Effect of aluminium oxide nanoparticles on the enzymatic activity on microorganisms of activated sludge

Nina Doskocz[1,*], *Katarzyna* Affek[1], and *Monika* Załęska-Radziwiłł[1]

[1]Warsaw University of Technology, Faculty of Building Services, Hydro and Environmental Engineering, Department of Biology, Nowowiejska 20, 00-653 Warsaw, Poland

Abstract. The increased production and commercial use of nanoparticles (NPs), combined with a lack of regulation regarding their disposal, may result in the unwanted introduction of NPs to wastewater. Wastewater nutrient removal depends on the metabolisms of activated sludge bacteria and their related key enzymes. Therefore, the aim of this work was to determine the effect of aluminium oxide nanoparticles concentrations on the activated sludge enzymatic activity of microorganisms. Tested nanoparticles inhibition cellular respiration in TTC method in the four highest tested concentrations. Moreover, in most samples observed increase dehydrogenase activity. In this study, nano-Al_2O_3 also caused a clear stimulation of the activity of hydrolytic enzymes microorganisms of activate sludge. Effects of aluminum oxide (compound in bulk forms) on enzymatic activity were different than in the case of the nano from of Al_2O_3.

1 Introduction

Wastewater may contain wide variety chemical substances, which can inhibit microorganism's growth and cause various problems during biological treatment. Many harmful substances, depending on their physical and chemical properties, can be not readily biodegradable and remain toxic during the entire wastewater treatment process [1]. Examples of such compounds are nanoparticles, which are used in agriculture, industrial products and in medicine [2]. Investments for their production in 2005 amounted to about 10 billion dollars, and in the years 2011–2015 they were to reach USD 1.000 billion [3].

The intensive growth of nanotechnology triggers the increase in nanoparticle content in sewage and waste, which as a consequence makes them enter the surface waters and water intended for human consumption [4]. Literature data showed, that predicted concentrations in sludge, for engineered nanoparticles (nano-TiO_2, nano-Ag, nano-SiO_2, nano-Au) that are being used or could be used in cosmetics and personal care products and coatings, they ranged from 0.01 to 40.7 mg/kg [5].

These data indicate, nanoparticles can have some negative influence on microorganisms in activated sludge, which is a commonly used method for pollutants removal from

* Corresponding author: nina.doskocz@pw.edu.pl

wastewater and depends on many biochemical reactions, which are catalysed by some key enzymes in activated sludge bacteria. However, current knowledge about these effects is limited.

The inhibition effect of the nanoparticles on microorganism's growth in activated sludge has been reported [4, 6, 7]. In the literature there are also a few reports about influence NPs on biological processes of nitrogen and phosphorus removal as well as on denitrifying bacteria and microorganisms responsible for phosphorus elimination, the so-called PAO (polyphosphate accumulating organisms). Zheng et al. (2011) showed that, nano-ZnO can induce serious trouble in self-purification of water as well as in biological wastewater treatment [8].

However, current knowledge about impacts of NPs on enzymatic activates which are the excellent indicators of activated sludge microbial function is limited. Especially, dehydrogenase activity is commonly assessed as a general indicator of the oxidative capacity of microorganisms [9–12].

Therefore, the aim of the work was to determine the effect of aluminium oxide nanoparticles concentrations on the activated sludge dehydrogenase and hydrolytic activity. The scope of the investigations covers also experiments with bulk materials (compounds of the macro-form $–Al_2O_3$).

Little information is available on the destiny, transport, and effects of nanomaterials including metal-based particles such as nanosized Al_2O_3, in the environment. Nanosized aluminum is currently being used by the military and commercial industries in many applications including coatings, thermites, and propellants. The use of aluminium oxide nanoparticles in various applications may cause a release of the oxidized form of nano-Al_2O_3, into the environment. As utilization of nanomaterials is on the rise, it is increasingly important to determine their potential environmental destiny and the effects [2, 7].

