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Research Article

METAGENOMICS EXPLORATIONS OF EARTHWORM GUT MICRO-BIOME TO DETOXIFY NPS IN SOIL SYSTEM

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ABSTRACT

Our current understanding of the potential impact of nanomaterials and their potentiality to remediate/nullify their toxic forms by naturally available scavenger is limited. Nature has its ways of resolving imbalances in the environment and organisms are one of the best tools of nature to eliminate toxic pollutants. The biological process of eliminating pollutants (bioremediation) with activities of earthworms and associated gut micro-biome may translate to improve bioremediation process and to improve soil health. Metagenomics is a recent field of science came into lime light since a decade and offers a potential way to access microbial diversity. The development of metagenomics stemmed from the evidence that yet difficult to cultivate microorganisms represents the vast majority of organisms. The field of metagenomics opens way to study genetic material directly from the environmental samples. DNA sequencing and synthesis technologies are making it possible to read and write and the huge amount of data obtained from the genome sequencing inevitably require bioinformatics tools to handle and further process them for analysis. This make possible to identify unculturable microorganism of earthworms that may possible to clean-up environment.

INTRODUCTION

Nanotechnology is rapidly expanding field of science with development of various nanaomaterials. In parallel to technological benefits from the impressive development of nanotechnologies, the arrival in the market of nano-products raises crucial issues dealing with human/environmental risk assessment and potential associated contamination. However, particles of nano-sized range have been present on earth for millions of year and have been used by mankind for thousands of years. But, recently their production and application has been increased because of our increasing ability to synthesize and manipulate their property. Every person is supposed to expose with nanometer sized foreign particles either through air or water. In truth, every organism on earth continuously encounters nano-meter sized entities. The majority causes little ill affect, and goes unnoticed, but intruder causes harm to organism (Buzea *et al*., 2007). Free nanoparticles(NPs) can be easily be released into environment and may pose serious health risk however, fixed NPs does not show serious ill effects (Oberdoster *et al*., 2005; Kreyling *et al*., 2006; Nel *et al*., 2006).Nanoparticles may be released from various products through normal use and then enter into wastewater stream. A major portion of these nanoparticles may release into sewage sludge those are disposed of in landfills, incinerated or applied to agriculture lands. Thus, soil system is an alternative sink for large portion of nanoparticles (Gottschalk *et al*., 2009). Unrine *et al*., (2010) and Gupta *et al*., (2014) reported that intact nanoparticles in the soil have potential to enter in terrestrial food webs and also absorbed by earthworms and bio-distributed to tissues remote from portal of entry.

Earthworms and nanoparticles

Earthworms are known as ecological receptor that plays an important role in structure and function of terrestrial ecosystem. Therefore, there is a need to investigate accumulation/aggregation property of nanoparticles in food chain and their trophic-transfer when assessing the ecological risks of nanaomaterials. As earthworms occupy major invertebrate biomass (>80%) in terrestrial ecosystem and have over 600 million years of experience as environmental managers in the ecosystem as ‘waste managers’ as ‘soil managers’ & ‘fertility improvers’ and ‘plant growth promoters’ for long time. But some comparatively new discoveries about their role in bioremediation of industrial wastes, chemically contaminated soil, dairy industry waste material, and detergent

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industries have revolutionized the understanding of functioning of this unheralded soldier of mankind. Darwin wrote, "*No other creature on earth has done so much for mankind*" as the earthworms. Proposed study may give an overview on behavior of NPs and their potential remediation by earthworm micro-biota in lines of Dr Anatoly Igonin who said "Earthworms create soil & improve soil fertility and provides critical biosphere's functions, disinfecting, neutralizing protective and productive for human welfare". Addressing this documentation problems present article, is proposed to identify robust new methods of decontaminating nano-waste at lower cost and with less energy, while at the same time minimizing their impact on the environment.

Earthworms'gut microbiota to nullify toxicity of NPs

Symbiotic organisms and earthworms are a driving force of bioremediation (Lin, 2016) and, therefore, may be an important tool to clean-up environment. The micro-biome in association to earthworms might contribute to:

1. The bioaccumulation and bio-flocculation of nanomaterials in coelomic fluid of earthworms using bacteria and fungus (Gultom and Hu 2013; Leitao, 2009) to deposit homo and hetero-aggregates of nanoparticles and reduce their toxic impact in soil system.
2. Facilitate trapping properties to capture nanoparticles from contaminated soil using mucus glycoproteins/ and or glycans with intestinal or microbial origin. The glycoprotein molecules possess a vatiety of charges(positive N-bonds or negative O or P or S or C-O bonds) which allow particles to link together, resulting in a Zero point of charge as pH~2 for SiO_2; pH~6.5 for TiO_2; pH~8 for Fe_2O_3(Patwa *et al*.,2015) that may facilitate strong interactions between NP surfaces and glycoprotein. It is known that nanoparticles may adsorb on weak polyelectrolytes.The charge distribution in the polymers,molecular weight (Mabire *et al*., 1984) and polymer coformation (Liu, 2013) may play an ipmortant role in formation and density/stability of aggregates of nanoparticles.
3. An immunity boost (Jinek *et al.* 2012) that allow earthworms to live in the "hostile environments" with full contaminants with synthetics nanoparticles-polysaccharide interactions.

