Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2023.29447

288 (2023) 130–139
March

Genotoxicity assessment of aluminum oxide nanoparticles using *in vitro* methods

Nina Doskocz*, Monika Załęska-Radziwiłł

Warsaw University of Technology, Faculty of Building Services, Hydro and Environmental Engineering, Nowowiejska 20, 00-653 Warsaw, Poland, Tel.: +48 22 234 7903; emails: nina.doskocz@pw.edu.pl (N. Doskocz), monika.radziwill@pw.edu.pl (M. Załęska-Radziwiłł)

Received 4 January 2023; Accepted 26 February 2023

ABSTRACT

The widespread use of nanoparticles leads to their presence in wastewater, landfills and migration to the environment, especially to water and soil. However, while the lists of known toxic effects of nanoparticles continue to grow, there is still a vast gap in our knowledge about their harmfulness. In this study, SOS Chromotest and comet assay were used to the genotoxicity of aluminum oxide nanoparticles (Al_2O_3 NPs) using *Escherichia coli* bacteria and erythrocytes of *Cyprinus carpio*. Al_2O_3 NPs are used by military and commercial industries in many applications. The analysis of the obtained results showed that the tested nanoparticles can potentially cause changes in the genetic material of the bioindicators. Results of genotoxicity induction coefficient (*I*) values in the SOS Chromotest clearly showed the genotoxicity of Al_2O_3 NPs, both in the presence and in the absence of S9 fraction. In turn, the statistical analysis of the comet assay test results showed a significant increase of '% DNA in tail' and 'tail length' of comets in cells populations incubated in solutions of Al_2O_3 NPs compared to the negative control. Furthermore, the obtained results showed that the nanoparticles had a greater effect on the genetic material of bacteria and eukaryotic cells than their bulk counterparts.

Keywords: Nanoparticles; SOS Chromotest; Comet assay; Genotoxicity; DNA

1. Introduction

Nanoparticles (NPs) are products of nanotechnology that have dimensions smaller than 100 nm, which is within the size range of colloidal particles and smaller than bacterial and eukaryotic cells. Their unique physicochemical properties, such as increased surface area per unit of mass and reactivity, mean that there are many possibilities for their use in a range of consumer applications. Indeed, NPs can be used in printable inks for flexible electronics, biomedical assays, drug delivery, colorants and paints, solar cells, stain-resistant clothing, tires, and semiconductors. However, the same properties that make these particles attractive for use in technology also increases concerns about their hazardous effects on biological systems [1–3]. Extensive use of NPs has led to their accumulation in the environment, especially in water and soil, as well as in landfills and their water effluents. It may be assumed that the tendency for NPs to accumulate, and their low solubility, would limit the exposure of living organisms to these particles. However, some studies have demonstrated high accumulation of NPs, especially metal oxide NPs, in aquatic organisms [4].

In the literature, there are data demonstrating the effect of different types of NPs on various functional aspects of aquatic and terrestrial organisms [5–11]. However, there is still a lack of data concerning the hazards of NPs, with little information available on their genotoxicity in aquatic organisms in particular. Due to their small size, NPs can cross biological barriers such as cell membranes, where they can potentially reach the nucleus and can cause many harmful

* Corresponding author.

effects, including deoxyribonucleic acid (DNA) damage [12,13]. Genotoxicity can be defined as destructive genetic alterations involving gene mutations, structural chromosomal aberrations, and recombination, that are induced by genotoxins. Such damage to DNA and chromosomal structure is mediated through a number of mechanisms, including addition, deletion, duplication, and ring formation. Due to the major role NPs play in the initiation and progression of the above-mentioned abnormalities, concern has arisen over their use [14]. Interaction of genotoxins dissolved in water with the genetic material of aquatic organisms can trigger changes that impact on one generation, such as cancer, teratogenicity, and embryotoxicity. Furthermore, constant exposure can affect populations of organisms through a number of mechanisms, including reduced fertility and viability of offspring, or by inhibiting growth [15–18]. Genotoxic events can be transient, meaning that the damage can be repaired, or can lead to permanent mutations in the genetic material of a cell. When a mutation is present in a germ cell it is inherited by the next generation, and can cause a genetic disorder [19]. Moreover, genotoxicity is considered an important aspect of carcinogenesis, which may be a hazard to human health. Indeed, it should be emphasized that, due to the role of DNA in cell functionality, alterations to DNA structure/function can severely compromise the lives of organisms at different levels of the food chain [12]. Therefore, genotoxic evaluation of NPs is necessary in order to learn more about the risks they pose to the environment and living organisms.

