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Bio-safety assessment of nanozeolites of varying size and doses on soil beneficial microorganisms

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Abstract

Aim: To evaluate the toxic effects of nano-zeolites on soil beneficial microorganisms.

Methodology: This study involved dry milling of zeolites at varying parameters to obtain dry-milled zeolites of four size regimes such as 10-100 nm, 200-400 nm, 500-1000 nm and 1000-2000 nm that were tested at incremental concentrations such as 100 ppm, 1000 ppm and 2000 ppm along with control on various soil beneficial microorganisms. Three replications were done for each treatment with factorial completely randomized design. The poison food technique, growth curve assessment followed by cytotoxicity and genotoxicity studies on all four bacterial genera treated with zeolites of varying sizes and doses were undertaken. The effects of zeolite on average linear growth rate (ALGR) of biocontrol agent, *Trichoderma viride* were also studied.

Results: The effect of zeolites tested on four bacterial genera viz. *Azotobacter chroococcum*, *Rhizobium leguminosarum*, *Bacillus megaterium* and *Pseudomonas fluorescens* using poison food technique and growth curve revealed that zeolites regardless of size or concentration had positively influenced the growth dynamics of all four bacteria tested. The effect of zeolite on average linear growth rate (ALGR) of *Trichoderma viride* also indicated that incremental dose of zeolite had a positive effect. Lactose dehydrogenase revealed that 2000 ppm nano-zeolite exhibited cytotoxic effects on soil beneficial micro-organisms tested. On the other hand, comet assay demonstrated no quantifiable DNA damage in nano-zeolite treated cells in comparison to control cultures.

Interpretation: This study unequivocally demonstrated that zeolites of size greater than 200-400 nm, irrespective of doses even up to 2000 ppm are quite safe for soil beneficial microbes.

Key words: Bacteria, Comet assay, Cytotoxicity, Nano-zeolite, *Trichoderma viride*

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Introduction

Interventions in the size regime of 'nano' are being exploited in varied sectors for resolving several issues that cannot be achieved either through conventional methods or by bulk materials (Subramanian and Tarafdar, 2011). High surface area to mass ratio is a unique property that a material acquires when converted into nanomaterials. This characteristic feature indicates changes in other properties such as electrical, optical, mechanical etc., that makes the nanomaterials best candidates for novel applications (Vuong and Do, 2015; Kolhatkar et al., 2013). Naturally occurring minerals like zeolites are promising material that are employed in a wide range of application. Zeolites are naturally occurring crystalline aluminosilicates with unique properties such as large surface area, defined pores and structures, high thermal and mechanical resistance with tunable chemical composition and most importantly, they are recoverable and reusable (Chen et al., 2012; Mintova et al., 2013). The uniqueness of zeolite comes from its ordered three-dimensional structures with microporous channels of interconnected tetrahedral, comprised of Al, Si and oxygen atoms.

The general formula of zeolite is $\text{Me}_{2n}\text{O} \cdot \text{Al}_2\text{O}_3 \cdot x\text{SiO}_2 \cdot y\text{H}_2\text{O}$ (Bogdanov et al., 2009). Agricultural scientists find nano-zeolites as a promising material for the development of better soil conditioners, growth medium in nurseries and nano fertilizers (Subramanian and Sharmila Rahale, 2012; Subramanian et al., 2015; Subramanian and Thirunavukkarasu, 2017; Suratman et al., 2020). Nano zeolites are widely researched in several fields including medicine (Derakhshankhah et al., 2020) and hence, large quantities are likely to be produced in near future. Though nanoparticles in general exhibit novel properties as a result of increased surface area and reactivity, it is this transformation that is also a cause for concern owing to increasing reports of toxicity pronounced by several nanomaterials (Rajkishore et al., 2013). Thus, this study was focused on assessing the effect of size reduced zeolites at varying concentrations on growth, cytotoxicity and genotoxicity of four beneficial bacteria and average linear growth curve of *Trichoderma viride*.

Materials and Methods

Experimental site and source materials: This investigation was undertaken at the Department of Nano Science & Technology, TNAU, Coimbatore to assess the biosafety of nano-zeolites on soil beneficial microorganisms. Zeolites used for the study was fine textured naturally occurring (Clinoptilolite) that was obtained from GM Chemicals, Ahmedabad and the beneficial microorganisms (*Trichoderma viride*, *Bacillus megaterium*, *Pseudomonas fluorescens*, *Rhizobium leguminosarum*) were collected from Microbial Type Culture Collection and Gene bank (MTCC), Chandigarh and *Azotobacter chroococcum* was obtained from the Department of Microbiology, TNAU, Coimbatore.

