62.24	1	62.24	22.83	0.0020	
C^2	45.07	1	45.07	16.53	0.0048	
Residual	19.08	7	2.73			
Lack of fit	14.78	3	4.93	4.58	0.0877	Not significant
Pure error	4.30	4	1.07			
Cor total	1564.12	16				

df degree of freedom

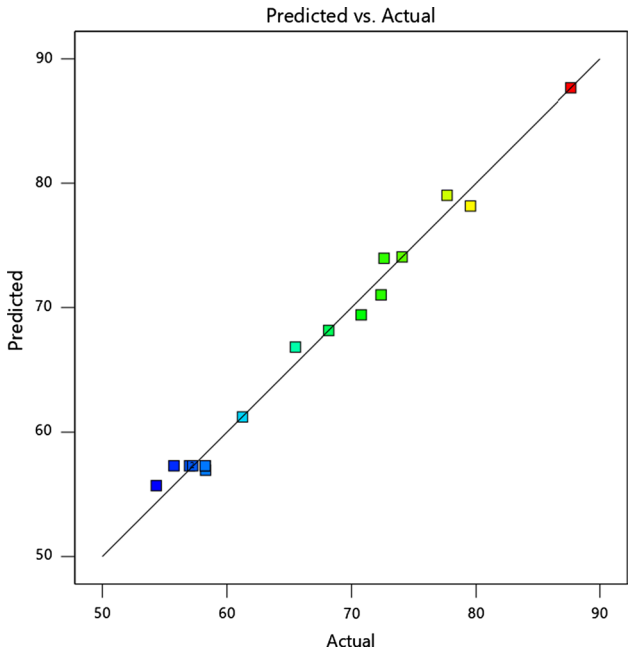


Fig. 4 Actual versus predicted graph for % HMX degradation

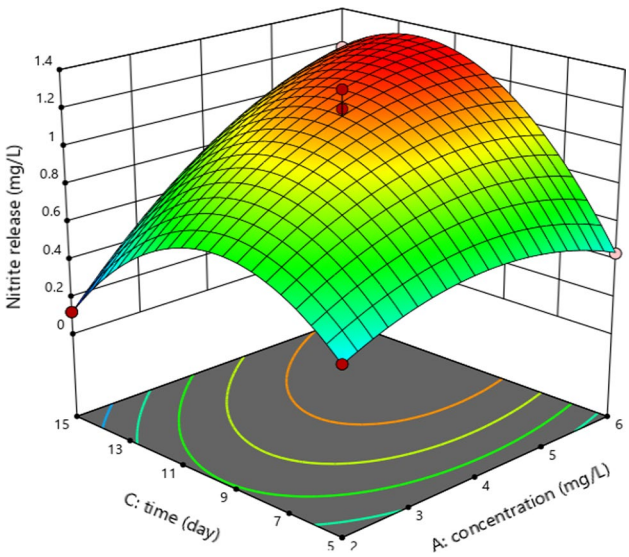


Fig. 5 Nitrite release (mg/L) with varying initial HMX concentration (mg/L) and time (days)

Williams et al. 2001; Singh et al. 2009). Effect of different variables (inoculum size, exposure time and initial HMX concentration) on nitrate production during HMX transformation is shown with model graphs (Figs. 6 and 7). In Fig. 7, it is showed that at constant volume of inoculum size with increasing time of degradation and increased concentration of HMX, there is an increase in nitrate release. Nitrate concentration increased from 0.34 to 1.15 mg/L from 5th to 15th day at 4 mg/L HMX concentration with 6% inoculum size. The maximum nitrate was observed at 15th day (1.51 mg/L) with 6 mg/L HMX initial concentration.

The significance of variables and their interaction with response at different probability levels is explained by ANOVA. Table 7 shows that after fitting the data with the model, a quadratic model with significant *F*-value (28.97) and *P*-value (0.0001) was obtained. The terms of the model are explained on the basis of *P*-value, which should be less than 0.05 for the model. The significant model terms having less *P*-value (0.05) are *A*, *C*, *AC*, A^2 and C^2, which have positive effect with the variable–variable interaction. Correction total (sum of squares = 3.57) explains that the variation

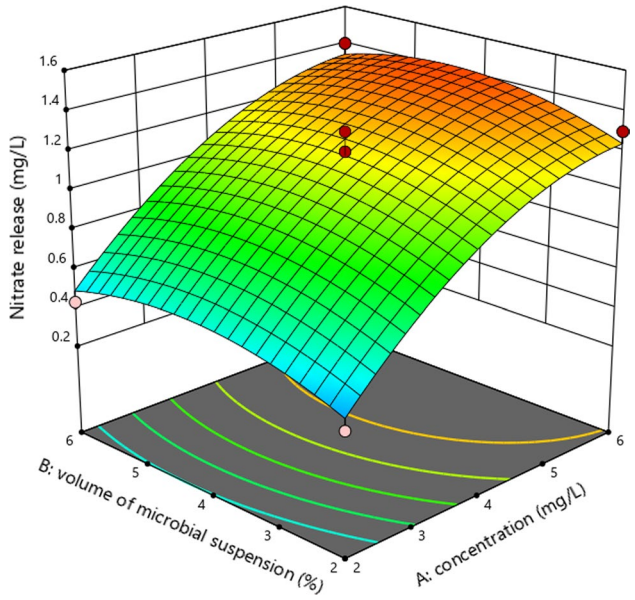


Fig. 6 Nitrate release (mg/L) with varying initial HMX concentration (mg/L) and microbial suspension volume (%)

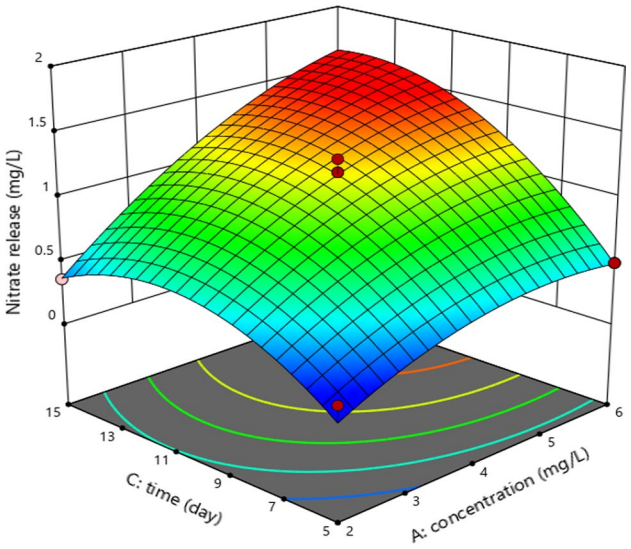


Fig. 7 Nitrate release (mg/L) with varying initial HMX concentration (mg/L) and time (days)

around the mean was less and the model was significant for the response. So, ANOVA explains the significance of model, which can be applied to optimize nitrate release during the HMX degradation process. All the parameters were fitted for second-order polynomial equation as follows

$$Y = 1.15 + 0.4200A + 0.0450B + 0.3625C + 0.0050AB + 0.2150AC - 0.0250BC - 0.1700A^2 - 0.1100B^2 - 0.3400C^2 \quad (3)$$

Table 5 ANOVA for quadratic model for nitrite release

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value	
Model	2.18	9	0.2420	17.15	0.0006	Significant
A-concentration	0.6216	1	0.6216	44.04	0.0003	
B-volume of microbial suspension	0.0000	1	0.0000	0.0035	0.9542	
C-time	0.1035	1	0.1035	7.33	0.0303	
AB	0.0552	1	0.0552	3.91	0.0884	
AC	0.2601	1	0.2601	18.43	0.0036	
BC	0.0156	1	0.0156	1.11	0.3277	
A^2	0.1342	1	0.1342	9.50	0.0177	
B^2	0.0616	1	0.0616	4.37	0.0750	
C^2	0.8470	1	0.8470	60.00	0.0001	
Residual	0.0988	7	0.0141			
Lack of fit	0.0099	3	0.0033	0.1489	0.9252	Not significant
Pure error	0.0889	4	0.0222			
Cor total	2.28	16				

df Degree of freedom

Table 6 Fit statistics of the model

Model	R^2	Adjusted R^2	Predicted R^2	Mean	SD	CV %
% degradation of HMX	0.9878	0.9721	0.8445	66.40	1.65	2.49
Nitrite release	0.9566	0.9008	0.8693	0.8100	0.1188	14.67
Nitrate release	0.9739	0.9402	0.6950	0.8671	0.1154	13.31

Table 7 ANOVA for quadratic model for nitrate release

Source	Sum of squares	*df*	Mean square	*F*-value	*P*-value	
Model	3.47	9	0.3860	28.97	0.0001	Significant
A-concentration	1.41	1	1.41	105.93	< 0.0001	
B-volume of microbial suspension	0.0162	1	0.0162	1.22	0.3066	
C-time	1.05	1	1.05	78.91	< 0.0001	
AB	0.0001	1	0.0001	0.0075	0.9334	
AC	0.1849	1	0.1849	13.88	0.0074	
BC	0.0025	1	0.0025	0.1877	0.6779	
A^2	0.1441	1	0.1441	10.82	0.0133	
B^2	0.0658	1	0.0658	4.94	0.0617	
C^2	0.5306	1	0.5306	39.83	0.0004	
Residual	0.0932	7	0.0133			
Lack of fit	0.0652	3	0.0217	3.11	0.1510	Not significant
Pure error	0.0280	4	0.0070			
Cor total	3.57	16				

df degree of freedom

where Y is nitrate release; A, B and C are coded values for initial concentration of HMX, volume of microbial suspension and time of degradation, respectively.

Conclusion

In this study, response surface methodology was used to optimize process parameters for degradation of HMX (exposure time, inoculum size and initial HMX concentration) with outputs such as % degradation, nitrite and nitrate release. It helped in the optimization of large experimental domains during the HMX degradation process with *Bacillus toyonensis*. Maximum 87.7 ± 4.6% degradation was achieved for HMX at 2 mg/L concentration with 4% volume of microbial suspension on 15th day.