A number of studies have demonstrated the genotoxic effects of NPs. Wise et al. [3] used a medaka (*Oryzias latipes*) cell line to investigate the cytotoxicity and genotoxicity of 30 nm diameter silver (Ag) nanospheres. Treatments of 0.05, 0.3, 0.5, 3 and 5 μg/cm^2 induced 80%, 45.7%, 24.3%, 1% and 0.1% survival, respectively, in a colony forming assay. Additionally, these Ag NPs induced chromosomal aberrations and aneuploidy. Guo et al. [20] demonstrated that the smaller the Ag NPs, the greater their cytotoxicity and genotoxicity. Meanwhile, Ghosh et al. [21] confirmed the genotoxicity of titanium oxide NPs, with results of the comet assay demonstrating that they induced DNA damage in the nuclei of *Allium cepa* roots. Furthermore, Güneş et al. [22] observed significant DNA damage in *Eisenia hortensis* in the presence of cerium oxide and magnesium oxide NPs. Studies on the embryonic development of fish have also been used for genotoxicity evaluation, as the embryo is more sensitive to NPs than adults. It was observed that Ag, copper, platinum, and metal oxide NPs such as titanium dioxide, copper oxide, nickel oxide, and zinc oxide, showed toxicity to embryos of zebrafish [23].

Understanding of the detailed cellular and molecular mechanisms that lead to the biological effects of NPs must be gained in order to develop safe nanoscale engineered materials and standardized assays of their toxicity risk [1,15]. Although several groups have contributed data towards elucidating genotoxicity pathways, the associated molecular mechanisms and correlations still remain unclear. Auffan et al. [24] showed that chemically stable metallic NPs had no significant cellular toxicity, whereas NPs that were able to be oxidized, reduced, or dissolved, were cytotoxic and genotoxic to cellular organisms. After being ingested by an organism, NPs can dissolve into ions and trigger several signaling pathways and cascades that may lead to increased calcium influx or gene upregulation. Therefore, the solubility of Ag NPs, copper NPs, and zinc oxide NPs, is one of the main contributors to their toxicity [25]. Also, Sayes et al. [26] and Goodman et al. [27] demonstrated that particle size and composition can affect genotoxicity, with some sizes and forms being highly toxic and others being nontoxic. Nonetheless, factors other than NP properties are important determinants of their genotoxicity. Kononenko et al. [28] showed that NP concentration and the type of cell exposed, as well as the cell's genetic and proteomic properties, also contributed to genotoxicity.

Due to the fact that most data available in the literature concerns Ag NPs, and that there is a lack of data on the genotoxicity of NPs in aquatic organisms, more research is required. Furthermore, the European Union has called for accelerated large-scale research into the development of standardized tests and the creation of ecotoxicological databases for new and current nanomaterials. Therefore, this paper focused on the genotoxic effects of aluminum oxide NPs (Al_2O_3 NPs) on *Escherichia coli* and erythrocytes from *Cyprinus carpio*, using the SOS Chromotest and comet assay. This substance is currently being used by the military and commercial industries in many applications, including coatings, thermites, and propellants, however, little information is available on its fate, transport, and effects on the environment [9–11,29]. Additionally, the genotoxic effects of Al_2O_3 NPs on the bioindicators evaluated were compared with its bulk counterpart (macro form of Al_2O_3).

2. Materials and methods

2.1. Chemicals

Commercial samples of Al_2O_3 NPs (nanopowder < 50 nm with a specific surface area > 40 m^2/g) and Al_2O_3 (Al_2O_3, purity over 98%) were obtained (CAS no. 1344-28-1, Merck Life Science, Poznan, Poland), and the size of NPs was determined by scanning electron microscopy (LEO 1430VP, Warsaw, Poland) (Fig. 1). Stock suspensions of 500 mg/L (for SOS Chromotest) and 200 mg/L (for comet assay) of nano and macro forms of Al_2O_3 were prepared in deionized water. To avoid the formation of aggregates, stock suspensions were sonicated (0.4 kW, 20 kHz) for 30 min before being diluted to the exposure concentrations.

2.2. Genotoxicity tests

In order to study the genotoxicity of nanoparticles in relation to prokaryotic and eukaryotic cells, two tests were used: SOS Chromotest and comet assay. The SOS Chromotest measures the primary response of a bacterial cell to genetic damage. In just a few hours, provides a clear, completely objective measurement of the genotoxicity of a sample by a visual qualitative evaluation of the degree of DNA damage the cell experiences. Comet assay is a microgel electrophoresis technique, which detects DNA damage and repair in individual cells. The assay measures DNA damage (i.e., strand breaks, DNA adducts, excision repair sites, and cross-links) at the single-cell level. The main advantages of the comet

assay is sensitivity for detecting low levels of genetic material damage.

Genotoxicity of the Al_2O_3 compounds was assessed using prokaryotic cells, *E. coli PQ37* mutant (SOS Chromotest), and eukaryotic cells, *C. carpio* erythrocytes (comet assay). Lyophilized bacteria were provided in the test kit, while erythrocytes were acquired from the Inland Fisheries Institute Department in Zabieniec, Olsztyn, Poland.

