SUMMARY

The present study attempts to elucidate the prokaryotic diversity from explosive contaminated sites in India by using a combination of culture dependent and independent approaches. Since, the literature pertaining to investigations of prokaryotic diversity from these environments in our country are rudimentary with respect to geographical and biogeochemical parameters, therefore, , in this work different soil and water samples were collected from two distinct explosives manufacturing/contaminated sites. Moreover, soil and water samples were also collected from the vicinity of these establishments to get an extensive perception of the shift in prokaryotic diversity. The collected samples were plated on different media formulations with an intent to isolate a diverse bacterial community and identify explosives degrading bacteria. Further a thorough 16S rRNA gene based and whole genome metagenome sequencing approaches were applied to understand the prokaryotic community structure and functions outside the limitations of conventional approaches. Some highlights of the present work are detailed below:

1. The soil and water samples collected from two geographically distinct explosives contaminated sites (Bhandara, Nagpur [site 1] and Panchkula, Haryana [site 2]) were subjected to physiochemical analysis with respect to salinity, pH, metals and concentration of explosives (RDX and HMX). The soil samples from both sites and those collected from outside the explosives manufacturing facility showed high levels of RDX/HMX contamination, which surpasses both the U.S, EPA residential soil screening levels (5.6 mg/kg) and industrial screening levels (24 mg/kg) (U.S, EPA., 2015) limits (Section 4, Table 4.2). Additionally, the concentration of potassium was more in vicinity soil and site 2 samples (199 & 166 mg/kg respectively) (Section 4, Table 4.1) which is a characteristic profile for an ecosystem with innate bioremediation potential Walker *et al.*, 2001). The hierarchal clustering of samples based on physiochemical parameters (Euclidian distances) suggested that site 1(Nagpur) soil sample clustered differently compared to site 2 (Panchkula) and Panchkula vicinity soil sample thus indicating different physio-chemical factors in the two manufacturing sites (Fig 5.1).

2. A total of 11 soil samples (site 1: S2, S4, SL1, WS2, WS4 & site 2: S1, S5, S6, S8, S9, S10) were used for culture dependent analysis, which were subjected to serial dilution and plated on 5 different microbiological media (Section 4, Table 4.3 & Fig 4.2). The maximum number of CFU/g was observed in site 1 samples (WS2 & WS4). Overall, the number of colonies observed in nutrient rich media was more compared to nutrient deficient media (except S2 and S4 samples). The more number of observed colonies in nutrient rich media could be due to high carbon and nitrogen availability and better adaptability of viable cells in the sample towards such media. However, higher CFU number does not guarantee a diverse nature of cell types within the limitations of media conditions utilized in this study as low nutrient conditions (explosives supplemented as a sole nitrogen source) have been previously documented to target explosives degrading bacteria (Binks et al., 1995; Coleman et al., 1997).A total of 425 bacterial isolates were purified and preserved from the two sites and 397 strains were characterized using partial 16S rRNA gene sequencing. All 397 strains were analysed for their taxonomic affiliation using EZtaxon server (Chun et al., 2007) and subsequently analysed using RDPclassifier (Wang et al., 2007) tool from comparative analysis providing taxonomic placement till genus level and facilitated identification of putative novel taxa.

3. In culture dependent analysis, the two sites were constituted by 5 bacterial phyla i.e., *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and *Deinococcus-Thermus*. The phylum *Firmicutes* was the most abundant taxon constituting ~55 % of overall bacterial diversity, followed by *Proteobacteria* (~20 %) and *Actinobacteria* (~20 %). However, *Deinococcus-Thermus* (2 %) was exclusively identified in sample WS2 while the phylum *Bacteroidetes* (3 %) was observed only in the samples S8, WS2 and WS4 (Fig 5.3). The phyla *Firmicutes* and *Actinobacteria* constituted the core culturable bacterial community. Around 77 % isolates were Gram positive that included all the members of core phyla (Fig 5.4) while 33 % isolates were Gram negative. The overall class level distribution in 11 soil samples (Section 4, Fig 4.21) suggested that class *Bacilli* (~55 %) was most predominant and common to all samples, while among 57 genera identified, *Bacillus* was the most

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predominant. Moreover in the PCoA analysis, samples from Nagpur and Panchkula showed variation in clustering wherein sample SL1, WS2 and WS4 of Nagpur clustered together (Section 4, Fig 4.24) and samples S2 and S4 showed a shift towards Panchkula samples.

