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ORIGINAL RESEARCH PAPER

Effect of dosage and particle size of natural zeolite on the survival of *Escherichia coli* in soil

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ABSTRACT

Survival of enteropathogenic bacteria in soil is a key factor to control waterborne diseases. In this study, we evaluated the significance of zeolite nanoparticles (nano zeolite) in comparison with natural size particles on the survival of Escherichia coli in the soil incubated in sterile and unsterile conditions to examine the effectiveness of nanosizing on the behavior of natural amendments in the environment. The experimental mixtures prepared by adding zeolite and nano zeolite at levels of 0, 5, 15% w/w to various amounts of a loam textural soil that. Then every mixture inoculated by a nalidixic acid resistance Escherichia coli (*E.coli* NAR) at a rate of 10_6 cells per gr soil. Results showed that in the unsterile soils, adding 5% zeolite had no significant effect on survival of bacteria in soil and 15% nano zeolite reduced bacteria survival in soil especially at initial days of inoculation (about 3 log-unit). While adding 15% zeolite and 5% nano zeolite had a significantly positive effect on bacteria's time need to reach the detection limit (td). Sterilization of soil mixtures significantly enhanced bacteria survival in all treatments. The highest value of ***td*** obtained in sterile soil amended with 15% zeolite (46 days). In sterile mixtures adding nano zeolite caused an increasing in bacteria population at initial days after inoculation (about 1-1.5 log-units). Decreasing in the size of natural zeolite particles to nano scale had a negative effect on survival of the studied bacterium in unsterile mixtures and *E.coli* NAR survived more in zeolite amended mixtures. While this negative effect was not observed in sterile soil. These results clearly showed that the negative biological interaction is the main factor that controls enteropathogenic bacteria's survival in soil.

Keywords: *Escherichia coli, Nanozeolite, Zeolite, Weibull survival model*

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INTRODUCTION

Land application of manure is a common disposal method in agricultural practices. Manures are the main source for the release of pathogenic bacteria to the environment and consequently a serious risk to human health [1].

The use of manures in agriculture is the most common reason for reaching pathogens to environments [2, 3]. Unc and Goss [1], reported that animal manure application to the soil can readily lead to groundwater contamination with fecal bacteria, especially under moist soil conditions. Fecal contamination of groundwater is potentially leading to waterborne disease; because In many arid regions especially in developing countries, the main source of drinking water is groundwater that abstracted from drilled wells [4]. Therefore, the study of surviving of pathogenic bacteria derived from manures in the environment is very important.

Nanotechnology is a current modern approach, that provided new kinds of materials that exposure novel solutions to the limitations of other conventional materials and have vast applications. Because of their unique characteristics, nanoparticles can be used as amendments to improve soil quality, control soil contaminations, land–application of the conventional amendment

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materials safety and enhance soil erosion control [5]. Using nanotechnology in agricultural practices is one of the existing options to increase agricultural production, solve environmental problems, and providing foods for the world's growing population [6].

There are many reports about the effects of nanoparticles on the fate of enteropathogenic indicator bacteria and soil quality. But most of them have been done on the effects of engineered nanoparticles (ENPs) on soil microorganisms as a good index of soil quality because of the response to environmental stresses such as heavy metals and antimicrobial agents sensitively [7]. ENPs can be classified into carbonaceous nanomaterials, metal and metal oxides, zero-valent metals and nanopolymers [8]. The main reason for the focus on ENPs is that they are applied to various industrial products and so maybe released to soils [9]. ENPs are so reactive particles due to their size and their specific surface area to volume ratio [8, 9], therefore, because of these features ENPs (especially metal and metal oxides types) can leading to heavy metals pollution of soil [9]. So using ENPs for soil remediation or control contaminations maybe not appreciated especially under field conditions, because of the high cost of their creature and environmental usage consequences duo to their ecotoxicity effects on soil biota [10]. Therefore, using natural nanoparticles with compare to ENPs is safer and more reliable.

Among the various conventional amendments that have been used in agricultural practices, natural zeolites are more interested because of their inimitable characteristics. Zeolites are crystalline, hydrated aluminosilicates of alkaline and alkaline-earth minerals [11], which have an ability to hydrate/dehydrate reversibly and to exchange some of their constituent elements with aqueous solutions without a major change in their structure [12, 13]. In agricultural practices, zeolites have been used as slow-release fertilizers, zeoponic substrates, mitigation of soil contaminations, soil conditioner and remediation agents [14], but the usage of zeolites are enormous.

According to previews studies, the effectiveness of soil conditioners is upon their structural components and particle size distributions [5]. Huang and Petrovic [15] concluded that when clinoptilolite particles decreased in size and increased in the amendment dosage, the water available to plants increased in a sand medium. Therefore, according to importance of particle size on efficiency of conditioners in soil, this study was carried out to evaluate the efficiency of nano-size particles of natural zeolite in comparison with normal size particles on *Escherichia coli* survival, as a key factor to determine enteropathogenic contamination risk in soil, in order to control its fate in the environment. This study for the first time shows the effect of the particle sizes of natural zeolite on survival and multiplication of an index fecal bacterium introduced into soil.

