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Research Article

ACUTE EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES IN SOIL BACTERIA, FUNGI AND ACTINOMYCETES

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ABSTRACT

The Nanotechnology industry is growing rapidly, leading to concerns about the potential ecological consequences of the release of nonmaterial to the environment. Titanium dioxide nanoparticles (TiO2 NPs) are widely used in commercial products such as sunscreens and toothpastes, industrial products like paints, lacquers and paper, and in photocatalytic processes such as water treatment. Also, TiO2 NPs are indirectly discharged in agricultural soils through irrigation or sewage-sludge application and directly as nanofertilizers or nanopesticides. Soil microorganisms are key contributors to nutrient cycling and are essential for the maintenance of healthy soils and sustainable agriculture. Although the antimicrobial effects of a broad range of nanoparticulate substances have been characterised in vitro, little is known about the impact of these compounds on microbial communities in environments such as soil. This study focused on the acute effects of TiO2 NPs on soil microbial communities such as bacteria, Fungi and Actinomycetes. This research revealed substantial shifts in bacterial, fungal and actinomycetes community composition in soils amended with TiO2. The TiO2 NPs exerted an adverse effect on the microbial population, causing the reduction of bacteria, Fungi and Actinomycetes in the substrate. The viability of the microbial population was reduced at the high concentration (50 mg kg^{-1}) of TiO2. Results demonstrate that microbial communities differed in their sensitivity to TiO2 NPs with its various concentration and the release of TiO2 NPs to the environment has the potential to alter the composition of these microbial communities, which could have implications for the stability and function of soil ecosystems.

Keywords: Ecotoxicology, Nanoparticles, TiO2, Antibacterial effects, Microbial, Inhibition.

INTRODUCTION

Nanoparticles have specific nanotechnological properties in terms of size, properties, behavior etc. The main reasons why materials built of NPs have different optical, electrical, magnetic, chemical and mechanical properties from their bulk counterparts are that in this size-range (between 1 and 100 nm) quantum effects start to predominate and the surface-area-to-volume ratio (sa/vol) becomes very large. The sa/vol of most materials increases gradually as their particles become smaller, which results in increased adsorption of the surrounding atoms and subsequently change their properties and behavior. Once particles become small enough, they start to obey the quantum mechanical laws. Materials reduced to the nanoscale can suddenly show very different properties, compared to what they exhibit on the macro-scale, which enables unique applications. For example, opaque substances become transparent (copper); stable materials become combustible (aluminum); inert materials become catalysts (platinum); insulators become conductors (silicon); solids turn into liquids at room temperature (gold) (Hristozov and Malsch, 2009). NPs can be made of single elements like carbon (C) or silver (Ag) or a mixture of elements/molecules.

The increasing entry of these NPs will inevitably lead to their accumulation in soil, which has raised concerns about their potential adverse effects on soil microbial activity and diversity. Currently very little information is available on how these NPs affect the soil microbial community. They may have an impact on soil microorganisms via a direct effect (toxicity), changes in the bioavailability of toxins or nutrients, indirect effects resulting from their interaction with natural organic compounds and interaction with toxic organic compounds which would amplify or alleviate their toxicity (Simonet

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and Valcárcel, 2009). While toxicity mechanisms have not yet been completely elucidated for most NPs, possible mechanisms include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species (ROS), and release of toxic constituents (Klaine *et al*., 2008). However, close contact is necessary for membrane disruption to occur, and it is unlikely that NPs cross into the cytoplasm although accumulation within the cytoplasm, probably after membrane disruption, is often observed (Neal, 2008). Raghupathi *et al*. (2011) reported that the antibacterial activity of NPs might involve both the production of ROS and the accumulation of NPs in the cytoplasm or on the outer membranes. The NPs also appear to cause structural changes to the microbial cell surface that may eventually lead to cell death (Suresh *et al*., 2010). It is, therefore, apparent that NPs stimulate the production of ROS in organisms and cause damage in possibly every cell component (Bhatt and Tripathi, 2011). Soil quality is defined as the capacity of a soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation (Karlen *et al*., 1997). Among the factors influencing soil quality, biological indicators are reported as critically important (Doran and Zeiss, 2000) because soil organisms directly influence soil ecosystem processes, especially the decomposition of soil organic matter and the cycling of nutrients (Kennedy and Smith, 1995). Therefore, protection of soil microbial biomass and diversity is one of the major challenges for sustainable resource use because greater levels of microbial biomass and diversity mean greater nutrient turnover and disease suppressiveness of the soil (Janvier *et al*., 2007). The opposite being true for a sick soil with low nutrient and carbon reserves and greater levels of contaminants caused by the presence of xenobiotic (humanmade) chemicals or other alterations in the soil environment. Among the xenobiotics are the staggering numbers of new nanoparticles engineered for industrial and environmental applications or formed as by-products of human activity, which are already finding their way into soils (Maurice and Hochella, 2008). While the concentrations of most NPs in the environments still remain unknown, exposure modeling suggests that soil could be a major sink of NPs released into the environment and that NP concentrations in soil are higher than in water or air (Gottschalk *et al*., 2009; Klaine *et al*., 2008; Tiede *et al*., 2009).b The unregulated deposition of metal-based nanoparticles in terrestrial ecosystems particularly in agricultural systems has alarmingly threatened the sustainability of the environment and diversity of beneficial microbial populations such as soil bacteria and fungi. Continuous deposition, low biodegradability, and longer persistence of metal nanoparticles in soils adversely impact the population of soil beneficial bacteria and fungi.

Among the factors influencing soil quality, biological indicators are reported as critically important because soil organisms directly influence soil ecosystem processes, especially the decomposition of soil organic matter and the cycling of nutrients. Hence, any factor that affects soil microbial biomass, activity and populations would necessarily affect soil quality and sustainability. The antimicrobial activity of these NPs has been extensively studied with human pathogenic bacteria. Similarly, studies also exist on the affect of NPs on beneficial microbes *in vitro* under controlled conditions. But very little information is available on how these NPs affect microbial communities in soil. The overproduction, use, and abuse of nanoparticles have rapidly introduced their discharge into various environmental compartments (Gonz´alez-G´alvez *et al*., 2017; Gottschalk and Nowack, 2011). Initially, research was focused on the behavior and impact of the nanoparticles on aquatic systems (Klaine *et al*., 2008; Maurer-Jones *et al*., 2013), however, more recently, penetration of nanoparticles in soil system from various sources and their impact on soil organisms such as plants, bacteria, fungi, and nematodes have been studied (Abbas *et al*., 2020; Ahmed *et al*., 2018a; Lead *et al*., 2018; Rajput *et al*., 2019). Also, the impact of nanoparticles on soil organisms is subject to soil properties and complexity such as its buffering capacity, natural organic matter, aggregation and immobilization of nanoparticles, deposition of nanoparticles, and environmental corona formation around nanoparticles (Zhang *et al*., 2020). In soils, nanoparticles have shown adverse effects on soil fertility (Fayiga, 2017), soil microbiota (Yanga *et al*., 2017), and agricultural crops (Pittol *et al*., 2017). Due to these, the impact of nanoparticles on the growth and physiology of soil microorganisms especially those beneficial for soil and plant health is imperative to be assessed. Beneficial soil microbes like bacteria and fungi play vital roles in maintaining both soil and plant health (Jacoby *et al*., 2017; Lambers *et al*., 2009). These include biogeochemical cycling of nutrients and carbon, detoxification/minimization/degradation of soil contaminants/pollutants, and direct or indirect plant growth promotion by plant growth-promoting rhizobacteria (PGPR) (Kumar and Verma, 2019; Rizvi and Khan, 2018; Wilpiszeski *et al*., 2019). Similarly, soil beneficial fungi (asymbiotic or symbiotic like mycorrhiza) performs nutrient cycling, conversion of soil organic matter in simpler forms thus making them available to plants, beneficially shaping the rhizospheric microbial population, and protecting the resilience and functionality of agro-ecosystems (Hashem *et al*., 2018; Talbot *et al*., 2015.