Synthesis and characterization of nano-zeolite by ball milling: Nano-zeolites were synthesized using ball milling, a top-

down approach (Mechanical synthesis). Process was optimized and our developed standard operational protocol was adopted (Sivashankari et al., 2019). Ball milling was performed by utilizing the planetary ball milling (FRITSCH, Germany, Model: Pulverisette 7) available at the Department of Nano Science and Technology, TNAU, Coimbatore. Ball milling at 400 rpm for 5 hrs produced nano-zeolite under the size regime of 10-100 nm. Particle size was observed using Nano Particle Size Analyzer (Model: HORIBA-SZ-100), Scanning Electron Microscope (FEI, Quanta 250) and Transmission Electron Microscope (FEI Technai Spirit).

The particle size distribution of zeolite milled at 2 hr, 3 hr, 4 hr and 5 hr were in the range of 300-550, 200-400, 100-200 and 10-100 nm, respectively. The zeta potential was measured between -30mV to -60mV and all the milled samples including natural zeolites (H0) were found stable. SEM and TEM imaging of samples revealed that natural zeolites were cubic shaped and nano-zeolites as spherical. EDAX data showed that the zeolites have Si/Al ratio of 4.14 indicating that the study material is clinoptilolite type of zeolite. FTIR peaks clearly indicates a slight frequency shift in nano-zeolites. In XRD spectra of nano-zeolite, eight prominent peaks that were recorded in natural zeolites were not observed, indicating that there was an intense reduction in crystalline nature of zeolite after ball milling process. SEM image showing zeolites milled at different hours are presented in Fig.1.

Biosafety studies: Pour plate method was adopted to assess the effects of varied size and concentration of zeolites on microbes. A fixed amount of inoculum from 10^{-5} dilution was taken and placed in the center of sterile petriplate to which approximately, 15 ml of warm/partially cooled melted nutrient agar containing zeolites at different concentrations (100ppm, 1000ppm and 2000ppm) and varied size range (10-100nm, 200-400nm, 500-1000 nm, and natural zeolite of around 1-2µm) was added and mixed well with the inoculum. Once the medium was solidified, the plates were kept inverted in an incubator at 30°C for 18-24 hr. Then each colony was carefully counted using magnifying lens (at 18 and 24 hr) and each of colony represents a colony forming unit (CFU). The number of colonies counted in each treated test samples were further determined by the formula: $\text{CFU/ml} = \text{No. of colonies} \times \text{dilution factor}$

The effect of zeolites on the growth dynamics of microbial population was determined by turbidometric technique by analyzing the growth trend of bacterial cell in a liquid media. Spectrophotometer was employed to observe changes in the optical density (OD) over the period of time for plotting a growth curve. A 60 ml sterilized broth was taken in 100 ml conical flasks to which zeolites (respective sizes) were added. Then it was mixed well to achieve homogenous dispersion. Subsequently, 1ml of 10^{-5} diluted culture grown in liquid medium was inoculated in each of the conical flask. Immediately, the inoculated samples (4 ml of it transferred to a clean cuvette) were analyzed for OD value at zero hour. The sterile broth alone was used as blank. The same procedure was repeated for all the bacterial cultures at a time interval of 3 hrs upto 32 hrs. All the readings were recorded at

660nm. The readings obtained were plotted in the graph with time on X-axis and OD value as Y-axis. Lactate Dehydrogenase Assay (Decker and Lohmann, 1988) was performed to assess the cytotoxicity effects of experimental treatments (Nano and Natural Zeolites) on four beneficial microorganisms viz., *Pseudomonas*, *Bacillus*, *Azotobacter* and *Rhizobium*. Cultured cells were incubated in individual wells of ELISA plate that were exposed to different forms of zeolites to assess cytotoxicity potential.