MATERIALS AND METHODS

Soil sampling and analysis

The soil sample was collected from the surface layer (0-15 cm) of an agricultural site in Hamadan, western Iran. The region has a semi-arid climate, with annual precipitation of 328 mm and a mean annual temperature of 13°C.

The collected soil was air-dried and sieved (2-mm mesh sieve) by hand for laboratory experiments. Among all soil characteristics, soil particle-size was analyzed using the hydrometer method [16], soil pH and EC were determined in a 1:2 soil: water extract [17, 18], calcium carbonate equivalent (CCE) was measured by the back titration procedure [19], and organic carbon content was determined by the wet-oxidation method [20].

For biological analysis of the collected soil, some amount of soil stored in a sterile plastic bag and kept at 4□. Among all of the biological features, heterotrophic bacterial and fungal population and basal respiration measured in soil. The total soil bacteria, actinomycetes and fungi populations in the soil were estimated by the plate count method using nutrient agar (NA), Rose Bengal starch casein nitrate agar (RBSCNA), and potato dextrose agar (PDA) respectively and were expressed as log10 colony-forming units (CFU) per gram dry mass of soil sample ($\log_{10}$ CFU g^{-1}). Basal respiration (BR) was measured as CO_2 rate vented over 5 days per gram of soil dry mass and was determined at the field capacity for water content at 25□. [21].

Characteristics of zeolite and nano zeolite

The natural zeolite used in this study purchased from Afrazand mineral Company (Semnan-Iran) and obtained from the Mianeh regions in the northwest of Iran. Based on the XRD analysis, the purchased zeolite was composed of clinoptilolite

(Fig. 1). Clinoptilolite nanoparticles were obtained by the mechanical ball-milling method (600 rpm, 6 h) of natural clinoptilolite powder (According to the company's claim (. Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analyses of nano zeolite clearly showed that nano zeolite didn't have homogeneous surfaces and have no harmful elements for bacteria survival (Fig. 2). The main characteristics and features of zeolite and nano zeolite previously reported by Aminiyan, Sinegani [22] According to their study, the particle size of zeolite was in the range of 0.1 to 0.4mm and the particle size of nano zeolite was in range of 90 to 120 nm.

Bacterial inoculums preparation

A nalidixic acid-resistant *Escherichia coli* strain (*E. coli* NAR), kindly provided by the Pasteur Institute of Iran, used as fecal coliform indicator bacteria. This strain, rarely found in natural environments, and has survival characteristics similar to other *E. coli* strains [23]. The advantage leads to the use of *E. coli* NAR by many researchers is the easiness of tracing, because other kinds of bacteria cannot grow in media containing nalidixic acid [24].

Bacteria inoculums prepared by transferring a loopful of bacterial colonies formed on nalidixic acid amended EMB agar (Eosin Methylene Blue) to an Erlenmeyer flask containing 100 mL Trypticase Soy Broth (TSB). The inoculated flask was incubated at 37°C on an orbital shaker (150 rpm) for 14-16 h overnight [23] due to achieving bacteria cells in stationary phase because the most of bacteria exist in this condition in the natural environment [25]. Then, cells were harvested by centrifugation at 5000 × g for 20 min. Cells were washed twice with sterile distilled water in order