The few published literature does suggest that among the NPs, fullerenes and their derivatives are less toxic, while small size metal and metal oxide ENPs are detrimental to soil microbial communities. However, under field conditions, soil organic matter and related components like humic and fulvic acids could possibly negate the toxic effects of these NPs through various mechanisms. Also, the resistance and resilience of soil microbial communities to such perturbations cannot be discounted. The paper also stresses the need for more information on interaction of NPs with soil microorganisms under field conditions.

MATERIALS AND METHODS

Titanium Dioxide nanoparticles

Titanium dioxide (TiO_2) nanoparticles were purchased from Sigma Aldrich, Chennai. The TiO2-NPs (80% anatase, 20% rutile) were provided by Sigma Aldrich (St Louis, USA) with a particle size of 21 nm in powder and ≥ 99.5% purity. The aggregated size and surface charge of TiO2-NPs in water were previously characterized by Dynamic Light Scattering (DLS) using a NanoZS (Malvern) 25. the average aggregated size and zeta potential of TiO2-NPs in the ultrapure water spiking suspension were 160} 7.2 nm and − 13.4} 0.5 mV, respectively.

Collection of Soil, Earthworm and Cowdung

Top soil is collected from Periyar University garden not exceeding a depth of five inches. Then the collected soil was sun dried by spreading it on a flat, clean broad surface for 48h. The dried soil was sieved using a 2mm diameter sieve to remove debris adopted by Khan *et al*. (2012) and also was done soil spiking stainless steel spoon method (Doick et al. 2003). Physico-chemical characteristics of soil: pH: 7.2 ± 0.01; EC: 1.8 ± 0.05 (dsm^{-1}); OC: 6.8 ± 0.05 (%); N: 0.3 ± 0.02 (%); K: 0.1 ± 0.01 (%); P: 0.04 ± 0.01 (%); C:N Ratio: 20.3. Vermicompost with Earthworms *Eudrilus eugeniae* were used for the study. These earthworms were purchased from Mani Organic Farm, Salem District, Tamil Nadu, and India and cultured in cement tanks for further studies. The earthworms were reared in garden soil and garden waste in a vermibed of dimension 4 x 2 x 4.4 feet (length x breadth x height) sufficient for 2,000-3,000 worms with a controlled moisture content of 60-70% and temperature between 26 and 28ºC. Nylon net was used to cover the bed to prevent the entry of predators. Adequate watering was done daily to maintain optimal moisture conditions in the vermibed. Cow dung (CD) was collected from a Cowshed in Karuppur, Salem, Tamil Nadu, India. CD was freshly used for further experimentations and Physico-Chemical characteristics of CD: pH: 8.2 ± 0.10; EC: 0.11 ± 0.02 (dsm^{-1}); OC: 4.9 ± 0.15 (%); N: 0.3±0.01 (%); K: 0.008 ± 0.005 (%); P: 0.03 ± 0.004 (%); C: N Ratio: 15.6.

Experimental design

The earthworms were acclimatized to the laboratory conditions for a period of 60 days before the commencement of the experiment. Six circular buckets (33 cm length × 24 cm height) were used for the present study. The circular bucket was weighed with a Digital Sensitive Weighing balance (Model-CG203). The test substrates were prepared according to the ISO guidelines for earthworm toxicity testing (ISO 11268-1, 1993). The soil sample collected was sieved (≤ 5 mm) to remove coarse stones and to homogenize. 1 kg of soil was weighed into a bucket. The soil was made up21 to 60% water holding capacity using deionised water. The soil samples were contaminated with various concentrations of the TiO2 (Table-1). The mixture was thoroughly mixed manually. Furthermore, the buckets were marked as T1, T2, T3 and T4. Soil and TiO2 were taken in the following proportion.

Table 1. Titanium Dioxide concentration in various vermicompost soil sample.

S.No	Treatments	Earthworms *Eudrilus eugeniae-(n)*	Soil (kg)	Cow dung(g)	TiO2 mg/kg
1	T1	10	1	50	0
2	T2	10	1	50	5
3	T3	10	1	50	50
4	T4	10	1	50	500

The above treatments with TiO_2-contaminated soil were left for 10 days in the laboratory exposed to the elements. After 10 days, freshly collected cow dung of about 50g for each treatment was thoroughly mixed into the bucket with TiO2-contaminated soil. Immediately after addition of additives, earthworms were sorted out from the holding containers, washed with clean water and ten earthworms of the species *Eudrilus eugeniae* measured and weighed were inoculated into each container with contaminated soil. A netting material was placed on top of each of the containers and the cover lid frame was used to hold the containers firmly. This is done to avoid escape of the earthworms and to allow free flow of oxygen into the treatment during the course of the experiment. The setup was placed inside the laboratory and checked morning and evening on a daily basis for 21 days.

pH and EC

pH and EC of vermin compost samples were measured using pH (Elico, Model-Li 120) and EC meter (Amber science inc. Model 1056). 20 ± 0.1 g of vermicompost was added in 100 ml of distilled water and then stirred for 10 min and left for 30 min and pH was measured in supernatant liquid using pH meter. For electrical conductivity, the above mixture was left for 1 hour and then measured for conductivity using an electrical conductivity meter.

Analysis

The first step in characterizing bacterial communities in soil is to estimate the viable numbers of microbes present in a sample. This will be accomplished by plating a sample of the soil that has been serially diluted in sterile saline and using the number of visibly growing colonies to calculate the original colony forming units per milliliter of diluted sample plated, or CFU/mL. Identifying the species of cultured bacteria begins with an examination of their macromorphology, or visible appearance of their growth colonies without the aid of a microscope. Then quadrant streak for isolation is performed on one of these colonies. After having a pure culture (only one type of bacteria) a Gram stain, biochemical assays and a genetic analysis of the isolates is done. The purpose of this experiment is to calculate the CFU/mL and describe each of the macromorphologies observed from soil samples taken with four different concentrations of TiO2.

