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Effect of aluminum oxide nanoparticles on aquatic organisms – a microcosm study

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ABSTRACT

The study examined structural and functional changes in the microcosm, inhabited by representatives of all trophic levels of the aquatic food chain, in the presence of aluminum oxide nanoparticles and their bulk counterparts (100 mg/L). The 28 d experiment showed negative effects of nano-Al_2O_3 on aquatic ecosystems, as evidenced by reduced biodiversity of microbenthic and plankton organisms. Furthermore, exposure to nanoparticles contributed to the increase of the activity of antioxidative enzymes of benthos. Cytochemical analysis of *Daphnia magna* crustacean cells showed accumulation of nanoparticles on the surface of organisms and in their mitochondria, causing swelling of mitochondrial cristae, as well as disturbances of their system with a clear matrix in the centre of organelles. However, there were no significant changes in microcosms with bulk counterparts of nanoparticles, as well as no accumulation of aluminum oxide on the surface, nor in the mitochondria of *D. magna,* was detected.

Keywords: Nanoparticles; Aluminum oxide; Microcosms; Ecotoxicity; Biodiversity

1. Introduction

Nanoparticles (NPs) have numerous applications, including the use in various domestic products, such as textiles and personal care products. Therefore, there is a high potential of release of NPs to the aquatic ecosystems, where they are affected by abiotic factors, such as temperature, pH, and ionic strength. Those agents alter the chemistry of NPs and influence their fate in the environment. Furthermore, NPs tend to quickly aggregate in the water column through sedimentation, which promotes exposure of benthic organisms to this type of compounds [1]. As a result, NPs can affect broader systems because many benthic microorganisms or invertebrates, are the basis of food webs or play a principal role in litter decomposition and organic matter release.

Despite ongoing research on the toxicity of NPs, there is still a lack of data concerning risk and fate of nanocompounds in the natural environment [2,3]. Most studies are currently performed using single species and poorly reflect environmental conditions [1,4–9]. There is little information in the literature about the long-term impact of NPs on the aquatic environment. Multispecies studies in model aquatic ecosystems – microcosms, differing in volume, organisms and, criteria for test endpoints assessment, are recommended by researchers. Such experiments are much more laborious, but, at the same time, they give a more reliable assessment of probability of phenomena that can occur in natural biocenosis. Moreover, microcosmic studies allow a deeper understanding of ecological processes, such as species interactions, biomass production, and organic matter decomposition [10]. Multispecies

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research has been carried out on several types of NPs, but little information could be found about the effects of nano-Al_2O_3. Some studies have confirmed the difference in toxicity of nano- and micro-sized NPs [2,11]. Laboratory studies on algal populations (*Desmodesmus* sp., *Scenedesmus* sp., *Chlorella* sp., and *Pseudokirchnella* sp.,) have shown that bulk aluminum is less toxic than nano-sized aluminum [2,4,5,12]. The toxicity of bulk counterparts of NPs to invertebrates and higher plants have also been reported [5,13]. Therefore, we made a hypothesis, that the basis for ecotoxicological assessment of the impact of nanoparticles on organisms of aquatic ecosystems should be a set of conventional tests (single species tests) supplemented with multi-species microcosm studies. In addition, we think that Al_2O_3 nanoparticles due to their specific properties may be more toxic than compounds in the macro form in multispecies studies.

Due to the fact that the literature data indicate that nanoparticles have completely different properties than substances in the macro form, such as, they are characterized by low solubility (tendency to form agglomerates), exhibit low degradability and can occur in the environment in high concentrations reaching values in milligrams, the paper presents on more assumption. Criteria set by the US EPA or the European Union declaring the compounds to be non-toxic, when the determined $LC(EC)_{50}$ is higher than 100 mg/L, and other commonly accepted and included in risk assessment for "normal" compounds, are not sufficient to adequately characterize the risks associated with such specific compounds as nanoparticles [14,15]. Furthermore, the hypothesis was tested that the lack of visible acute toxic effects [4,5] in the presence of nano-Al_2O_3 at 100 mg/L does not necessarily mean no change at the cytogenetic level. Sun et al. [16], showed that PEC for zinc oxide nanoparticles was very high and reached 200 mg/kg (in bottom sediment). Literature data has also shown that nanoparticles used in cosmetics are detected in wastewater in an amount of about 50 g per capita and day [17]. Nano-Al_2O_3 are currently mostly used in chemical and military industry. They find their application also in medicine, among others in anti-cancer or immunological therapy. Extensive use of nano-Al_2O_3 may lead to their widespread distribution in the environment, and hence, to unpredictable changes in aquatic ecosystems [18–20].

Hence, the aim of the work was to study structural and functional changes in the microcosm, in the presence of aluminum oxide NPs (nano-Al_2O_3). Additionally, to investigate the dependence of Al_2O_3 toxicity on the form of the occurrence of this compound, toxic effects were compared in separate microcosms, contaminated with nano- and macro-Al_2O_3, respectively.

2. Materials and methods

2.1. Chemicals

Commercial samples of nano-Al_2O_3 (nanopowder < 50 nm with a specific surface area > 40 m^2/g) and its bulk counterpart (macro-Al_2O_3, purity over 98%) were obtained from Sigma-Aldrich. CAS no. of compounds containing Al_2O_3 is 1344-28-1. The stock suspensions (1,000 mg/L) of nano- and macro-forms were prepared in deionized water. To avoid formation of the aggregates, the stock suspensions were sonicated (0.4 kW, 20 kHz) for 30 min before being diluted to the exposure concentrations.