2.2.1. SOS Chromotest

The colorimetric SOS Chromotest assay, used to assess the mutant *PQ37 E. coli* strain, was conducted in accordance with the manufacturer's instructions (Environmental Bio-Detection Products Inc., ON, Canada) [30]. The DNA repair system of the *PQ37* strain has been altered by a series of mutations so that even limited DNA damage will not be repaired, and the SOS promoter does not activate the SOS system. Instead, it induces the synthesis of a readily detectable enzyme which, when in contact with a chromogenic substrate, catalyzes the formation of a colored product. The assay is quantitative and uses two standard genotoxic solutions, 4-nitroquinoline 1-oxide (4NQO) and 2-aminoanthracene (2AA).

Assays were conducted both with and without metabolic activation (S9 fraction). S9 mix contained S9 extract of sensitized rat liver and was prepared according to the kit instructions [30]. Aliquots of samples and controls (10 μL) were dispensed into appropriate wells of a microplate. Subsequently, 100 μL of the bacterial suspension (culture diluted overnight) and 100 μL of bacterial suspension with S9 were added to wells, and the microplate was incubated at 37°C for 2 h. Alkaline phosphatase chromogen was then added, and the mix was incubated at 37°C for 90 min using a MB100-4A Thermo Shaker with rotatable platform (Hangzhou Allsheng Instruments Co., Ltd., Hangzhou, China), for the development of the enzymatic activity. Genotoxic activity was measured at 620 nm and viability was detected at 405 nm using a LT-4500 microplate reader (Beijing LabTech Instruments Co., Ltd., Beijing, China). Readings were taken immediately after the colorimetric incubation. Genotoxicity assessment was based on the SOS induction factors (SOSIF), which were calculated by Eq. (1):

$$\mathrm{SOSIF} = \frac{\left(\frac{A_{620}S - A_{620}B}{A_{620}N - A_{620}B}\right)}{\left(\frac{A_{405}S - A_{405}B}{A_{405}N - A_{405}B}\right)} \quad (1)$$

where $A_{620}S$ – absorbance readings at λ = 620 nm for sample wells; $A_{405}S$ – absorbance readings at λ = 405 nm for sample wells; $A_{620}N$ – absorbance readings at λ = 620 nm for negative control wells; $A_{405}N$ – absorbance readings at λ = 405 nm for negative control wells; $A_{620}B$ – averaged absorbance readings at λ = 620 nm for reagent blank wells; $A_{620}N$ – averaged absorbance readings at λ = 405 nm for reagent blank wells

Genotoxicity assessment was performed according to the criteria presented in Table 1 [31].

Cytotoxicity assessment was based on survival rate (SR, %), which were calculated according to Eq. (2):

$$\mathrm{SR} = \frac{A_{405}S}{A_{405}N} \times 100\% \quad (2)$$

A survival rate of 80% was required to confirm a positive result of genotoxicity. Stock solutions ranging from 0.24–500 mg/L were diluted in descending order with a geometric series of quotient.

2.2.2. Comet assay

Five concentrations of the Al_2O_3 compounds were prepared, in triplicate, in Hanks' Balanced Salt Solution (HBSS) with 1 mM cytosine β-D-arabinofuranoside (*Ara-C*). *Ara-C* was added for its ability to inhibit DNA repair following nucleotide excision. The negative control was HBSS solution with *Ara-C*, whilst the positive control solution contained hydrogen peroxide at a concentration of 500 mM. Incubation of erythrocytes was carried out in the dark at room temperature for 1 h.

2.2.2.1. Fluorochrome-mediated viability test

Cell viability was routinely determined using the 5(6)-carboxyfluorescein (CFDA) assay according to Strauss [32]. Immediately after treatment, 25 μL of cell sample was mixed with 25 μL of staining solution (30 μg/mL CFDA, in phosphate-buffered saline [PBS]), spread on a microscope slide and covered with a coverslip [32]. Comet experiments

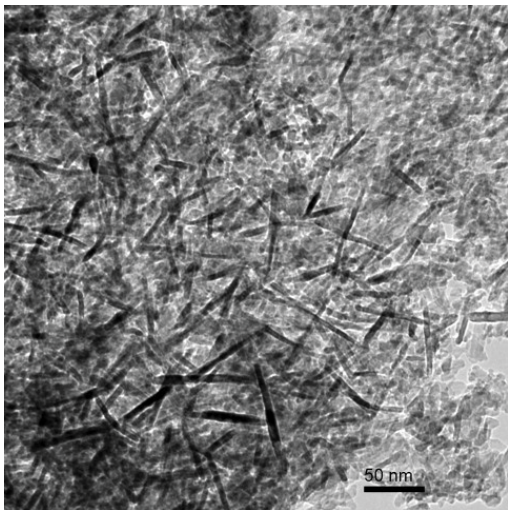

Fig. 1. Scanning electron microscopy visualization of Al_2O_3 NPs.