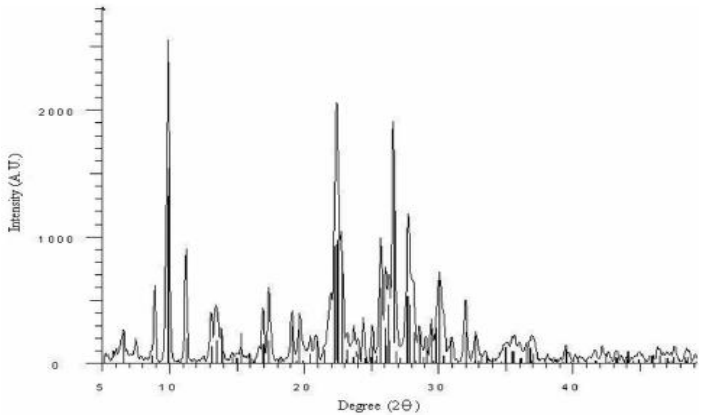

Fig. 1. XRD analysis of Miyaneh clinoptilolite

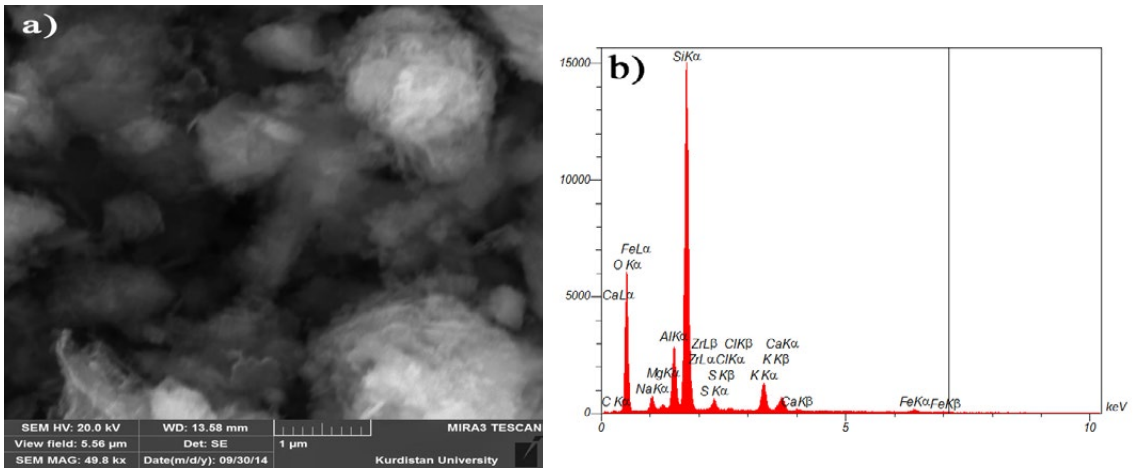

Fig. 2. Scanning electron microscopy (SEM) image (a) and EDX analysis (b) of nanozeolite

to exclude nutritional particles and re-suspended in distilled water and have been diluted to get a suspension with approximately 1 to 2×10^7 cells per mL; that was determined via measuring the absorbance at 600 nm (OD_{600}=1 by Varian, Cari 100 UV-vis spectrophotometer).

Mixtures preparation, sampling, and enumeration of E. coli NAR

Mixtures were prepared by adding zeolite and nano zeolite (separately) at levels of 5 and 15 g per100g soil, using the method described by Taha and Taha [26]. Briefly, each soil sample divided into 10 sub-samples and each sub-sample was amended with the required amount of zeolite\nanozeolite and mixed separately. Then all sub-samples mixed and compounded together. Unamended soil samples were also considered as control. Incubation carried out in both unsterile and sterile conditions. For sterile conditions, half of each mixture was sterilized over 5 d through a 2 h daily autoclaving at 121°C [27]. This allowed to evaluating the definite effect of abiotic soil properties on *E. coli* NAR survival.

After preparing mixtures, all mixtures inoculated with 10^6 cell g^{-1} and the water content of all mixtures adjusted to field capacity (FC) to obtain the optimum condition of bacteria growth and kept at a fixed temperature of 25±2°C in darkness. The FC water content of each mixture determined at 33 kPa using a pressure-membrane extraction apparatus [28]. During incubation, mixtures water status was checked and distilled water was added as necessary to maintain the soil water content around field capacity.

The counts of *E. coli* NAR in each treatment were determined at days 0, 1, 2, 3 and 8 after inoculation (DAI), then every 5 d for the first month, every 10 d during the second month and eventually every 15 d afterward. Spread plate method used for determining the counts of *E. coli* NAR population during incubation. So, 1 g dry mass of each soil mixture sample was removed and added to 99 ml of sterile solution ($Na_4P_2O_7$, 0.2 % in water) and being homogenized by agitating on a rotary shaker for 20 min (200 rpm) [29]. Further decimal dilutions were prepared as necessary to produce 30 to 300 colonies per plate from a 0.1 mL dilution aliquot. The plates of EMB agar contained 100 mg mL^{-1} nalidixic acid [24] used for enumeration, following 24 h of incubation at 37°C. Sampling was performed in triplicate and enumeration of *E. coli* NAR in mixtures continued until reaching the detection limit. The detection limit of the spread plate method was 100 CFU g^{-1} soil [30], but sampling continued after reaching the detection limit to check the contingency of bacterial re-growth.

Data Analysis

The population of *E. coli* NAR at each sampling event was expressed as colony-forming units (CFU) per gram of oven-dry weight and were converted to log10 (CFU g^{-1}) before statistical analysis. The general linear model (GLM) was employed to evaluate the significance of amendments (0, 5, 15 g per 100g soil) on the survival of *E. coli* NAR in different sterility status (unsterile and sterile) separately. For some sampling events, witch data were not normally distributed (Shapiro-Wilk test), we used the nonparametric Kruskal-Wallis model for better understanding the significance of amendments usage.

The survival data were analyzed by fitting to the Weibull survival model (Eq. (1)) [31], using GInaFiT version 1.6 [32].

$$logN_t = logN_0 - \left(\frac{t}{\delta}\right)^p \quad (1)$$

Where N_t is the number of survivors at time t, N_0 is the initial inoculums population size (both in CFU g^{-1}); t is the time (day); p is the shape parameter (-), and δ is the scale parameter representing the time needed for first decimal reduction (day). Convex and concave die-off curves result in $p>1$ and $p<1$ respectively, and the linear curve is observed when $p=1$. A very important and useful parameter, *td* (the time when N_t reaches the detection limit of 100 CFU g^{-1}) can also be calculated from Eq. (1). Mean comparisons for model parameters were done by Student's t-test and Duncan method to compute the significance of treatments. Data analyses and graphing were performed on SAS 9.12 [33] and MS-Excel.