2.2. Microcosms preparation

The experiment was carried out in 10 L aquariums, filled with water treated in a biofilter, and inhabited by representatives of the food chain – higher plants (*Lemna minor*), algae (*Desmodesmus quadricauda*, *Scenedesmus obliquus*, *Raphidocelis subcapitata*, *Chlorella vulgaris*), cyanobacteria (*Microcystis aeruginosa*), and crustaceans (*Daphnia magna* and *Heterocypris incongruens*). Bottom sediment was prepared as mixture of sand and activated sludge inhabited by oligochaete *Tubifex tubifex* and was placed in separate crystallizers. After 2 weeks of adaptation of organisms, the tested compounds were introduced to the microcosms to obtain a final exposure concentration of 100 mg/L. The concentration was selected based on the results of single species ecotoxicity tests carried out on aquatic organisms [4,5,20]. Nano-Al_2O_3 and macro-forms of aluminum oxide were tested in separate aquariums in triplicates, and control microcosms without the added compounds were also prepared. Light was provided in 12:12 h light–dark cycles and constant temperature was maintained (21°C ± 1°C). The tank was constantly aerated, the study was carried out for 4 weeks. Control analyses were carried out at the beginning of experiments and after 14 and 28 d.

2.3. Physico-chemical analyses

Ammonium, nitrite, nitrate, total nitrogen, soluble orthophosphate, total phosphorus, chlorides, and chemical oxygen demand (COD) were determined by standard LCK vial test kits and sachet tests using DR3900 spectrophotometer (HACH-Lange). Dissolved organic carbon (DOC) was determined by TOC-5000 analyzer (Shimadzu, Japan) [21–28]. Temperature, pH, and dissolved oxygen concentration were measured in accordance with Standard Methods (1999) [29].

2.4. Biological analyses

Quantitative analyzes of plankton and bottom sediment organisms were carried out using light microscopy in Sedgwick–Rafter chambers at 100× magnification (phytoplankton and bottom sediment organisms) and 4× (zooplankton). The results were expressed as the number of organisms in 1 mL of the sample (phytoplankton and bottom sediment organisms) or as the number of organisms in the total volume of the model ecosystem (zooplankton). Abundance of filamentous bacteria was assessed by comparing the microscopic image of the samples with the reference photographs and expressed as filament index (F0–F5) [30].

The obtained results were used to calculate the biodiversity index [31]. The general species diversity index of Shannon (H') (1948) was calculated according to the equation:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i \quad (1)$$

where S is the number of species (taxa) in the sample; p_i is a fraction of the total number of individuals (N) of the i-th species (taxon), that is,

$$p_i = \frac{n_i}{N} \tag{2}$$

During the experiment observations of *D. magna* organisms were carried out in in the scanning electron microscopy (SEM) LEO 435VP and the transmission electron microscope (TEM) Libra 120. Photographic documentation was made with a Slow Scane CCD camera (Proscane) using the EsiVision Pro 3.2 software (Soft Imaging Systems GmbH). Measurements were made with the analySIS® 3.0 image analysis software (Soft Imaging Systems GmbH) (the analysis was carried out in the Laboratory of Electron Microscopy, Faculty of Biology, University of Warsaw).

The *L. minor* growth inhibition test was performed in accordance with PN-EN ISO 20079:2004 methodology. Growth inhibition assessments were performed based on surface area measurement and number of leaves count. Measurements were made using UTHSCSA ImageTool digital image analysis software version 3.0 [32].

Enumeration of culturable bacteria (CFU/mL) in water samples was performed by the colony count method using TSA medium (PN-EN ISO 6222: 2004) [33].

Catalase activity in tissues of the whole organisms of *T. tubifex* was determined by the Goth method, which uses the ability to form a stable complex by the substrate (H_2O_2) and ammonium molybdate formed in the analyzed sample [34]. Specific activity of the enzyme was expressed in mM/min/mg protein. In order to determine the protein content by the Lowry method [35], 15 individuals of *T. tubifex* were washed in phosphate buffer (pH 7.4) to remove residual sediment. The oligochaetes were immobilized on lignin in an ice-water bath and homogenized for about 1.5 min. The obtained homogenates were centrifuged for 15 min at 12,000 g at 4°C to remove cell debris, aliquoted, and stored in a freezer at –45°C.

Determination of dehydrogenase activity of the microorganisms in bottom sediment was performed by the triphenyltetrazolium chloride (TTC) test in accordance with PN-C-04616-8 2008 [36]. Specific enzyme activity was determined at 490 nm by measuring the concentration of triphenyl formazan (TF) formed from TTC reduction and expressed as micromoles of TF per second and kilogram of protein mass (μkatal/kg). Two types of samples were prepared – containing an additional carbon source (glucose) and without exogenous substrate. Samples incubation was carried out within 24 h in mixing conditions (120 rpm) at 26°C. The protein content was determined by the Lowry method in cell-free extracts obtained after ultrasonic disintegration.