Table 1
Classification of genotoxic intensity according to significance level of the response [31]

SOSIF < 1.5	No genotoxicity	–
1.5 < SOSIF < 2.0	Slight genotoxicity	+
2.0 ≤ SOSIF < 5.0	Moderate genotoxicity	++
SOSIF ≥ 5.0	Strong genotoxicity	+++

were only carried out on cell suspension preparations with more than 90% viable cells.

2.2.2.2. Electrophoresis on microgel slides and evaluation of DNA damage

The alkaline comet assay was carried out according to the modified procedure described by Singh et al. [33] and Załęska-Radziwiłł et al. [15]. Twelve milliliters of isolated erythrocytes (or erythrocytes incubated with chemicals) were added to 65 mL of 0.5% low melting point agarose at 37°C and layered onto slides precoated with 1% regular agarose, covered with a coverslip, and left on a leveled frozen plate for 10 min to solidify the agarose. Coverslips were carefully removed, and the slides were immersed in a lysis solution (2.225 M NaCl; 89 mM ethylenediaminetetraacetic acid [EDTA]; 8.9 mM tris [pH 10]; 0.89% N-lauroylsarcosine sodium; 1% triton X-100 and 10% dimethyl sulfoxide) at 4°C for 1 h. Slides were then washed in PBS for 5 min, immersed in a freshly prepared alkaline buffer (10 M sodium hydroxide; 200 mM EDTA; pH > 13), and randomly distributed in a horizontal electrophoresis chamber. After the DNA unwinding period (20 min), electrophoresis was conducted at 18 V and 300 mA (1 V/cm) for 30 min at 4°C. Slides were then rinsed 3 times with neutralization buffer (0.4 M tris; pH 7.5) and stained with ethidium bromide solution (0.02 mg/L). Genotoxicity assessment was based on analysis of the comets formed from nuclear DNA, using a fluorescent microscope (Nikon Eclipse 50, PlanFluor 20£, Nikon, Tokyo, Japan). An increased amount of genetic material in comet tails causes a decrease in the DNA content of the comet heads. The percentage of DNA in the tail of the comets was compared using a Laboratory Universal Computer Image Analysis System (LUCIA Comet Assay). This method was chosen because it shows good linearity between dose and damage over a reasonable range. At least 30 randomly chosen cells were analyzed in duplicate per slide (at least 90 cells per sample).

2.3. Statistical analysis

Distribution of data was evaluated using the Kolmogorov–Smirnov goodness-of-fit test. Comet assay data were not normally distributed and were therefore analyzed using the nonparametric Mann–Whitney U test (one-tail; $P < 0.05$). Due to highly asymmetric distribution of data, the median was chosen instead of the mean as the central value to visualize differences on the plots. These analyses were performed using STATISTICA Software Package (version 10, StatSoft, Poland).

3. Results

3.1. Genotoxicity test with E. coli – SOS Chromotest

The SOS Chromotest was carried out with Al_2O_3 at concentrations in the range of 0.24–500 mg/L, with all concentrations resulting in at least an 80% survival rate. Values of SOSIF clearly showed Al_2O_3 NPs to be genotoxic, based on the criteria presented in Table 1. Strong genotoxicity was found for samples without the S9 fraction at concentrations between 31.25–500 mg/L. Concentrations ranging from 3.90–15.63 mg/L were moderately genotoxic, while no genotoxicity was observed for the other concentrations. Addition of the S9 fraction resulted in lower SOSIF coefficient values in comparison to samples incubated with the S9 fraction. Moderate genotoxicity was observed for concentrations ranging between 1.25–500 mg/L, while concentrations in the 1.95–7.81 mg/L range resulted in low genotoxicity. No genotoxicity was detected with the other concentrations tested. The minimum genotoxic concentration (MGC) was found to be affected by the S9 fraction. Indeed, the MGC without addition of the S9 fraction was 3.9 mg/L and was 1.95 mg/L with the S9 fraction added (Table 2). The Al_2O_3 did not induce genotoxic effects in *E. coli* mutants (Table 3).

Before the comet assay procedure was performed, the viability of erythrocytes was tested in order to verify that the cells were not damaged. Viable cells fluoresced green, whereas dead cells were indicated by orange stained nuclei and erythrocytes did not label. At least 200 cells were scored per data point, and their viability after chemical treatment was always >90% (Fig. 2).

The impact of Al_2O_3 NPs on the formation of double-strand breaks in the DNA of eukaryotic cells was assessed using a concentration range of 0.32–200 mg/L. Results from the comet assay were presented as percentage of DNA in the tail and tail length. Figs. 3 and 4 show comets obtained for the cells in the control group and for the cells exposed

Table 2
Results of SOS of Al_2O_3 NPs (SOSIF – SOS induction factor; SD – standard deviation)