2 Materials and Methods

2.1 Chemicals

Aluminum oxide nanoparticles (nano-Al_2O_3), nanopowder<50 nm of specific surface of > 40 m^2/g, aluminum oxide of purity of > 98%, were obtained from Sigma-Aldrich Company. Initial solutions of the tested compounds were prepared in deionized water. Because of the tendency of aggregate formation by the compounds, the obtained solutions of the compounds were sonicated for 30 min using an ultrasonic disintegrator of MDM-10 type (0.4 kW at a frequency of 20 kHz). These solutions of nano-Al_2O_3 and Al_2O_3 were diluted in a decreasing series with the ratio of geometric progression of dilutions of q = 2 obtaining solutions in the concentration range 500–0.97 mg/l.

Enzymatic activity of the microorganisms in the presence of tested compounds was determined by means of the triphenyl tetrazolium chloride (TTC) test for dehydrogenases activity and test for hydrolytic activity – fluorescein diacetate (FDA) determination. The TTC test was performed in accordance with the Polish Standard PN-C-04616-8 2008. Specific enzyme activity was estimated in a spectrophotometer at 490 nm by measuring the concentration of triphenyl formazan (TF) formed from TTC reduction.

Similarly, The TTC calibration curve was made as follows: introduced into tubes respectively 0.1; 0.2; 0, 3, 0.4; 0.5 ml of TF and made up to 5 ml with butanol. The obtained standard solutions contain respectively: 2.0; 4.0; 6.0; 8.0 and 10 μmol TF in a 5 ml sample. Absorbance measurement of the above solutions using a spectrophotometer at 490 nm.

Determination of TTC dehydrogenase activity was made as follows: introduced into tubes a suspension of bacterial cells in a volume of 5 ml. The cell suspension was prepared by inoculating 100 ml of broth medium with 0,5 ml of activated sludge after incubation with test compounds after 30 min. and 24 h. The cultures were incubated at 26°C in 48 h. After incubation, the cultures were centrifuged and suspended in buffered water. 7 mL Tris-HCl buffer, 1 ml Na_2SO_3 (0.36 %) were added into each tube. Incubation was carried out at 37°C under shaking conditions (120 rpm) in the dark for 30 min. 2 ml sulfuric acid was added to terminate enzyme reaction. 10 ml of butanol was added to the samples, mixed thoroughly and placed in a water bath with a temperature of 90°C for 5 min. Then 5 ml of butanol extract from each tube were centrifuged at 6000 rpm for 5 min. The TF was measured at 490 nm in the spectrophotometer. The results were given in μmol of TF per s and kg of dry weight (d.w.) [13].

The FDA test was performed in accordance with the Schnürer and Rosswall method [14]. The bacterial suspensions in the phosphate buffer (pH 7.6) with FDA in acetone (20 mg cm^{-3}) were incubated at 26°C under shaking conditions. The enzymatic reaction was stopped by the addition of acetone in a volume ratio of 1:1. The concentration of produced fluorescein was determined spectrophotometrically at 490 nm. The enzymatic activity is reported as mg of fluorescein s^{-1} g^{-1} d.w. [15].

To perform TTC and FDA tests the appropriate chemicals and activated sludge working in laboratory conditions, in Department of Biology, Faculty of Building Services, Hydro and Environmental Engineering, Warsaw University of Technology were used.

Assessment of inhibition activity of the hydrolase was made after 30 min and 24 hours of activated sludge incubation with the tested compounds. Each experiment was done in 3 replicates. The mean values and standard deviations was then calculated for each experimental group exposed to tested compounds. Samples containing activated sludge that had not previously been treated with the test compounds were used as control. To clear the presentation of the results, the enzyme activity data of treated activated sludge samples were expressed as the percentage of the enzyme activity compared to the control sample.

