



Research article

Nanopore-based metagenomic analysis of the impact of nanoparticles on soil microbial communities[☆]Sangeeta Chavan^{a,*}, Vishwas Sarangdhar^a, Nadanathangam Vigneshwaran^b^a Caius Research Laboratory, St Xavier's College, Mumbai, India^b Nanotechnology Research Group, CIRCOT, Mumbai, India

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ABSTRACT

The current trend of using nanotechnology products in all spheres of human life, including for crop improvement may have a possible impact on soil microorganisms which influence soil and plant health. Nanopore-based metagenomic study reported here used full-length 16S rRNA gene sequences to assess shifts in community composition of soil microorganisms when treated with silver, titanium dioxide and zinc oxide nanoparticles (S-NP, T-NP, Z-NP, respectively). Firmicutes and Proteobacteria were the two dominant phyla in this soil, and there were no significant differences ($p < 0.05$) observed in these phyla across treatments. However, in the phylum Firmicutes, the abundance of the order Clostridiales showed a significant decrease ($p < 0.05$) in the presence of S-NP. Similarly, in the phylum Proteobacteria, a significant decrease in the presence of S-NP was seen for two orders, Vibrionales ($p < 0.05$) and Rhodobacterales ($p < 0.01$). Analysis at a further depth revealed that abundance of the genus *Clostridium* (order Clostridiales) decreased in the presence of both S-NP ($p < 0.01$) and T-NP ($p < 0.05$). The abundance of the genus *Vibrio* (order Vibrionales) was likewise impacted in the presence of all the three NPs — S-NP ($p < 0.01$), T-NP ($p < 0.05$) and Z-NP ($p < 0.05$). Analyses at high taxon ranks such as phyla may not give a good representation of the nature of microbial community shifts, and at times may paint an erroneous picture. The use of full-length 16S rRNA gene sequences here yielded a greater taxonomic depth, and some shifts at the lower ranks were discernible.

1. Introduction

Advances in nanotechnology in the last two decades have been innovative and almost revolutionary (Roco, 2011). Nanomaterial applications have expanded and touched almost every sphere of human life including agriculture (Hristozov and Malsch, 2009; Usman et al., 2020). Today there are more than 1800 nanomaterial-based products in use (<http://www.nanoproject.org/cpi/>). Silver, titanium dioxide and zinc oxide nanoparticles (S-NP, T-NP, Z-NP, respectively) find a prominent place in this list of consumer products. In the context of agriculture, these three nanomaterials among others have been reported to improve crop yields (Hojjat, 2015; Kumar et al., 2019; Liu and Lal, 2015; Melika et al., 2015; Mishra and Singh, 2014; Prasad et al., 2012). The varied applications of nanomaterials have consequently led to a boost in the production of engineered nanomaterials (Piccinno et al., 2012). This will lead to an increase in the release of NPs into the environment during the production, use and disposal of the nanomaterial-containing commodities.

When the nanoparticulate formulations are used as agrochemicals, these nanomaterials would be directly introduced into soil. With the continuous increase in production and applications of these, the likelihood is high that nanomaterial concentrations will eventually reach detrimental levels in the environment. Alleviating their harmful effects could become very challenging at that late stage. Therefore, there is an urgency to study how soil microbiota is impacted by metal/metal oxide NPs.

To carry out any study about effect of nanomaterials on the environment, it is important to know their environmental concentrations. Existing analytical techniques have not been able to accurately determine nanomaterial concentrations in the environment (Maurer-Jones et al., 2013). As a result, different models have been developed to estimate these. These models have predicted a very wide range of nanomaterial concentrations in different environmental compartments; in surface water—0.08 ng/L (S-NP), 21 ng/L (T-NP), 1–10000 ng/L (Z-NP); and in waste-water treatment plant sludge—1.29–39 mg/kg (S-NP), 100–2000 mg/kg (T-NP), 13.6–64.7 mg/kg (Z-NP) (Maurer-Jones et al., 2013). In another review,