Metagenomics Exploration

Metagenomics exploration of earthworm microbial communities (combined with the traditional approaches and with the theoretical modeling of evolutionary strategies) with strategic approach that might lead to development of bioremediation technology of nano-waste. The comparison of study of different lineages of the model species identification and screening of metagenome from nano-waste contaminants of the earthworms'gut may be crucial to explain the approaches of metagenomics in different contaminated soils' environments. That may be followed with different sequences and function –based metagenomic strategies and multiple advanced sequencing strategies dealing with the prevalent metagenomes in bioremediation of nanoparticles and to provide wide data of metagenome to neutralize /reduce their impact in environment. That also indicates that the micro-biota play key role in forming hetero and homo aggregates of nanoparticles to reduce toxic impact of nano-materials. In order to get a comprehensive picture of bioremediation/nullify toxic impact of nano-materials through earthworm in association of micro-biota approach combined field observations may help to decontaminate systems for specific nano-waste treatments in factories using nanotechnologies.

Metagenomics of earthworm gut and bioremediation techniques

This new branch of science opens the doors to a tremendous amount of scientific exploration however metagenomics of earthworms gut still have certain key questions:(1) Is earthworms' metagenomics may boost the development of improved strategies for monitoring the impact of nano-waste on ecosystems and help to clean-up contaminated environments?;(2) Is understanding of microbial communities of earthworms'gut cope-up with nanoparticles (*in-vivo)* and could help in pre-assessment of contaminated sites to recover from nano-waste with biological processes/ increase the chances of bioaugmentation or biostimulation ?;(3) may direct access to the pool of environmental genomes without bias of cultivation, offers the possibility to explore the vast diversity of degradation pathways of nano-waste materials using environmental microorganisms, which remain to a large extent poorly characterized or totally unknown?

A case study of Metagenomics of earthworm treated with nanoparticles

Collection of earthworms

Eisenia foetida were collected from vermibed Sagar, India (Latitude $23^0 50' 2'' N$; Longitude $78^0 47' 1'' E$; 550m elevation) during August - September, 2015. For identification, collected specimens were preserved in ethyl alcohol for molecular characterization, and also fixed in 4% formalin for morpho-anatomical study.

Characterization of earthworms

Collected earthworms were identified with the help of available literature (Gates, 1972); later re-confirmed with amplified 683 bp cytochrome oxidase *coi*-I gene. The universal primers, LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*., 1994) were used to amplify *coi*-I gene sequences. Master mix used for PCR reactions contained 1 U Taq polymerase (JonakiTaq, CCMB, Hyderabad, India), 1.5mM $MgCl_2$, 0.2mM of each primer, 0.125mM of each deoxynucleotide. Thermal cycling was done in the ABI thermocycler with following conditions of PCR; 4 min initial denaturation at 94°C, 33 cycles of 1 min denaturation at 94°C, 1 min annealing at 45°C, 1 min elongation at 72°C, and a final elongation at 72°C for 10 min followed by 4°C for 10 min. The PCR products were visualized on 1.0 % agarose gels with 1 X TAE buffer and 0.5 µg/mL EtBr. The PCR products of the expected size were purified using the QIAquick Gel Purification Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturers' protocols. Purified PCR products were sequenced using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster

City, California) on an ABI3500 with LCO 1490 - HCO2198 primers (Xcelris Genomics Pvt. Ltd., Ahmedabad, India). The electropherograms were processed and analyzed with Bio-edit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and phylogenetic analyses was conducted using MEGA v6 and BLAST on NCBI platform.

Exposure of nanoparticles and collection of samples

Collected worms were thoroughly washed in running tap water before rinsing in distilled water and were not subjected to any control condition. Worms were placed on wet cotton to ensure complete defecation in order to avoid contamination. Worms were exposed to ZnO nanoparticles (mg/kg) for 28 hrs following OECD guidelines. After 28 days of exposure the gut swab were collected in cryovials. These vials were then immediately frozen into liquid nitrogen.

DNA extraction

DNA was extracted using QIAamp DNA extraction kit (Qiagen, USA) and metagenomes was quantified using the Qubit spectrophotometer (Fig 1, Table 1).