Estimation of total bacteria, Fungi and Actinomycetes in the substrate

One gram of sample was taken into sterilized test tubes with distilled water and mixed carefully using a shaker for 30 min. Furthermore, the mixture was diluted serially and 1-mL aliquots were pour-plated in Nutrient Agar, Rose Bengal Agar and Kenknight's media for estimation of bacteria, fungi and actinomycetes population. Plates were incubated for 24 h (bacteria), 72 h (fungi) and one week (actinomycetes) to count the colony forming units of microbes. Bacteria, Fungi and Actinomycetes are often counted in the laboratory by the viable plate method, where a dilution of the culture is plated onto an agar medium. Following incubation, plates containing 30–300 colonies are counted. This range was chosen to include enough colonies for statistical accuracy, but not too many that colonies compete for nutrients, or that you can accurately count. Counts are then used to calculate the number of colony forming units per ml of diluted culture plated, or CFU/ml.

RESULTS AND DISCUSSION

Microbial toxicity has been reported for metal NPs, like oxides of Ti. These NPs raise serious environmental concerns because of their unique dissolution properties and electronic charges, in addition to their small sizes and large surface-to-mass ratios (Wang *et al*., 2010). Even water suspension of nanosized TiO2 was found to be harmful to varying degrees, with antibacterial activity increasing with particle concentration (Adams *et al*., 2006). They also found that the antibacterial activity generally increased from SiO2 toTiO2 to ZnO, and *B. subtilis* was most susceptible to their effects. Likewise, oxides of Ti NPs have been reported to be toxic to the microalgae *Pseudokirchneriella subcapitata* (Aruoja *et al*., 2009). Because of their size, TiO2 NPs can easily reach the nuclear content of bacteria and they present the greatest surface area; therefore the contact with bacteria is the greatest (Lok *et al*., 2006). This could be the reason why they present the best antibacterial activity. Basically, the smaller size they are, the greater their surface area to volume ratio and the higher their microbial contacting efficiency (Jeong *et al*., 2005; Lok *et al*., 2007; Thiel *et al*., 2007; Wong *et al*., 2010).

Although the toxicity of is reported to be dependent on various factors such as particle size, shape and capping agent, surface charge may also one of the most important factors that govern the toxicity of TiO2 NPs. Possible effects of TiO2 NP include interaction with the bacterial membrane, causing pitting of the cell wall, dissipation of the proton motive force, and finally cell death. TiO2 NP would also bind with bacterial DNA, and this might compromise the DNAs replication fidelity (Rai *et al*., 2009; Yang *et al*., 2009). More interesting is the fact that these metal oxide NPs may act as 'Trojan-Horses',entering cells and releasing ions intracellularly (Limbach *et al*., 2007). In a study to model the quantities of ENPs released into the environment, Mueller and Nowack (2008) found that the predicted environmental concentrations (PEC) values for nano-TiO2 in water are 0.7−16 μg L−1 and close to or higher than the predicted no effect concentrations (PNEC) value for nano-TiO2 (b1 μg L−1). In a review done by Kahru and Dubourguier (2010) to evaluate the currently existing data on toxicity (L(E)C50 values) of synthetic NPs on organism groups representing main food-chain levels (bacteria, algae, crustaceans, ciliates, fish, yeasts and nematodes), TiO2 NP was classified as "harmful", (L(E)C50 10–100 mg L−1). Soil micro-organisms play key roles in immobilization/cycling of nutrients/carbon and detoxification/degradation of contaminants leading eventually to enhanced soil health (Pajuelo *et al*., 2011; Sacc´a *et al*., 2017). Among variously distributed heterotrophic microflora, bacterial populations belonging to different species form about 15% of the total microbial populations (Govindasamy *et al*., 2010) which directly or indirectly improve the plant growth (Etesami and Maheshwari, 2018; Navarro-Torre *et al*., 2016; Shameer and Prasad, 2018). These bacterial populations inhabiting the rhizosphere, generally termed as plant growth-promoting rhizobacteria (PGPR), are competent enough in colonizing plant roots (Zablotowicz *et al*., 1991). The notable PGPR fostering plant growth belongs to genera *Rhizobium*, *Bradyrhizobium*, *Azotobacter*, *Bacillus*, *Thiobacillus*, *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Arthrobacter*, *Acinetobacter*, *Agrobacterium*, *Serratia* etc. (Gopalakrishnan *et al*., 2015; Manzanera *et al*., 2015; Rangasamy *et al*., 2014; Seneviratne *et al*., 2016). Despite such a varied group, only 2–5% of rhizosphere bacteria have been found as potent PGPR (Jha *et al*., 2010; Siddikee *et al*., 2010). Given the importance of PGPR to plant health, the interactions of NPs-PGPR are crucial (Mesa-Marín *et al*., 2018). Similar to the other xenobiotics, the negative effect of NPs on soil beneficial microbes is gradually emerging and still not well understood. Hence, the assessment of NPs-bacteria interactions is also imperative due to the increasing release of nano-enabled agricultural products such as nanoparticle-based pesticides, fertilizers, and herbicides (Duhan *et al*., 2017; Wagner *et al*., 2016). For instance, direct entry of Fe-NPs and TiO2-NPs used in environmental remediation and water treatment inhibit and stimulate the growth of target organisms (Lecoanet and Wiesner, 2004; Mueller and Nowack, 2010; Yavuz *et al*., 2006). Whereas, at the same doses Fe-NPs and TiO2-NPs also exert toxicity to non-target microbes and other biological entities. On the contrary, nZVI exerted only adverse effects on soil microorganisms (Cullen et al., 2011). Some other NPs such as ZnO-NPs, CuO-NPs, Ag-NPs, FeO-NPs, and TiO2-NPs have shown variable chronic and acute toxic effects on pure microbial cultures and soil microbes (Table 1). Other factors influencing NPs-bacteria interaction include size, surface charges, capping agent and the presence of divalent anions/cations, the composition and charge of the bacterial cell wall (Acharya *et al*., 2018; Sondi and Salopek-Sondi, 2004).

Table 2. Bacterial, Fungal and Actinomycetes population during the experimental period.