RESULTS AND DISCUSSION

Physical, chemical and biological characteristics of the studied soil

The soil texture was loam (43.4, 46.2 and 10.4 g per 100 g^{-1} for sand, silt and clay respectively) with low clay content and had pH and EC of 7.2 and 0.11 $dS.m^{-1}$ respectively. The organic carbon content of the studied soil was 0.33 g $100g^{-1}$ and soil CCE content was in the range of 2.5 g $100g^{-1}$.

Table 1. Analysis of variance (χ^2-value) of culturable *E. coli* NAR in soil as affected by zeolite and nanozeolite

Sampling events (day)	Chi-square parameter (χ^2)	
	unsterile	sterile
8	13.40**	13.50**
40	-	11.51*
50	-	10.57*

Values marked by ** and * are significant at $P < 0.01$ and $P < 0.05$ respectively.

According to Farhangi, Safari Sinegani [34], CCE content of the soil was not at a level to influence *E. coli* survival in soil negatively. The soil microbial counts for bacteria, fungi, and actinomycetes were 5.98, 3.55 and 4.15 $\log_{10}$ CFU g^{-1} respectively and soil basal respiration was in the range of 0.13 mg CO_2 d^{-1} g^{-1}.

Effect of zeolite and nano zeolite application on the survival of E. coli NAR

Analyses of variance showed a significant effect ($P < 0.01$) for using both amendments in unsterile mixtures on counts of *E. coli* NAR for 1, 2 and 3d sampling. In unsterile mixtures, Shapiro-Wilk test has shown that the log10-transformed counts of *E. coli* NAR were normally distributed ($P < 0.05$) at all sampling times except for days 8. Non-parametric statistical tests have shown that the effects of using amendments to be significant also for days 8 (Table 1). In sterilized mixtures, log10 counts of *E. coli* NAR were normally distributed (Shapiro-Wilk test; $P < 0.05$) for all sampling events except for 8th, 40th and 50th day. For all non-normally distributed datasets, nonparametric statistics were used and showed that amendments indicate a significant role for these sampling events (Table 1).

Survival dynamics of E. coli NAR in soil amended with zeolite and nano zeolite

The counts of culturable *E. coli* NAR in unsterile mixtures generally decreased with time over the incubation period (Fig. 3a). There was a relatively

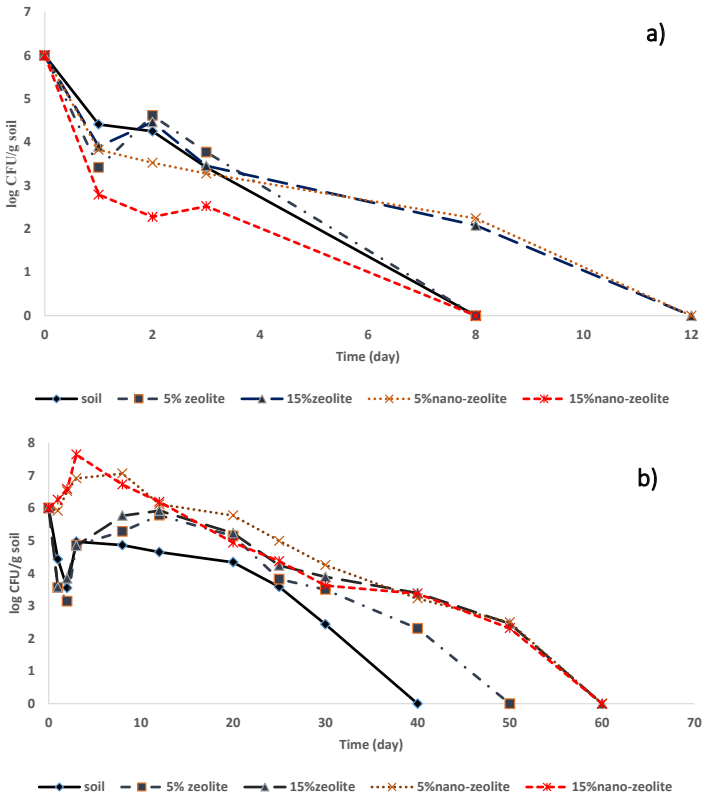

Fig. 3. Changes in culturable *E. coli* NAR over time in unsterile (a) and sterile (b) mixtures.