4. In site 1 samples, 5 species identified as *Acinetobacter junii* (Fuller and Manning, 1997), *Arthrobacter globiformis* (Kalafut et al., 1998), *Bacillus cereus, Bacillus subtilis* (Fuller and Manning, 1997; Kalafut et al., 1998) and *Rhizobium* sp. (Labidi et al., 2001) have been earlier reported to be involved in degradation of TNT under aerobic conditions. In site 2 samples, 4 species identified as *Arthrobacter globiformis* (Kalafut et al., 1998), *Bacillus cereus, Bacillus subtilis* (Fuller and Manning, 1997; Kalafut et al., 1998) were observed and are known to be involved in TNT degradation while, *Gordonia terrae* (Thompson et al., 2005; Indest et al., 2010) is previously found to be involved in degradation of RDX.

5. All the characterized isolated (397) from the two sites were subjected to primary screening from degradation of explosive by using Griess reagent (coloured reaction) assay which resulted in identification38 potential explosives degrading strains (RDX/HMX) (Section 4, Table 4.19). All these isolates were characterized using a combination of 16S rRNA gene sequencing, FAME and MALDI-TOF based analysis to reveal their identity up to species level (Section 4, Table 4.19). The phylum level distribution of these strains revealed that majority of the identified strains belonged to phylum *Firmicutes*. Interestingly, 3 strains identified as *Bacillus cereus* (WS4-TSB-1), *Acinetobacter junii* (WS4-TSB-15) and *Arthrobacter globiformis* (S9-M2-9) (Section 4, Table 4.19, Fig 4.36) were well documented for their role in explosives degradation (Fuller and Manning. 1997; Kalafut et al., 1998; Litake et al., 2005). From these 38 strains, 4 strains WS2-TSB-10 (*Pseudomonas entomophila*), WS2-TSB-13 (*Kinnertia asaccharophila*), WS2-TSB-28 (*Bacillus oceanisediminis*) and S5-TSA-19 (*Planomicrobium soli*) were able to degrade high concentrations of RDX and HMX and were further selected for whole genome based analysis that revealed genes for metabolism of xenobiotics degradation, gentisate, biphenyl degradation and cytP450 genes which is known for its role in explosives degradation capability.

6. Based on 16S rRNA gene sequencing and phylogenetic analysis a total of 11 strains seemed potentially novel (section 4.5) at genus and species level. One strain designated as S5-TSA-19^T (isolated from site 1, S5 sample) has been described as a novel explosive degrading genus within the family *Planococcaceae* as *Indiicoccus explosivorum* gen. nov, sp. nov. (Pal *et al.*, 2019) (based on phenotypic, phylogenetic, chemotaxonomic and genomic characteristics) (Section 4.5.1). The results of explosives degradation analysis suggested that it is able to degrade up to 80% of 150 µM RDX concentration in 20 days with concomitant release of nitrate and formaldehyde (section 4.6). Furthermore, cytochrome P450 152A1 (EC 1.14.14.1)/unspecific monooxygenase consisting of ferredoxin domain upstream of cyt P450 (Fig 4.37, S5-TSA-19), cyclohydrolase II (EC 3.5.4.25), acetamidase (EC 3.5.1.4) and creatinine amidohydrolase (EC 3.5.2.10) were identified through genome analysis and a putative RDX degradation pathway was proposed (Fig 5.11). Additionally, polyphasic taxonomic characterization of strain S9-TSA-12 identified it as a novel species of the genus *Monashia* (section 4. 4.5.2).