3. Results and discussion

No significant differences in the physico-chemical parameters were observed between the tested microcosms and the control microcosm at various sampling time (Table 1). However, an increase in COD and DOC was observed in both the test and control samples – these parameters reached the highest value on the last day of the study, which was probably related to the presence of metabolic products of organisms and animal excrements. In addition, a continuous increase in total phosphorus concentration was observed in microcosms. Bour et al. [1] also did not detect any significant changes in the physico-chemical parameters of the microcosm system with CeO_2 NPs. Only the pH fluctuated (decrease in the 2nd week of the study to 7.8, when the mean pH was 8.5), while in our study, the pH was in the range of 8.76–8.08 in all samples tested.

Microscopic analysis of bottom sediment (Table 2) revealed the presence of numerous protozoa in both the control and the test samples. Highest organism counts on day 28 belonged to control and macro-Al_2O_3 contaminated microcosms. Flagellates and ciliates, including *Paramecium* sp., were particularly abundant (6.5×10^3–9.4×10^3, 5.7×10^3–9.2×10^3, and 8.0×10^3–2.1×10^4 individuals in mL, respectively). The total number of benthic animals detected in the microcosm with nano-Al_2O_3 was significantly lower than in two other microcosms. Nematodes and filamentous bacteria were present in all microcosms throughout the study, but rotifers were only detected in both microcosms contaminated with Al_2O_3 at the end of the experiment. The presence of rotifers in tested samples can be explained by the fact that their main role in the sediment is the eating of bacteria and small protozoa, which helps rejuvenate the population and maintain biological balance. The obtained results indicate that in the samples tested there were definitely fewer protozoa and bacteria than in the control sample, for which the rotifers may be responsible.

Analysis of the biodiversity of the microbenthos community based on the Shannon index showed that in all microcosms, biodiversity increased after 2 weeks and was the highest in the control sample. On day 28 of the experiment, biodiversity in the sample with nano-Al_2O_3 was significantly lower than that observed in the control sample. In turn, biodiversity in the microcosm with macro-Al_2O_3 on the last day of the study was similar to the control sample (Table 3). Nanoparticles are characterized by low solubility, due to which they tend to form agglomerates and are slightly degradable. Both aspects promote accumulation in biological systems and accessibility to the organisms present there [37]. This may explain why in our microcosms with nano-Al_2O_3, there is almost half of the biodiversity of the microbenthos in comparison to the control microcosm.

No significant effect of nano-Al_2O_3 on dehydrogenase activity was observed. The enzymatic activity of bottom sediment microorganisms decreased during the experiment in all microcosms and changed depending on the availability of nutrients (Table 4). In addition, the activity of dehydrogenases increased with the increase of organic substance introduced into a given environment, indicating more intense mineralization processes [38]. This shows that in the examined microcosms, there was little organic substance that microorganisms could use, and thus the enzymatic activity decreased. These results stand in line with our study on bacterial abundance in the microcosms (Table 9). In all microcosms, the enzymatic activity in the samples with the addition of glucose was higher than in the samples without glucose, which indicates a shortage of carbon sources that could be easily utilized by microorganisms.

Table 1
Physical and chemical parameters of water in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Parameters	Sampling time (d)		
		0	14	28
With nano-Al_2O_3	*T*, °C	20 ± 0.00	20 ± 0.00	20 ± 0.00
	Oxygen, mg/L	8.70 ± 0.02	8.20 ± 0.01	7.40 ± 0.2
	pH	8.76 ± 0.06	8.41 ± 0.11	8.29 ± 0.01
	COD, mg O_2/L	52.2 ± 0.6	128 ± 1	163 ± 1.00
	DOC, mg C/L	20.9 ± 0.3	24.1 ± 0.1	29.1 ± 0.30
	N, mg N/L	8.78 ± 0.06	9.57 ± 0.81	9.54 ± 0.01
	NH_4^+, mg N_{NH_4}/L	0.00 ± 0.00	0.04 ± 0.04	0.36 ± 0.26
	NO_2^-, mg N_{NO_2}/L	0.002 ± 0.001	0.036 ± 0.001	0.05 ± 0.07
	NO_3^-, mg N_{NO_3}/L	2.38 ± 0.51	1.65 ± 0.80	1.80 ± 0.94
	P, mg P/L	0.105 ± 0.07	0.16 ± 0.03	0.34 ± 0.05
With macro-Al_2O_3	*T*, °C	20 ± 0.00	20 ± 0.00	20 ± 0.00
	Oxygen, mg/L	8.70 ± 0.02	8.55 ± 0.24	8.60 ± 0.11
	pH	8.76 ± 0.06	8.08 ± 0.58	8.50 ± 0.27
	COD, mg O_2/L	52.2 ± 0.6	173 ± 1	317 ± 1
	DOC, mg C/L	20.9 ± 0.3	22.3 ± 0.9	31.2 ± 0.2
	N, mg N/L	8.78 ± 0.06	8.97 ± 0.05	11.3 ± 0.1
	NH_4^+, mg N_{NH_4}/L	0.00 ± 0.00	0.05 ± 0.04	0.045 ± 0.061
	NO_2^-, mg N_{NO_2}/L	0.002 ± 0.001	0.04 ± 0.01	0.047 ± 0.017
	NO_3^-, mg N_{NO_3}/L	2.38 ± 0.51	2.15 ± 0.62	2.10 ± 0.14
	P, mg P/L	0.105 ± 0.07	0.159 ± 0.012	0.185 ± 0.184
Control	*T*, °C	20 ± 0.00	20 ± 0.00	20 ± 0.00
	Oxygen, mg/L	8.70 ± 0.02	8.80 ± 0.10	8.78 ± 0.09
	pH	8.76 ± 0.06	8.76 ± 0.08	8.57 ± 0.07
	COD, mg O_2/L	52.2 ± 0.6	173 ± 1	364 ± 1
	DOC, mg C/L	20.9 ± 0.3	23.1 ± 0.6	29.4 ± 0.4
	N, mg N/L	8.78 ± 0.06	4.00 ± 0.11	5.32 ± 0.36
	NH_4^+, mg N_{NH_4}/L	0.00 ± 0.00	0.04 ± 0.26	0.05 ± 0.04
	NO_2^-, mg N_{NO_2}/L	0.002 ± 0.001	0.004 ± 0.006	0.003 ± 0.009
	NO_3^-, mg N_{NO_3}/L	2.38 ± 0.51	1.20 ± 0.04	1.00 ± 0.05
	P, mg P/L	0.105 ± 0.07	0.028 ± 0.033	0.134 ± 0.018