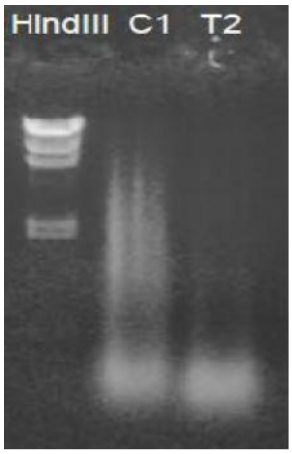

Fig 1 QC of gDNA on 0.8%

Table 1 Quantification by Qubit Flurometer

Sl No.	Sample Id	Concentration (ng/ µl)	Volume(µl)	Yield(ng)
01.	C1 (control)	38.8	20	796
02.	T2(Treated)	29.8	20	596

Amplicon Sequencing

16S rRNA amplicon Sequencing on Illumina MiSeq Platform was used at Xcelris Genomics Pvt. Ltd., Ahmedabad, India. The V3-V4 (Product size ~459bp) region of extracted 16s RNA PCR amplified product was reamplified using specific V3-F and V4-R primers with overhang adapter via PCR. Afterwards, PCR products was purified by using AmPure XP beads and checked on DNA-1000 chip on Agilent Bioanlyzer 2100, and also by running in 1.5% agarose gel. The purified amplicons were undergone Indexing PCR with Nextera XT Indices. And, resulting Index PCR products was purified using AmPure XP beads and run on DNA- 1000 chip on Agilent Bioanlyzer 2100. Libraries was quantified using Qubit HS and qPCR. The pooled PCR products (library) was loaded on MiSeq for cluster generation by hybridization of onto the oligonucleotide-coated surface of the flowcell. Immobilized DNA template copies was amplified by bridge amplification to generate clonal DNA clusters. The kit reagents was used for binding samples to complementary adapter oligos on paired-end flowcell. MiSeq Reagent Kit was used for sequencing metagenome library (2×300bp; PE) on Illumina MiSeq platform.

Data Analysis

QIIME was used for 16S/ITS2 for the assignment of taxonomic data using a naive bayesian classifier.

Microbial exploration of nanoparticles treated earthworms

Next generation sequencing has lead characterization and identification of the functional capacity of the nanoparticles treated worms. The taxonomy abundance was reported 65.7 % Firmcutes, 18 % Bacteroidetes, 7.63 % Spirochaetes; 3.17 % Proteobacteria; 2.05 % Actinobacteria; 1.64 %unassigned (Fig 2). The heat map of samples in Fig 3, corresponds to an OUT expressed in each column corresponds to a sample. The higher the relative abundance of an OTU in a sample indicated the more intense the color at the corresponding position in the heatmap.

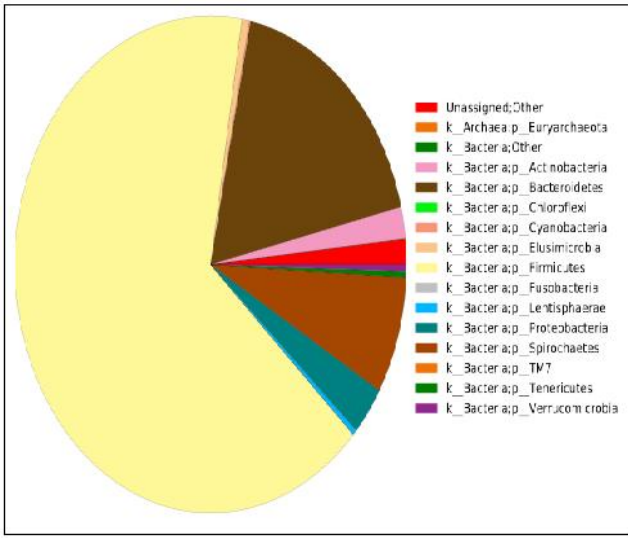

Fig 2 Taxonomic distribution phylum level Agarose gel

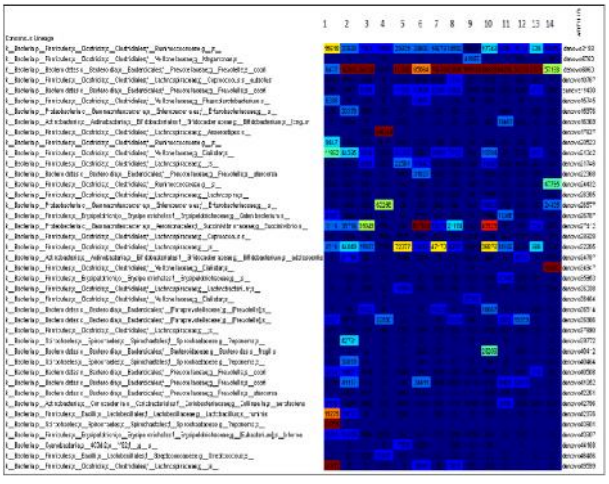

Fig 3 An OTU showing taxonomy assignment, T he coloured counts based on contribution of each OUT counts in samples(blue: contributes low percentage of OTUs to sample; red: contributes high percentage of OTUs).

CONCLUSIONS

Especially, three aspects were taken in consideration to profile micro-biomes either non-targeted characterization of sequences derived from microbial genome or targeted amplification, sequencing of bacteria, fungi, archaea and their relations between these various dimensions of genomic data, integrated with the earthworm exposed with nanoparticles and metadata including mining of discriminatory microbiome profiles in terms to nullify impact of toxicity of nanoparticles. However, their potentiality of nullify toxicity of nanoparticles need to further investigate. The present information provided first hand information of survival of large no of microbiota in gut of earthworm at exposure of nanoparticles.

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