It was observed that initial HMX concentration, time and inoculum size are the variables which readily affect the removal of HMX, nitrite release and conversion to nitrate. Analysis of variance (ANOVA) with different significant values (i.e., *P*-value, *F*-value, R^2 etc.) showed the significance for the model and also explained the effect of each variable on the different responses. Model obtained for each response can be used for the optimization of degradation process and release of each product. *Bacillus toyonensis* isolated from actual HMX contaminated site seems to be a potential and suitable microbe for the degradation of HMX. Further investigations related to its metabolic pathways for aerobic degradation are yet to be explored and also pilot scale studies need to be conducted for its field scale implementation.

Acknowledgements The authors acknowledge funding support provided to Arjun Meda by UGC, New Delhi. Funding organization has not played any role in study design, decision to publish or preparation of the manuscript. Authors are thankful to Defence Research and Development Organization (DRDO), New Delhi and Indian Institute of Technology Indore, for encouraging research and providing necessary facilities.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Adrian NR, Arnett CM (2004) Anaerobic biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) by *Acetobacterium malicum* strain HAAP-1 isolated from a methanogenic mixed culture. Curr Microbiol 48(5):332–340

An CJ, He YL, Huang GH, Liu YH (2010) Performance of mesophilic anaerobic granules for removal of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) from aqueous solution. J Hazard Mat 179(1–3):526–532

Bernstein RZ (2012) Biodegradation of the explosives TNT, RDX and HMX. Microbial degradation of xenobiotics. Springer, Berlin, pp 135–176

Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA (2008) Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76(5):965–977

Bhushan B, Paquet L, Halasz A, Spain JC, Hawari J (2003) Mechanism of xanthine oxidase catalyzed biotransformation of HMX under anaerobic conditions. Biochem Biophys Res Commun 306(2):509–515

Chatterjee S, Deb U, Datta S, Walther C, Gupta DK (2017) Common explosives (TNT, RDX, HMX) and their fate in the environment: emphasizing bioremediation. Chemosphere 184:438–451

Chaudhary P, Beniwal V, Kaur R, Kumar R, Kumar A, Chhokar V (2019) Efficacy of *Aspergillus fumigatus* MCC 1175 for bioremediation of Tannery Wastewater. CLEAN–Soil, Air, Water 1900131

Cook AM, Huetter R (1981) s-Triazines as nitrogen sources for bacteria. J Agric Food Chem 29(6):1135–1143

Crocker FH, Indest KJ, Fredrickson HL (2006) Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20. Appl Microbiol Biotechnol 73(2):274–290

Cuthbert JA, D'Arcy-Burt KJ, Carr SM (1985) HMX: acute toxicity tests in laboratory animals. US. Army Medical Research and Development Command. Report No. 2051, Ft. Detrick, MD

Draper NR, John JA (1988) Response-surface designs for quantitative and qualitative variables. Technometrics 30(4):423–428

Environmental Protection Agency (EPA). Validated method 8330B: nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC) (revised 2006)

Environmental Protection Agency (EPA) (2007) Validated method 8330A: nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC). Environmental Protection Agency (EPA), Washington, DC

Environmental Protection Agency (EPA) (2018) Office of Drinking Water Health Advisories, Drinking Water Health Advisory table (EPA 822-F-18-001)

Fournier D, Halasz A, Thiboutot S, Ampleman G, Manno D, Hawari J (2004) Biodegradation of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) by *Phanerochaete chrysosporium:* new insight into the degradation pathway. Environ Sci Technol 38(15):4130–4133

Fuller ME, Schaefer CE, Lowey JM (2007) Degradation of explosives-related compounds using nickel catalysts. Chemosphere 67(3):419–427

Garg SK, Tripathi M, Lal N (2015) Response surface methodology for optimization of process variable for reactive orange 4 dye discoloration by *Pseudomonas putida* SKG-1 strain and bioreactor trial for its possible use in large-scale bioremediation. Desalin Water Treat 54(11):3122–3133

Gong P, Hawari J, Thiboutot S, Ampleman G, Sunahara GI (2002) Toxicity of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX) to soil microbes. Bull Environ Contam Toxicol 69(1):97–103

Greenhough RJ, McDonald P (1985) HMX: 14 day toxicity study in mice by dietary administration. Research and Development Command. Army Medical Bioengineering Research and Development Laboratory, Ft. Detrick

Harkins VR, Mollhagen T, Heintz C, Rainwater K (1999) Aerobic biodegradation of high explosives, phase 1—HMX. Bioremed J 3:285–290

Hawari J, Beaudet S, Halasz A, Thiboutot S, Ampleman G (2000) Microbial degradation of explosives: biotransformation versus mineralization. J Appl Microbiol Biotechnol 54:605–618

Johnson MS, Reddy G (2015) Wildlife toxicity assessment for high melting explosive (HMX). In: Williams M, Reddy G, Quinn M, Johnson MS (eds) Wildlife toxicity assessments for chemicals of military concern. Elsevier, Amsterdam, pp 87–105

Kalafut T, Wales ME, Rastogi VK, Naumova RP, Zaripova SK, Wild JR (1998) Biotransformation patterns of 2, 4, 6-trinitrotoluene by aerobic bacteria. Curr Microbiol 36(1):45–54

Kim JS, Shea PJ, Yang JE, Kim JE (2007) Halide salts accelerate degradation of high explosives by zerovalent iron. Environ Pollut 147(3):634–641

Kiran B, Kaushik A, Kaushik CP (2007) Response surface methodological approach for optimizing removal of Cr(VI) from aqueous solution using immobilized cyanobacterium. Chem Eng J 126(2–3):147–153

Lotufo GR, Nipper M, Carr RS, Conder JM (2009) Fate and toxicity of explosives in sediments. In: Sunahara GI, Lotufo G, Kuperman RG, Hawari J (eds) Ecototoxicology of explosives. CRC Press, Boca Raton

MacFaddin JF (1985) Media for isolation-identification-cultivation-maintenance of medical bacteria, vol I. Williams and Wilkins, Baltimore

Mäkelä M (2017) Experimental design and response surface methodology in energy applications: a tutorial review. Energy Convers Manag 151:630–640

Mohanty SS, Jena HM (2018) Process optimization of butachlor bioremediation by *Enterobacter cloacae* using Plackett Burman design and response surface methodology. Process Saf Environ Protect 119:198–206

Myers RH, Montgomery DC, Anderson-cook CM (2002) Response surface methodology. Wiley, New York

Nagar S, Shaw AK, Anand S, Celin SM, Rai PK (2018) Aerobic biodegradation of HMX by *Planomicrobium flavidum*. 3 Biotech 8(11):455

Pichtel J (2012) Distribution and fate of military explosives and propellants in soil: a review. Appl Environ Soil Sci 20:1–33

Sangwan P, Mary Celin S, Hooda L (2015) Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using *Acinetobacter noscomialis*. Eur J Adv Eng Technol 2(4):51–56

Singh R, Soni P, Kumar P, Purohit S, Singh A (2009) Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria. World J Microbiol Biotechnol 25(2):269–275

Thompson KT, Crocker FH, Fredrickson HL (2005) Mineralization of the cyclic nitramine explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by *Gordonia* and *Williamsia* sp. Appl Environ Microbiol 71(12):8265–8272

U.S. Environmental Protection Agency, Integrated Risk Information System (IRIS), Chemical Assessment Summary National Center for Environmental Assessment (1998) U.S. Army Medical Research and Development Command. 1977. DAMD 17-76-C-6013. Ft. Deitrick, Frederick, p 21701

Williams RE, Rathbone DA, Moody PC, Scrutton NS, Bruce NC (2001) Degradation of explosives by nitrate ester reductases. In: Biochemical society symposia, vol 68. Portland Press Limited, pp 143–153

Wilson AB (1985) Determination of the acute and subchronic mammalian toxicity of HMX. US Army Medical Research and Development Command, Ft. Detrick

Zhao JS, Manno D, Hawari J (2007) Abundance and diversity of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX)-metabolizing bacteria in UXO-contaminated marine sediments. FEMS Microbiol Ecol 59(3):706–717

Zheng W, Lichwa J, D'Alessio M, Ray C (2009) Fate and transport of TNT, RDX, and HMX in streambed sediments: implications for riverbank filtration. Chemosphere 76(9):1167–1177

Zoh KD, Stenstrom MK (2002) Fenton oxidation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX). Water Res 36(5):1331–1341

Environmental Pollution 285 (2021) 117394

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

In-vessel composting of HMX and RDX contaminated sludge using microbes isolated from contaminated site

Arjun Meda [a,b], Pritam Sangwan [a,*], Kiran Bala [b]

[a] Centre for Fire, Explosive and Environment Safety, Defence Research & Development Organization, New Delhi, India
[b] Department of Biosciences and Biomedical Engineering, Indian Institute of Technology Indore, India

ARTICLE INFO

Keywords:
Sludge
Explosive
In-vessel composting
Contamination
Bioreactor
Half-life

ABSTRACT

Current study was carried out with an objective to remediate highly contaminated sludge with HMX and RDX obtained from an explosive manufacturing facility in North India employing indigenous microbes, *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (isolate no. S8-TSB-4) which were isolated from the same contaminated site. In-vessel composting of the explosive contaminated sludge was performed in 12 different bioreactors using cow manure and garden waste as bulking agents. 78.5% degradation of HMX was observed in reactor no. 2 with *Bacillus sonorensis* having combination of 10% sludge, 70% cow manure and 20% garden waste on 80th day. Two secondary metabolites Bis(hydroxymethyl)nitramine and methylene dinitramine were identified while studying the degradation pathway. Similarly, degradation of 91.2% was observed for RDX in reactor no. 11 with consortia of *Arthrobacter subterraneus* and *Bacillus sonorensis* on 80th day. During the study, release of significant nitrate and nitrite ions were observed. It has already been established that RDX and HMX degradation leads to release of nitrite/nitrate ions. The highest nitrite (reactor no. 11) and nitrate (reactor no. 2) release observed were 24.02 ± 0.05 mg/kg and 30.65 ± 0.99 mg/kg on 50th and 70th day, respectively. Scanning electron microscopic studies confirmed the attachment and presence of microbes with solid surface and no deformation in structure was observed in the microbial cells due to contamination stress. Findings of the study concluded that in-vessel composting assisted with native bacterial species can be a potential technology for the treatment of explosive contaminated sludge at the contaminated sites.

1. Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are the nitrogen containing energetic compounds, which are mainly used for military purposes. HMX is manufactured by the process of nitration of hexamine with nitric acid and ammonium nitrate using acetic acid as a catalyst. As, HMX is higher analogue of RDX, so during the manufacturing of HMX, RDX is always also present as an impurity. During the manufacturing of these compounds, a large quantity of wastewater is generated, which contains residues of RDX and HMX. The conventional method to dispose-off this wastewater is to discharge into unlined lagoons or pits after neutralization which leads to the formation of large amount of contaminated sludge sediments (Garg et al., 1991; Emery and Faessler. 1997). Generally, no further treatment of sludge is done before disposal at dumping sites. As RDX and HMX have low soil adsorption coefficient, they may leach down to the ground water table and contaminate it (Zheng et al., 2009; Sheremata et al., 2001). Due to the slow biological degradation and toxic nature, these contaminants are great threat to humans, other organisms and environment (Lapointe et al., 2017; ASTDR, 2012). Exposure of HMX can lead to malfunctioning of liver, central nervous system and kidney (Johnson and Reddy., 2015; Cuthbert et al., 1985). On the other hand, RDX is classified as group 'C' human carcinogen by US environmental protection agency (ASTDR, 2012). RDX exposure can lead to seizure, convulsions, vomiting and nausea (ASTDR, 2012; EPA, 2005). Apart from that RDX can also cross blood brain barrier and cause change in expression of genes (Williams et al., 2012; Gust et al., 2011). USEPA has recommended the life time drinking water standards for both RDX and HMX as 0.002 mg/L and 0.4 mg/L, respectively (USEPA, 2018). Due to toxic nature of both the explosives on abiotic and biotic environment, it is very important to remediate the sites contaminated with these explosives.

Various methods have been proposed and tested by different researchers to remediate the RDX and HMX contamination in water and

* Corresponding author.
E-mail address: pritam@cfees.drdo.in (P. Sangwan).

https://doi.org/10.1016/j.envpol.2021.117394
Received 15 February 2021; Received in revised form 30 April 2021; Accepted 13 May 2021
Available online 19 May 2021

soil, but very few studies have been focused on the sludge decontamination. Some of the methods which involve the explosive sludge treatment are incineration (Van Ham., 1998; Garg et al., 1991), composting (Doyle et al., 1986; Williams et al., 1988), and aqueous thermal decomposition (Garg et al., 1991). The conventional methods such as incineration require the sophisticated instrumentation and very high energy to operate. Therefore, these methods are not cost efficient and also generate waste such as ash which is again difficult to dispose-off (Chatterjee et al., 2017). To overcome these problems, green technologies viz. composting is gaining much attention, as it is eco-friendly, cost-effective and no fuel/energy is required during the process. There is also an opportunity for co-metabolism of contaminant with the bulking agents during the process. At the same time bulking agents, which are also a waste material, rich in organic content, easily available, can also be utilized to enhance the quality of the product during composting. To the best of our knowledge, very few studies have focussed on the composting of the explosives contaminated sludge. Composting of explosive contaminated sediments has been performed at the Louisiana Army Ammunition Plant and 98% of total explosive was removed with half-life of 42 days for HMX and 30 days for RDX (Williams et al., 1988). Emery and Faessler (1997) developed composting techniques at Umatilla Army Depot Activity (UMDA) to remediate explosive contamination. They observed that composting process was low cost and highly efficient. Apart from that, Clark and Boopathy (2007) studied land-farming for the explosive contaminated soil. They observed 82% removal of TNT, whereas removal of RDX and HMX was very low. Also, the composting techniques have already been established and well understood for the treatment of oil sludge, municipal waste and polycyclic aromatic hydrocarbons (Koolivand et al., 2019; Vasudevan et al., 2019; Antizar-Ladislao et al., 2004). Microbial remediation of explosives in soil and water has been studied by various researchers (Meda et al., 2020; Sangwan et al., 2015) and seems a possible option to treat explosive contaminated sludge also.

This study was planned with an objective to investigate the potential of two unexplored native microbes, *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (isolate no. S8-TSB-4) isolated from explosive contaminated site to decontaminate the RDX and HMX contaminated sludge, using in-vessel composting technique in a 80 days experiment and to study the reduction in contaminants, degradation mechanism and half-life of both compounds during the composting process.

2. Materials and methods

2.1. Composting materials and chemicals

Explosive contaminated sludge and soil samples were collected from a HMX manufacturing facility in North India. The samples were collected as per standard protocol (EPA, 2006). Sludge was dried in air and large particles were hand-picked. The physico-chemical analysis of the sludge was carried out as shown in Table 1. The cow manure was collected from cattle shed in Delhi, air-dried and pre-composted in open air to remove any volatile substances. The garden waste was collected from the premises of laboratory and mainly contains the grass cuttings, *Azadirachta indica* leaves and small amount of other types of leaves.

Table 1
Initial physico-chemical parameters of explosives contaminated sludge.

Parameters	Explosive contaminated sludge
pH	3.50 ± 0.02
E.C	0.5 ± 0.1 mS
Moisture	51.13 ± 2.0%
Nitrate	6.25 ± 0.1 mg/kg
Phosphate	29.9 ± 0.4 mg/kg
Potassium	22.1 ± 0.4 mg/kg
C:N ratio	25.6 ± 3.7
HMX concentration	95594.4 ± 2.3 mg/kg
RDX concentration	9638.4 ± 5.9 mg/kg

High performance liquid chromatography (HPLC) grade chemicals were purchased from Sigma Aldrich. All other chemicals used in the study were also of analytical grade and purchased from standard manufactures.

2.2. In-vessel reactor design

In-vessel bioreactors of 3 L capacity made up with acrylic material and moulded into cylindrical shape were designed and get manufactured. The whole experimental set up is shown in Fig. 1. Total 12 reactors were used in the study to carry out the experiment. All reactors were connected to an air compressor (Fig. 1). The air from the compressor was passed through the moisture vessel to make it humid and to keep the compost moist. Air flow was maintained at 50 mL/min with the help of air flow meters connected in series with each reactor. Air flow was maintained for 8 h each day. The moisture of each vessel was maintained at 60 ± 10% by adding the autoclaved water to compost on weekly basis and checked by gravimetric method. Temperature of the reactors was maintained at 32±3 °C and the compost was turned weekly to maintain aerobic conditions.

2.3. Microbial culture

Soil and water samples were collected from explosive contaminated site and handed over to Institute of microbial technology (IMTECH), CSIR, Chandigarh. The microbes were isolated and identified from the samples by Institute of microbial technology (IMTECH) and were obtained in lyophilized form. Out of various microbial species isolated, *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (MTCC no. 12855 isolate no. S8-TSB-4) were selected for this study after preliminary screening. Both the microbes were revived in tryptic soya broth (TSB) and grown in shaking flasks (120 rpm) at 32±2 °C to achieve an optical density of approx. 1.5 (1 mL ≈ 10^{12} cells/mL). As per the selected combinations, 10 mL of each microbial suspension was used as seed for reactor number 2 to 9. For reactor number 10–12, a consortium of two microbes were used which was prepared using 10 mL broth of each species. Different combinations were made with varying composition of contaminated sludge, cow manure and garden waste with both the bacteria as shown in Table 2. All the constituents of composting were taken on the dry weight basis. The whole experiment was conducted under aerobic conditions for 80 days.

2.4. Analysis

The analysis of explosives concentration was carried out as per the USEPA 8330A method (EPA, 2007). In brief, 2 g of sample was taken in amber colour sonication vial and mixed with 10 mL of acetonitrile. It was ultra-sonicated for 18 h at 18 °C after vortex. The sonicated samples were filtered through the Teflon filter of 0.45 μm. Supernatant was used for analysing concentration of explosive with the help of the High-performance liquid chromatography (HPLC). The HPLC system (Flexar™ LC, PerkinElmer, Waltham, MA, USA) was run at a flow rate of 1 mL/min of solvent consisting of acetonitrile and triple distilled water at ratio of 50:50. C-18 column was used as stationary phase for the explosive detection. The explosives were identified and quantified by photo diode array (PDA) detector at 254 nm by the system based on their retention time and peak area. As the contaminant's concentration in sludge as well as in compost was very high, the samples were diluted 100 times for the identification and quantification of the analyte. The calibration range used for the standardization was from 10 to 100 mg/L.

Mass spectroscopy (MS) was used to identify the intermediate products formed due to cleavage of cyclic ring and to study the mechanism of degradation during the process. Mass spectrometric (MS) analysis was performed on microTOF-Q (Bruker Daltonics, United

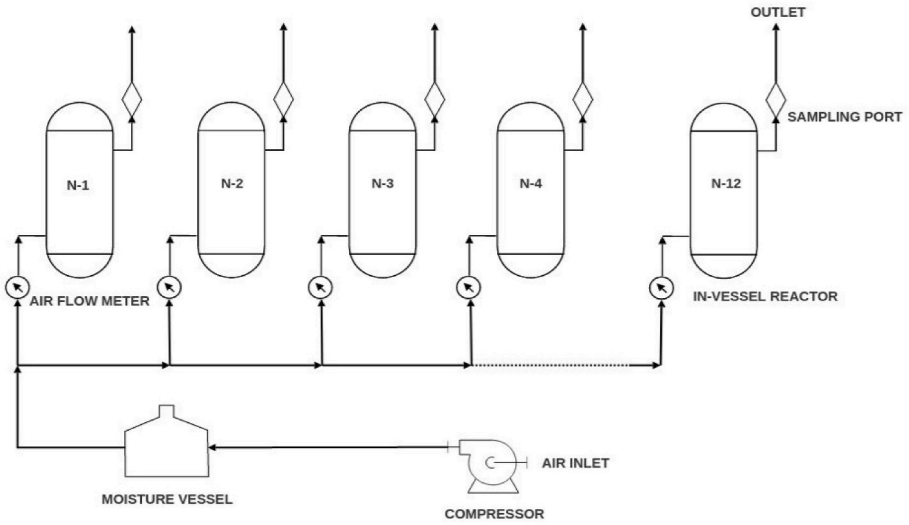


Fig. 1. The schematic diagram for in-vessel composting reactors.