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T-NP concentrations ranging from 305 to 6000 mg/kg of biosolids have been reported (Luo et al., 2014). Very few studies have predicted concentrations of nanomaterials in soil, and there is a great deal of variation in the estimated values. For example, one study predicted that the concentrations of S-NP in the EU region would be 20 ng/kg of soil and 2310 ng/kg of sludge-treated soil (Sun et al., 2016), whereas another study predicted that agricultural soil concentration of S-NP world-wide would be 0.5 ng/kg and in sludge-treated soil 22.66 ng/kg (Giese et al., 2018). For T-NP, the predicted total concentration is more than a million times higher at 50 mg/kg of soil in the EU region (Meesters, 2016).

If soil microbiota is altered by nanomaterials, the ecosystem services such as soil formation, crop production, waste decomposition, groundwater quality improvement, etc. provided by soil microbial communities will be affected (Saccá et al., 2017). S-NP, T-NP and Z-NP have all been shown to have antibacterial activities (Dizaj et al., 2014; Gold et al., 2018). This could greatly influence agricultural productivity of soils.

To examine the antibacterial effects of NPs, pure cultures of model organisms such as *E. coli*, *S. aureus*, etc., or bacteria of clinical significance have been used historically (Gold et al., 2018; Hachicho et al., 2014; Hsueh et al., 2015; Ramalingam et al., 2014). Even though antimicrobial effects of NPs can be studied using laboratory cultures, probing their effects directly on naturally occurring microbial communities would yield a more relevant picture of their environmental toxicity.

Culture-dependent techniques reflect behaviour of less than 1% of the total microbial population in soil, and therefore culture-independent metagenomic techniques employing 16S rRNA genes have proved to be especially valuable in revealing bacterial diversity (Rastogi and Sani, 2011).

Earlier metagenomic analyses based on the 16S rRNA sequences have revealed shifts in environmental bacterial community composition on exposure to S-NP, T-NP, Z-NP, especially S-NPs (Chavan and Vigneshwaran, 2019; Grun and Emmerling, 2018; Meli et al., 2016; Moll et al., 2016; Samarajeewa et al., 2019). Short-amplicon 16S rRNA gene sequencing has been the method of choice in almost all of these studies. However, it has been shown that the sequencing of short stretches of 16S rRNA gene sequences does not provide as much taxonomic depth as provided by the full length rRNA sequence (Johnson et al., 2019; Martínez-Porchas et al., 2016).

In this exploratory study, impact of S-NP, T-NP, Z-NP on microbial communities in soil was evaluated. Full-length 16S rRNA gene sequences generated with the Oxford Nanopore system were employed for metagenomic analysis. This was expected to enable a more detailed delineation of taxonomic differences at the genus and/or species levels and therefore, lead to a better assessment of impact of NPs.

2. Materials and methods

2.1. Soil

Soil sample was collected from Vasai, Maharashtra, India, 19.403092N, 72.800142E. This region has dark soil with 31% sand, 29% silt and 40% clay, and belongs to the soil texture class 'clay'. It is mainly used for rice cultivation. Soil was collected from 8-10 random points at a depth of 10–15 cm. Debris (plant matter, rocks, and wood chips) was removed manually and the samples from the different points were mixed to give a single composite sample which was preserved at 4 °C until use. The pH of the composite soil sample was 6.7.

2.2. Nanoparticles

S-NP, T-NP, Z-NP were synthesized as described (Chavan and Vigneshwaran, 2020; 2019).

2.3. Experiments

The concentrations of the three nanoparticles that are known to impact bacterial communities in soil were selected based on our own

work and literature (Asadishad et al., 2018; Chavan and Vigneshwaran, 2019; Collins et al., 2012; Ge et al., 2011; Simonin and Richaume, 2015).

Four types of microcosms were set up for this study, viz., C (control) – 50 g soil without nanoparticles; A – 50 g soil with S-NP to give a concentration of 10 µg/g of soil; T – 50 g soil containing T-NP at 500 µg/g of soil; Z – 50 g soil plus Z-NP at 500 µg/g of soil. These microcosms in triplicates were maintained at ambient temperature for ten days. After ten days, DNA samples from the different microcosms were used for Nanopore analysis.

