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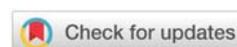
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***Corresponding author:** Delia Teresa Sponza, Department of Environmental Engineering, Engineering Faculty, Dokuz Eylül University, Buca, Kaynaklar Campus, 35160, İzmir, Turkey, E-mail: delya.sponza@deu.edu.tr

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Research Article

Ecotoxicity of HfO₂ and SiO₂ Nanoparticles on Bacteria (anaerobic methane Archaea); Yeast (*Candida albicans*) and Biodegradability Tests

Delia Teresa Sponza* and Nefise Erdinçmer

Department of Environmental Engineering, Engineering Faculty, Dokuz Eylül University, Buca, Kaynaklar Campus, 35160, İzmir, Turkey

Abstract

The applications nano-metal oxides (NMOs) are used in very common in industrial and consumer products because of the advantages of nanotechnology. The use of these NMOs cause the release of NMOs throughout the life cycle of nanoproducts to air, soil, water, and sediments. Knowledge of potential toxicity of nanoparticles to organisms is limited. To determine the toxicological effects of nano-HfO₂ and nano-SiO₂ on the anaerobic methane Archaea from bacteria and to *Candida albicans* from yeast, some toxicity analyses were performed to detect the EC₅₀ values (nanoparticle concentration inhibiting 50 % of the organisms). These values were calculated from the inhibitions of NMOs versus exposure time (24, 48 hours and 28 days). Anaerobic methane Archaea bacteria are very sensitive to nano-SiO₂. While the inhibition rate is 100 % for nano-SiO₂, nano-HfO₂ is less toxic to anaerobic bacteria because of the 13.97 % inhibition rate after 48 h. Compare to nano-HfO₂, the EC₅₀ value of nano-SiO₂ were low and showed high toxicity towards to *C. albicans* (24 h EC₅₀=119.68 mg/L; 48 h EC₅₀=98.06 mg/L). From the 28 days biodegradability tests of NMOs, it was found that the percentage of removal efficiencies are 11.2 % and 55.9 % for 100 mg/L nano-HfO₂ and nano-SiO₂, respectively after 28 days.

Introduction

Nanoparticles (NPs) are wide class of materials that include particulate substance, which have one dimension less than 100 nm at least [Khan et al., 2019]. Different sizes, different structures, one-element or multi-element structure can be formed in different shapes and formats, or desired NPs have wide potential: in the short term in the textile, cosmetics and dye in the long-term medications are used in drug delivery systems to send the requested body [1-12]. NMOs often have special properties, which are more likely to induce hazardous effects compared to conventional materials (Wang, 2018). Also NMOs are widely used in the treatment of industrial wastewater [2,3]. This widespread production and use of nanoparticles in nature means intense accumulation. NMOs because of can easily be synthesized chemically and can easily be modified consumer products; industrial products, machinery industry, military applications, in wastewater treatment and medicine widely

used [4-19]. In particular, the development of wastewater treatment technology that uses NP is seen as an alternative solution to the growing worldwide water pollution problems. Examples of this work in the treatment of heavy metals; nano zinc oxide were used for the removal of copper from industrial waste water and the maximum adsorption capacity obtained for nano-ZnO are 226 mg/g [5-21]. Among the inorganic oxide NPs, silica (SiO₂), is among the most commonly utilized NMOs, and this oxide included in the Organization for Economic Cooperation and Development's (OECD) priority list of NMOs requiring urgent testing for human health and environmental safety [6]. Hafnium oxide (HfO₂) is a suitable replacement for silicon oxide. HfO₂ has a dielectric constant of about 14, compared to silicon oxide with a dielectric constant of 3.9 [7-22]. Studies about the environmental toxicity of these NMOs is very limited. The most commonly used Nano-ZnO d creates high toxicity on bacteria (EC₅₀ value for *E.coli*: 0,048 mg/l). Zinc ions (Zn⁺²) connect to bacterial cells and reported that damage

to physiological function of the defeated cell to osmotic shock [8].