sharpen die-off during the first day of incubation (Fig. 3a) ranging between 1 and 2 orders of magnitude among the treatments. The bacterium die-off was more accelerated in treatment with a larger amount of nano zeolite and the counts of culturable *E. coli* NAR declined to 3 order of magnitude and reached to half of the initial inoculated counts. According to Farhangi, Safari Sinegani [34], this diminution in counts of culturability that happened among all treatments was presumably a result of the initial inoculation shock, caused by introducing bacteria to a new environment. As, culturability declined across treatments over time, in zeolite amended treatments, a slight recovery was observed in *E. coli* NAR culturability (1-1.5 log CFU g^{-1}) on the second day of incubation. While in the nano zeolite amended the soil, especially in the soil containing 15% nano zeolite, the bacteria not able to recover, and the counts of culturability not only didn't increased but also at 2 days after inoculation (DAI), counts of culturability were less than the first day (Fig. 3a). However, there is a slight recovery in culturability for soil amended with 15% nano zeolite at day 3, no more bacteria detected from unamended soil and those contain 5% zeolite and 15% nano zeolite at 8 DAI. While for soil contain 15% zeolite and those contain 5% nano zeolite, *E. coli* NAR reached to detection limit at 12 DAI (Fig. 3a). There are many reports on microbial toxicity of nano components, but most of them reported for engineered nanoparticles (ENPs), such as Ag, Au, Fe and TiO, FeO, ZnO, etc. For example, Rai, Yadav [35] reported the toxicity of Ag nanoparticles on *E. coli* and *Staphylococcus aureus*. Also, nanoparticles of CuO and ZnO inhibited *E. coli* and *S. aureus* [36] and *Pseudomonas putida* [37]. Baek and An [38], reported that CuO nanoparticles were the most toxic ENP to *E. coli*, *B. subtilis*, and *Streptococcus aureus*, among nanoparticles of CuO, NiO, ZnO, and Sb_2O_3. While, Jiang, Mashayekhi [39] reported that among metal oxide ENPs of Zn, Al, Si and Ti, the most toxic ENP for *B. subtilis*, *E. coli* and *P. fluorescens* was ZnO. According to Suresh, Pelletier [40], ENPs are associated with microbial cell surface changes that may finally lead to cell death. But the point here is that, contrary to ENPs, nano zeolite is a natural and native compound of soil and has no harmful effects on soil organisms. However, in a higher content of nano zeolite, *E.coli* NAR die-off rate was more accelerated and had a significantly negative effect on the survival of the studied bacterium but this phenomenon may not be related to the toxicity of these particles to *E.coli* NAR.

Khati, Sharma [41] studied the effects of nano clay, nano chitosan, and nano zeolite on the basis of physicochemical characters, microbial activities, indicator enzymes and total extractable protein of soil. They reported that in all the soil samples treated with nano compounds, available phosphorus, organic carbon, ammoniacal nitrogen, and total protein concentration were higher than the control and all the nano compounds had significant positive effects on the activities of dehydrogenase, fluorescein diacetate, alkaline phosphatase, amylase, arylesterase, and β-glucosidase. Higher enzyme activities were observed in soil samples treated with these natural nano compounds. They also concluded that among all nano compounds, nano zeolite was more beneficial for microbial activities and soil health.

There are many biotic and abiotic factors controlling fecal bacteria survival in soil. Among abiotic factors, soil moisture content [42, 43], soil pH [44], soil electrical conductivity [45], temperature [34, 46], soil texture [47] , organic matter content [46, 48], calcium carbonate equivalent [34], and manure medium characteristics [27, 49], are considered to be effective on fecal bacteria survival in soil. Among biotic factors, predation by soil protozoa [50], and competition with soil-dwelling bacteria [51] have also influenced the bacteria survival. Hence, the lowest survival time of *E. coli* NAR in unsterile soil treated with a higher amount of nano zeolite may be associated with these effective factors.

Khati, Sharma [52] studied the impact of nano zeolite treatment on species richness and evenness of soil metagenome. They reported that adding nano zeolite improved the soil microbial population and increased the survival time of soil microorganisms especially bacteria occurred in nano zeolite treatments. A higher abundance of proteobacteria, which is the most diverse group of prokaryotes in soil, was reported in the nano zeolite treated soil. They also reported that nano zeolite entraps water and nutrients and allow their slow-release, which causes better growth of the microbial population.

So according to this study clearly noted that nano zeolite has no harmful elements for the microbial community in sterile condition and the decreasing in counts of E.coli NAR in soil amended with 15% nano zeolite especially in unsterile

condition may be related to other factors.

Low OC content of studied soil, which is most common in arid and semi-arid regions, (0.33 g 100g soil) may induce competition between soil indigenous bacteria and *E. coli* NAR for carbon and energy sources. As *E. coli* is a bacterium that belonged to warm-blooded animal's digestive tract, it may fail to survive for a long period in the soil environment. So, to make a better understanding of the fate of *E. coli* NAR in treated soils, we examined the effect of competition between soil indigenous bacteria and *E. coli* NAR by inducing the sterility condition.

In fact, the main drawback of sterility is that it never occurs naturally and so any results obtained under sterile conditions may be exaggerated [34]. However, it is very useful for exploring the net effects of abiotic parameters. The only obvious difference between the sterile and unsterile mixtures is the absence of competitors [53, 54].