Inhibition of enzymatic activity was calculated according to Eq. 1.

$$I = \frac{B_c - B_n}{B_c} \cdot 100[\%] \tag{1}$$

I_c – Inhibition of enzymatic activity
B_c – Optical density of suspension in control sample after time t
B_n– Optical density of suspension in the sample examined after time t

3 Results and discussion

The enzymatic activities in activated sludge treated with two different compounds: aluminium oxide NPs, and their bulk form – Al_2O_3 were measured after 30 min and 24 h of exposure. In enzymatic tests (TTC and FDA tests) with microorganisms from activated sludge, were found inhibitions and stimulation of enzyme activity.

The dehydrogenase activity was changing rapidly. The activity in samples with nano-Al_2O_3 after 30 minutes ranged from 42.2 to almost 337.4 μmol TF/kg d.w· s^{-1} and increased with decreasing in the concentration of the tested NPs. After 24 hours observed increased of dehydrogenase activity with increasing concentration of tested NPs – from 82.2 to almost 192.4 μmol TF/kg d.w. s^{-1} (table 1).

Aluminum oxide nanoparticles, after 30 minutes contact with microorganisms in concentrations from 500–31 mg/l, inhibited the activity of dehydrogenases from 66%

to 0.39%, respectively. At lower concentrations NPs clearly, stimulated activity of this enzyme.

After 24 h, in concentrations of 500–15 mg/L, stimulation of dehydrogenase activity was also found, while in the lower concentration, inhibition of enzymatic activity was observed (figure 1).

Hydrolytic activity was slowly decreasing from the beginning till the end of the duration of the test (table 1). Nano-Al_2O_3 regardless of the time of action on activated sludge microorganisms caused a clear stimulation of the activity of hydrolytic enzymes in these microorganisms (figure 3).

The obtained results also show that the tested nanoparticle a different impact on dehydrogenase and hydrolytic activities than the same compound in the bulk form (table 2), (figure 2 and 4). Aluminium oxide greater stimulated dehydrogenase activity and to a lesser extent stimulated hydrolytic activity than compound in nano form.

Table 1. Dehydrogenase and hydrolytic activity in the presence of nano-Al_2O_3.

Tested compound	**Concentration [mg/l]**	**Dehydrogenase activity [µmol TF/kg d.w.]** (standard deviation, SD)		**Hydrolytic activity [µg fluorescein/kg of d.w. s^{-1}]** (standard deviation, SD)	
		after 30 min	**after 24 h**	**after 30 min**	**after 24 h**
Nano–Al_2O_3	500	42.2 (42.7–42.5)	192.4 (192.2–193.0)	704.0 (74.9–704.2)	16.6 (16.3–16.1)
	250	82.4 (82.5–81.7)	193.1 (193.6–194.2)	406.9 (406.4–406.5)	15.9 (15.1–16.7)
	125	88.5 (88.8–89.1)	161.8 (161.5–161.4)	384.7 (384.9–384.1)	15.8 (15.8–15.1)
	62.5	118.8 (118.6–119.5)	135.0 (135.0–136.4)	357.6 (358.2–357.8)	11.2 (11.4–11.5)
	31.3	123.9 (123.7–122.9)	126.1 (125.7–126.9)	319.4 (319.9–320.8)	10.9 (11.1–10.7)
	15.6	138.1 (138.1–138.6)	115.9 (115.9–116.3)	188.9 (188.6–188.9)	10.6 (10.6–10.7)
	7.8	195.0 (195.2–196.0)	110.6 (123.7–122.9)	155.7	10.4 (10.3–10.3)
	3.9	203.4 (203.9–203.4)	98.7 (98.8–98.7)	117.8 (117.7–118.9)	8.8 (8.6–8.4)
	1.9	231.4 (231.7–232.3)	96.7 (96.3–96.3)	74.3 (73.9–74.5)	8.6 (8.7–9.1)
	0.97	337.4 (338.3–337.8)	82.2 (82.8–81.7)	69.2 (69.4–68.7)	4.3 (4.2–4.7)
	Control	124.4 (124.7–124.3)	110.8 (110.8–111.1)	19.6 (19.8–19.3)	0.7 (0.6–0.7)

Table 2. Dehydrogenase and hydrolytic activity in the presence of Al_2O_3.