In this study the effects of increasing nano-SiO₂ and nano-HfO₂ concentrations (from 1 mg/l to 100 mg/l) were studied on two trophic levels (bacteria, yeast) and some toxicity analyses were performed to detect the EC₅₀ values (nanoparticle concentration inhibiting 50 % of the organisms). Furthermore, their biodegradability tests were determined in an aquatic environment during 28 days based on the soluble COD concentrations.

Materials and methods

Properties and preparation of NMOs

The environmental toxicity of nanoparticles was studied using three different NMOs. These nanoparticles are nano-SiO₂ (Sigma-Aldrich, 637238, Lot:MKBL8542V) and nano-HfO₂ (Sigma-Aldrich, 202118, Lot:MKBH3310V). The ranges of studied nanoparticles concentrations were determined by considering the acute toxicity test in the recent literature. NMOs were suspended in deionized water at 100 mg/l. In order to improve the dispersion of NPs, suspensions were sonicated for 60 min at sonicator. Then serial dilutions (10⁻¹, 10⁻², 10⁻³, 10⁻⁴) were prepared to obtain a range of concentrations for further toxicity testing. The diameter of nano-SiO₂, nano-HfO₂ were 10–20 nm, 5–7 nm, respectively.

Toxicity tests

Yeast acute toxicity tests: *Candida albicans* from yeast was studied to study the toxic effect of nano-HfO₂ and nano-SiO₂. Reference culture used this toxicity test that provided from Public Health Agency of Turkey. Lyophilized cultures were incubated in SD broth (sabouraud dextrose broth) during 4 hour at 30 °C for both organisms, then they were transferred by pour plate technique to petri plates containing PDA (potato dextrose agar) and they were incubated during 72 hours at 30 °C. When the organisms are in log phase (3,6×10⁻⁵ cfu/ml) for *C. albicans*, the acute toxicity of NMOs were performed. NMOs stock solutions (100 mg/L) were sonicated during 60 min. From the stock NMO solutions serial dilutions were performed. 5 ml of the different NMOs dilutions and 1 ml of *Candida albicans* culture staying in log phase (5×10⁻⁵ cfu/ml) for *Candida albicans* were placed on the steril tubes and they were incubated during 24h and 48h at 30°C temperature. The numbers of yeast were enumerated and compared with control groups containing no NMOs. From the inhibition percentages of the organisms, the values affecting the 50 % of the *C.albicans* was accepted as EC₅₀ values.

Anaerobic toxicity test ATA: Anaerobic toxicity assays (ATA) were performed at 35°C and volume of 150 ml amber bottle reactors [9]. Anaerobic sludge used for this test was obtained from Pakmaya Baker's Yeast Producing Factory (Izmir) providing 3000 mg/L anaerobic VSS (volatile suspended solids) concentration. Vanderbilt Mineral Medium containing 3000 mg/L glucose-COD, 30 ml sodium thioglycollate (to maintain the anaerobic environment) and 5 ml NaHCO₃ (to keep the

neutral pH) were added into a sterile 5 Liter flask (A solution). 1 ; 5 ; 10 ; 25 ; 50 and 100 mg/L NMOs concentrations were added into the amber bottle reactors. 75 ml from A solution were distributed to each bottle reactor and they were stirred in a sonicator for 1 hour. 40 mg/L of the anaerobic sludge containing 750 mg/L anaerobic VSS was added and the mouths of the bottles were sealed with rubber stoppers. Before the toxicity experiments, serum bottles were operated until the variation in daily gas production was less than 15% for at least 2 days. After 24 and 48 hours incubation period the methane gas was measured by passing the gas in the bottles from a solution containing 3 % NaOH solution by liquid displacement method [10]. Methane gas production of the samples containing NMOs and the control groups was determined and the degree of inhibition effect on anaerobic Archaea was calculated by comparison with the control samples and test groups. This inhibition was defined as a decrease in methane gas compared to the control samples.