Table 2
Different combinations of explosive sludge, cow manure, garden waste and microbes during the in-vessel composting process.

In-vessel reactor no.	Sludge (%)	Cow manure (%)	Garden waste (%)	Microbes
1	100 (control)	0	0	No microbes inoculated
2	10	70	20	*Bacillus sonorensis (B.S.)*
3	20	60	20	*Bacillus sonorensis (B.S.)*
4	30	50	20	*Bacillus sonorensis (B.S.)*
5	50	30	20	*Bacillus sonorensis (B.S.)*
6	10	70	20	*Arthrobacter subterraneus (A.S)*
7	20	60	20	*Arthrobacter subterraneus (A.S)*
8	30	50	20	*Arthrobacter subterraneus (A.S)*
9	50	30	20	*Arthrobacter subterraneus (A.S)*
10	10	70	20	*Arthrobacter subterraneus and Bacillus sonorensis (A. S. + B.S.)*
11	20	60	20	*Arthrobacter subterraneus* and *Bacillus sonorensis (A. S. + B.S.)*
12	30	50	20	*Arthrobacter subterraneus* and *Bacillus sonorensis (A. S. + B.S.)*

States) MS system using electron spray ionization in the positive ion mode (+ve ESI). C-18 column was used to separate the degradation products. The flow rate was 1 mL/min with a run time of 5 min. The solvent system consisted of 0.1% formic acid, acetonitrile (50%) and triple distilled water (49.9%).

Nitrite and nitrate analysis was done using colorimetric method (Nyanhongo et al., 2006; APHA, 1985) based on absorption at 540 nm and 220 nm, respectively using spectrophotometer (PerkinElmer, Model Lambda 650S, USA). Total organic carbon (TOC) analysis was done by Walkley & Black method (1934). Temperature and moisture was measured using hygrometer (Hanna HI9565, US) and Total Kjeldahl Nitrogen (TKN) was analysed by Kjeldahl method using nitrogen analyser (Pelican equipment, kelplusEmVa, Chennai, India).

Scanning electron microscopy (SEM) was also carried out using SEM (Supra55 Zeiss, Oberkochen, Germany). Samples were prepared following different steps like fixation, dehydration and post fixation steps as per standard method (Murtey and Ramasamy., 2016). The samples were fixed at different aluminium stubs and sputter coated with gold particles. The instrument was operated at 10–12 mA current under vacuum with the voltage of 5 kV to increase the image quality.

3. Results and discussion

3.1. Changes in physico-chemical properties

Initial-physico-chemical properties of the actual explosive contaminated sludge obtained from manufacturing site are shown in Table 1.

It was observed that there was high concentration of HMX (95594.4 ± 2.3 mg/kg) and RDX (9638.4 ± 5.9 mg/kg) in the sludge. The moisture, nitrate, phosphate, potassium and pH of the sludge were found in the range of 51.13 ± 2.0%, 6.25 ± 0.1 mg/kg, 29.9 ± 0.4 mg/kg, 22.1 ± 0.4 mg/kg and 3.50 ± 0.02, respectively. Table 2 shows different combinations of contaminated sludge, cow manure and garden waste with both the microbes used in the study.

Different physico-chemical parameters were studied to assess the properties of the compost, which are given in Table 3. The initial pH of sludge was acidic having a value of 3.5 ± 0.02, whereas after mixing with the bulking agents it got increased towards alkalinity having a range from 6.86 to 8.49 for different combinations. During the process of composting, pH got inclined towards neutrality ranging from 6.96 to 7.23 for all the combinations. The initial phosphate and potassium concentration in the compost were in the range of 20.1 ± 0.1 to 27.2 ± 0.1 mg/kg and 15.2 ± 0.2 to 20.3 ± 0.4 mg/kg, respectively. The phosphate and potassium concentrations were decreased on 80th day in each combination. Decrease in phosphate and potassium concentration can be due to their up-take by microbes for growth and metabolism. The initial C:N ratio was in the range from 23.3 to 39.8 for different in-vessel reactors. Whereas, at the end of 80 days the final C:N ratio of the compost in each combination was in the range of 20.5–35.9. The initial Total Kjeldahl Nitrogen (TKN) values for different combinations were 0.7–1.3%, which were increased at the end of 80th day from 0.8 to 1.5%. Increase in TKN shows the release of nitrogen containing compounds into the compost, which were accessible to microbes. An increase in TOC of different reactors was observed from 22.8 to 30.7% to 21.89–39.28% on final day which may be due to the release of carbon metabolites during the process. The whole experiment was performed with three replicates.

3.2. Degradation of explosives during in-vessel composting

Degradation pattern of HMX and RDX during the in-vessel

Table 3
Initial and final physico-chemical characterization of different waste combinations during in-vessel composting (n = 3).

In-vessel Reactor no.	pH		Phosphate (mg/kg)		Potassium (mg/kg)		TKN (%)		TOC (%)		C:N ratio	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
1	3.50 ± 0.02	3.53 ± 0.02	29.9 ± 0.4	12.34 ± 0.2	22.1 ± 0.4	11.34 ± 0.4	0.9 ± 0.1	0.9 ± 0.1	22.8 ± 0.2	21.89 ± 0.1	25.62 ± 3.7	24.63 ± 4.0
2	7.42 ± 0.02	6.96 ± 0.02	21.3 ± 0.4	10.0 ± 0.2	19.8 ± 0.2	15.25 ± 0.4	0.9 ± 0.1	1.3 ± 0.2	30.7 ± 0.2	39.28 ± 0.4	34.51 ± 5.1	30.91 ± 6.3
3	6.86 ± 0.04	7.02 ± 0.02	22.4 ± 0.2	6.77 ± 0.2	17.3 ± 0.4	15.23 ± 0.2	0.7 ± 0.0	1.2 ± 0.1	27.9 ± 0.1	38.94 ± 0.2	39.85 ± 0.2	32.66 ± 3.6
4	7.30 ± 0.02	7.15 ± 0.01	25.7 ± 0.5	7.43 ± 0.1	16.9 ± 0.4	11.67 ± 0.2	0.8 ± 0.1	1.2 ± 0.2	27.8 ± 0.2	25.40 ± 0.5	35.26 ± 5.8	21.82 ± 5.6
5	7.43 ± 0.04	7.10 ± 0.02	26.2 ± 0.2	3.55 ± 0.4	16.2 ± 0.1	13.27 ± 0.2	0.7 ± 0.1	0.8 ± 0.0	25.5 ± 0.1	28.73 ± 0.5	37.16 ± 7.3	35.91 ± 0.7
6	7.26 ± 0.04	7.08 ± 0.01	22.9 ± 0.4	8.45 ± 0.2	18.5 ± 0.2	9.35 ± 0.1	1.2 ± 0.2	1.5 ± 0.1	27.3 ± 0.4	31.33 ± 0.4	23.35 ± 5.1	20.96 ± 1.6
7	7.52 ± 0.02	7.11 ± 0.02	23.6 ± 0.1	9.27 ± 0.1	17.9 ± 0.4	15.22 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	25.9 ± 0.1	30.52 ± 0.5	29.12 ± 4.4	26.10 ± 5.6
8	7.39 ± 0.02	7.23 ± 0.02	25.5 ± 0.2	10.59 ± 0.1	17.1 ± 0.1	12.34 ± 0.4	0.8 ± 0.1	1.3 ± 0.2	26.4 ± 0.1	37.27 ± 0.8	33.50 ± 5.7	29.29 ± 5.7
9	7.62 ± 0.04	7.07 ± 0.01	27.2 ± 0.1	10.19 ± 0.2	15.2 ± 0.2	10.56 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	24.7 ± 0.4	24.09 ± 0.4	31.31 ± 5.0	27.13 ± 4.7
10	7.60 ± 0.02	7.12 ± 0.02	20.1 ± 0.1	8.96 ± 0.2	20.3 ± 0.4	8.97 ± 0.4	1.3 ± 0.2	1.5 ± 0.1	30.4 ± 0.4	30.69 ± 0.5	23.91 ± 3.4	20.53 ± 1.5
11	8.49 ± 0.04	7.08 ± 0.04	24.2 ± 0.2	9.06 ± 0.4	18.6 ± 0.2	16.34 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	28.1 ± 0.2	35.54 ± 0.1	31.58 ± 4.6	27.50 ± 3.1
12	8.14 ± 0.02	7.01 ± 0.01	25.9 ± 0.1	11.22 ± 0.1	15.2 ± 0.2	15.23 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	27.3 ± 0.2	33.49 ± 0.7	34.63 ± 5.7	30.74 ± 4.5

composting of sludge over a time period of 80 days is given in Fig. 2(A) and (B), respectively.