Sample		Concentration (mg/L)	SOSIF ± SD	Genotoxicity assessment
Al_2O_3 NPs	Without S9 fraction	500	***18.25*** ± 0.04	+++
		250	***16.07*** ± 0.08	
		125	***14.09*** ± 0.04	
		62.50	***10.54*** ± 0.15	
		31.25	***6.76*** ± 0.03	
		15.63	***2.06*** ± 0.03	++
		7.81	***2.12*** ± 0.04	
		3.90	***2.03*** ± 0.09	
		1.95	1.00 ± 0.07	–
		0.98	0.94 ± 0.04	
		0.49	0.92 ± 0.13	
		0.24	0.88 ± 0.05	
	With S9 fraction	500	***3.10*** ± 0.05	++
		250	***3.08*** ± 0.09	
		125	***3.07*** ± 0.12	
		62.50	***2.76*** ± 0.10	
		31.25	***2.34*** ± 0.03	+
		15.63	***2.05*** ± 0.02	
		7.81	***1.85*** ± 0.09	
		3.90	***1.64*** ± 0.06	
		1.95	***1.55*** ± 0.09	–
		0.98	1.03 ± 0.06	
		0.49	0.99 ± 0.11	
		0.24	0.97 ± 0.08	

Genotoxic samples are indicated in bold-italic letters.

Table 3
Results of SOS of Al_2O_3 (SOSIF – SOS induction factor; SD – standard deviation)

Sample		Concentration (mg/L)	SOSIF ± SD	Genotoxicity assessment
Al_2O_3	Without S9 fraction	500	1.35 ± 0.02	–
		250	1.34 ± 0.09	
		125	1.23 ± 0.03	
		62.50	1.20 ± 0.04	
		31.25	1.16 ± 0.06	
		15.63	1.14 ± 0.17	
		7.81	1.12 ± 0.15	
		3.90	1.11 ± 0.09	
		1.95	1.07 ± 0.05	
		0.98	1.07 ± 0.04	
		0.49	1.07 ± 0.07	
		0.24	1.03 ± 0.05	
	With S9 fraction	500	1.12 ± 0.02	–
		250	1.12 ± 0.05	
		125	1.10 ± 0.10	
		62.50	1.10 ± 0.18	
		31.25	1.09 ± 0.02	
		15.63	1.08 ± 0.09	
		7.81	1.06 ± 0.06	
		3.90	1.04 ± 0.12	
		1.95	1.02 ± 0.03	
		0.98	1.01 ± 0.02	
		0.49	1.00 ± 0.01	
		0.24	0.93 ± 0.05	

to different concentrations of Al_2O_3. An increased amount of genetic material in comet tails caused a direct decrease in genetic material in comet heads.

The medians of the percentage of DNA in the tail of the comets were 7.8% for the negative control and 34.2%, 33.5%, 26.9%, 25.6%, 14.8%, for Al_2O_3 NPs. Statistical analysis indicated a significant increase in tail length of comets in populations of cells incubated with solutions of Al_2O_3 NPs. Comparisons between all of the concentrations of Al_2O_3 NPs assessed and the negative control are shown in Fig. 5. For the Al_2O_3 (macro form), the medians of the percentage of DNA in the tail of the comets were 15.1%, 14.3%, 12.0%, 8.0%, and 6.6%.

Median tail lengths of the comets were 19.85%, 19.36%, 16.94% and 8.71%, for Al_2O_3 NPs, and 7.26% for the negative control. Statistical analysis showed a significant increase

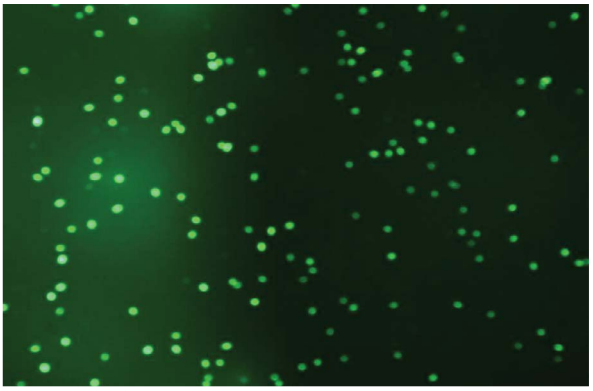

Fig. 2. Erythrocytes viability assay – Strauss method (light-green cells are live).