The released LDH enzyme was subsequently quantified using ThermoFisher Pierce LDH Cytotoxicity Assay Kit (Cat#88953), USA by following the manufacture's protocol. ELISA plates containing 50 ml of bacterial cultures incubated with zeolites (nano to microscale) and 50 ml of reaction buffer were kept undisturbed in dark condition for 30 min. Then, 50 ml of stop buffer was added to each wells of ELISA plates and absorbance value was recorded at 490 nm and 680 nm immediately after adding of stop buffers using ELISA plate reader (SpectraMax i3X) and data acquisition using SoftMax Pro ver.6.5.1 of Molecular Devices LLC, USA. The maximum LDH activity of controls and Spontaneous LDH activity controls for each microorganism were calculated based on the mean of three replicates. Per cent cytotoxic was determined by the formula:

$$\text{Percent Cytotoxicity} = \frac{\text{Nanotreated LDH activity} - \text{Spontaneous LDH activity}}{\text{Maximum LDH activity} - \text{Spontaneous LDH activity}} \times 100$$

COMET Assay was performed to assess the genotoxic effects of nano-zeolite on the *Rhizobium* cultures by the action of mobility of nano particles into the cell / nuclear membrane resulting in irreversible DNA damage (Tice et al., 2000). The magnitude of DNA damage was quantified using Gel Doc™ XR+, BioRad, USA and observation on DNA damage was recorded. The Average Linear Growth Rate (ALGR) of *Trichoderma viride* treated with zeolites was also measured. For all the treatments, 20 ml of medium containing measured amount of zeolites (of varied size and dose) was poured in each petriplate (36 + 3 plates for treatment and control). As soon as the medium was solidified, a 5 mm mycelium block was placed upside down at the center of each petriplate. The petriplates were then incubated in growth

chamber maintained at room temperature (27±2°C). In order to measure ALGR of *Trichoderma*, the linear growth (mm) of the fungi was measured by obtaining the average of three diameters taken from each plate. The standard formula used to measure the Average Linear Growth Rate is:

$$\text{“ALGR (mm/day) = (Colony diameter after 3 days of inoculum - Initial colony diameter of inoculum)/3”}$$

Statistical Analysis: The data obtained from these experiments were statistically analyzed using AGRES software.

Results and Discussion

Poison food technique was employed to study the effect of varied sizes and concentration of zeolites on population count of beneficial microorganisms (Table. 2 and Fig. 2). In general, the population counts were significantly higher in zeolite and nano-zeolites enriched medium compared to control. The plate count was significantly highest in the treatment T₃S₄ for two bacterial genera such as *Rhizobium* and *Pseudomonas*, while the highest counts were recorded under T3S3 (500-1000 nm @ 2000 ppm) for other two bacterial genera *Azotobacter* and *Bacillus* when compared with control plates. *A. chroococcum*, under the influence of T₃S₃ and T₃S₄ sized zeolites, recorded 39% and 34% more colonies compared with control plates in which no zeolites were added in the growth medium. For *B. megaterium*, the increase in plate count was 41% under T3S3 and 37% under T3S1, compared to control. Overall, both natural and nano-zeolites increased the microbial growth and among the four micro-organisms, the highest growth enhancement was recorded in *R. leguminosarum*, followed by *P. fluorescens*, *A. Chroococcum* and *B. megaterium*.

The effect of zeolite on growth dynamics of four different beneficial microorganisms are shown Fig. 3. The growth curve of all four bacterial genera followed the usual trend of lag phase, log or exponential phase, stationary phase and death phase and the trend was similar for all the treatments. The growth trend of all four bacteria showed that the zeolite irrespective of its size range had no toxic effects on bacterial cells. Instead, the growth in treated ones were found to be significantly higher than control. The maximum absorbance was observed in *P. fluorescens* after 32 hrs (0.788), followed by *A. chroococcum* (0.591), *R. leguminosarum* (0.587) and *B. megaterium* (0.587) in treatment T₃S₄. Such increase in microbial population counts and biomass in the zeolite treated medium suggests that substrate use efficiency was enhanced as result of zeolites. This is attributed to the fact that zeolites increase carbon incorporation into microbial biomass and our observations are in agreement with Chander and Joergensen (2002) and Heinrichs et al. (1986) who reported that zeolites promote glucose decomposition thereby increases microbial biomass. In addition, zeolite as a mineral might have triggered cell division and contributed for increased population counts. Similar responses as a result of clay mineral such as kaolinite have been reported by Courvoisier and Dukan (2009) who observed rapid cell division and increased microbial population after adding these materials. It is also speculated that

Table 1: Treatments conducted to study the toxic effects of zeolites of varying sizes at varying doses

T0	Control	Without zeolite
T1S1	100 ppm	10-100 nm
T1S2		200-400 nm
T1S3		500-1000 nm
T1S4		1000-2000 nm (Natural zeolite)
T2S1	1000 ppm	10-100 nm
T2S2		200-400 nm
T2S3		500-1000 nm
T2S4		1000-2000 nm (Natural zeolite)
T3S1	2000 ppm	10-100 nm
T3S2		200-400 nm
T3S3		500-1000 nm
T3S4		1000-2000 nm (Natural zeolite)