The activity of catalase in *T. tubifex* tissues fluctuated in all microcosms. After day 14 of the study, a decrease in the activity of this enzyme was observed, whereas the catalase activity increased on day 28. The increase in enzyme activity on the last day of the study in the microcosm with the tested nanocompounds was over 40% higher than in the control sample, whereas in the case of Al_2O_3 macro-form the catalase activity was similar to the control (Table 5).

Nanoparticles had little impact on *L. minor* growth and development (Table 6). After 14 d of experiments, tested nanocompounds stimulated plant growth. However, after 28 d, inhibition of the number and surface area of the leaves *L. minor* was observed. In the case of this metal oxide, stimulation of duckweed growth was observed after both 14 and 28 d of the test.

Hydrobiological studies of plankton showed that the number of algal cells fluctuated, reaching the highest level after 14 d of the experiment in all microcosms (Table 7). The number of algae depended on the number of zooplankton (Table 8) and decreased with the increase in the number of consumers.

The biodiversity of phytoplankton in the microcosms with macro-Al_2O_3 and without Al_2O_3 was at a similar level throughout the experiment. In the microcosm with nano-Al_2O_3, biodiversity dropped to 0.4 after 14 d and remained at this level until the end of the experiment (Table 3).

The number of crustaceans (Table 8) increased in all microcosms during the experiment, reaching 54–426 individuals in 10 L of water. However, in the presence of nano-Al_2O_3, the number of *D. magna*, and *H. incongruens* at the end of the experiment was 75% and 68% lower, respectively, than in the control. A definitively greater increase in the number of crustaceans was observed in the microcosm with macro-Al_2O_3, than with nano-Al_2O_3.

An increase in the number of bacteria from 20 to 760–960 CFU/mL was observed in all microcosms after 14 d

Table 2
Microorganisms in bottom sediment [individuals/mL] in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Organisms	Sampling time (d)		
		0	14	28
With nano-Al_2O_3	*Litonotus* sp.	0 ± 0.00	0 ± 0.00	0 ± 0.00
	Paramecium sp.	2,567 ± 67	0 ± 0.00	0 ± 0.00
	Plagiocampa rouxi	0 ± 0.00	0 ± 0.00	0 ± 0.00
	Pseudocohnilemsbus pussillus	0 ± 0.00	0 ± 0.00	0 ± 0.00
	Euglena sp.	0 ± 0.00	621 ± 32	585 ± 12
	Peranema sp.	0 ± 0.00	497 ± 58	473 ± 49
	Ciliata nd.	6,821 ± 129	8,510 ± 162	9,200 ± 188
	Flagellata nd.	7,544 ± 158	6,741 ± 128	8,965 ± 163
	Rotatoria nd.	0 ± 0.00	762 ± 38	1,300 ± 76
	Nematodes	1,342 ± 100	1,438 ± 129	4,000 ± 63
	Filamentous bacteria Type 021N	FI5	FI1	FI1
	Total microfauna	18,274 ± 355	18,569 ± 547	24 523 ± 552
With macro-Al_2O_3	*Litonotus* sp.	0 ± 0.00	0 ± 0.00	0 ± 0.00
	Paramecium sp.	2,567 ± 67	3,283 ± 97	8,000 ± 102
	Plagiocampa rouxi	0 ± 0.00	00 ± 0.00	00 ± 0.00
	Pseudocohnilemsbus pussillus	0 ± 0.00	00 ± 0.00	00 ± 0.00
	Euglena sp.	0 ± 0.00	677 ± 87	642 ± 73
	Peranema sp.	0 ± 0.00	569 ± 30	543 ± 42
	Ciliata nd.	6,821 ± 129	7,549 ± 201	8,160 ± 238
	Flagellata nd.	7,544 ± 158	8,643 ± 251	9,437 ± 266
	Rotatoria nd.	0 ± 0.00	00 ± 0.00	4,000 ± 114
	Nematodes	1,342 ± 100	1,564 ± 56	7,000 ± 140
	Filamentous bacteria Type 021N	FI5	FI1	FI1
	Total microfauna	18,274 ± 355	22,285 ± 723	37,782 ± 974
Control	*Litonotus* sp.	0 ± 0.00	6,312 ± 172	9,704 ± 261
	Paramecium sp.	2,567 ± 67	4,489 ± 117	20,794 ± 358
	Plagiocampa rouxi	0 ± 0.00	0 ± 0.00	0 ± 0.00
	Pseudocohnilemsbus pussillus	0 ± 0.00	621 ± 58	532 ± 30
	Euglena sp.	0 ± 0.00	1,476 ± 114	1,401 ± 155
	Ciliata nd.	0 ± 0.00	1,128 ± 88	1,063 ± 151
	Peranema sp.	6,821 ± 129	6,972 ± 142	5,876 ± 208
	Flagellata nd.	7,544 ± 158	7,941 ± 214	7,944 ± 251
	Rotatoria nd.	0 ± 0.00	0 ± 0.00	0 ± 0.00
	Nematodes	1,342 ± 100	2,764 ± 155	2,590 ± 171
	Filamentous bacteria Type 021N	FI5	FI1	FI1
	Total microfauna	18,274 ± 355	31,703 ± 1,059	49,904 ± 1,332