Biodegradability test: This method evaluates the biodegradability of NMOs in activated sludge (AS) according to OECD 314 [13]. Before studies, room temperature was adjusted 21 °C. NMOs solutions were prepared (10 mg/2L and 100 mg/2L) and sonicated during 60 minutes. This 2L solutions were transferred to glass reactors with volumes of 2000 ml and they were added 50 mg/L suspended solids (SS) containing aerobic sludge, 10 mg/L and 100 mg/L glucose-COD, separately. pH and dissolved oxygen (DS) were adjusted as 7 and 4 mg/L, respectively. Then it was started to the test. The soluble-COD values were measured for each NMOs in 5th, 10th, 15th, 20th and 28th days. Each NMOs of removal efficiencies were calculated by comparison with the COD value at 0th day.

Statistical analysis

The acute toxicity of NMOs to organisms with increasing doses has been studied by the statistical analysis of inhibition of organisms whether it is time-dependent or dose-dependent. The relationships between the variables of time and inhibition percentages were investigated with multiple regression analysis using the ANOVA program (JMP 10). r² and p (<0.05) parameters were used to describe the statistical significance between dependent and independent variables.

Results and discussion

Effect of NMOs to Yeast – *Candida albicans*

During 24 h and 48 h incubation period *C.albicans* colonies were exposed to increasing NMOs concentrations (1 mg/L; 10 mg/L; 50 mg/L and 100 mg/L). *C.albicans* colonies were enumerated and were calculated as percent inhibition compared to the control groups. Table 1 showed that when the NMOs doses were increased from 1 to 100 mg/L, there is more toxicity for nano-SiO₂ compare to nano-HfO₂. After 24 h, the inhibition increased from 4.54 % to 40.91 % and also this toxicity continues after 48 h increasing 12.17 % to 46.95 % for nano-SiO₂. As we compare nano-HfO₂ to nano-SiO₂, this NMOs showed that less toxicity. The highest inhibition rate is 23.62 % after 48 h at 100 mg/L concentrations (Table



2). ANOVA tests statistics for yeast – *Candida albicans* acute toxicity tests revealed that there is a linear relationship between NMOs concentrations and incubation period (for nano-HfO₂, Rsquare=0.982 ; for nano-SiO₂, Rsquare=0,991). It was found that regression analysis between time (for nano-HfO₂, p=0,0145< 0,05 ; for nano-SiO₂, p=0,0185< 0,05) and doses (for nano-HfO₂, p=0,0052< 0,05 ; for nano-SiO₂, p=0,0013< 0,05) was significant as a results of ANOVA tests (α=0,05).

After acute expose time, the EC₅₀ value evaluated (effective concentrations inhibiting the 50 % of the yeast) at increasing NMOs concentrations (from 1 mg/L to 100 mg/L). The EC₅₀ values are given in Table 2. After 48h, for both of them the EC₅₀ values were decreased and this showed that the sensitivity of organism to the increasing NMOs concentrations during incubation period. High EC₅₀ values also indicated that low toxicity after expose NMOs for nano-HfO₂ (24 h EC₅₀=251.21 mg/L; 48 h EC₅₀= 250.16 mg/L) (Table 2). Compare to nano-HfO₂, the EC₅₀ value of nano-SiO₂ were low and showed high toxicity towards to *C.albicans* (24 h EC₅₀=119.68 mg/L; 48 h EC₅₀=98.06 mg/L) (Table 2). ANOVA tests statistics revealed that there is a linear relationship between NMOs concentrations and incubation period (for nano-HfO₂, Rsquare=0.992; for nano-SiO₂, Rsquare=0,993). It was found that regression analysis between time (for nano-HfO₂, p=0,0251< 0,05 ; for nano-SiO₂, p=0,01766< 0,05) and doses (for nano-HfO₂, p=0,0011< 0,05 ; for nano-SiO₂, p=0,0009< 0,05) was significant as a results of ANOVA tests (α=0,05).