7. Interestingly, genera involved in degradation of explosives, crude oil, aromatic hydrocarbon, polyaromatic hydrocarbons, heavy metals, phenanthrene, endosulfan, DDT, hexadecane and vinyl chloride, nitrophenolic compounds etc were frequently isolated in both sites (Section 5, Table 5.2 & 5.3). Furthermore, many genera were found to be involved in nitrate reduction, denitrification and sulphur metabolism, which clearly indicate the anthropogenic disturbances associated with both sites. Interestingly, based on RDPclassifier and MEGAN-LR analysis, many of the previously known xenobiotic and explosives degrading genera like *Flavobacterium* & *Deinococcus*, *Flectobacillus* and *Rhizobium*, *Pseudomonas* and *Enterobacter*, *Janibacter* and *Aeromonas* (Section 5, Table 5.2) were positively co-occurring in site 1 (Section 5, Fig 5.8), while, *Arthrobacter* & *Sterptomyces*, *Bacillus* and *Paenibacillus*, *Intrasporangium* and *Sphingomonas*, *Microbacterium* and *Sphingopyxis*, *Planomicrobium*, *Kocuria* and *Klebsiella* (Section 5, Table 5.3) were co-occurring in site 2 samples (Fig 5.8).

8. The culture independent metagenomic sequencing analysis based on 16S rRNA gene revealed a complex bacterial community composition in the

explosives contaminated environments with recovery of > 40 phyla from the 16 soil samples. In the present study a varied distribution of OTU's with predominance of the phyla *Proteobacteria* and *Firmicutes* was observed which have been previously reported to be the abundant communities in similar environments (Ronen *et al.*, 2008; Roh *et al.*, 2009; Kwon and Finneran., 2010; cho *et al.*, 2013; Jayamani & Cupples. 2015), *Actinobacteria* (Ringelberg *et al.*, 2008; Moshe *et al.*, 2009; Roh *et al.*, 2009; Fuller *et al.*, 2010; Cho *et al.*, 2013; Cupples *et al.*, 2013). It was also observed from the results of 16S rRNA gene targeted sequencing thatpopulations of *Proteobacteria* and *Bacteroidetes* increased with higher levels of RDX (Section 4, Fig. 4.44). Interestingly, members of the genera identified using culture dependent studies (*Enterobacter, Intrasporangium, Isoptericola, Janibacter, Klenkia, Knoellia, Lentzea, Lysinibacillus, Micrococcus , Monashia, Mycolicibacterium , Nonomuraea, and Paenarthrobacter*) were not observed in culture independent studies in both site 1 and site 2 (Section 5, Table 5.5).

9. The comparison of 16S rRNA gene based studies with previous reports, targeting profiling of bacterial composition in soil microcosms (soil samples artificially contaminated with explosives/RDX, Jayamani & Cupples (2015)) (Fig 5.12) revealed radical differences between soil samples from Indian and American continents. Majorly, at phylum level the community composition of soil samples from site 1 and site 2 showed predominance of *Proteobacteria* in all samples. Interestingly, some of the bacterial genera like *Acetobacter, Weissella, Tsukamureella* and *Acrobacter* showed site specificity and were exclusively retrieved in site 1 samples while *Xanthomonas, Vulgatibacter* and *Wenxinia* were exclusively present in site 2 soil samples. In addition, the most predominant genera observed in our study belonged to *Gemmatimonas, Paenibacillus* and *Gemmata* (Section 4, Fig. 4.45) which were previously reported to have major implications in biodegradation of aromatic hydrocarbons (Wu *et al.*, 2016)

10. Analysis of prokaryotic diversity in 4 water samples from site 2 (section 4.7.4.2.2) revealed that *Proteobacteria, Bacteroidetes, Firmicutes* and *Planctomycetes* together constituted $\geq$ 80 % of phylum. Interestingly, several

studies pertaining to RDX degrading bacterial communities in aquatic environments of the ammunition sites (groundwater, aquifer samples and water from wastewater treatment plants) have suggested that *Proteobacteria*, (with in the class *α, β, γ and δ-Proterobacteria*) (Fuller et al., 2010, Roh et al., 2009; Cho et al., 2013; Kwon et al., 2011; Kown and Finneran, 2010 and Livermore et al., 2013) were the most dominant genera in groundwater contaminated with explosives. Moreover, at the genus level, *Pseudomonas*, *Rhodoferax, Desulfovibrio, Geobacter* and *Prevotella* were observed that have been previously reported from similar habitat (groundwater, wastewater treatment plant) contaminated with explosives (Arnett et al., 2009; Roh et al., 2009; Jayamani et al., 2010; Fuller et al., 2010; Kown and Finneran, 2010; Eaton et al., 2011; Kwon et al., 2011; Perumbakkam & Craig., 2012; Cho et al., 2013; and Livermore et al., 2013).