(Table 9). However, at the end of the experiment, the abundance of bacteria dropped slightly to 300–430 CFU/mL, probably due to the low availability of growth substrates for microorganisms.

Concentration of 100 mg/L of Al_2O_3 NPs did not cause significant acute effects in single-species studies carried out by us previously [4,5,20].

Jiang et al. [39] demonstrated that in the presence of silver NPs (500 mg/L), biomass of phytoplankton, aquatic plant, and animals did not differ significantly between control and samples treated with Ag NPs for 90 d. Also, the diversity and richness of microbial communities were not affected by Ag NPs [39]. In turn, our research showed that biodiversity of phytoplankton was lower than in the control sample and the number of algae depended on the number of zooplankton. Moreover, after 28 d, inhibition of the number of crustaceans: *D. magna* and *H. incongruens* was observed. Aluminum oxide nanoparticles also inhibited the number and surface area of *L. minor* leaves (Tables 3 and 6–8). On the other hand, in research of Kumar et al. [40], zero-valent Fe NPs reduced algal population content in short exposure period (2–24 h), while in the case of longer exposure up to 180 d, a gradual increase in the algal abundance was noted. In the study

Table 3
Values of Shannon index showing changes in microbenthos and phytoplankton biodiversity in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control)

Organisms	Microcosm	Sampling time (d)		
		0	14	28
Microbenthos	With nano-Al_2O_3	0.52	0.55	0.59
	With macro-Al_2O_3	0.52	0.61	0.73
	Control	0.52	0.87	0.71
Phytoplankton	With nano-Al_2O_3	0.55	0.40	0.41
	With macro-Al_2O_3	0.55	0.46	0.52
	Control	0.55	0.47	0.56

Table 4
Dehydrogenase activity (μkatal/kg protein) of sediment organisms in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Glucose	Sampling time (d)		
		0	14	28
With nano-Al_2O_3	Not added	343 ± 13	90 ± 4	21 ± 4
	Added	356 ± 22	121 ± 7	33 ± 3
With macro-Al_2O_3	Not added	343 ± 13	157 ± 26	10 ± 1
	Added	356 ± 22	199 ± 31	12 ± 4
Control	Not added	343 ± 13	14 ± 3	3 ± 1
	Added	356 ± 22	62 ± 10	13 ± 1

Table 5
Catalase activity (mM/min/mg protein) in tissues of *T. tubifex* in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Sampling time (d)		
	0	14	28
With nano-Al_2O_3	61 ± 3	32 ± 3	57 ± 8
With macro-Al_2O_3	61 ± 3	35 ± 5	36 ± 6
Control	61 ± 3	26 ± 2	33 ± 5

conducted by Vijayaraj et al. [41] on TiO_2 NPs in aquatic and terrestrial microcosms, significant decrease in abundance of bacterial, and archaeal nitrifiers (–45% and –36%, respectively) were demonstrated. Furthermore, considerable translocation of Ti to *Medica gotruncatula* leaves (+422%) was detected, resulting in significant reductions in plant height (–17%), number of leaves (–29%), and aboveground biomass (–53%) [41]. In our 28 d experiment, a lower number of bacteria was found in the sample with nanoparticles than in the control sample (Table 9).

At the same time, in the microcosm with nano-Al_2O_3 increased activity of catalase (antioxidant enzyme) in the tissue *T. tubifex* was also observed. Interestingly, no