3.2.1. Degradation of HMX

Fig. 2(A) revealed that with the increase in time, there was significant reduction in HMX concentration for each composting reactor as compared to the control. However, in each reactor the rate of degradation with time was different. The reactors inoculated with *Bacillus sonorensis* (reactor no. 2–5) showed that with increase of sludge concentration, there was decrease in HMX removal efficiency. The degradation on 80th day in reactor no. 2, 3, 4 and 5 was 78.5 ± 0.12%, 62.01 ± 0.08%, 59.9 ± 0.06% and 49.6 ± 0.06%, respectively on 80th day. However, for reactors no. 6 to 9 which were inoculated with *Arthrobacter subterraneus* the maximum degradation observed was 74.7 ± 0.17% in reactor no. 7 with 20% explosive sludge and 80% bulking agents. Further, the reactors inoculated with consortium of the microbes (reactor 10, 11 and 12) showed low HMX degradation values (62.7 ± 0.03, 63.85 ± 0.14 and 56.18 ± 0.23%, respectively) on 80th day. This may be due to the fact that the microbes did not interacted positively with each other to remove significant amount of contaminant in the compost. Maximum HMX degradation (78.5 ± 0.12%) was observed with *Bacillus sonorensis* (isolate no. S8-TSB-4) in combination having 10% sludge, 70% cow manure and 20% garden waste in reactor no. 2. It was followed by reactor no. 7 which observed 74.7 ± 0.17% HMX reduction with microbe, *Arthrobacter subterraneus* (isolate no. S2-TSB-17) having combination of 20% sludge, 60% cow manure and 20% garden waste. The minimum degradation 48.5±0.37% was observed in reactor no. 6 with isolate no. S2-TSB-17 having 10% sludge, 70% cow manure and 20% garden waste which was approximately 37% lesser than the maximum degradation. In other compostersdegradation observed was in the range of 48.5–78.5%. HMX removal was higher at lower sludge concentration which may be due to lower toxicity at lower contaminant concentrations.

Bacillus sonorensis seems to be performed better at lower concentration of HMX (10% sludge), however, *Arthrobacter subterraneus* performed better at 20 and 30% sludge concentration. Further consortium of microbes showed significant results at 10 and 20% sludge concentrations as compared to 30% sludge. Earlier studies showed that cyclic ring of nitramines (HMX & RDX) is less stable in nature and get broken in other intermediate products by different mechanisms. Single nitrite ion (NO_2^-) elimination is one of the identified routes. The current study also observed the reduction in concentration of RDX and HMX with formation of nitrite ions. During the in-vessel composting, microbes utilized nitrogen and carbon present in the explosive sludge as a source of nutrition for their growth. The nitro-reductase enzymes are known to break complex ring structure into simpler secondary metabolites before complete mineralization.

It was also observed that the degradation of HMX/RDX and microbial growth for individual microbes at 50% sludge concentrations were very low which may be due to more toxicity at higher concentration. Williams et al. (1992) performed the composting of soil contaminated with explosives under thermophilic and mesophilic conditions. They observed that the RDX and HMX were decreased below the detection level from the initial concentration, 17900 mg/kg and 2390 mg/kg in the compost in 153 days with the help of indigenous microbes present in the compost. US Army Environmental Center (1993), carried out windrow composting of explosive contaminated soil under aerated conditions and observed 76.6% removal of HMX from the initial concentration (199.5 mg/kg). Authors obtained similar results during the study. Further, there was negligible degradation of 2.8% in HMX concentration in control which may be due to photolysis of the contaminants. It also showed that the microbes played an important role in degradation of contaminants during the study. ANOVA (single factor) was performed to check the significance of degradation efficiency between different reactors and the reactor having least degradation (reactor 6) on 80th day. A p-value less than 0.0001 was observed for reactor 2, 3, 4, 7, 8, 10 and 11 and a p-value less than 0.01 was observed for reactor 9 and 12 indicating significant degradation of HMX in these reactors. The combination with maximum degradation was further evaluated for half-life of HMX during the composting process. Pseudo first order kinetic model was applied which is represented in Eq. (1).

$$\ln A = -kt + \ln A_0 \quad (1)$$

Where, A is concentration after incubation, A_0 is the initial concentration of HMX, $t_{1/2}$ is the time for 50% HMX degradation and k is the degradation rate. The observed rate of degradation (k) and half-life ($t_{1/2} = 0.693/K$) of the selected combination was 0.0223 day^{-1} and 31 days, respectively with the correlation coefficient (R^2) of 0.99 (supplementary

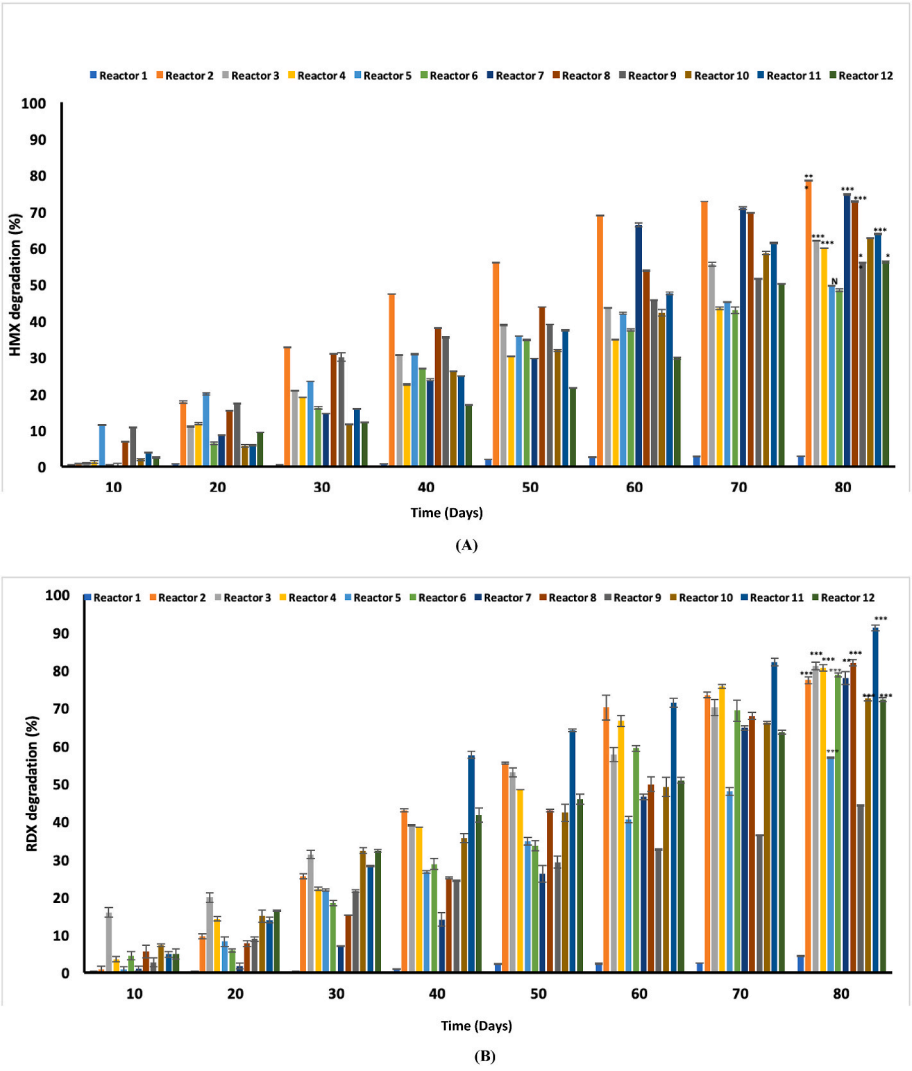


Fig. 2. (A).Degradation of HMX in different reactors during in-vessel composting with time. (B). Degradation of RDX in different reactors during in-vessel composting with time. *shows significance level of p-value (*** indicate p value $\leq$ 0.0001 and ** indicate p value $\leq$ 0.01 on 80th day).

Fig. 1). The high half-life of HMX shows, its recalcitrant nature. Garg et al. (1991) have done a sludge composting study and found that the half-life of HMX was nearly 42 days.

3.2.2. Degradation of RDX

RDX was present in the sludge as a co-contaminant of HMX and its degradation was also evaluated during the composting process as shown in Fig. 2(B). It was observed that both microbes were well adapted to utilize RDX during composting process. The reactor number 2, 3, 4 and 5 inoculated with *Bacillus sonorensis* showed a degradation of 77.36 $\pm$ 0.95%, 81.09 $\pm$ 1.02%, 80.60 $\pm$ 0.79% and 56 $\pm$ 0.17%, respectively at a concentration of 10, 20, 30 and 50% sludge concentration. Maximum degradation achieved with *Bacillus sonorensis* was 81.09 $\pm$ 1.02%, in reactor number 3 with 20% explosive sludge concentration. Similarly, in the reactors no. 6, 7, 8 and 9 which were seeded with *Arthrobacter subterraneus* maximum degradation of 81.96 $\pm$ 0.77% was achieved in reactor no. 8 which was 54.56% higher than the minimum degradation in reactor no. 9. Out of all reactors, the maximum reduction observed in the RDX concentration was 91.2 $\pm$ 0.80% in reactor no. 11 having 20% sludge, 60% cow manure and 20% garden waste in the presence of both the microbes. The presence of both microbes together seems to have positive impact on the removal of RDX. During RDX degradation, both the microbes performed very well at 10, 20 and 30% sludge concentration whereas, 50% sludge concentration tends to decrease the degradation efficiency, which can be due to high toxic effect of RDX on microbes. The degradation achieved was more with RDX in comparison with HMX which may be due to the high concentration of HMX in the contaminated sludge and less of RDX. The concentration of HMX was nearly 9–10 times higher as compared to the RDX concentration in explosive sludge (Table 1). It is well proven that quantity and concentration of the contaminant plays an important role in microbial degradation.

Apart from this, based on equation (1), half-life for RDX degradation was also calculated for the combination having maximum degradation. The rate constant (k) for the RDX in the combination having 20% sludge, 60% cow manure and 20% garden waste with consortia was found 0.0327 day^{-1} with a half-life ($t_{1/2}$) of 21 days with the correlation coefficient of 0.95 (supplementary Fig. 2). Further, a negligible degradation of 4.3% RDX was observed in control which may be due to environmental factors and showed that the indigenous microbes present in the sludge were unable to uptake and mineralize the RDX. ANOVA single factor was performed and significant RDX degradation was achieved for reactor no. 2, 3, 4, 5, 6, 8, 10, 11 and 12 with a p-value of less than 0.0001 and reactor no. 7 with a p-value less than 0.01.