Tested compound	Concentration [mg/l]	Dehydrogenase activity [µmol TF/kg d.w.] (standard deviation, SD)		Hydrolytic activity [µg fluorescein/kg of d.w. s^{-1}] (standard deviation, SD)	
		after 30 min	after 24 h	after 30 min	after 24 h
Al_2O_3	500	68.2 (68.7–69.5)	265.8 (265.9–265.8)	744.3 (744.9–745.1)	20.5 (20.3–20.9)
	250	72.5 (73.4–72.9)	239.8 (239.7–239.3)	542.8 (5455.1–541.9)	19.6 (19.3–20.1)
	125	98.5 (98.8–99.7)	211.8 (211.1–212.2)	486.2 (485.2–485.8)	18.6 (20.3–20.4)
	62.5	120.9 (120.1–120.9)	208.4 (207.9–210.4)	457.4 (457.9–457.8)	17.0 (17.8–18.4)
	31.3	178.3 (178.2–179.6)	206. 9 (204.9–206.6)	419.2 (419.6–420.1)	16.0 (16.5–16.5)
	15.6	209.3 (208,9–209.2)	195.7 (214.9–212.5)	378.6 (265.9–265.8)	14.3 (14.8–15.1)
	7.8	213.7 (214.9–212.8)	171.7 (172.5–171.8)	275.3 (274.9–275.5)	11.7 (11.6–11.7)
	3.9	241.7 (241.8–242.1)	153.4 (153.2–153.7)	217.8 (218.4–217.2)	10.4 (17.8–18.4)
	1.9	271.8 (272.8–270.9)	137.8 (214.9–212.8)	104.3 (104.9–104.9)	9.8 (9.1–8.8)
	0.97	367.2 (366.8–367.1)	92.2 (92.1–93.6)	85.1 (82.8–85.6)	6.3 (6.2–6.6)
	Control	125.8 (126.1–125.4)	115.6 (115.4–115.2)	20.1 (20.3–20.4)	1.6 (1.7–1.5)

Table 3. Percentage of inhibition and stimulation of dehydrogenase activity in the presence of nano-Al_2O_3 and Al_2O_3.

Tested Compounds	Concentration [mg/l]	Percentage of inhibition and stimulation of dehydrogenase activity [%] (standard deviation, SD)	
		after 30 min	after 24 h
Nano–Al_2O_3	500	66.07 (64.87–59.75)	–78.19 (–76.72–(–78.46)
	250	33.72 (29.69,87–34.55)	–74.32 (–75.35–(–74.61)
	125	28.82 (29.71–31.89)	–46.01 (–46.23–(–45.33)
	62.5	4.50 (4.22–6.43)	–21.89 (–46.23–(–45.33)
	31.3	0.39 (0,76–0,38)	–13.80 (–12.97–(–13.84)
	15.6	–11.04 (–14.39–(–16.37)	–4.65 (–4.23–(–4.57)
	7.8	–56.8 (–49.92–(–56.74)	0.13 (–4.23–(–4.57)
	3.9	–63.53	10.86

		(–64.37–(–63.25)	(–10.54–(–10.29)
	1.9	–86.02 (–86.72–(–86.46)	12.71 (–12.66–(–13.01)
	0.97	–171.24 (–172.36–(–170.63)	25.77 (–4.23–(–4.57)
	Control	0.00	0.00
Al_2O_3	500	45.80 (45.71–34.79)	–129.82 (–128.92–(–129.58)
	250	42.37 (41.63–42.09)	–107.37 (–104.76–(–107.81)
	125	21.70 (21.87–22.32)	–83.14 (–84.03–(–83.63)
	62.5	3.95 (2.79–3.61)	–80.26 (–80.25–(–79.83)
	31.3	–41.67 (–41.94–(–41.75)	–78.93 (–75.35–(–74.61)
	15.6	–66.3 (–66.75–(–66.74)	–69.28 (–68.37–(–69.03)
	7.8	–69.75 (–69.72–(–70.40)	–48.44 (–48.62–(–48.57)
	3.9	–92.04 (–92.86–(–91.39)	–32.63 (–31.99–(–32.53)
	1.9	–115.95 (–114.72–(–115.58)	–19.19 (–41.94–(–41.75)
	0.97	–191.72 (–192.37–(–191.55)	14.19 (–14.66–(–14.37)
	Control	0.00	0.00