Table 6
Inhibition (%) of the surface area and the number of *L. minor* leaves in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Parameter	Sampling time (d)		
		0	14	28
With nano-Al_2O_3	Number of leaves	0.0 ± 0.0	–(35.0 ± 5.0)	19.0 ± 3.0
	Surface area	0.0 ± 0.0	–(27.0 ± 6.0)	9.0 ± 0.8
With macro-Al_2O_3	Number of leaves	0.0 ± 0.0	–(7.0 ± 0.6)	–(1.0 ± 0.3)
	Surface area	0.0 ± 0.0	–(1.0 ± 0.7)	–(7.0 ± 0.9)
Control	Number of leaves	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Surface area	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 7
Number of phytoplankton organisms (cells/mL) in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Organisms	Sampling time [d] (Standard deviation, SD)		
		0	14	28
With nano-Al_2O_3	*Microcystis aeruginosa*	2,200 ± 220	425,000 ± 229	71,000 ± 325
	Chlorella vulgaris	11,500 ± 123	7,983 ± 130	6,348 ± 263
	Desmodesmus quadricauda	200 ± 22	29,000 ± 155	5,000 ± 203
	Scenedesmus obliquus	8,800 ± 184	313,000 ± 192	48,000 ± 128
	Raphidocelis subcapitata	4,500 ± 79	10,000 ± 176	14,000 ± 121
	Total phytoplankton	27,200 ± 627	784,983 ± 881	144,348 ± 1,040
With macro-Al_2O_3	*Microcystis aeruginosa*	2,200 ± 220	700,000 ± 550	175,000 ± 460
	Chlorella vulgaris	11,500 ± 123	8,125 ± 290	9,718 ± 134
	Desmodesmus quadricauda	200 ± 22	50,000 ± 391	5,000 ± 94
	Scenedesmus obliquus	8,800 ± 184	110,000 ± 627	50,000 ± 302
	Raphidocelis subcapitata	4,500 ± 79	400,000 ± 467	15,000 ± 223
	Total phytoplankton	27,200 ± 627	1,268,125 ± 1,094	254,718 ± 1,211
Control	*Microcystis aeruginosa*	2,200 ± 220	1,175,000 ± 722	150,000 ± 223
	Chlorella vulgaris	11,500 ± 123	49,976 ± 134	55,440 ± 150
	Desmodesmus quadricauda	200 ± 22	99,000 ± 229	85,000 ± 240
	Scenedesmus obliquus	8,800 ± 184	1,525,000 ± 452	475,000 ± 328
	Raphidocelis subcapitata	4,500 ± 79	225,000 ± 220	108,000 ± 165
	Total phytoplankton	27,200 ± 627	3,073,976 ± 1,757	873,440 ± 1,107

Table 8
Number of crustaceans (individuals/10 L) in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Organism	Sampling time (d)		
		0	14	28
With nano-Al_2O_3	*Daphnia magna*	11 ± 2	26 ± 7	98 ± 10
	Heterocypris incongruens	80 ± 4	120 ± 5	54 ± 3
With macro-Al_2O_3	*Daphnia magna*	11 ± 2	131 ± 6	190 ± 10
	Heterocypris incongruens	80 ± 4	72 ± 12	122 ± 9
Control	*Daphnia magna*	11 ± 2	281 ± 16	426 ± 24
	Heterocypris incongruens	80 ± 4	78 ± 8	173 ± 17

Table 9
Number of bacteria (CFU/mL) in microcosms with nano-Al_2O_3 (100 mg/L), macro-Al_2O_3 (100 mg/L), and without Al_2O_3 (control) – mean values from three replicates ± standard errors

Microcosm	Sampling time (d)		
	0	14	28
With nano-Al_2O_3	$0.2 \times 10^2 \pm 0.02 \times 10^2$	$0.76 \times 10^3 \pm 0.08 \times 10^3$	$0.30 \times 10^3 \pm 0.05 \times 10^3$
With macro-Al_2O_3	$0.2 \times 10^2 \pm 0.02 \times 10^2$	$0.94 \times 10^3 \pm 0.09 \times 10^3$	$0.33 \times 10^3 \pm 0.04 \times 10^3$
Control	$0.2 \times 10^2 \pm 0.02 \times 10^2$	$0.96 \times 10^3 \pm 0.17 \times 10^3$	$0.43 \times 10^3 \pm 0.23 \times 10^3$

significant similar changes were observed in relation to the biodiversity of organisms in the microcosm with macro-Al_2O_3.

NPs can trigger direct and indirect effects on algal population in the ecosystem. Bioavailability of NPs may induce oxidative stress, which affects the algal growth and cause adverse impacts on phytoplankton. NPs, like zero-valent iron, can form a coating around the algae, leading to the shutdown of photo system II, thereby inhibiting

photosynthesis, which eventually affects cell growth [40]. Furthermore, NPs may accumulate and move in plant tissues. Moreover, the negative consequences for plants may not only concern growth inhibition, but also its stimulation [4].

The influence of NPs on bacterial communities is associated with many factors like the shape and coating of NPs. Contact of bacteria with NPs may result in lipid peroxidation, membrane damage, and reactive oxygen species (ROS) [1]. NPs with many corners and edges may be cytotoxic and induce cellular damage [1,42]. Furthermore, the changes in bacterial communities may be due to the different sensitivities of different bacterial species to NPs. The tested Al_2O_3 NPs in this study affected bacterial communities only after a long period of time, during the 1st weeks of the experiment the NPs concentrations were not high enough to influence bacteria. These observations and in line with those made by Bour et al. [1].

This research showed that NPs did not significantly affect the enzymatic activity of microorganisms for 28 d (Table 4). Our previous studies [43] on the impact of Al_2O_3 NPs on the enzymatic activity on microorganisms in activated sludge have shown that 500 mg/L of NPs can influence metabolism of bacteria in suspension. After 24 h, the tested nanocompound reduced the dehydrogenase activity, suggesting the biocidal properties of these NPs. This may be related with the role of potentially released Al^{3+} ions which can affect the active site of the enzyme and have a negative effect on the dehydrogenase activity [44,45]. However, ions released from NPs can only partially be responsible for the toxic or stimulating effects of NPs [43–45]. The present study showed that microcosms can be used as valuable tool particularly for the assessment of NPs ecotoxicity. Moreover, they appeared to be more sensitive test than single species tests, such as based on growth inhibition. Nevertheless, more work is still needed to explore and confirm these hypotheses.