Changes in *E. coli* NAR number over time in sterilized soils are presented in Fig. 3b. Obviously, sterilization increased *E. coli* NAR survival period in comparison with unsterile mixtures. This result strongly indicated that competition is the main factor the negative biological interactions are very important and specially the controlling *E. coli* NAR survival in soil. In the zeolite amended soil and unamended soil, after initial adaptation shock, bacteria recovered itself. In the zeolite contained mixtures increasing in *E. coli* NAR number continued until day 12 and reached about the initial inoculated level. In the unamended soil, the number of bacteria declined after 3 DAI and after 40 DAI no bacteria detected from the unamended soil. Whereas in zeolite amended mixtures bacteria survived more. In all sampling events in a sterile condition, mixture contained 15%zeolite had the highest number of *E. coli* NAR (Fig. 3b). Anyway, bacteria have been recovered from 5 and 15% zeolite amended mixtures till 40 and 50 DAI respectively. The higher survival time of the bacterium in sterile soil treated with zeolite may be related to the suitable effects of zeolite and sterilization on soil properties for the survival of the bacterium.

Zeolite, as a soil conditioner, has many effects on soil properties. For example, Ming and Allen [14] reported that adding zeolite to coarse textural soil can decrease the bulk density of soil. They also reported that adding zeolite to the soil can improve the soil quality by increasing the water holding capacity, increasing the clay-silt fractions, improving nutrient levels, and removing toxins. Kátai, Sándor [55] reported that zeolite influenced significantly the examined soil microbiological parameters and the total number of bacteria, the microscopic fungi, and nitrifying bacteria increased in the zeolite treatments.

The heat of sterilization can change the physical and chemical environment of the soil and may also increase the concentration of extractable OC significantly. Razavi and Lakzian [56] found a significant increase in the concentration of extractable OC in autoclaved soil in comparison with control using the chloroform fumigation extraction (CFE) technique. This increase was attributed to the breakdown of humic substances and the death of microorganisms. Increased OC availability, beside the omission of rival microflora, and also the presence of lysed cells' residues, make a favorable environment for fecal organisms to survive in the sterilized soil by wet heating [57].

An interesting thing to note here is the unexpected survival dynamics in sterilized nano zeolite treatments (Fig. 3b). In contrary with unsterile treatments, in the sterilized nano zeolite treatments, the number of *E. coli* NAR not only didn't decreased over the initial days of inoculation but also there is an obvious sharp increase in counts of *E.coli* NAR (about 1-1.5 log-units) and mixtures containing larger amounts of nano zeolite have a higher counts of bacteria at initial days after inoculation (Fig. 3b). After this increase in counts of *E. coli* NAR in sterilized nano zeolite treatments, the number of bacteria decreased with time over the incubation period and after 60 DAI no bacteria detected from these treatments.

Nanozeolite has substantial calcium ion content [58] which acts as a potential cationic bridge between organic and inorganic colloids surface. Aminiyan, Sinegani [22] reported that adding nano zeolite improved the aggregation process in soil and acts an important role in improving soil physical characteristics and soil carbon sequestration. Nanozeolite owning complex structure, small size, and high cation exchange capacity, which may chelate nutrients and trap moisture, which may help improve the availability of resources in soil [41, 59].

Among all soil features, soil moisture is a key parameter that can obscure the impacts of other factors. Safari Sinegani and Maghsoudi [43], reported that *E. coli* survival increased by increasing soil moisture content and survival time extended to over 90 days in saturated soils treated

with cow manure. Improving aggregating features of soil beside the high moisture content that creates the best micro niches for fecal coliforms along with the absence of competitors and abundance of readily substances remained from lysed cells, together, enhanced *E. coli* NAR growth in nano zeolite sterilized treatments. This phenomenon is the probable reason for the sharp increase in counts of bacteria in these treatments in comparison with zeolite amended soils. Here the nano zeolite treated soil had higher moisture content at 33 kPa (unpublished data). So, soil with higher zeolite and nano zeolite had higher moisture content in FC which may provide better soil conditions for the studied bacterium.

Modeling of E. coli NAR survival in treatments

Analysis of variance showed that both zeolite and nano zeolite had a significant effect on Weibull survival model's parameters and time needed to reach the detection limit (*td*) in both sterile and unsterile mixtures ($P<0.01$). Duncan's multiple range tests for mean comparison of model parameters and the time (day) needed to reach detection limit *(td)* obviously showed the effect of amendments in sterile and unsterile conditions separately (Figs. 4 and 5).