DNA extractions were carried out for all microcosm soils on day zero and day ten. Samples (1 g) were taken from each plate and DNA extracted using EXpure Microbial DNA isolation kit developed by Bogar Biobee stores Pvt Ltd, India, as per manufacturer's instructions.

PCR amplification and nanopore sequencing was done at Yaazh Xenomics Private Ltd, Coimbatore, Tamil Nadu, India and briefly it included the following steps. The primers used were 27F 5'AGAGT TTGATCMTGGCTCAG 3' and 1492R 5'AAGGAGGTGATCCAGCCGCA 3'. PCR was performed using the following thermal cycling conditions – initial denaturation 95 °C–2 min, followed by 25 cycles of denaturation 95 °C–30 s, annealing 60 °C–30 s, extension 72 °C–2 min, and a final extension 72 °C–10 min, then hold at 4 °C. Montage PCR Clean up kit (Millipore Corporation, USA) was used to remove unincorporated PCR primers and dNTPs from PCR products. The quality and quantity of the PCR product was checked using Qubit Fluorometer 3.0. Nanopore sequencing was performed with 1 µg of the amplified DNA. The sequencing workflow had the following steps – end repair/dA tailing, ligation of barcode, adapter barcoding, PCR end repair/dA tailing, blunt-end adapter ligation, purification using AMPure XP bead binding, priming and loading the SpotON flow cell.

The sequence data were analysed using the WIMP (What's in My Pot) identification workflow available in Metrichor's EPI2ME bioinformatics platform (nanoporetech.com) for Nanopore data (Juul et al., 2015; Kim et al., 2016). The workflow is designed to search the 'basecalled' sequence in the NCBI 16S rRNA bacterial database. The classification of reads depends on identity and percent coverage. EPI2ME 16S rRNA analysis function was used to identify genera; with access for detailed search at the species and sub-species level. These sequences are available in the NCBI Sequence Read Archives (SAMN23897201).

2.4. Data analysis

Since microbiome data are compositional in nature, the analysis was carried out using compositional analysis tools (Gloor et al., 2017). All statistical analysis were performed using R version 4.1.2. The total number of reads per sample were variable; hence, normalization by log transformation was carried out prior to the analysis to make microbiome abundances comparable. Principal Coordinate Analysis (PCoA) was performed using the normalized data at the genus level using the Bray-Curtis distance metric. Alpha diversity indices, viz., Richness, Evenness, Shannon and Simpson were calculated. Results are reported as means ± SD. The control and the treated samples were evaluated using the Kruskal-Wallis test and the Dunnett's post hoc test for significant differences. Values of $p < 0.05$ were considered statistically significant.

3. Results

A total of 168719 bacterial 16S rRNA reads were analyzed from the fifteen microcosms. Out of these, 100486 reads were classified, and 865 bacterial genera were identified across all the microcosms. Richness, Evenness, Shannon and Simpson diversity indices were calculated and were not significantly different (Table 1).

PCoA showed no obvious dissimilarities between the control sample and treated samples (Figure 1).

Percentage abundance at three taxonomic levels – phylum, order and genus – were calculated in the untreated and each of the treated soils.

Table 1. Alpha diversity indices.

Sample/Index	Richness	Evenness	Shannon	Simpson
Untreated soil (C)				
C1	580	0.148	4.146	0.941
C2	225	0.173	3.992	0.937
C3	481	0.151	4.059	0.934
S-NP treated soil (A)				
A1	208	0.184	4.811	0.984
A2	575	0.147	4.268	0.936
A3	125	0.189	3.559	0.914
T-NP treated soil (T)				
T1	403	0.153	3.910	0.917
T2	179	0.175	3.688	0.910
T3	510	0.145	3.820	0.904
Z-NP treated soil (Z)				
Z1	514	0.148	4.036	0.921
Z2	671	0.144	4.390	0.939
Z3	315	0.158	3.768	0.911

Firmicutes (25%) and Proteobacteria (25%) were found to be the two dominant phyla in this soil (Table 2). The other three phyla that were present at more than one percent level of abundance were Actinobacteria, Bacteroidetes, and Acidobacteria.