Table 2: Effect of zeolite on population count of beneficial microorganisms (24th hour) (Xx10³ cfus ml⁻¹)

Treatment		<i>Azotobacter chroococcum</i>	<i>Bacillus megaterium</i>	<i>Pseudomonas fluorescens</i>	<i>Rhizobium leguminosarum</i>
Dose	Size				
T0	Control	321.15	308.15	324.00	338.00
T1	S1	334.26	294.33	367.68	315.63
	S2	356.00	312.00	392.70	367.60
	S3	347.66	302.44	365.00	374.80
	S4	362.33	323.15	343.33	377.34
T2	S1	359.00	383.00	332.33	443.00
	S2	363.00	378.22	367.33	452.66
	S3	347.66	416.66	374.00	438.00
	S4	362.33	402.09	399.56	465.73
T3	S1	437.66	425.12	433.00	454.22
	S2	424.00	419.22	426.00	458.66
	S3	453.00	448.00	448.66	451.00
	S4	449.00	437.00	458.00	472.00
SEd	0.224	0.206	0.249	0.242	
CD (0.05%)	0.463	0.425	0.515	0.499	

Table 3: Effect of zeolite on linear growth rate of *Trichoderma viride*

Treatment		Average linear growth rate (mm per day)	
Dose	Size		
T0	Control	21.50	
T1	S1	21.85	
	S2	22.91	
	S3	22.88	
	S4	21.03	
T2	S1	20.87	
	S2	23.32	
	S3	22.64	
	S4	21.91	
T3	S1	22.50	
	S2	22.17	
	S3	23.71	
	S4	23.50	
	SEd		CD (5%)
Dose		0.2645	0.5819
Size		0.3054	0.6718
Dose × Size		0.5290	1.1638
Ctrl vs Trtd		0.3893	0.8564

leaching of metal nutrients from the zeolite framework might have also provided additional nutrients in the medium favouring microbial growth. Furthermore, impurities as nutrients over the surface of zeolites could have contributed for microbial metabolism since zeolites used in this study for milling process were also natural zeolites. These statements are in accordance with the reports of Hrenovic *et al.* (2003) who reported that metals leached from zeolites may play a role as nutrients. Such discussions may also dispel the fact that zeolites are inert carriers of nutrients, since studies on microbe-mineral interactions highlight that micro-organisms develop several strategies to

attack minerals for extracting nutrients from them for metabolism (Cuadros, 2017). On the other hand, zeolite might have facilitated for adherence/immobilization of microbial cells on its framework thereby providing a conducive platform for nutrient uptake and metabolism. This reason is in conformity with the reports of Vieira and Melo (1995) who observed that positively charged ion exchange sites over zeolite enhanced the bacterial growth. Though zeolites in general were found to enhance the growth in micro-organisms, the increase in population count and biomass by nano-zeolites was relatively lower compared to zeolites of the size range 1000-2000 nm. This is attributable to the fact that ball milling process had changed the structure of nano-zeolites which could be the reason for reduction in the bacterial colonies compared to natural zeolites. In addition, the mechanical synthesis of nano-zeolites might have disturbed the framework of zeolites leading to release of alumina and this component could have decreased the positive effects. Similar observations have been reported by Kihara *et al.* (2011). Overall, the positive effects of natural zeolites might have been offset by the size reduction to nano range.

The cytotoxicity effects of treatments amended with natural zeolite and nano-zeolite were quantified using Lactose Dehydrogenase Assay and the results obtained are presented in Fig.4. In comparison of all the treatments exposed to zeolites of varying sizes and concentration, treatments T₃S₁ showed the presence of toxicity with varying degree among the four beneficial micro organisms. The maximum cytotoxicity (%) was recorded in *P. fluorescens* (51.0%), followed by *R. leguminosarum* (46.4%), *B. megaterium* (44.5%) and *A. chroococcum* (25.8%). Among four bacterial species that were exposed to zeolite, *Pseudomonas* sp. was more prone to cytotoxicity irrespective of treatments (varying size and concentration) which is evident from the highest level of LDH content in the culture broth of all treatments. Further, *Azotobacter* sp. demonstrated comparatively higher level of resistance to cytotoxicity regardless of size or concentration. It

Fig.1: SEM image showing zeolite milled at different time duration: (a) milled for 2 hr (b) milled for 3 hr (c) milled for 4 hr and (d) milled for 5 hr.