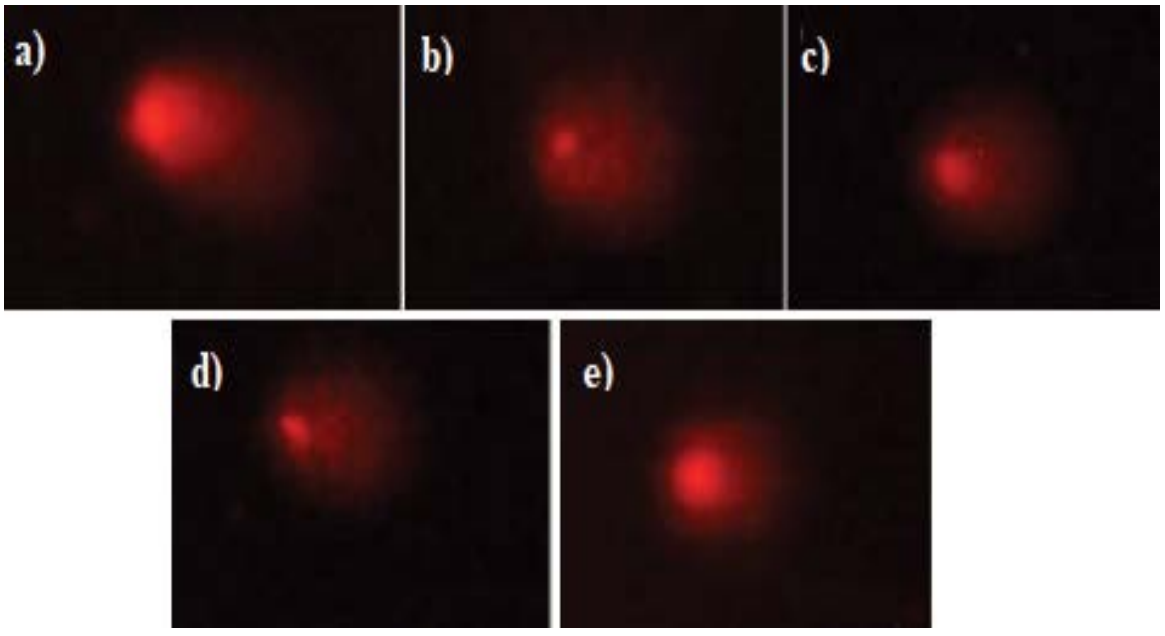

Fig. 3. DNA damage in *Cyprinus carpio* erythrocytes exposed to Al_2O_3 NPs – comet assay. (a) 200 mg/L of Al_2O_3 NPs, (b) 40 mg/L of Al_2O_3 NPs, (c) 8 mg/L of Al_2O_3 NPs, (d) 1.6 mg/L of Al_2O_3 NPs and (e) 0.32 mg/L of Al_2O_3 NPs.

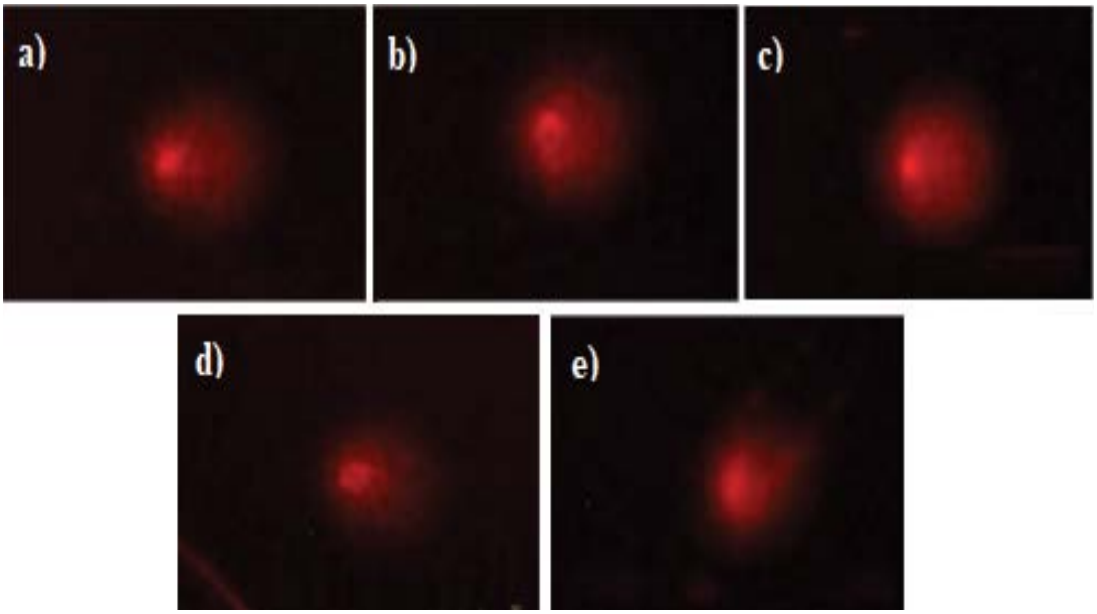

Fig. 4. DNA damage in *Cyprinus carpio* erythrocytes exposed to Al_2O_3 – comet assay. (a) 200 mg/L of Al_2O_3, (b) 40 mg/L of Al_2O_3, (c) 8 mg/L of Al_2O_3, (d) 1.6 mg/L of Al_2O_3 and (e) 0.32 mg/L of Al_2O_3.