The findings of the work were in line with the previous studies done by the US Army Environmental division in 1993, where windrow

composting of soil contaminated with explosives was carried out. Also, Griest et al. (1991) studied static pile composting for the explosive contaminated soil and found up to 97% reduction in the RDX concentrations. Similar degradation efficiency was observed for *Pseudomonas fluorescens, Rhodococcus* sp., *Gordonia* sp. and *Willamsia* sp. during the RDX degradation in soil (Lorenz et al., 2013; Thompson et al., 2005; Fournier et al., 2002).

Some intermediate products peaks were also observed while quantifying the concentration of remaining explosives using HPLC. The samples showing those peaks were chosen and subjected to mass spectroscopic analysis using LC-MS/MS to elucidate the HMX degradation intermediates formed during in-vessel composting of explosive sludge due to cleavage of HMX and RDX ring. Under the Positive electron spray ionization (ESI) mode, the presence of intermediate molecular ions were identified having the major $[M+H]^+$ peaks at ~ 205.09 Da and 177.04 Da m/z ratio (Fig. 3). Different molecular ion fragments identified, indicate the presence of two different intermediate products which were Bis(hydroxymethyl)nitramine ($C_2N_6H_8O_3$, M + nCH_3CN) (Hawari et al., 2001) and Methylene dinitramine ($CH_4N_4O_4$, M + nCH_3CN) (Zhang et al., 2003) having m/z ratio of 205.09 and 177.04, respectively. The microbes may have utilized HMX and RDX as a source of nitrogen during their growth and metabolic activities. With the help of nitro-reductase enzyme microbes tends to break the HMX ring structure into nitroso-derivatives of HMX like Bis(hydroxymethyl)nitramine, CO_2, nitrous oxide, formaldehyde, nitrate and nitrite (Hawari et al., 2001; Zhang et al., 2003; Fournier et al., 2004). The sequential hydroxylation and de-nitration of the ring may lead to the breakdown of HMX ring structure.

The Scanning Electron Microscope (SEM) imaging of the microbes was carried out at high magnification and the attachment and presence of microbes with substrate was confirmed (Fig. 4). SEM image of isolate S2-TSB-17 and isolate no. S8-TSB-4) with explosive treatment and without explosive treatment (Fig. 4) also illustrated the isolates to be rod-shaped cells and affirmed that there was no deformation in their shape in presence of explosives. Microbes were not affected adversely by the toxic environment of HMX and RDX. Upswing growth of both microbes showed that they could easily tolerate high concentration ofthe contaminants present in the sludge during the composting process and were also able to maintain their originality at the same time.

3.3. Release of nitrite and nitrate during the composting

Nitrite is the by-product of HMX and RDX degradation. So, nitrite and nitrates were estimated to understand the degradation mechanism of contaminants. The changes in concentration of nitrite and nitrate were also observed during the in-vessel composting of explosive sludge over 80 days time period which is mentioned in Fig. 5 (A) and Fig. 5 (B) respectively. During the composting of sludge, the concentration of nitrite increased till 50th day and after that a decrease was observed. The increasing nitrite concentration can be co-related with the degradation of RDX and HMX during the composting process. However, further decrease in nitrite concentration may be due to uptake of it by microbes or conversion into nitrate. It is due to the fact that nitrite ions are less stable under aerobic conditions and readily get converted into nitrate. The maximum nitrite release observed was 24.02 ± 0.05 mg/L in reactor no. 11 with *Bacillus sonorensis* and *Arthrobacter subterraneus* having 20% sludge concentration on 50th day. This was followed by reactor no. 10 (20.48±0.07 mg/L) having 10% sludge with *Bacillus sonorensis* and *Arthrobacter subterraneus,* and reactor no. 3 (20.41±0.05 mg/L) with 20% sludge having *Bacillus sonorensis* on 50th day.

The change in nitrate concentration observed during the composting process is shown in Fig. 5 (B). *Bacillus sonorensis* in reactor no. 2 with 10% sludge, 70% cow manure and 20% garden waste showed accumulation of nitrate ions till 80th day and maximum nitrate concentration observed was 30.65 ± 0.9 mg/L on 70th day. Whereas, in other combinations the nitrate concentration increased till 40–50 days and then it started to decline. As from the earlier work it has already been established that during RDX and HMX degradation, there is a release of nitrite and nitrate ions (Singh et al., 2009; Bhushan et al., 2006; Zoh and Stenstrom, 2002) and same was confirmed in the current study.ANOVA showed significant p-values for the reactors having maximum nitrite (reactor 11) and nitrate (reactor 2) released on 50th and 70th day respectively.

The ring cleavage of HMX and RDX follows two different steps which involves de-nitration and hydrolytic cleavage. It has been found that during the degradation of mono-nitroso derivatives of the explosives,

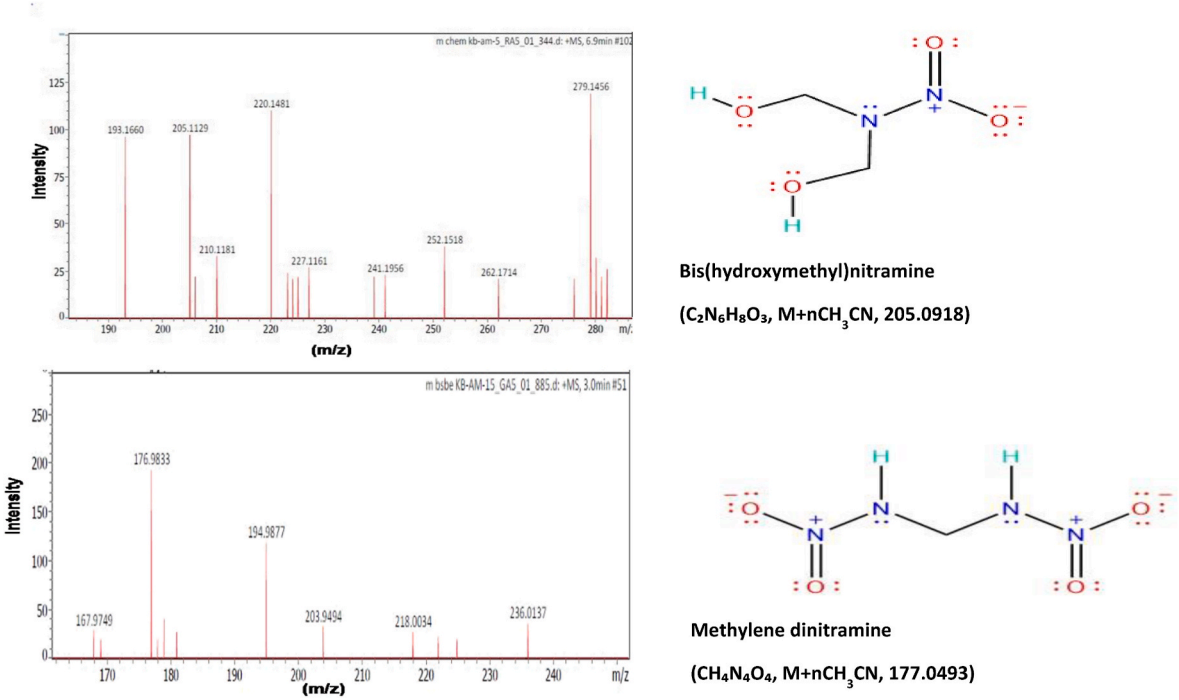


Fig. 3. Mass spectrometric analysis of secondary metabolites formed during explosive sludge degradation process in reactor no. 2 on 50th day and their structures.

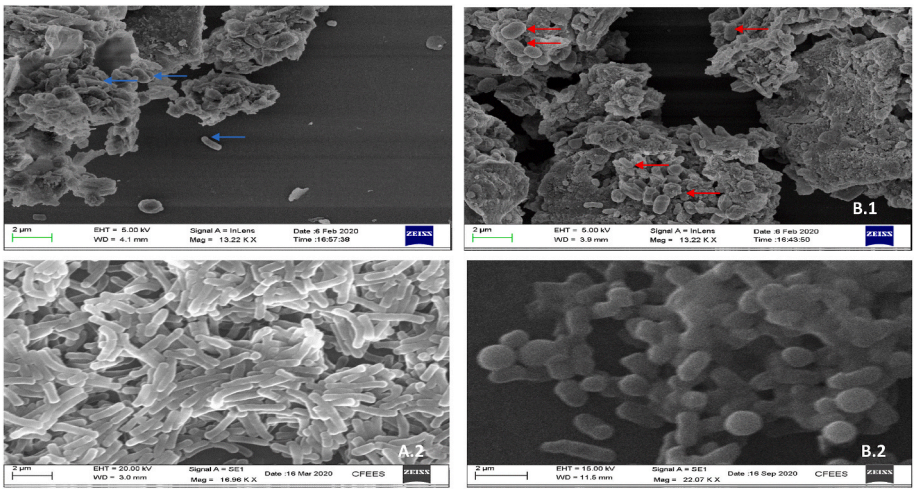


Fig. 4. Scanning electron micrograph of microbes, *Arthrobacter subterraneus* (isolate no. S2-TSB-17) with treatment (A.1) and without treatment (A.2) and *Bacillus sonorensis* (isolate no. S8-TSB-4) with treatment (B.1) and without treatment (B.2) in reactor 2 and 6, respectively.