Though there were no significant differences ($p < 0.05$) observed in the phyla across treatments, phylum Firmicutes showed decrease (33%) in abundance in the presence of S-NP ($p = 0.12$), and the phylum Proteobacteria showed a marginal decrease (12–16%) in abundance on exposure to all the three nanoparticles ($p = 0.21$).

Though overall phyla abundances remained largely unchanged, it is possible that shifts at lower ranks may have been masked. Hence, the most dominant orders belonging to the phyla Firmicutes and Proteobacteria were analyzed. The orders belonging to phylum Firmicutes were Clostridiales, Lactobacillales, Bacillales, Veillonellales, and those belonging to Proteobacteria were Enterobacterales, Vibrionales,

Table 2. Relative abundance (%) of the top five bacterial phyla identified in treated and untreated soil samples.

Phyla	C (%)	A (%)	T (%)	Z (%)
Firmicutes	24.72 ± 1.24	16.52 ± 7.48	25.49 ± 0.92	24.31 ± 1.98
Proteobacteria	24.46 ± 1.65	20.33 ± 7.63	20.25 ± 1.92	21.26 ± 1.01
Actinobacteria	1.76 ± 0.11	1.94 ± 0.36	1.99 ± 0.22	2.07 ± 0.14
Bacteroidetes	1.34 ± 0.29	1.09 ± 1.09	1.03 ± 0.20	1.06 ± 0.07
Acidobacteria	0.43 ± 0.19	1.24 ± 0.78	0.36 ± 0.11	0.70 ± 0.32

C – control (untreated soil); A – S-NP-treated soil; T – T-NP-treated soil; Z – Z-NP-treated soil.

Rhodospirales, Burkholderiales, Sphingomonadales, Rhodobacterales, Alteromonadales, Rhizobiales, Rickettsiales, Pseudomonadales.

Order Clostridiales (phylum Firmicutes) showed a decrease in abundance in the presence of all the three NPs, but the decrease was significant ($p < 0.05$) in the presence of S-NP. Similarly, orders Enterobacterales, Vibrionales, Rhodobacterales, Alteromonadales, Rickettsiales and Pseudomonadales (phylum Proteobacteria) decreased in abundance in the presence of the three NPs. However, a significant decrease was seen only for two orders, Vibrionales ($p < 0.05$) and Rhodobacterales ($p < 0.01$) in the presence of S-NP (Figure 2).

Since the Nanopore sequencing yielded classified reads up to the species level, analysis was carried out at the genus level for the 14 orders listed above.

It was observed that the decrease in abundance of the three orders could be attributed to significant decrease ($p < 0.05$) in the abundance of the genera *Clostridium* (order Clostridiales), *Vibrio* (order Vibrionales) and *Gluconobacter* (order Rhodobacterales). *Clostridium* abundance decreased in the presence of both S-NP ($p < 0.01$) and T-NP ($p < 0.05$), and the abundance of the genus *Vibrio* was impacted in presence of all three NPs, S-NP ($p < 0.01$), T-NP ($p < 0.05$) and Z-NP ($p < 0.05$). Though only S-NP was found to bring a significant reduction at the order rank, examining change at the genus rank showed that some genera were sensitive to T-NP and Z-NP as well.

4. Discussion

Considering the compositional nature of the data presented here, Principal Coordinate Analysis was performed to summarize and check for similarity/dissimilarity between untreated and NP-treated soil. With PCoA, no obvious dissimilarities were found amongst the soils. Even though no significant change was observed at the level of phyla, abundance analysis at the order and genus level within these phyla presented a divergent picture. Thus, the direction and the extent of shifts seen at the level of phylum are not necessarily the same for every order or genus in that phylum. On examining influence of treatments at the different ranks of taxa, it can be seen that abundances of some taxa show significant changes ($p < 0.05$).