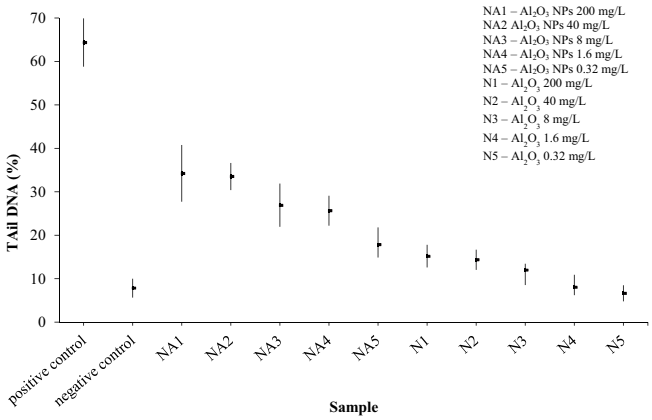

Fig. 5. "% DNA in tail" (median) in erythrocytes populations exposed to Al_2O_3 NPs and Al_2O_3. 95% confidence intervals are shown.

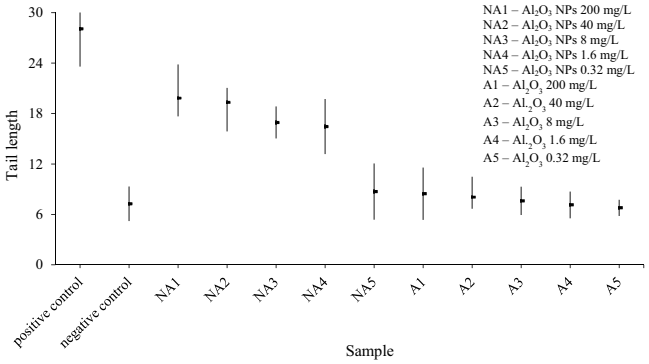

Fig. 6. "Tail length" (median) in erythrocytes populations exposed to Al_2O_3 NPs and Al_2O_3. 95% confidence intervals are shown.

in tail length of comets in populations of cells incubated in solutions of Al_2O_3 NPs at concentrations of 200, 40, 8 and 1.6 mg/L, in comparison with the negative control. (Fig. 6). For the Al_2O_3 (macro form), median tail length of the comets was 8.45%, 8.07%, 7.61%, 7.13%, and 6.78%, respectively.

4. Discussion

Dynamic expansion of the nanotechnology industry has led to the widespread use of NPs in various domestic products, such as textiles and personal care products, and has resulted in the appearance of such materials in elements of the environment [1–3,9,34,35]. Increasing research activities in this area are partly driven by emerging evidence that substances previously considered to be biologically inert may become toxic in the nanoparticulate state, due to their increased reactivity and the possibility of increased cellular uptake. Given that nanotechnology industries plan future large-scale production, it is inevitable that these products and their by-products will have an influence on living organisms and may cause significant changes to the structure and functioning of biocenoses [36]. Furthermore, according to criteria set by the Environmental Protection Agency in the United States, and the European Union, existing toxicological and ecotoxicological methods for risk assessment of NPs are insufficient. Indeed, current methods do not provide a complete profile of the environmental risks associated with the presence of these materials in ecosystems [9,14,36]. Size, concentration, stability, and duration of exposure to NPs, are some of the primary factors that can cause genotoxic effects to exposed organisms and must be considered during risk assessment [35,37].

Genotoxicity is a phenomenon that affects all aspects of ecosystem functioning, and may determine population and species fitness in their changing habitat. As such, determining its effects in ecologically relevant organisms outside the scope of laboratory models is paramount. Even though the adequacy and value of genotoxicity detection methods is beyond dispute, a protocol that could efficiently detect alterations to the genome at the DNA strand level in the presence of NPs (and other chemical substances) is still lacking [14,38]. Thus the main purpose of the present investigation was to test the hypothesis that engineered NPs exert genotoxicity on bacteria and erythrocytes. This was achieved using two separate tests that measure different mechanisms of action of NPs. Given that little is known about the chronic ecotoxicological effects from multispecies studies and studies on the molecular level, particularly genotoxicity, the genotoxicity tests performed in this study (SOS Chromotest on prokaryotic cells and comet assay on eukaryotic cells) correspond with these knowledge gaps.

The SOS Chromotest is based on the induction of a bacterial (*E. coli*) SOS repair system, by genotoxic compounds, that is conjugated to the β-galactosidase gene and the subsequent measurement of the enzyme's expression. The test was performed to assess whether or not Al_2O_3 could induce sufficient DNA damage to block DNA synthesis, which is referred to as the SOS response [30].

A test more commonly used to assess DNA damage is the single cell gel electrophoresis assay (SCGE), commonly known as the comet assay. Depending on the pH used, the comet assay is able to detect a wide variety of DNA damage such as single and double strand breaks, incomplete excision repair sites, cross-links (by decreased comet tail), alkali-labile sites (e.g., a basic sites) and oxidized DNA lesions [32,34,39,40]. Strand breaks (if not repaired) lead to chromosomal aberrations and cell death. They can also be inherited and lead to carcinogenesis. Electrophoresis at high pH results in structures resembling comets that can be observed by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks [4]. Erythrocytes of fish are a common and convenient model organism as they possess nuclei and are very numerous (up to 97% of all cellular components of fish blood), while their biochemical and physiological characteristics are well understood [4,39,41]. There are also numerous software packages available for comet image analysis, and the output includes a variety of different parameters. The most commonly used parameters are the tail length and the percentage of DNA in the tail. Tail length can only be used at low levels of DNA damage since it does not tend to change once the tail is established. Subsequently, the intensity of the tail increases as the damage is enhanced. The percentage of DNA in the comet tail is another useful parameter, as it is linearly related to the breaking frequency [42–44]. These parameters were used in the present study to evaluate genetic damage due to Al_2O_3 exposure.