Cytochemical analysis of *D. magna* cells from the microcosm with nano-Al_2O_3 indicated that the tested NPs were adsorbed on the body covering of this crustacean (Figs. 1 and 2). Disturbed mitochondrial morphology was demonstrated by the swelling of mitochondrial combs and a disorder of their system with a clear matrix at the centre of this organelle (Figs. 3–5). Proteins, which are parts of complexes in the respiratory chain, are located on the surface of the mitochondrial combs, therefore the tested NPs can have negative effects on the mitochondrial respiratory activity or may increase the formation of ROS. The free radicals formed in the mitochondria can have destructive effects as they may damage mitochondrial membranes and lead to cell death [46].

Possible negative effects of nano-Al_2O_3 on the respiratory activity of *D. magna* cells, may have caused the situation in which the number of *D. magna* in the presence of aluminum NPs was over 75% lower than in the control sample (see Table 8). It is possible that, once ingested, nano-Al_2O_3 clog the gut, as has been demonstrated for TiO_2 NPs, carbon nanotubes, and colloidal clay, thus producing decreased nutrient absorption and physiological disturbances [47–49]. The obtained results confirmed our hypothesis that, nano-Al_2O_3 at a concentration of 100 mg/L may cause changes at the cytogenetic level, despite the lack of visible toxic effects in single species studies. However, complementary experiments (chronic and molecular research) should be carried out to answer that hypothesis. No accumulation of macro-Al_2O_3 in shellfish mitochondria was detected, either. To the best of our knowledge, no data on the effects of NPs on the mitochondria of aquatic organisms exists in the literature. Few studies focus on the impact of NPs on mammalian mitochondria. Xue at al. [50] showed that SiO_2 NPs induced mitochondrial damage accompanied by decrease in mitochondrial dehydrogenase activity, mitochondrial membrane potential, enzymatic expression in the Krebs cycle and activity of the mitochondrial respiratory chain complexes I, III, and IV [50].

This work also proved that the risk assessment procedures followed for "normal" chemical compounds are inadequate to properly characterize the environmental risks associated with such specific compounds like nanoparticles.

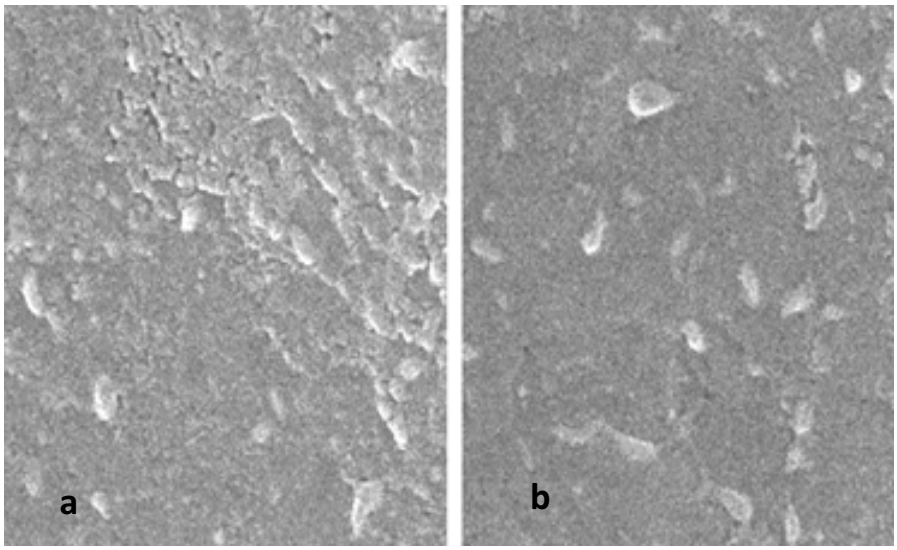

Fig. 2. Body covering of *D. magna* in the control microcosm (a) and in the microcosm with macro-Al_2O_3 (b) (SEM).

According to the European Parliament resolution of 2008, the existing toxicological and ecotoxicological methods for risk assessment of nanoparticles are insufficient [51].

The results obtained in this study showed that impacts of nano-Al_2O_3 on structural and functional changes in the model aquatic ecosystem (microcosm) were different than those observed for their bulk counterparts (Tables 1–9; Figs. 2 and 5). These may be a result of many specific properties of NPs, such as high surface to volume ratio, high chemical reactivity, the ability to form aggregates, diffusivity, and mechanical strength. Moreover, NPs due to their small size (1–100 nm) can more easily penetrate into tissues and cells of an organism than their bulk counterparts, and they can cause various types of dysfunctions [4,5,20]. Thus, it can be hypothesized that bulk counterparts of nano-Al_2O_3, in the form of large aggregates, are less bioavailable for *D. magna* once ingested. In contrast, Al_2O_3 NPs are small, less likely to be aggregated, and may be more easily digestible, although further work is required to confirm their passage through the intestinal membrane.