Microbial Population		T1	T2	T3	T4
Bacteria (CFU×10^4 g^{-1})	Initial	24.08±0.12	24.65±0.05	25.16±0.65	25.98±0.01
	Final	36.87±0.04	35.76±0.07	35.97±0.21	31.87±0.05
Fungi (CFU×10^3 g^{-1})	Initial	28.09±0.01	28.45±0.56	28.31±0.08	27.45±0.02
	Final	43.76±0.04	41.18±0.02	41.98±0.01	36.43±0.08
Actinomycetes (CFU×10^4 g^{-1})	Initial	17.87±0.02	18.54±0.03	18.98±0.01	18.12±0.09
	Final	29.76±0.06	28.34±0.02	28.62±0.06	24.15±0.02

While there is little doubt on the toxicity of NPs to microorganisms the issue that raises serious concern is their toxicity to microorganisms that promote plant growth and those that benefit nutrient cycling in soils. Plant growth promoting rhizobacteria (PGPR) like *P.aeruginosa*, *P. putida*, *P.* f*luorescens*, *B. subtilis* and soil N cycle bacteria *viz.*, nitrifying bacteria and denitrifying bacteria have shown varying degrees of inhibition when exposed to NPs in pure culture conditions or aqueous suspensions (Mishra and Kumar, 2009). These released amounts in the environment may pose severe toxicological consequences. Additionally, the lack of knowledge regarding NPs physicochemical properties limits the development of systematized approaches for the analysis of NPs in complex environmental media. A massive NPs production will inevitably lead to their accumulation in the environment. For example, the TiO2-NPs were reported to accumulate in the environment via urban and industrial effluents (Ottofuelling *et al*., 2011). Furthermore, up to 75 and 100 µg L The use of NPs in personal grooming products also poses significant environmental implications (Keller *et al*., 2013; Keller and Lazareva, 2013). By 2014, from the use of personal care products in the United States, TiO2-NPs, with 0.87–1.0 × 103 metric tons/year and ZnO-NPs, with 1.8-2.1 × 103 metric tons/year, representing 94% of NPs were discharged into the landfills and environment. Among them, 36-43% of NPs from personal care products (PCPs) were estimated to be discharged in landfills, 0.7-0.8% to air, 28–32% to water bodies, and 24-36% released to soils system. The NPs of ZnO and TiO2 as ultraviolet blocking agents in sunscreen represent around 81-82% of the total discharge, followed by facial moisturizer (7.5%) and foundation (5.7%) (Keller *et al*., 2013). In particular, TiO2-NPs, owing to its huge demand in nanotechnology industries and regular consumption in everyday life, such as cosmetics, pharmaceuticals, food additives, and paints are frequently discharged into the local environment (Frazier *et al*., 2014). An inventory of NPs-enabled product applications in Woodrow Wilson Database (2016). suggested that > 1814 nano-enabled products have been manufactured and projected to increase three times by the end of 2020 (www.nanoproduct.org/inventories/consumer). However, > 8800 nanotechnology-based products are now in the market place from 60 countries and > 2300 manufacturers (Figure 4). The increased applications of nano-enabled products, however, may result in unintentional deposition of NPs in the environment through various routes with unknown impacts on water, soil, and biota (Eduok and Coulon, 2017). At the end of the product life cycle, it is unlikely that NPs will remain bound to the products (Cao and Liu, 2016). For example, empirical evidence shows that NPs are present in wastewater effluents (Azimzada *et al*., 2017; Jiang *et al*., 2018), sewage sludge (Wigger *et al*., 2015), and landfill leachates (Hennebert *et al*., 2013). In contrast, 55% of sewage sludge is used in agriculture and soil amendment, whereas, 25% is used in thermal energy generation (25%). Due to these, the use of wastewater may act as a primary entry point of aged-NPs input into the environment (Eduok and Coulon, 2017). Apart from sewage sludge, the use of nano-based pesticides/fertilizers in agriculture to effectively control the growth of plant pathogenic microbes and hence, to optimize plant growth/yields have also been the major source of NPs in soil ecosystems (Dwivedi *et al*., 2016; Mukherjee *et al*., 2016).

Once in the environment as aerosolized sprays, dry powders, associated with biosolids/effluents/waste, the soil system becomes a major sink foraged as well as pristine NPs (Cornelis *et al*., 2014; Ju-Nam and Lead, 2016; Keller *et al*., 2013). Though the natural concentrations of NPs in soils are low the inadvertent release of engineered NPs and the continuous use and abuse increase their concentration in different environments (Nowack *et al*., 2015). Out of the total production, 63-91% of NPs end up in landfills worldwide (Figure 3), which paves the way for their release in water, soil, and atmosphere (Rizwan *et al*., 2017). In soil, the major route of NPs entry includes the use of NPs based agrochemicals and NPs used in soil remediation. Also, NPs are used as additives in pesticides to enhance the solubility of essential ingredients or to protect its ingredients from premature degradation (Chhipa, 2017). Apart from these, the accidental transport of NPs from other environmental compartments also adds NPs to the soil system. Due to the deposition of NPs in soils, the interaction between metal-based NPs and soil microorganisms is certain which may affect the soil beneficial bacteria and fungi adversely. Therefore, it becomes imperative to assess the overall impact of NPs on beneficial soil bacteria and fungi.

Jiang *et al*. (2009) reported that the TiO2–NPs (50 nm) without photo-activation at a dose rate of 418 mM were non-toxic to bacteria. In contrast, glass surfaces coated with photo-activated TiO2-NPs (5.6 nm) resulted in a more than 99% reduction in viability of *P. putida* biofilms (Jalvo *et al*., 2017). Recently, the impact of TiO2-NPs on PGPR metabolism in soils was examined and observed the enhancement in the growth of *T. aestivum* when inoculated with *Paenibacillus polymyxa* A26, *Alcaligenes faecalis*, *Bacillus thuringiensis* AZP2 and a mutant strain of *P. polymyxa* A26Dsfp alone or in different combinations (Timmusk *et al*., 2018). The effects of TiO2-NPs and PGPR on drought, salt, and pathogen responses of wheat were also assessed simultaneously. Based on the accumulation of shoot biomass of wheat, it has been suggested that TiO2-NPs can enhance the growth of PGPR when plants are co-inoculated with *P. polymyxa* A26, *B. thuringiensis* AZP2, or *A. faecalis*. However, no growth enhancement was reported under TiO2-NPs exposure when plants were raised in the sand (Timmusk *et al*., 2018). The symbiotic bacterium *R. leguminosarum* bv. viciae 3841 was also affected severely by TiO2-NPs that resulted in morphological damage. Moreover, the symbiotic relationship between *R. leguminosarum* and *P. sativum* was disrupted by TiO2-NPs. As a result, nodule formation and subsequent nitrogen fixation were impeded. Further, a systemic response in the host (*P. sativum*) was initiated and the polysaccharide composition of nodules was altered (Fan et al., 2014). Also, TiO2-NPs impact plant growth by reducing the quantity of secondary lateral roots (Fan *et al*., 2014). Similarly, in *Trifolium pretense*, TiO2-NPs reduced nitrogen fixation by an endosymbiont (*Rhizobium trifolii*) maximally by 54% (Moll *et al*., 2016).