Members of the order Clostridiales which decreased in abundance in the presence of S-NP are commonly found in soil. Many species of the genus *Clostridium* are free-living nitrogen fixers and contribute significantly to nitrogen fixation, especially in paddy fields (Meurial et al., 2017). Since *Clostridium* abundance was negatively impacted in the presence of S-NP and T-NP, this could lead to detrimental changes in the agricultural productivity of soils.

The dominant genus of the order Vibrionales, *Vibrio* was inhibited significantly in the presence of the three NPs. These organisms are biofilm formers and by adhering to roots will allow other PGPR in the biofilm matrix to exert their beneficial effects (Santoyo et al., 2021). In addition, *Vibrio* have been reported to produce the phytohormone IAA and siderophores thus promoting plant growth themselves (Gutierrez et al., 2009; Pramanik and Vibhuti, 2022). Biofilm production is especially useful if there is an environmental stress. A decrease in the abundance of *Vibrio* may affect plant growth.

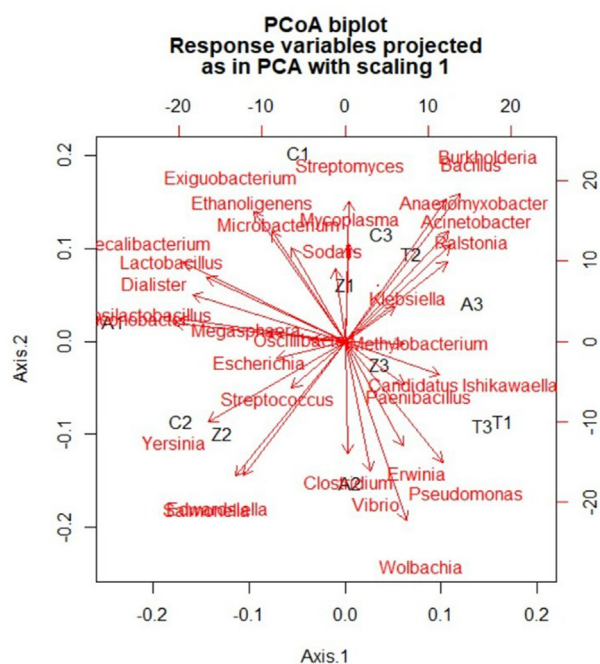


Figure 1. Principal Coordinate Analysis of the tested samples— C1, C2, C3 – control (untreated soil); A1, A2, A3 – S-NP-treated soil; T1, T2, T3 – T-NP-treated soil; Z1, Z2, Z3 – Z-NP-treated soil.