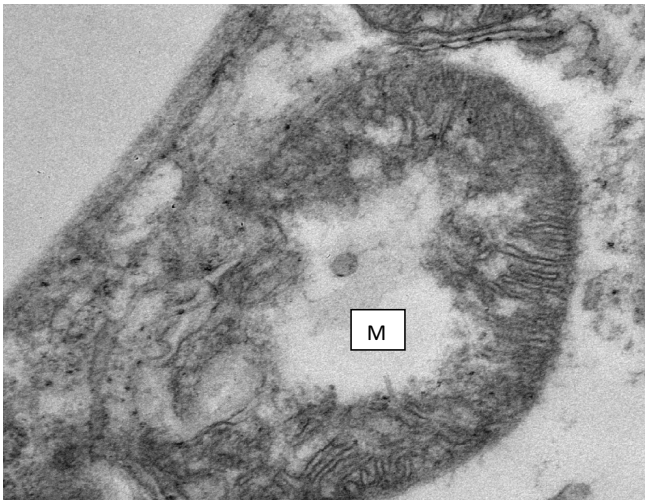

Fig. 3. *D. magna* cells from the control microcosm. (M) – mitochondrion (TEM).

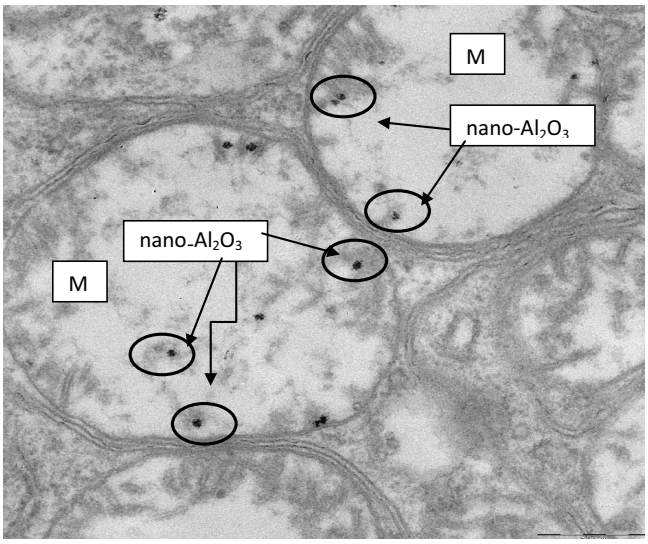

Fig. 4. *D. magna* cells from the microcosm with nano-Al_2O_3. Mitochondria (M) with nano-Al_2O_3 (TEM).

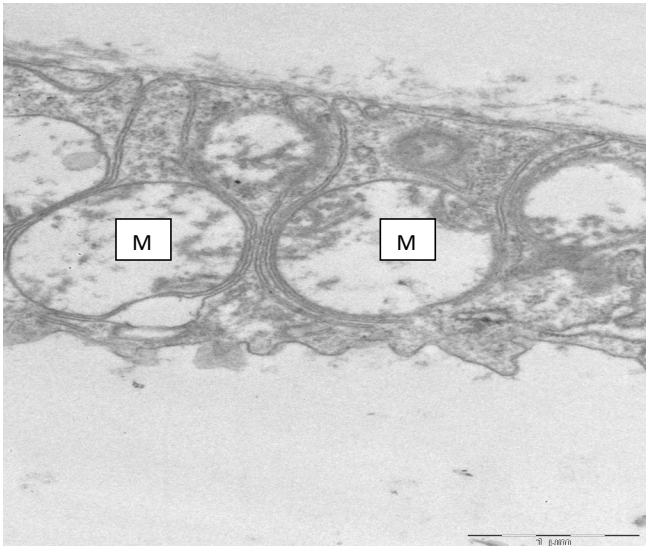

Fig. 5. *D. magna* cells from the microcosm with macro-Al_2O_3. Mitochondria (M) do not contain Al_2O_3 (TEM).

4. Conclusion

Multi-species microcosm type studies were used to assess structural and functional changes in the model aquatic ecosystem triggered by nano-Al_2O_3. This type of research is becoming more and more popular, but there is still little data available about the impact of NPs on aquatic organisms.

This study attempted to answer important questions concerning fate and toxicity of nano-Al_2O_3. The potential long-term negative impact of nano-Al_2O_3 on the aquatic environment, resulting from sub-lethal toxicity to bioindicators, was presented. This is demonstrated by reduced biodiversity of microbenthic and plankton organisms and increase of the activity of antioxidative enzymes of benthos. Cytochemical analysis also showed that the tested nanoparticles significantly affect crustaceans found in the microcosm, causing changes that could lead to their death. The parameters and variables tested in this study are sensitive, compatible biomarkers, and confirm the validity of using microcosms for NPs ecotoxicity assessments. The simultaneous application of microcosms and sensitive toxicity biomarkers are postulated to be used on a wider scale in (nano)ecotoxicology. Authors of Nanotechnology White Paper (EPA Nanotechnology, 2007) postulate acceleration of extensive research aiming at the need to develop test methods and their standardization as well as creating ecotoxicological databases for used and newly produced nanomaterials [37]. The obtained results indicate that, the basis for ecotoxicological assessment of the effect of nanoparticles on organisms of water ecosystems should be not only single species studies, but also a set of multi-species microcosm tests supplemented with chronic and molecular

tests. Various hypotheses still need to be confirmed but have opened many perspectives for future research.

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