The cellular toxicity of nanoparticles to beneficial strains of soil bacteria generally depends on the shape, size, chemical composition, and concentration of nanoparticles, and time of exposure. In due course, the bacterial cells may become partially or fully resistant to some nanoparticles but no to all. For example, four plant growth-promoting rhizobacterial species *Bacillus thuringiensis*, *Pseudomonas mosselii*, *Azotobacter chroococcum*, and *Sinorhizobium meliloti* tolerated up to 3000 µg/ml dose of CuO, TiO2, and Al2O3 nanoparticles, however, these bacteria were found sensitive towards < 1500 µg/ml of Ag and ZnO nanoparticles (Ahmed *et al*., 2020). This could be because the release of free metal ions from CuO, TiO2, and Al2O3 nanoparticles was negligible. Besides, some other reasons for tolerance could be (i) evolution of new defense mechanisms, (ii) structural changes in cell envelope (Sedlak *et al*., 2012), (ii) beneficial physiological alterations such as accumulation of electron-dense particles or granules at subcellular locations (Woo *et al*., 2008), (iii) pumping out of the metal ions released from nanoparticles by membrane-embedded efflux pumps (Salas Orozco *et al*., 2019), and (iv) point mutations (Tripathi *et al*., 2017). On the other hand toxicity of Ag and ZnO nanoparticles could be due to the following reasons: (i) loss of bacterial cell respiration (Choi *et al*., 2008) and (ii) oxidative damage and cellular disintegration (Zhang *et al*., 2018). The nanoparticles prepared from Ag and ZnO also enhanced surface roughness in beneficial bacteria, reduced the bacterial adherence on a solid surface, extracellular polymeric substance (EPS), and bacterial colonization as compared to untreated control (Ahmed *et al*., 2020). Similarly, the toxicity of AgNPs has been reported on two beneficial rhizobacteria *Azotobacter vinelandii* (Zhang et al., 2018) and *Bacillus subtilis* (Gambino *et al*., 2015). Apart from destructing the cell morphology and number of viable cells, nanoparticles also inhibit the production of some bioactive molecules vital for plant growth and soil fertility. As an example, the production of indole-3-acetic-acid by three PGPR strains was reduced by ZnO and Ag nanoparticles in a concentration-dependent manner which was completely abolished at a concentration of 1000 µg/ml. In similar experiments, heavy metals reduced the secretion of indole-3-acetic-acid by a symbiotic and nitrogen-fixing bacteria *Bradyrhizobium japonicum* which was found highest at 500 µg/ml (Seneviratne *et al*., 2016). This reduced synthesis of indole-3-acetic-acid could be due to slow bacteria growth and altered physiology under nanoparticle stress.

A plethora of literature embodied the significance of fungi in soil health (Frac *et al*., 2018). Herein, the overall soil health instead of soil quality is important which defines soils' capacity to sustain biological productivity and plant health (Doran, 2002). Researchers have endorsed the role of fungal diversity in improving soil health, crop productivity, and overall improved agricultural system (Nielsen *et al*., 2015). Despite being only consumers of energy, fungi play a crucial role in the maintenance of terrestrial life through their widespread activity on and in the soil. Soil fungi are typically the pioneer colonizers of dead plant tissues breaking-up and decompose the dead vegetation on and within the soil. Indeed, fungi are naturally endowed with the ability to decompose dead materials due to their physical organization into a network of mycelium, composed of branching, rigid tubes (hyphae), filled with protoplasm. Thus, fungal population with simultaneous coordination of other soil organisms decomposes the soil organic matter and makes the nutrients available for plant growth. This role becomes so important when it comes to the safeguarding of crops against pathogenic microbes. For example, arbuscular mycorrhizal fungi (AMF) are the most important class of beneficial microorganisms in agri- and horticultural soils (Gosling *et al*., 2006) exhibiting significant increases in plant's performance and crop yield, by improving the rooting, nutrient cycling, stress tolerance, and uptake of ions

(Azc´on *et al*., 2009). Besides these, some antagonistic fungi such as *Glomus* sp. or *Trichoderma* sp. suppressing fungal pathogens to protect the crops plants from plant diseases (Dawidziuk et al., 2016). Also, some biostimulants or biocontrol formulations contain *Trichoderma* sp. (*T. asperellum, T. atroviride, T. harzianum, T. virens,* and *T. viride*) frequently for horticulture crops (Guzm´an-Guzm´an *et al*., 2019). Beyond this, the recent developments within the landscape of plant growth promotion and protection underscore the significance of nanotechnology in traditional agricultural practices. Concisely, nanoparticles (NPs) based formulations/strategies offer a diverse set of magnificent application such as nano-fertilizers, nano-pesticides, NPs (TiO2NPs, SiO2NPs, and CNTs) based nano-carriers for targeted delivery and controlled release of agrochemicals and nano-sensors programmed to detect biotic/abiotic stresses in plants have revolutionized the existing agricultural systems (Fraceto *et al*., 2016; P´erez-de-Luque and Carmen Hermosín, 2013). The nano-agro-chemicals/-agro-formulations are devised for crop growth promotion and protection while mitigating the undesired wastage and environmental pollution. The NPs based growth stimulators are potentially more efficient as compared to their conventional analogs (Fraceto *et al*., 2016). The exposure of Ag-NPs, TiO2-NPs, and ZnO-NPs to soil has resulted in mixed responses of plant growth according to experimental conditions such as the type, size, and dose of NPs (Ahmed *et al*., 2018b; Hatami *et al*., 2016). In parallel, the antimicrobial applications of these NPs against a wide range of bacteria and fungi are acknowledged adequately. Owing to their size and type, NPs can penetrate fungal hyphae to deform and damage native morphology. However, NPs exploited for plant growth promotion showed controversial opinions and which makes it crucial to evaluate the potential impact of nanomaterials/nanoformulations on key plant-microbe symbioses such as mycorrhizas and rhizobia. The interaction between various NPs and mycorrhizal fungi was found to influence its growth and showed both positive and negative effects, which are vital for the health, functioning, and sustainability of both natural and agricultural ecosystems. Some types of NPs help in the colonization of fungi, whereas some negatively affect the colonization. Therefore, it is necessary to establish understandings of the possible mechanism of interaction between fungi.