was quite interesting to observe that cytotoxicity assay could provide better insights on the size and concentration dependent toxicity of zeolites. Poison plate technique revealed that zeolites regardless of size or concentration significantly enhanced the microbial population. In contrary, the data on LDH assay suggested that 2000 ppm nano-zeolite exhibited cytotoxic effects on soil beneficial micro-organisms. This is evident from the increased surface area and change in crystal shapes of nano-zeolites might have contributed for cytotoxic effects. Our observations are in accordance with the studies of Kihara *et al.* (2011) and Thomassen *et al.* (2012) who have reported *in-vitro*

cytotoxicity caused by nano-zeolites. It is also due to increased concentration (2000ppm) of any material could possibly increase the cell lysis process (Amachi and Hussain, 2010). Furthermore, it is understandable that microbes in nano-zeolite treated petriplates might have overcome the toxic effects since nutrient medium could have supported better cell proliferation. Nevertheless, the bacterial cells incubated for LDH assay had higher interactions with nano-zeolites, especially at the highest dose (2000 ppm) and it could capture the cytotoxicity suggesting that this assay is more reliable to quantify even the minimal events of cell lysis.

Fig. 2: Total plate counts (CFU) in zeolite treated test organisms: (a) *Azotobacter chroococcum*; (b) *Bacillus megaterium*; (c) *Pseudomonas fluorescens* and (d) *Rhizobium leguminosarum*.

Fig. 3: Growth dynamics of beneficial microorganisms; (a) *Azotobacter chroococcum*; (b) *Bacillus megaterium*; (c) *Pseudomonas fluorescens* and (d) *Rhizobium leguminosarum* treated with zeolites.

Fig. 4: Assessment of cytotoxicity of zeolite (nano and natural) on beneficial microorganisms using LDH assay.

Fig. 5: Zeolites of size regime 500-1000 nm and 200-400 nm showing rapid sporulation.

The data suggest that poison plate technique could not account for the dead cells that might have been generated as an initial exposure of nano-zeolites. This may be attributed to the fact that live cells unexposed to nano-zeolites in the nutrient medium shall mask the cytotoxic effects. Such observation is supported with our data on genotoxic assay in which the micro slides depicted that there was no quantifiable DNA damage in nano zeolite treated cells as compared to the control cultures. These results corroborates with the recent findings of Wu *et al.*, (2020) who reported zeolite enriched compost showed enhanced microbial growth indicating that these materials have no toxic effects.

The Average Linear Growth Rate of *Trichoderma viride* was recorded by measuring the average of three diameters and the results are presented in Table.3. In general, incremental dose of zeolites recorded significantly highest linear growth when compared with control. Zeolites at 2000 ppm recorded the maximum ALGR and the lowest in control. Among different sizes of zeolites, 500-1000nm (S2), 200-400 nm (S3), 1000-2000 nm (S4) recorded significantly higher ALGR (22.8, 23.07 and 22.44 mm per day) compared with control (21.50 mm per day) whereas nano-zeolite (S1) treated ones were not significantly higher with a mean value of 21.74 mm per day. The data indicated that incremental concentration of zeolite had a positive effect. In addition, it was also observed that the zeolites in the size regime of 500-1000 nm and 200-400 nm promoted rapid sporulation. Our data is in conformity with the previous studies (Badalova 2014; Kivanc, 2005; Sayit Sargin, 2013) where zeolites promoted hyphal growth and triggered rapid sporulation as a result of better cellulose activity in the medium.

Overall, this study unequivocally demonstrated that zeolites of size greater than 200-400 nm irrespective of concentration upto 2000 ppm were quite safe for soil beneficial microbes. Though biosafety data of nano-zeolites (1-100 nm) showed enhanced microbial population and increased biomass irrespective of the concentration through poison food technique, LDH assay suggested marginal cytotoxic effects at 2000 ppm, besides Comet assay indicated no genotoxic effects. Hence, zeolite based nanoproducs (below 100 nm) should necessarily undergo high throughput screening and advanced biosafety studies with wide array of biological systems to provide conclusive biosafety information to ensure environmental health.

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Add-on Information

Authors' contribution: L. Sivashankari: Student thesis work. Carried out all the laboratory experiments; S.K. Rajkishore: As a guide framed methodology and designed the

experiments; A. Lakshmanan: Advisory member for result interpretation of zeolite effects on microorganisms; K.S. Subramanian: Mentor for overall research and result interpretation; M. Praghadeesh: Standardized cytotoxicity and genotoxicity experiments.

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