In unsterile mixtures, the *td* among all treatments significantly varied (Fig. 4a) and the highest value of *td* were for soil containing 15% zeolite (7.8) that obviously showed the positive effect of adding zeolite on the survival of *E.coli* NAR in soil. While in unsterile soil treated with 15% nano zeolite the *td* value decreased significantly to the lowest value (2.7) among all treatments and it was lower than that in untreated soil. As discussed in the preview section, there are some reports that concluded about the bonding soil organic matter with nano zeolite and act as slow-release sink for SOM and enhanced carbon sequestration in soil [22, 52]. So, because of low OM content of soil (which is common in arid and semi-arid soils) in one side and the incapability of *E.coli* NAR to compete with soil born microorganisms on the carbon and energy resources on other side lead to elimination of *E.coli* NAR and low *td* value for bacterium obtained in mixtures with higher nano zeolite content. According to Fig. 4a, unlike mixture with 15% nano zeolite, mixture containing 5% nano zeolite was more like those contained 15% zeolite and *E.coli* NAR survived more in these treatments and *td* was higher than 15%nanozeolite

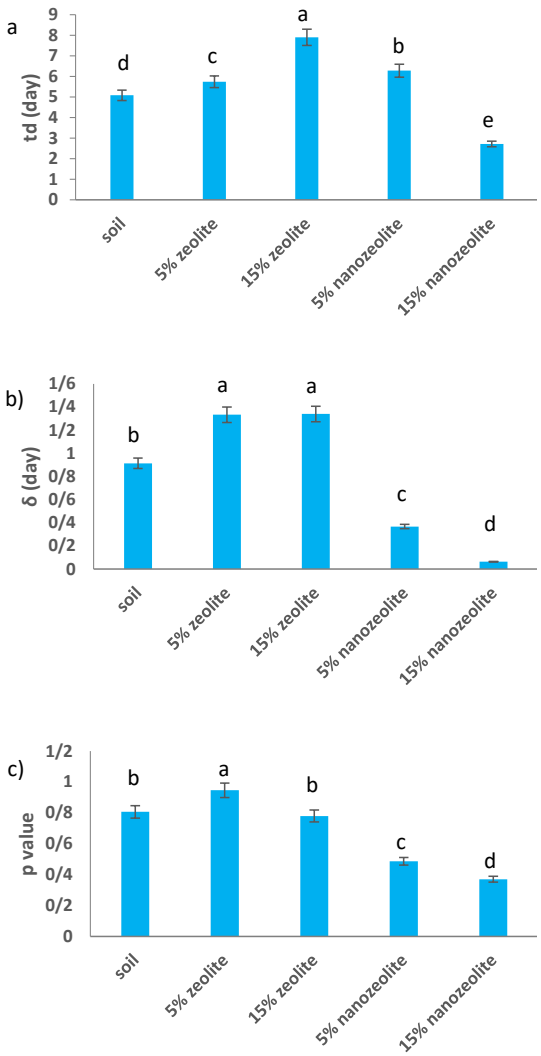

Fig. 4. The Weibull model parameters td (a), δ (b) and p (c) for *E. coli* NAR survival in the unsterile treatments. Bars are ± the standard deviation of means. Means followed by the same letter on the columns indicates no significantly different ($P<0.05$) by Duncan's multiple range tests.

treatments (6.28). This phenomenon may be in association with the exaggerated results obtained from 15% nano zeolite treatments. The amounts of nanoparticles used in these treatments may extremely bond the SOM in soil and lead to intensive competition between *E.coli* NAR and soil indigenous bacteria on the carbon and energy resources that caused vanishing *E.coli* NAR from competition. But lower amounts of nano zeolite were more favorable and *E.coli* NAR survived more in these mixtures. This different result may also be related to the specific effect of zeolite and nano zeolite on soil microbial interaction. Elucidation of

these effects may need more studies.

In unsterile mixtures, the fitted values of the first decimal reduction time (δ) followed the same pattern discussed for *td* (Fig. 4b). The highest value of δ obtained for mixtures contain15% zeolite (1.34) and there is no difference between mixtures contain 5 and 15% zeolite. Also, the lowest value of δ obtained for the mixture contained 15% nano zeolite (0.06). According to Ma, Ibekwe [60], the *td* positively correlated with the shape parameter (*p*), and a concave ($p<1$) survival shape may be associated with shorter *td*, while a convex ($p>1$) survival shape may be associated with a longer *td*. And because of the positive correlation between δ and *p* [31, 32], the highest value of δ can lead to higher *td* value and contrary the lowest value of δ lead to lower value for *td*. The shape parameter (*p*) in all unsterile mixtures was lower than 1 ($p<1$) which means that all survival curves had a concave shape (Fig. 4c).

Sterilizing intensively increased *E.coli* NAR survival in treatments and the *td* values are higher than unsterile treatments (Fig. 5a). This procedure undoubtedly indicated that competition with soil indigenous bacteria is a critical parameter that controls *E.coli* NAR survival in soil. Among all treatments, the highest value of *td* was for soil containing 15% zeolite (56) and untreated soil had the lower one (37). These results strongly confirmed that both zeolite and nano zeolite had a significantly positive effect on survival of *E.coli* NAR survival in sterile soil. According to Fig. 5a, in nano zeolite amended mixtures, *td* values were lower than zeolite mixtures. This phenomenon may be associated with increases in bacteria population at initial days in nano zeolite treatments. This increase in bacteria population in nano zeolite amendments may be caused an inner competition between *E.coli* NAR population on the carbon and energy resources that caused a rapid reduction in *E.coli* NAR counts in nano zeolite mixtures. However, there are no significant differences among *td* values of 5% nano zeolite and 5% zeolite, but the first decimal reduction time (δ) of these treatments are significantly different (Fig. 5b). As expected, with increasing in *td* value the value of δ increased and this increase was higher in zeolite amended mixtures. In zeolite amended mixtures, and also in untreated soil, there are two reduction phases. The first phase was at initial days of inoculation, because of introducing bacteria to the new environment, followed by an increasing phase