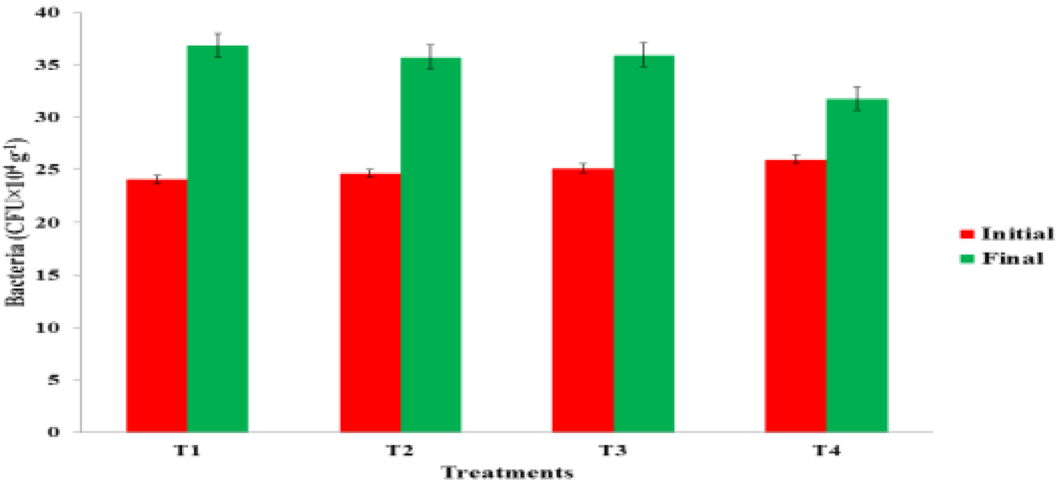

Figure 1. Bacterial population during the experimental period**.**

The primary research published on the direct exposure of metal NPs witnessed both positive and adverse effects in soil fungi. The classical example of the influence of various metal NPs on the growth and plant growth-promoting activities of soil fungi is arbuscular mycorrhizal fungi (AMF). Concisely, both groups i.e., endo-mycorrhizal and ectomycorrhizal of AMF are found associated with over 90% of higher plants (shrubs and most herbaceous plants) as root colonizers and developed a symbiotic or mutualistic association called "mycorrhiza" (Sharma and Sharma, 2014). AMF is also known as vesicular-arbuscular mycorrhizae (VAM) due to their hyphal insertion into the cell wall and development of branched structure (i.e., arbuscules) within the cortical root of the host plant. The plants inoculated with AMF exhibit increase resistance to the fungal root-rot disease (George *et al*., 2016) and significant elevation in nutritional uptakes such as absorption of phosphorus (P) and other nutrients that are relatively immobile and available in low concentration in the soil (Begum *et al*., 2019). On one hand, a diverse array of metallic NPs has been reported to have a great influence on the growth of soil fungi beneficial for plant growth. To date, various metallic NPs based formulations such as nano-herbicides, nano-pesticides, nano-fertilizers (Iavicoli *et al*., 2017; Makarenko and Makarenko, 2019) and as a vehicle for the target-specific delivery in plants have been used and explored their role in plant growth promotion. The evaluation of comparative dose-dependent biological effects of nano and bulk forms nanoparticles on AMF mycorrhizal clover (*Trifolium repens*) has been reported (Feng *et al*., 2013). Results demonstrated significant positive effects on AMF colonization/infection to enhance plant growth-promoting activities; however, their bulk counterparts did not show AMF colonization. On the contrary, Cao *et al*. (2017) observed a significant decrease ($p < 0.05$) in AMF growth and ecological function at NPs exposure which was characterized by root mycorrhizal colonization rate, soil alkaline phosphatase activity, available phosphorus (P) content, and P nutrition in plants. In this line, a study shows a significant percentage reduction in mycorrhizal colonization observed at the NPs (2 nm) (Noori *et al*., 2017).

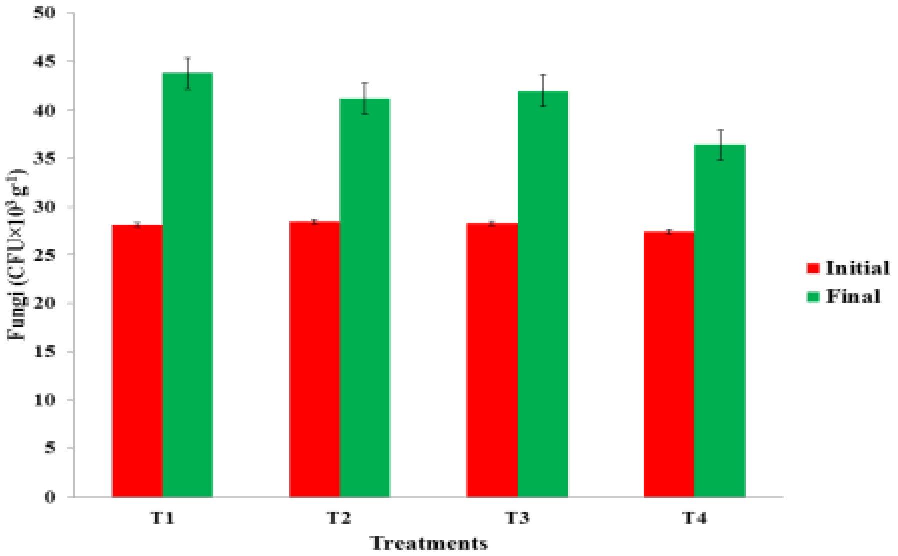

Figure 2. Fungal population.

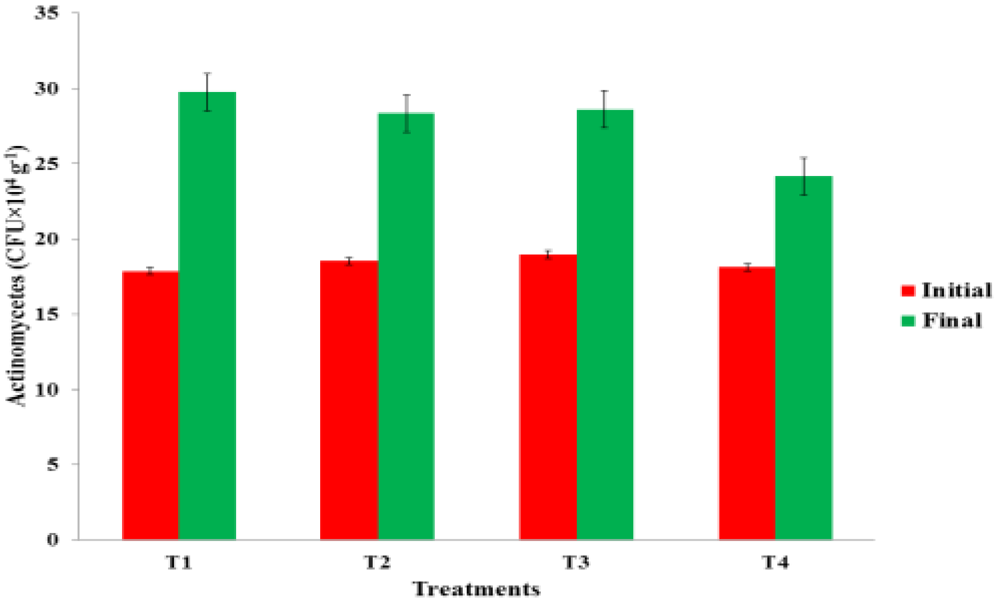

Figure 3. Actinomycetes population.