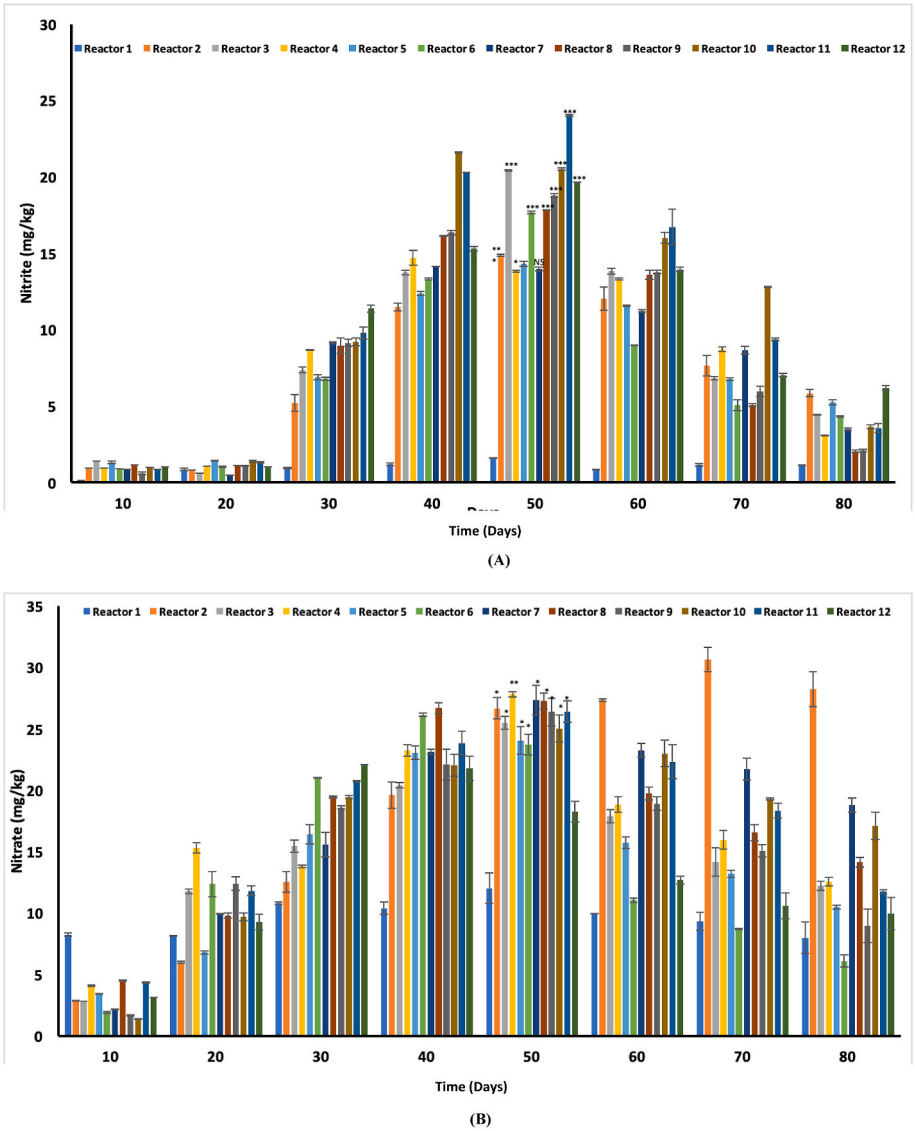


Fig. 5. (A).Release of nitrite during the RDX and HMX degradation in different reactors with time. (B).Release of nitrate during the RDX and HMX degradation in different reactors with time. *shows the significance level of p-value (*** indicate p value $\leq$ 0.0005, ** indicate p value $\leq$ 0.005, * indicate p value $\leq$ 0.01 and NS indicates non-significant values on 50th day).

nitrite and nitrate are released (Fournier et al., 2004; Khan et al., 2015).

4. Conclusions

In this study, the contaminated sludge was collected from a HMX manufacturing facility in the North India. High concentration of HMX and RDX were present in the sludge. In-vessel composting of HMX and RDX contaminated sludge was carried out using microbes *Arthrobacter subterraneus* (isolate no. S2-TSB-17) and *Bacillus sonorensis* (isolate no. S8-TSB-4) which were isolated from the same contaminated site in a 80 days experiment under aerobic conditions. The maximum HMX degradation (78.5%) was observed with *Bacillus sonorensis* having 10% sludge, 70% cow manure and 20% garden waste in reactor no. 2. Further, the degradation of RDX was maximum (91.2%) in reactor no. 11 inoculated with consortium of microbes having 20% sludge, 60% cow manure and 20% garden waste. The half-life for HMX and RDX in the combinations having highest degradations was found 33 days ($k = 0.223day^{-1}$) and 21 days ($k = 0.0327day^{-1}$), respectively. One way ANOVA showed significant results for in-vessel composting process in treatment of explosive contaminated sludge. The intermediate products for the HMX during degradation were identified as bis(hydroxymethyl)nitramine and methylene dinitramine. The HMX and RDX breakdown was further confirmed by analysing the release of nitrite and nitrate during the composting process. The results concluded that both indigenous microbes have the potential to degrade RDX and HMX contaminated sludge efficiently. Also, it is the first In-vessel composting study using these microbes for degradation of explosive contaminated sludge. This study can be extended for pilot scale composting process to decontaminate the sludge at actual contaminated sites. Furthermore, the detailed mechanism and the metabolic pathway for the aerobic degradation of RDX and HMX required to be studied explicitly. There is also a need for the further investigation of enzymes involved in the process of degradation of explosive contaminated sludge.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors acknowledge fellowship support provided to Arjun Meda by University Grant Commission (UGC), New Delhi. Funding organization has not played any role in study design, decision to publish or in preparation of the manuscript. Authors are thankful to Centre for Fire Explosive and Environmental Safety (CFEES),Defence Research and Development Organisation (DRDO), New Delhi and Indian Institute of Technology Indore for encouraging research and providing necessary facilities. Authors acknowledge IMTECH, Chandigarh for support in identification of microbial species. Authors would also like to acknowledge Dr. V.K Bind, Scientist 'E', CFEES, DRDO for helping in designing of in-vessel composting reactors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117394.

Author statement

All the authors agree to submission of manuscript.

References

Antizar-Ladislao, B., Lopez-Real, J., Beck, A., 2004. Bioremediation of polycyclic aromatic hydrocarbon (PAH)-contaminated waste using composting approaches. Crit. Rev. Environ. Sci. Technol. 34 (3), 249–289. https://doi.org/10.1080/10643380490434119.

American Public Health Association (APHA), 1985. Standard Methods for the Examination of Water and Wastewater. APHA.

Agency for Toxic Substances and Disease Registry (ATSDR), 2012. Toxicological Profile for RDX. www.atsdr.cdc.gov/toxprofiles/tp78.pdf.

Bhushan, B., Halasz, A., Hawari, J., 2006. Effect of iron (III), humic acids and anthraquinone-2, 6-disulfonate on biodegradation of cyclic nitramines by Clostridium sp. EDB2. J. App. Microbiol. 100 (3), 555–563. https://doi.org/10.1111/j.1365-2672.2005.02819.x.

Chatterjee, S., Deb, U., Datta, S., Walther, C., Gupta, D.K., 2017. Common explosives (TNT, RDX, HMX) and their fate in the environment: emphasizing bioremediation. Chemosphere 184, 438–451. https://doi.org/10.1016/j.chemosphere.2017.06.008.

Clark, B., Boopathy, R., 2007. Evaluation of bioremediation methods for the treatment of soil contaminated with explosives in Louisiana Army Ammunition Plant, Minden, Louisiana. J. Hazard Mater. 143 (3), 643–648. https://doi.org/10.1016/j.jhazmat.2007.01.034.

Cuthbert, J.A., D'Arcy-Burt, K.J., Carr, S.M., 1985. HMX: Acute Toxicity Tests in Laboratory Animals (No. IRI-2051). Inveresk Research International LTD Musselburgh (Scotland).

Doyle, R.C., Isbister, J.D., Anspach, G.L., Kitchens, J.F., 1986. Composting Explosives/organics Contaminated Soils. U. S. Army Toxic and Hazardous Materials Agency, Aberdeen Proving Ground, Maryland, p. 21010.

Emery, D.D., Faessler, P.C., 1997. First production-level bioremediation of explosives-contaminated soil in the United States. Ann. N. Y. Acad. Sci. 829, 326–340. https://doi.org/10.1111/j.1749-6632.1997.tb48586.x.

Environmental Protection Agency (EPA), 2005. Handbook on the Management of Munitions Response Actions. EPA505-B-01-001.nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P100304J.txt.

Environmental Protection Agency (EPA), 2006. Validated Method 8330B: Nitroaromatics, Nitramines, and Nitrate Esters by High Performance Liquid Chromatography (HPLC).

Environmental Protection Agency (EPA), 2007. Validated Method 8330A: Nitroaromatics, Nitramines, and Nitrate Esters by High Performance Liquid Chromatography (HPLC).

Fournier, D., Halasz, A., Spain, J., Fiurasek, P., Hawari, J., 2002. Determination of key metabolites during biodegradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine with Rhodococcus sp. strain DN22. Appl. Environ. Microbiol. 68 (1), 166–172. https://doi.org/10.1128/AEM.68.1.166-172.2002.

Fournier, D., Halasz, A., Spain, J., Spanggord, R.J., Bottaro, J.C., Zhang, J., 2004. Biodegradation of the hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine ring cleavage product 4-nitro-2, 4-diazabutanal by Phanerochaete chrysosporium. Appl. Environ. Microbiol. 70 (2), 1123–1128. https://doi.org/10.1128/AEM.70.2.1123-1128.2004.

Garg, R., Grasso, D., Hoag, G., 1991. Treatment of explosives contaminated lagoon sludge. Hazard Waste Hazard. Mater. 8 (4), 319–340. https://doi.org/10.1089/hwm.1991.8.319.

Griest, W.H., Tyndall, R.L., Stewart, A.J., Ho, C.H., Ironside, K.S., Caton, J.E., et al., 1991. Characterization of Explosives Processing Waste Decomposition Due to Composting (No. ORNL/TM-12029). Oak Ridge National Lab., TN (United States) https://doi.org/10.2172/5598569.

Gust, K.A., Wilbanks, M.S., Guan, X., Pirooznia, M., Habib, T., Yoo, L., Perkins, E.J., 2011. Investigations of transcript expression in fathead minnow (Pimephalespromelas) brain tissue reveal toxicological impacts of RDX exposure. Aquat. Toxicol. (Amst.) 101 (1), 135–145. https://doi.org/10.1016/j.aquatox.2010.09.011.

Hawari, J., Halasz, A., Beaudet, S., Paquet, L., Ampleman, G., Thiboutot, S., 2001. Biotransformation routes of octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine by municipal anaerobic sludge. Environ. Sci. Technol. 35 (1), 70–75. https://doi.org/10.1021/es0013531.