Garcia-Suedo et al., 2011 studied nano-SiO₂ and nano-HfO₂ toxicity towards yeast *Saccharomyces cerevisiae* and the results demonstrated that this NMOs were not toxic at O₂ uptake, when NMOs were at concentrations as high as 1000 mg/L [X]. But Kahdum et al., (2017) [10] exhibited the antimicrobial activity of nano-SiO₂ and nano-ZnO and this NMOs could inhibit most of the important pathogenic bacteria. The MIC concentration of nano-SiO₂ was determined 0.625 µg/ml. Lipovsky et al (2011) found that a concentration-dependent effect of ZnO on the viability of *C.albicans* and at 1 mg/ml. Almost complete killing of 99.5 % of *C.albicans* was observed [17]. Kasemets and coworkers (2011) found that the EC₅₀ value

of yeast – *Saccharomyces cerevisiae* was 131 mg/l for nano-ZnO after 24 hours [18].

Results of Anaerobic Toxicity Assay (ATA)

Anaerobic toxicity assay was performed on methane production during 24 h and 48 h incubation periods at different NMOs concentrations. In this test methane gas production of each assay bottle were measured and inhibition percentages were calculated with the control groups no containing NMOs. The ATA test results indicated that increasing of NMOs doses caused adverse effect to methane productions from the anaerobic Archaea (Table 3). After 24 and 48 h incubation period, there is low toxicity to anaerobic methane Archaea for 1 mg/L nano-HfO₂ (I=0.35 %; I=1.79 %, respectively). As the nano-HfO₂ doses increased, the toxicity were increased 0.35 % to 14.23 % for 24 h and 1.79 % to 13.97 % for 48h (Table 3). Otherwise nano-SiO₂ is very toxic to anaerobic methane Archaea as seen in Table 3. After 48 h exposed to nano-SiO₂, bacteria were inhibited 100 % at 100 mg/L nano-SiO₂ dose (Table 3). ANOVA tests statistics for anaerobic toxicity assay (ATA) tests revealed that there is a linear relationship between NMOs concentrations and incubation period (for nano-HfO₂, Rsquare=0.095; for nano-SiO₂, Rsquare=0,92). It was found that regression analysis between time (for nano-HfO₂, p=0,0119<0,05; for nano-SiO₂, p=0,0143< 0,05) and doses (for nano-HfO₂, p=0,0003< 0,05 ; for nano-SiO₂, p=0,0101< 0,05) was significant as a results of ANOVA tests (α=0,05).

Table 3: Methane production and inhibition percentages of anaerobic Archaea after exposed to NMOs during 24h and 48h.

NMOs Concentrations (mg/L)	Nano-SiO ₂				Nano-HfO ₂			
	24 h		48 h		24 h		48 h	
	CH ₄ g (ml) *	I (%)**	CH ₄ g (ml)	I (%)	CH ₄ g (ml)	I (%)	CH ₄ g (ml)	I (%)
Control Group	25.3	0	26.7	0	28.1	0	27.9	0
1 mg/L	24.1	4.74	23.9	10.48	28	0.35	27.4	1.79
5 mg/L	22.8	9.88	23.6	10.49	27.1	3.55	27	3.22
10 mg/L	19.4	23.32	18.7	29.96	26.4	6.04	26.2	6.09
25 mg/L	13.4	47.03	9.4	64.79	25.1	10.67	24.9	10.75
50 mg/L	9.4	62.84	5.7	78.65	24.8	11.74	24.3	12.90
100 mg/L	2.1	91.69	0	100	24.1	14.23	24	13.97

* CH₄ g: Methane gas (ml) **I (%): Inhibition percentages (%)

Table 1: The number of *Candida albicans* colonies after 24 h and 48 h exposed to NMOs

	Nano-SiO ₂				Nano-HfO ₂			
	24 h		48 h		24 h		48 h	
	cfu/ml	I* (%)	cfu/ml	I (%)	cfu/ml	I (%)	cfu/ml	I (%)
Control Group	110	-	115	-	122	-	127	-
1 mg/L	105	4.54	101	12.17	120	1.63	118	7.08
10 mg/L	94	14.54	91	20.86	117	4.09	114	10.23
50 mg/L	77	30	68	40.86	102	16.39	102	19.68
100 mg/L	65	40.91	61	46.5	98	19.67	97	23.62

*I (%) : Inhibition percentages (%)

Table 2: EC₅₀ values of yeast – *Candida albicans*.