The term bioremediation refers to the elimination or decomposition of contaminants, pollutants, or unwanted substances/products from the environment (e.g., soil and aquatic) by living organisms e.g., microorganisms. In this line, filamentous fungi are an excellent example that possesses the intrinsic ability to metabolize complex lignin and the polysaccharide components of wood and litters in both surfaces and beneath the soil. Besides, various lignin-degrading white-rot fungi such as *Phanerochaete chrysosporium, P. sordid*, and *Trametes hirsute* have been reported to deplete soil contaminants such as pentachlorophenol (PCP) and creosote (Lamar *et al*., 1994, 1993). Precisely, *P. chrysosporium* is a member of the white-rot fungi group and well recognized for its cellulolytic applications therefore employed extensively for bioremediation of lead-contaminated soil and degradation of various xenobiotic compounds (Huang *et al*., 2018a; Yildirim *et al*., 2011). Thus, the bioremediation applications of white-rot fungi are also applied in the decontamination of various pollutants (Hatakka, 1994). On the other hand, nanoparticulate release from the NPs based agrochemical formulations directly interact with the lignin-degrading fungi and alter their metabolic activities. In this context, various researchers have focused to evaluate the toxicological effects and interaction of metal NPs with white-rot fungi. A recent study demonstrated low-level toxicological effects in white-rot fungi, which augmented due to direct accumulation of nanoparticles in fungus balls by break of the cell wall and the loss of cytoplasm (Ma *et al*., 2020). The uptake of NPs reduced the ligninolytic enzyme production affecting the mechanism of decomposition, including laccase (Lac), manganese peroxidase (MnP), and ligninase in white-rot fungi. Andries *et al*., (2016) have shown that *P. chrysosporium* protects itself from reactive chemical species produced by light-sensitive AuNPs stressors. In brief, the results of the study revealed the enhanced enzymatic activities e.g., catalase, superoxide dismutase, lipid peroxidase, and malondialdehyde enzymes, when AuNPs treated *P. chrysosporium* exposed to white, blue, green, and yellow light wavelengths. Recently, Du *et al*., (2020) have conducted an experiment to investigate the ramifications of NPs exposure on the aquatic fungi community associated with leaf litter decomposition. The exposure of NPs to the fungi community reduced the litter decomposition rate significantly. This reduction in litter decomposition rate likely occurred due to NPs inducing suppression of activities of N-acetylglucosaminidase, glycine-aminopeptidase, aryl-sulfatase, polyphenol oxidase, and peroxidase .

Our pyrosequencing data demonstrate that nano-TiO_2 induced soil bacterial community shifts through either direct toxicity or indirect effects on some sensitive taxa shows that these NPs can reduce some specific soil bacterial populations. Since these data were counts determined from a constrained number of sequences, for one species to decrease in this data set, others would necessarily increase. However, as there were many taxa that could respond a significant response at the taxon level would suggest a strong enough specific effect that it would likely have been due to a distinct target mechanism. As the overall biomass and activity of this community declined as a result of the NPs, significant negative responses are most likely due to toxicity. Increases could result from a specific enhancement due to either the NP or a release from competition from taxa that were repressed. Previous studies also demonstrated distinct effects of nano-TiO_2 on phyllosphere microbial communities, activated sludge bacterial communities, and microbial biofilms in stream microcosms, which partially support our observations in the terrestrial system studied here. The potential mechanism could be the direct toxicity of NPs on soil bacteria through the release of metal ions or attachment-related cell damage. NPs may also indirectly affect soil bacteria by changing nutrient availability or the bioavailability of co-occurring contaminants and by changing physical properties of the soil due to their large surface area and high reactivity. Further research is needed to partition the relative importance of different factors in influencing soil microbial communities. Previous studies have found significant correlations between quantitative PCR and pyrosequencing-based abundance estimates, and the relative abundance has been used in characterizing the relationship between environmental gradients and bacterial responses.

A number of taxa susceptible to nano-TiO_2 exposure were identified, and the slopes of dose-response curves varied among taxa. Considering that nano-TiO_2 reduced total soil microbial biomass, taxa that declined in relative abundance almost certainly declined in absolute abundance, but it is hard to evaluate the changes in absolute abundance of those that increased their relative abundance. They may have actually increased in absolute terms, remained unchanged while other organisms declined, or even declined but to a lesser degree than other taxa, thereby increasing as a proportion of the community. Notably, some of the taxa sensitive to metal oxide NP exposure are known to be functionally significant in organic matter decomposition, N_2 fixation, and methane oxidation, indicating that specific ecosystem processes carried out by such bacteria may have changed. For example, the relative abundance of the family *Sphingo monadaceae*, known as a decomposer of recalcitrant organic pollutants, increased in the presence of both nanoparticles. It has been reported that *Sphingomonas paucimobilis* maintained a higher population density in hexachlorocyclohexane-contaminated soil than in uncontaminated soil, which may indicate the relative success of this genus in contaminated environments. The family *Strepto mycetaceae* and the genus *Streptomyces* also responded positively to both nanoparticles. Bacteria in these taxa metabolize biopolymers including protein, cellulose, chitin, and lignocellulose. The order *Rhizobiales*, the family *Brady rhizobiaceae*, and the genus *Brady rhizobium*, which contain symbiotic N_2-fixing bacteria, declined in response to nanoparticles. Thus, these ENPs could interfere with symbiotic N_2 fixation in exposed legume crops, such as soybean (an important food crop). The family *Methylo bacteriaceae*, which contains methanotrophs and other taxa that use one-carbon compounds as their sole source of carbon and energy, decreased in response to both nanoparticles. Methanotrophs are vital for methane oxidation to CO_2 and hence contribute to reducing methane emissions from terrestrial ecosystems.

The association of nanoparticles with biomass is likely to occur in one or two steps. In the first step, the nanoparticles are adsorbed to bacterial surfaces. Some previous works suggested that the phenomenon is driven by electrostatic attraction . However, up to the present time, the specific mechanism(s) responsible for the adsorption of nanoparticles to bacterial surfaces is still unknown. After the adsorption of nanoparticles to the cell surface, a possible second step is the uptake of nanoparticles into the cell. Many mechanisms such as passive diffusion or facilitated transport across an intact membrane or diffusion across a disrupted membrane may play a role in this step. Ge *et al*., (2011) have reported that metal oxide NPs may measurably and negatively impact soil bacterial communities. They exposed a grassland soil to different doses of NPs of TiO2 (0, 0.5, 1.0, and 2.0 mg g^{-1} soil) and ZnO (0.05, 0.1, and 0.5 mg g^{-1} soil) in microcosms over 60 days. They found that NPs reduced microbial biomass (as indicated by declines in both substrate induced respiration, SIR and total extractable DNA) and bacterial diversity and composition (as indicated by T-RFLP analysis).This is well supported by the findings of Du *et al*., (2011). They studied the effects of TiO2 (10 g in 110 kg soil) on wheat growth and soil enzyme activities under field conditions and found that these NPs did not affect urease activity but significantly inhibited soil protease, catalase, and peroxidase activities which suggested that these NPs themselves or their dissolved ions were clearly toxic for the soil ecosystem.