Table 4. Percentage of inhibition and stimulation of hydrolytic activity in the presence of nano-Al_2O_3 and Al_2O_3.

Tested Compounds	**Concentration [mg/l]**	**Percentage of inhibition and stimulation of hydrolytic activity [%]** (standard deviation, SD)	
		after 30 min	**after 24 h**
Nano–Al_2O_3	500	–3489.94 (–3488.92–(–3529.51)	–2339.63 (–2338.34–(–2327.56)
	250	–1974.90 (–1976.33–(–1965.75)	–2248.21 (–2234.45–(–2248.21)
	125	–1861.55 (–1931.91–(–1868.73)	–2228.63 (–2233.07–(–2246.45)
	62.5	–1723.27 (–1753.66–(–1739.38)	–1545.75 (–1544.54–(–1574.22)
	31.3	–1528.73 (–1531.78–(–1561.44)	–1517.99 (–1538.67–(–1527.51)
	15.6	–863.05 (–864.49–(–863.55)	–1461.89 (–1437.78–(–1483.98)
	7.8	–693.85 (–691.40–(–694.02)	–1438.83 (–1421.73–(–1481.56)
	3.9	–500.76 (–501.41–(–500.41)	–1202.13 (–1266.12–(–12,34.16)
	1.9	–278.85 (–280.64–(–277.57)	–1162.73 (–1144.54–(–1176.69)

	0.97	–252.88 (–251.19–(–252.27)	–542.33 (–541.46–(–543.22)
	Control	0.00	0.00
Al_2O_3	500	–3595.78 (–3596.44–(–3595.84)	–1052.72 (–1052.67–(–1053.17)
	250	–2595.13 (–2595.85–(–2595.26)	–999.89 (–998.76–(–999.89)
	125	–2313.95 (–2312.39–(–2327.56)	–943.13 (–944.77–(–941.65)
	62.5	–2171.25 (–2171.68–(–2171.77)	–856.58 (–956.13–(–855.880
	31.3	–1981.58 (–1982.93–(–1982.43)	–801.50 (–801.27–(–802.06)
	15.6	–1779.64 (–1779.54–(–1779.22)	–705.95 (–705.74–(–706.28)
	7.8	–1266.88 (–1254.55–(–1274.45)	–558.14 (–558.53–(–558.87)
	3.9	–981.28 (–984.76–(–981.89)	–483.95 (–483.04–(–482.74)
	1.9	–418.12 (–418.27–(–429.06)	–449.67 (–449.64–(–449.57)
	0.97	–322.79 (–324.04–(–321.94)	–256.33 (–256.58–(–256.39)
	Control	0.00	0.00

Rapidly developing industry raises concerns about the environmental impacts of nanoparticles, but the effects of nanoparticles on bacterial metabolism in wastewater treatment remain unclear. The comparison of dehydrogenases' and hydrolytic activity between samples with activated sludge containing nano-Al_2O_3 and the control samples (activated sludge without NPs) proved that tested nanoparticles can influence on metabolism of the bacteria in the suspension. After 24h in high tested concentration, nano-Al_2O_3 caused decrease in dehydrogenase activity, suggesting biocidal properties of these nanoparticles. In most samples we observed increase enzyme activities. This may be related with the role of potentially released Al ions which are a part of the active site of the enzyme [16]. Therefore, the presence of Al can have a positive effect on the activity of that enzyme [17]. Ions released from the NPs can only partially be responsible for the toxic or stimulated effect NPs. Stimulation of enzymatic activity of microorganisms in activated sludge may be disadvantageous. May lead to an increased demand for oxygen in biomass biodegradation process and can lead to the formation of anaerobic zones. These results may suggest that the presence of nanoparticles might negatively influence the communities of micro-organisms participating in biological processes.