11. The archaeal diversity in samples from site 1 and site 2 were dominated by phylum *Euryarchaeota* and *Thaumarchaeota*. The predominance of *Euryarchaeota* in was not surprising considering the fact that it forms about ~25% of total archaeal population in environmental samples (Porat *et al.*, 2010) while, abundance of *Thaumarchaeota* (Section 4, Fig 4.49) is related to its potential to oxidize ammonia (Pester *et al.*, 2011) in contaminated environments (explosives contamination in our study) hence playing an important role in nitrogen cycle.

12. Samples collected from the vicinity of site 2 (6 samples) were analysed by 16S rRNA gene targeted metagenomic sequencing and a total of 8 phyla (*Proteobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Acidobacteria, Actinobacteria, Verrucomicrobia* and *Frimicutes*) were observed to be part of the core bacterial population in these samples while, *Proteobacteria* was found to be the most predominant phyla in all samples. Interestingly, samples PSS2 and PSS3 (heavily contaminated with explosives) showed abundance in *Proteobacteria* and *Bacteroidetes* population, Moreover *Chloroflexi* which is previously been reported to be involved in rejuvenation of contaminated soil samples are more abundant (~12 %) in uncontaminated soil compared to contaminated samples (~3 %) (PSS2 & 3).

13. The whole genome metagenome based analysis was conducted on 4 samples collected from site 1, site 2 and vicinity of site 2 (PWGS2 & PWGS4). The analysis of prokaryotic community structure suggested that majority of OTU's belonged to phylum *Euryarchaeota* (within the class *Thermoprotei*), *Proteobacteria* (within *γ-Proteobacteria*) *and Crenarchaetoa* (within the class *Methanopyri*) (Section 4, Fig 4.71 & 4.73). The analysis of metabolic profiling (KEGG, COG) suggested that genes responsible for xenobiotic biodegradation and metabolism, carbohydrate metabolism, biosynthesis of secondary metabolites, signal transduction, environmental adaptation etc (Section 4, Fig. 4.76) were present in all sites. Moreover the functional profiling suggested that cyt P450, acyl-coA-dehydrogenase, aminotransferase, alpha/beta hydrolase, bacterial regulatory proteins and aminase were major functional genes

14. The samples with higher concentration of explosives (i.e, PWGS1, PWGS3) exhibited greater frequency of xenA/B, xplA/B gene copies (Fig 5.18) as observed in similar studies carried out by Wilson & Cupples (2016) quantifying functional genes in RDX-degrading microcosms implying that enzymes encoded by these gene are critical for prokaryotic population to thrive in such conditions. Further, the reconstruction of whole genome reads from whole genome metagenome based analysis resulted in a total of 22 MAGs (Metagenome assembled genomes) (Section 4, Table 28) from which 7 MAGs were selected (less than 50 % contamination level) that corresponded to the taxa *Desulfuromonas thiophila*, *Rhodanobacter* sp., *Paenibacillus riograndensis*, *Marinobacterium jannaschii*, *Cellvibrio* sp., *Rheinheimera* sp. KL1, *Chitinophaga* sp. YR627, respectively.

15. Based on annotation of all the observed MAGs a scheme for role in explosives contaminated habitat was sketched (Fig 5.19) wherein, MAGs corresponding to *Leptolyngbya sp.*, *Massilia sp.*, and *Cellvibrio mixtus* were identified to contain genes involved in sulfur and nitrogen cycling and also for the metabolism of xenobiotics (Fig 5.19). While MAGs corresponding to *Xanthomonas sp.*, and *Blastococcus* sp. were involved in nitrogen, sulfur cycling and metabolism of xenbiotics, respectively (Fig 5.19) suggesting a possible role of such microbial communities in explosives contaminated sites

i.e. processing of complex compounds through biogeochemical cycling. Collectively, the data from present study suggested that explosives contaminated sites harbour and subsequently aid in development of a core prokaryotic community which are relatively different from natural and pristine habitats.