CONCLUSION

The unregulated deposition of metal-based nanoparticles in terrestrial ecosystems particularly in agricultural systems has alarmingly threatened the sustainability of the environment and diversity of beneficial microbial populations such as soil bacteria and fungi. This occurs due to the poor treatment of biosolids during wastewater treatment and their application in agricultural fields to enhance the fertility of soils. Continuous deposition, low biodegradability, and longer persistence of metal nanoparticles in soils adversely impact the population of soil beneficial bacteria and fungi. In recent years the behavior and properties of nanoparticles released to the environment have been studied extensively to better assess the potential consequences of their broad use in commercial products. The fate, transport and mobility of nanoparticles in soil were shown to be strongly dependent on environmental conditions. However, little is known about the possible effects of nanoparticles on soil chemical, physical and biological properties. In this study, the effects of the nanoparticles on various soil microorganisms were assessed. The nanoparticles affected the soil bacterial community composition, based on denaturing gradient gel electrophoresis (DGGE) fingerprinting, but had little impact on the macroscopic properties of the soil. Increased application of nanoparticles threatens communities as well as plants, terrestrial and aquatic animals. Thus, it is important to explore whether nanoparticles could compromise soil biodiversity and the important functions maintained by soil communities. TiO2-NPs impose strong perturbations of the nitrogen cycle and a modification of the bacterial community structure in an agricultural soil, even at low realistic concentration (1 mg kg^{-1} dry soil). Surprisingly, the two TiO2-NPs concentrations used (1 and 500 mg kg^{-1} dry soil) resulted in similar effects on the soil microbial activities and AOA abundance. Non classical dose-response seems to be rather common with NPs and has been observed several times on soil microbial activities. The current hypothesis is that the NPs homo- and hetero-aggregation processes (i.e. the aggregation of NPs with themselves and the aggregation of NP with other environmental constituents) vary according to NP concentration at time of exposure, resulting in variable NP bioavailability and toxicity for microorganisms. Moreover, the initial particle size, coating and phase composition can affect NPs reactivity and aggregation. Therefore, further research on the physicochemical properties of NPs in soils as it relates to the applied concentration is necessary to clarify this assumption. No functional resilience was observed during the time course of the experiment, which raises concerns about the ecotoxicity of TiO2-NPs in soils. The greatest effects of TiO2-NPs appeared 90 d after the exposure suggesting that aged NPs can affect microorganisms even at low concentrations and after a long exposure. This should be considered with regards to transport experiments suggesting that TiO2-NPs exhibit a low mobility in soils and would have a long residence time in this ecosystem. Most studies to date are based on shorter incubations no longer than 60 d and simulate exposures to exceptionally high NPs concentrations (> 100 mg kg−1). Therefore, our results demonstrate that shorter-term experiments may not accurately reflect the toxic potential of NPs in soil over the long term, suggesting that further research should be conducted under more realistic NP concentrations and assessed over longer periods.

Nanomaterial based sustainable agricultural approach largely relies on the compatibility of integration between nanotechnology and agriculture. To date, several NPs based agro-chemicals/-formulations including nanofertilizer, nanopesticide, nanoherbicide, nanosensor have offered potential applications in the sustainable agriculture landscape (Campos et al., 2014; de Oliveira et al., 2014; Grillo et al., 2016; Salamanca-Buentello et al., 2005). Ultimately, NPs introduced in the environment in these ways are accumulated in the soil and affect the soil characteristics for native inhabitants. Thus, it is often argued that this integrated approach, i.e., agri-nanotechnology has several limitations to get accepted in each aspect including transport, bioavailability, and toxicity of NPs. In the present context, agricultural scientists are taking attempts to fill the gaps in the existing knowledge of agri-nanotechnologies answering the controversies regarding the interaction of NPs with crucial components of agro-ecosystem such as plant, soil, and soil biota. Since the last decade, a plethora of research literature published focused on the direct impact of NPs on soil microbial community structure (H¨ansch and Emmerling, 2010; Simonin and Richaume, 2015). In this context, a comprehensive yet interesting report was published highlighting the TiO2 and ZnONPs induced alteration in two key player soil bacterial communities i.e., *Rhizobiales, Bradyrhizobiaceae,* and *Bradyrhizobium* (related to nitrogen fixation) and *Sphingomonadaceae* and *Streptomycetaceae* (related to decomposition process of organic pollutants and biopolymers). Precisely, Ge et al. (2012) had observed in DNA-fingerprinting analysis that there was an apparent reduction in *Rhizobiales, Bradyrhizobiaceae,* and *Bradyrhizobium* and escalation in *Sphingomonadaceae* and *Streptomycetaceae* bacterial taxa at dose-dependent manner in response to TiO2.

Nano-TiO_2 altered soil bacterial community diversity, including abundances of specific functional groups, but it is possible that over the long term such community changes could reverse, for example, if the pressures driving the community shift are removed. However, in a somewhat comparable study, microbial communities in soils experimentally polluted with various heavy metals were different from those in uncontaminated soil 34 months after the exposures. Another study showed that microbial communities did not recover even 12 months after the metal stress was removed. Both studies suggested that some ecological niches might have been taken over by tolerant species and that pollution-induced community shifts could be preserved for a long time. Since most NPs are chronically released into the environment and are not biodegradable, such materials are likely to accumulate in soil and thus could cause long-term effects. The study revealed substantial shifts in bacterial community composition in soils amended with TiO2 . The published literature suggests that among the NPs, the antimicrobial activity of metal NPs to soil microbial communities holds great significance. Though such negative effects of pollutants, especially heavy metal contamination on microbial activity, biomass and diversity in soil have been amply demonstrated (Gremion et al.,2004), little information is available on how metal NPs act in the soil matrices especially their adsorption to clay minerals, organic fractions,toxic substances, organic pollutants etc. Such interactions between organic pollutants and NPs may result in a pollutant with increased toxicity or reduced toxicity or anything in-between. However, if the microbial cells adsorb the ENPs containing the adsorbed pollutant a toxic effect may result from the pollutant, the NPs or from both (Nowack and Bucheli, 2007). Also, one of the main drawbacks of current investigations is the lack of information on transformations of NPs in soil and detection in the presence of natural NPs like nano-clays, minerals, oxides and hydroxides of Al, Fe, and Mn, enzymes, organic fractions like humic substances, viruses and mobile colloids. This highlights the need for more information on interaction of NPs with soil components and more quantitative assessments of aggregation/dispersion, adsorption/desorption, precipitation/dissolution, decomposition, and mobility of NPs in the soil environment (Klaine et al., 2008). Mobility in soil is dependent on the size of the NPs, although it is the agglomerate size, not the primary size that is correlated with transportability. Many factors influence the mobility of NPs in the soil, but size, charge, and agglomeration rate in the transport medium are predictive of NP mobility in soil (Darlington *et al*., 2009). The existence and speciation of metal NPs in soil solution and knowledge on the interaction between their active sites and soil solution or other ions are essential for a better understanding of the interactions between metal NPs and microorganisms in the soil. However, the solution chemistry of metal NPs is quite limited and thermodynamic data such as solubility and reaction constants of NPs are unavailable.

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