		Nano-SiO ₂	Nano-HfO ₂
		EC (mg/L)	EC (mg/L)
Yeast – <i>Candida albicans</i>	24 h	EC ₅₀ = 119.68	EC ₅₀ = 251.21
	48 h	EC ₅₀ = 98.06	EC ₅₀ = 250.16

Adsorption of nanoparticles to organic substance, precipitation of nanoparticles, or changes in surface charge are example of environmental influences that affect the toxicity of NMOs [15]. The study performed by Mu et al., (2011) showed that nano-TiO₂, nano-Al₂O₃ and nano-SiO₂ doses up to 150 mg/gr-TSS showed no inhibitory effect. But for nano-ZnO 22.8 % and 81.1 % methane gas inhibitions at 100 mg/l nano-ZnO containing 30 and 150 mg/g-TSS [16]. Releasing Zn²⁺ from nano-ZnO cause inhibitory effect. Furthermore, it was reported that the metabolic intermediates and key enzyme activities in sludge hydrolysis, acidification and methanation were inhibited by nano-SiO₂ [16]. Zhang et al., (2018) evaluated the effects of NMOs (nano-TiO₂, nano-SiO₂, nano-Al₂O₃, nano-CeO₂) on anammox process in waste water treatment plant and among these NMOs the most toxic NMO is nano-SiO₂ [X].



Biodegradability of NMOs

Table 4 showed that the soluble-COD concentrations measured for each NMOs and their removal efficiencies calculated at 5th, 10th, 15th, 20th and 28th days. The biodegradability of NMOs for 10 and 100 mg/L doses in activated sludge were measured during 28 days.

While the highest removal efficiency was 77.1 % for 10 mg/L nano-HfO₂ after 28 days, the lowest percentages was 11.2 % for 100 mg/L nano-SiO₂ after 28 days (Table 4). ANOVA tests statistics for biodegradability tests revealed that there is a linear relationship between NMOs concentrations and incubation period (for nano-HfO₂, Rsquare=0.952; for nano-SiO₂, Rsquare=0.935). It was found that regression analysis between time (for nano-HfO₂, p=0,0048< 0,05; for nano-SiO₂, p=0,0497< 0,05) and days (for nano-HfO₂, p=0,0046< 0,05; for nano-SiO₂, p=0,0006< 0,05) was significant as a results of ANOVA tests ($\alpha=0,05$).

Norani, et al. (2017) [10] evaluated the biodegradation behaviour of experimental particleboard bonded with modified 30 % of PVOH, 70 % of Oil palm starch and 3 % of nano-SiO₂ and nano-SiO₂ showed significant effect towards modified oil palm [X]. Dyshlyuk et al., (2020) studied the antimicrobial activity in protecting building materials from biodegradations. Nano-ZnO is more toxic for all bacteria and fungus species as compare to other NMOs (nano-SiO₂, nano-TiO₂) but the building materials biodegradated with NMOs [10].

Table 4: The biodegradability of NMOs during 28 days.

Days	Nano-SiO ₂		Nano-HfO ₂	
	10 mg/L-COD	100 mg/L-COD	10 mg/L-COD	100 mg/L-COD
t=0	0	0	0	0
t=5	11.34	3.1	23.9	18.4
t=10	28.4	4.8	46.8	27.6
t=15	30.1	6.8	53.7	51.7
t=20	34.7	9.7	72.7	54.3
t=28	36.9	11.2	77.1	55.9

Conclusions

In conclusion, among two nano-metal oxides (nano-SiO₂, nano-HfO₂) investigated in this study it was found that the less toxic NMO is nano-HfO₂ to yeast – *Candida albicans* because of the highest EC₅₀ value (250.16 mg/L) after 48 hours exposure time. While the more toxic NMO is nano-SiO₂ because of 100 % inhibition rate after 48 h at 100 mg/L doses, the less toxic NMO is nano-HfO₂ (I=13,97 %, after 4,8 h). The easiest biodegradable nanoparticles is nano-HfO₂ due to the highest removal efficiencies (77.1 %, at 10 mg/L concentration).

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