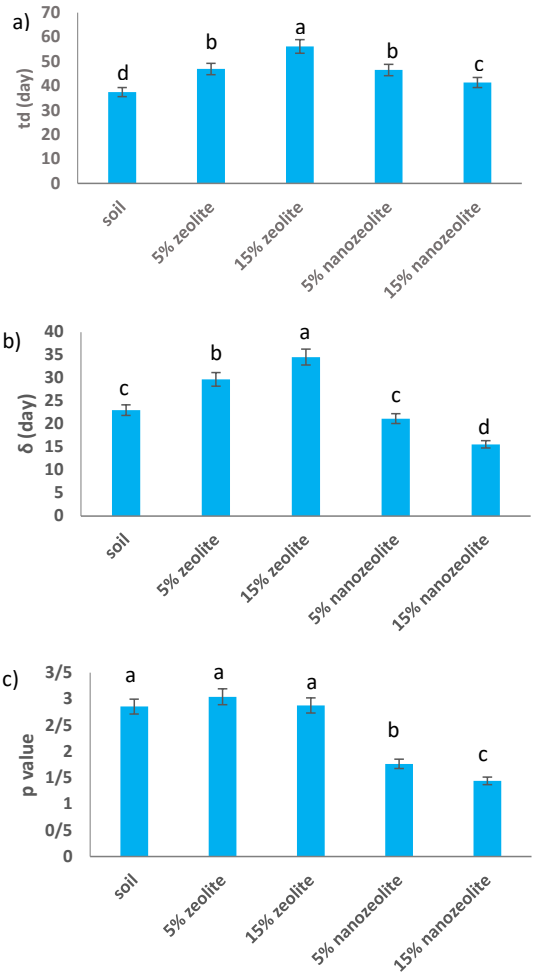

Fig. 5. The Weibull model parameters td (a), δ (b) and p (c) for *E. coli* NAR survival in the sterile treatments. Bars are ± the standard deviation of means. Means followed by the same letter on the columns indicates no significantly different (P<0.05) by Duncan's multiple range tests.

and then the second phase of reduction. The fitted δ in these treatments consider the second phase of reduction as the first decimal reduction time. The increasing in *E.coli* NAR population at initial days of inoculation in nano zeolite treatments caused the lower δ value for these treatments in compression with zeolite amended soil and the soil containing 15% nano zeolite had the lowest δ value, because of the higher peak for population increasing.

As discussed before, *td* value had a significant positive correlation with shape factor and longer *td* correspond to convex shape ($p>1$). In contrast to the unsterile condition, in the sterile condition, *E.coli* NAR showed convex pattern in survival dynamics and the values of shape factor increased

(Fig. 5c). As discussed before, the correlation of shape factor (p) and the first decimal reduction time (δ), higher p values calculated for those corresponding to the higher δ value and vice versa. So, the p values in zeolite mixtures are significantly higher than those in nano zeolite mixtures and there are no significant differences between those in zeolite amended mixtures along with each other and that in untreated soil (Fig. 5c).

CONCLUSION

Our study conducted at field capacity to evaluate the effectiveness of zeolite and nano zeolite on the survival of the *E.coli* NAR in soil, as a key factor to determine enteropathogenic contamination risk in soil. Our results suggest that survival of *E. coli* NAR is clearly effected by adding both zeolite and nano zeolite to soil. Adding 5% zeolite to soil had no significant effect on the survival of *E. coli* NAR in the soil while increasing in amendment amount to 15% significantly corresponded to higher *td* in soil. Because in this study we want to make a clear compression between zeolite and nano zeolite for better understanding of nano particle size impacts of natural amendments, we used as same as the amount of nano zeolite. Results showed that adding 5% nano zeolite obviously enhanced *E. coli* NAR survival in the soil while increasing the amount of nano zeolite to 15% had a negative effect on survival of *E. coli* NAR in unsterile soil. This negative effect clearly not due to the nature of nanoparticles because, in sterilized nano zeolite amendments, the population of *E.coli* NAR increased during the initial days. Results in sterile soil obviously showed that the negative biological interaction is the main factor that controls fecal bacteria's survival in soil. Our results showed that the addition of nanoparticles of zeolite (especially at the lower level) had no significant harmful effect on *E.coli* NAR survival. So, land application of zeolite and nano zeolite with fresh manure or sewage sludge may be associated with microbial contamination risks of groundwater sources. Because of the specific features of nanoparticles, land application of these particles should be investigated better.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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