Johnson, M.S., Reddy, G., 2015. Wildlife toxicity assessment for high melting explosive (HMX). In: Wildlife Toxicity Assessments for Chemicals of Military Concern. Elsevier, pp. 87–105. https://doi.org/10.1016/B978-0-12-800020-5.00005-3.

Khan, M.I., Yang, J., Yoo, B., Park, J., 2015. Improved RDX detoxification with starch addition using a novel nitrogen-fixing aerobic microbial consortium from soil contaminated with explosives. J. Hazard Mater. 287, 243–251. https://doi.org/10.1016/j.jhazmat.2015.01.058.

Koolivand, A., Godini, K., Saeedi, R., Abtahi, H., Ghamari, F., 2019. Oily sludge biodegradation using a new two-phase composting method: kinetics studies and effect of aeration rate and mode. Process Biochem. 79, 127–134. https://doi.org/10.1016/j.procbio.2018.12.003.

Lapointe, M.C., Martel, R., Diaz, E., 2017. A conceptual model of fate and transport processes for RDX deposited to surface soils of north american active demolition sites. J. Environ. Qual. 46 (6), 1444–1454. https://doi.org/10.2134/jeq2017.02.0069.

Lorenz, A., Rylott, E.L., Strand, S.E., Bruce, N.C., 2013. Towards engineering degradation of the explosive pollutant hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine in the rhizosphere. FEMS Microbiol. Lett. 340 (1), 49–54. https://doi.org/10.1111/1574-6968.12072.

Meda, A., Sangwan, P., Bala, K., 2020. Optimization of process parameters for degradation of HMX with Bacillus toyonensis using response surface methodology. Int. J. Environ. Sci. Technol. https://doi.org/10.1007/s13762-020-02783-0.

Murtey, M.D., Ramasamy, P., 2016. Sample preparations for scanning electron microscopy–life sciences. In: Modern Electron Microscopy in Physical and Life Sciences. IntechOpen. https://doi.org/10.5772/61720.

Nyanhongo, G.S., Erlacher, A., Schroeder, M., Gübitz, G.M., 2006. Enzymatic immobilization of 2, 4, 6-trinitrotoluene (TNT) biodegradation products onto model humic substances. Enzym. Microb. Technol. 39 (6), 1197–1204. https://doi.org/10.1016/j.enzmictec.2006.03.004.

Sangwan, P., Mary Celin, S., Hooda, L., 2015. Response surface methodological approach for optimizing process variables for biodegradation of 2, 4, 6-trinitrotoluene using Acinetobacter noscomialis. Eur. J. Adv. Engg. Technol. 2 (4), 51–56.

Sheremata, T.W., Halasz, A., Paquet, L., Thiboutot, S., Ampleman, G., Hawari, J., 2001. The fate of the cyclic nitramine explosive RDX in natural soil. Environ. Sci. Technol. 35 (6), 1037–1040. https://doi.org/10.1021/es001389t.

Singh, R., Soni, P., Kumar, P., Purohit, S., Singh, A., 2009. Biodegradation of high explosive production effluent containing RDX and HMX by denitrifying bacteria. World J. Microbiol. Biotechnol. 25 (2), 269–275. https://doi.org/10.1007/s11274-008-9889-x.

Thompson, K.T., Crocker, F.H., Fredrickson, H.L., 2005. Mineralization of the cyclic nitramine explosive hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine by Gordonia and Williamsia sp. Appl. Environ. Microbiol. 71 (12), 8265–8272. https://doi.org/10.1128/AEM.71.12.8265-8272.2005.

US Army Environmental Center, 1993. Windrow Composting of Explosives Contaminated Soil at Umatilla Army Depot Activity Hermiston, Oregon. http://erwiki.net/images/0/05/1993-AEC_Technology_Applicatons_Analysis.pdf.

United States Environmental Protection Agency (USEPA), 2018. Office of Drinking Water Health Advisories, Drinking Water Health Advisory Table. EPA 822-F-18-001.

Van Ham, N.H., 1998. Recycling and disposal of munitions and explosives. Waste Manag. 17 (2–3), 147–150. https://doi.org/10.1016/S0956-053X(97)10014-9.

Vasudevan, M., Karthika, K., Gowthaman, S., Karthick, K., Balaganesh, P., Suneeeth Kumar, S.M., Natarajan, N., 2019. Aerobic in-vessel co-composting of dewatered sewage sludge with mixed municipal wastes under subhumid and semiarid atmospheric conditions. Energy Sources, Part A Recovery, Util. Environ. Eff. 1–12. https://doi.org/10.1080/15567036.2019.1624888.

Walkley, A., Black, I.A., 1934. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci. 37 (1), 29–38.

Williams, R.T., Ziegenfuss, P.S., Marks, P.J., 1988. I. Nstallation Restoration Program. Environmental Technology Development Task Order-8. Field Demonstration-Composting of Explosives-Contaminated Sediments at the Louisiana Army Ammunition Plant (LAAP). Weston (Roy F) Inc West Chester PA.

Williams, R.T., Ziegenfuss, P.S., Sisk, W.E., 1992. Composting of explosives and propellant contaminated soils under thermophilic and mesophilic conditions. J. Ind. Microbiol. 9 (2), 137–144. https://doi.org/10.1007/BF01569746.

Williams, L.R., Wong, K., Stewart, A., Suciu, C., Gaikwad, S., Wu, N., et al., 2012. Behavioral and physiological effects of RDX on adult zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155 (1), 33–38. https://doi.org/10.1016/j.cbpc.2011.02.010.

Zhang, S., Nguyen, H.N., Truong, T.N., 2003. Theoretical study of mechanisms, thermodynamics, and kinetics of the decomposition of gas-phase α-HMX (octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine). J. Phys. Chem. 107 (16), 2981–2989. https://doi.org/10.1021/jp030032j.

Zheng, W., Lichwa, J., D'Alessio, M., Ray, C., 2009. Fate and transport of TNT, RDX, and HMX in streambed sediments: implications for riverbank filtration. Chemosphere 76 (9), 1167–1177. https://doi.org/10.1016/j.chemosphere.2009.06.043.

Zoh, K.D., Stenstrom, M.K., 2002. Fenton oxidation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tetrazocine (HMX). Water Res. 36 (5), 1331–1341. https://doi.org/10.1016/S0043-1354(01)00285-8.

CURRICULUM VITAE

Arjun Meda

Ph.D. Scholar

arjun.meda110@gmail.com, phd1701171016@iiti.ac.in

H.no.230, ward no.6, Vaibhav colony, village-Rajgarh

Th. Sardarpur, Dist. Dhar, (M.P) 454116

+918085001546, +919589389361

Career Objective

To work with commitment and passion and apply my skill and expertise for the accomplishment of goals.

Educational Qualification

CLASS/DEGREE	BOARD/UNIVERSITY	SCHOOL/COLLEGE	% / SGPA
Ph.D. (Biosciences and Biomedical Engineering	Indian Institute of Technology Indore (IIT Indore)	Department of Biosciences and Biomedical Engineering	**Thesis submitted**
M.Sc (Biochemistry)	Devi Ahilya University Indore	School of Biochemistry, Indore	**73.72 %**
B.Sc (Biotechnology)	Devi Ahilya University Indore	Holkar Science College , Indore	**57.6%**

Higher secondary	CBSE	JawaharNavodayaVidyalaya, Multhan	**60.6%**
Secondary school	CBSE	JawaharNavodayaVidyalaya, Multhan	**77.6 %**

- Qualified CSIR-UGC net 2015 December exam in life sciences [Reference no. 20/12/2015(ii)EU-V].

Professional Profile

- Six-month dissertation on the topic "cloning, expression, and purification of p450 oxidoreductase and its possible in the metabolism of environmental carcinogens."

Publications

1. Meda, A., Sangwan, P., & Bala, K. (2020). Optimization of process parameters for degradation of HMX with *Bacillus toyonensis* using response surface methodology. ISSN 1735-1472 International Journal of Environmental Science and Technology, Springer. DOI 10.1007/s13762-020-02783-0 (Impact factor 3.463).
2. Meda, A., Sangwan, P., & Bala, K. (2021). Optimization and Degradation Studies on Hexahydro-1, 3, 5-Trinitro-1, 3, 5-Triazine (RDX) with Selected Indigenous Microbes under Aerobic Conditions. Water, MDPI, 13(9), 1257, DOI 10.3390/w13091257 (Impact factor 3.530).
3. Meda, A., Sangwan, P., & Bala, K. (2021). In-Vessel composting of HMX and RDX contaminated sludge using microbes isolated from a contaminated site. Environmental Pollution, Elsevier, Vol. 285, 117394. DOI 10.1016/j.envpol.2021.117394 (Impact factor 9.988).

Conference

1. Meda, A., Sangwan, P., & Bala, K. Optimization and transformation studies on hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with selected indigenous microbes under aerobic conditions. **Best paper award** at 5th international conference on Bioenergy, Environment and Sustainable technology (BEST), 2021.

Research Interest

I have been working in the field of microbial degradation of the xenobiotic compound during my Ph.D. work and gained experience in wastewater and soil analysis. I have worked on High-performance liquid chromatography and LC-Mass spectroscopy. I am interested in developing efficient microbial formulation for the effective degradation of xenobiotic compounds at contaminated sites. I am interested in bioreactors for in-situ studies. In my current endeavors, I have performed optimization of degradation parameters for RDX and HMX. I am looking forward to any opportunity to work in this field further.

Reference

1. Dr. Kiran Bala
   Associate Professor
   Department of Bioscience and Biomedical Engineering
   Indian Institute of Technology, Indore
   E mail: kiranb@iiti.ac.in
2. Dr. Pritam Sangwan
   Scientiest 'E'
   Center for Fire, Explosive and Environmental Safety,
   Defense Research and Development Organization, Delhi
   E mail. pritamsangwan@gmail.com

DECLARATION

I hereby declare that the above information furnished by me is true to best of my knowledge

Place: Indore

Arjun Meda

07/08/2022