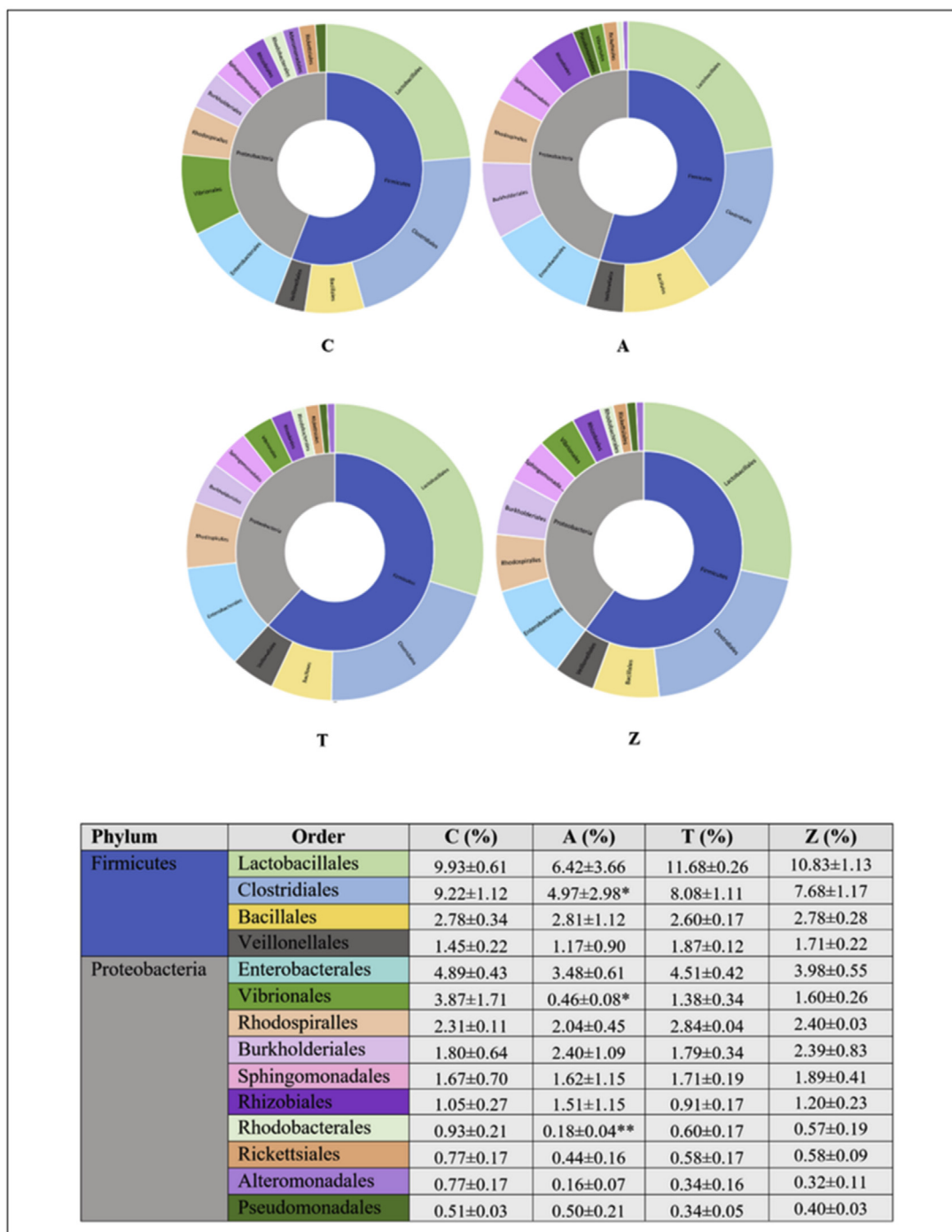


Figure 2. Percentage abundance of bacterial orders in the two dominant phyla. The sum of percentages is not 100 since orders of only two phyla are included. The relative abundance of prominent orders within the phylum are shown. C – control (untreated soil); A – S-NP-treated soil; T – T-NP-treated soil; Z – Z-NP-treated soil. *** $p < 0.01$, ** $p < 0.05$.

Previous studies including our own work employing short stretches of 16S rRNA gene sequences have reported inhibitory effects of S-NP on soil bacterial communities (Carbone et al., 2014; Chavan and Vigneshwaran, 2019; Grun and Emmerling, 2018; Liu et al., 2017; McGee et al., 2017; Wang et al., 2017). For example, Liu et al. have reported changes in bacterial community structure after 21 days of exposure to 1 µg/g of S-NP as determined by Principal Component Analysis (Liu et al., 2017). But the authors also reported that after 49 days, the bacterial community structure went back to normal. In another study, after a one-year exposure, it was found that the

abundances of common bacterial phyla found in soil showed a mixed response to S-NP concentration of 0.01 mg/kg of soil. Acidobacteria, Bacteroidetes and beta-Proteobacteria were significantly diminished but Actinobacteria and alpha-Proteobacteria were unaffected by S-NP treatments (Grun and Emmerling, 2018).

It has been shown that forest soil samples treated with S-NPs at two different concentrations (10, 100 µg/g) revealed a significant influence of the NPs on the soil microbial community after an incubation period of two months (Carbone et al., 2014). This study too used a short stretch of 16S rRNA gene sequence for analysis.