The data found in literary sources about effect of nanoparticles on enzymatic activity, confirm our studies. Chojniak et al. (2017) also not observed decrease in respiratory activity, moreover confirmed activity of silver NPs as anti-biofilm agent [18].

In turn, study of Asadishad et al. (2017) showed, that the nanoparticles (citrate-Gold 50 nm, PVP-Gold 50 nm, PVP-Gold 5 nm, PVP-Gold 100 nm) caused an initial decrease in enzyme activity after 2 hours of exposure. We also observed (after 24 h in high tested concentration) inhibition cellular respiration in TTC method [19].

Furthermore, our previous study [7] on NPs' influence on planktonic and biofilm bacteria participate in wastewater treatment, showed inhibitory effects of Al_2O_3 NPs in higher concentrations and stimulation of the bacteria growth in lower concentrations, which confirms that the tested NPS in this study (after 24 h at high concentrations), can

inhibit growth/amount of microorganisms of activated sludge, and thus, affect their metabolism and wastewater treatment processes.

The obtained results shown that the effects of nano-Al_2O_3 on bacteria and their metabolism were stronger/different than those observed for their bulk counterparts (table 2, figure 2 and 4).

NPs have greater specific surface area, and therefore greater reactivity and potential for generating ROS (reactive oxygen species) than their bulk counterparts, and thus their expected inhibition or stimulation of activity should be greater, however, the opposite tendency can also be observed. In our study we observed greater inhibition after 30 min and smaller stimulation after 24 h of dehydrogenase activity in the presence of the nano- than the bulk form of Al_2O_3. In the case of hydrolytic activity, Al_2O_3 caused greater less stimulation the nano form of Al_2O_3. Lower toxic effects for nano from in some samples may have been determined by their tendency to aggregation and solubility. Aggregation causes a decrease of specific surface area of nanoparticles and of their solubility, and hence, lowers reactivity [19, 20]. Similar conclusions were obtained by Kim et al. (2011) who also observed different dehydrogenase activity in the presence of the nano- than the bulk form of CuO [9].

4 Conclusions

The conducted studies concerning the impact of aluminum oxide nanoparticles (nano-Al_2O_3) on the enzymatic activity on microorganisms of activated sludge allowed formulating the following conclusions:

- Aluminum oxides nanoparticles caused decrease in dehydrogenase activity after 2 hours of exposure;
- Tested nanoparticles inhibition cellular respiration in TTC method only in the four highest tested concentrations;
- Nano-Al_2O_3 caused at high concentrations caused a clear stimulation of dehydrogenase activity after 30 minutes as well as after 24 hours in low concentrations;
- Nano-Al_2O_3 caused a clear stimulation of the activity of hydrolytic enzymes in microorganisms of activate sludge;
- Effects of aluminum oxide (Al_2O_3) on enzyme activity were different than in the case of the nano forms.

This research confirmed the data found in literary sources and showed that the presence of nanoparticles might influence on biological processes of wastewater treatment by negative effects on enzyme activity of microorganisms participating in this process

It was found that nano forms of the tested compounds were posing different risks to microorganisms involved in biological wastewater treatment than the same compounds in the bulk form. Therefore, available ecotoxicity data about these compounds cannot be used to assess the harmfulness of their nano form counterparts.

This study increased the ecotoxicological knowledge and database in relation to the effect of aluminum oxide nanoparticles on enzymes in activated sludge bacteria, which catalyze many biochemical reactions, involved in to pollutants removal from wastewater. Due to specific features which are characteristic for nanocompounds, it is also important to study the interaction mechanism of nanoparticles with microbial cells on molecular level.

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