High-throughput sequencing of the V4 region was used to investigate the effects of S-NPs (10, 50 and 100 mg/kg) on microbial community structure of soil during an exposure period of 7 days (Wang et al., 2017). The authors showed abundance of Proteobacteria and Planctomycetes increased significantly, whereas, Acidobacteria, Actinobacteria, Cyanobacteria, and Nitrospirae significantly decreased with increasing NP concentration.

Another study using V3, V4 and V5 regions of 16S rRNA gene found that, S-NPs (50 µg/g of soil) brought about a significant decrease in the relative abundance of the Acidobacteria, Verrucomicrobia and Planctomycetes coupled with an increase in the abundance of the Proteobacteria and the Bacteroidetes (McGee et al., 2017).

Identification of bacteria in soil is generally carried out by sequencing one or two of the nine hypervariable regions of the 16S rRNA gene (Bukin et al., 2019; García-López et al., 2020). Instead of the short stretches, this study, used the entire 16S rRNA gene sequence for the metagenomic analysis. The resultant improved accuracy in taxonomic identification will better illustrate the influence of NPs on soil bacterial communities. Regardless of the 16S rRNA gene region chosen, it is known that the choice of primers, reference databases and bioinformatics pipelines may lead to under-representation of some bacterial taxa.

(Abellan-Schneyder et al., 2021).

Notwithstanding the analytical approach used, physical properties of soil are known to modulate the effect of NPs as described here. Effect of S-NPs on bacterial communities of two soils from Thailand was studied using ARISA (Chunjaturas et al., 2014). Different concentrations of S-NPs were employed – 50, 100, 250, and 500 µg/g soil and incubation periods were 2, 4 and 8 weeks. The analysis showed that bacterial community structure shifted with increasing concentration of NPs and the incubation period in both the soils. The study further showed that the effect was influenced more by the soil type than by the concentration of NPs and/or the incubation period. It has also been shown that soil pH and clay content modulate NP toxicity (Rahmatpour et al., 2017; Schlich and Hund-Rinke, 2015). Simonin et al. have suggested that toxicity of TiO₂NPs may be influenced by organic content and pH rather than by the soil texture, which is a reflection of its clay content (Simonin et al., 2015). Therefore, any study that analyses the impact of nanoparticles on soil microbial communities should take soil properties into account.

There are a few limitations of the present work. The data contain a small number of replicates and the depth of sequencing in two samples is low. However, these reads were included in the analyses as the number of replicates was small. In addition, this study used a short exposure period of 10 days. Therefore, inferences drawn from this work can be extrapolated to long-term impacts only after further experimentation.

Linking changes in soil bacterial community composition to agricultural yield may seem premature, but it is a possibility that cannot be ignored. This can be addressed by carrying out field studies where the observed shifts can be correlated with changes in agricultural productivity. Moreover, there is an apprehension that, not being biodegradable, these metal-based nanomaterials will accumulate in soil and their effects in 'nano' and other forms will in all probability be long lasting. Framing guidelines that will regulate the release of these metal-based nanomaterials in the environment is therefore time critical (Romero-Franco et al., 2017). The findings reported here will add to the existing nano-toxicological data permitting better assessment of environmental hazards. This will help regulatory authorities to formulate adequate measures to preserve soil health in the long term.

5. Concluding remarks

The effects determined immediately after short exposure of soil microcosms to the three nanoparticles revealed some changes in soil bacterial abundance and diversity, not at the phylum level, but at deeper levels of 'orders' and 'genera'. Amongst the three nanoparticles, S-NP seems to have maximum inhibitory effect on bacteria present in soil.

By adding to the existing data, our data will help assessment of environmental risks associated with metal/metal oxide nanoparticles, more so in the Indian context where such data are not available. The promotion of such nanoparticles as agricultural formulations needs to be deliberated keeping in mind the effects they have on soil microbial diversity.

Declarations

Author contribution statement

Sangeeta Chavan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Vishwas Sarangdhar: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Nadanathangam Vigneshwaran: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at Sequence Read Archive, NCBI under the accession number SAMN23897201.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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