Results from the SOS Chromotest established the genotoxicity of Al_2O_3 NPs, both in the presence and in the absence of S9 fraction. The minimum concentrations that induced genotoxicity were 1.95 and 3.9 mg/L. Available SOS Chromotest data for NPs is extremely limited in the literature, with only two studies having used this test to date. Zakharenko et al. [45] showed that fullerene C60 NPs were not genotoxic, with Nam et al. [46] drawing a similar conclusion. They performed a SOS Chromotest and reported that gold, Ag, zinc oxide, and titanium dioxide NPs had no genotoxic potential. In turn, studies by Alhadrami and Shoudri [47] found that synthesized titanium dioxide NPs at a concentration of 800 μg/mL showed non-genotoxic and non-mutagenic effects. The divergent results obtained from bacterial assays might be due to the inability of the NPs tested to penetrate the bacterial cell wall. Of course, the discrepancy between our results and the literature is likely down to the type of NPs tested, concentrations assessed, and the type of solvent used. In this regard, Alhadrami and Shoudri [47] suggested that 50% dimethyl-sulfoxide, which is often used to dissolve NPs, may contribute to their genotoxic effect.

The research carried out here is one of the first applications of SCGE to the investigation of Al_2O_3 NPs in environmental research. The tail length and percentage of DNA in the tail within the comet significantly ($p < 0.05$) increased in a concentration-dependent manner after exposure to Al_2O_3 NPs, demonstrating an increase in DNA damage. These observations are in line with other studies using fish, where exposure to other NPs resulted in a positive response in the comet assay. Vidya and Chitra [48] observed genotoxicity in *Oreochromis mossambicus* after short-term (24, 72 and 96 h) and long-term exposure (15, 30 and 60 d) to silicon dioxide, titanium dioxide, and iron oxide. Research conducted by Vevers and Jha [49] on fish a cell line derived from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue (i.e., RTG-2 cells) showed that the highest concentration of titanium dioxide NPs did not cause elevation in DNA damage over 4 h (comet assay) and 24 h (modified comet assay) intervals. However, significantly increased levels of strand breaks were observed in combination with ultraviolet A radiation (3 kJ/m^2). The adverse impact of Ag NPs on fishes was confirmed by research carried out by Naguib et al. [50], with the NPs significantly affecting ($P < 0.0001$) all of the comet parameters. From research by Aziz et al. [35] it was concluded that sub-lethal concentrations of copper oxide NPs induced significant DNA damage in fish erythrocytes, with the percentage damage increasing as concentration and time of exposure increased. Further work by Shahzad et al. [51] evaluated the potential of applying the alkaline comet assay to the investigation of DNA strands break. They observed significant DNA damage in erythrocytes exposed to zinc oxide NPs, where the percentage of tail DNA increased with increased NP, and a similar pattern was shown for the olive tail moment.

In conclusion, the results from the present study suggest that Al_2O_3 NPs has the potential to induce DNA damage. Genotoxicity may be caused by the direct association of these NPs with DNA strands due to their small size. Indeed, they are able to cross cellular barriers, stimulate oxidation systems, and interfere with DNA repair proteins, to cause damage to the DNA strand. Additionally, they may have the ability to stimulate cellular apoptosis. Regardless, information currently available in the literature does not point to a coherent explanation as to how NPs induce such genotoxic damage. The appearance of genotoxic effects caused by NPs may be influenced by particle properties such as type, size, shape, and exposure scenarios [14,41].

This work demonstrated that the risk assessment procedures followed for "normal" chemical compounds are inadequate for proper characterization of the environmental risks associated with NPs specifically. Indeed, the impacts of Al_2O_3 NPs on the genetic material of *E. coli* and *C. carpio* were different than those observed for Al_2O_3 (Tables 2 and 3; Figs. 5 and 6). This difference 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, due to their small size (1–100 nm) NPs can more readily penetrate tissues and cells of an organism in comparison to the bulk form, meaning they have the potential to cause various types of dys-function [9–11]. Thus, it can be hypothesized that Al_2O_3 in the form of large aggregates are less bioavailable for the bioindicators used in the tests. In contrast, Al_2O_3 NPs are small, are less likely to aggregate, they may be more easily assimilable which would allow them to enter the cell nucleus by passing through the nuclear pore, or they may interact directly with DNA during mitosis [52].

5. Conclusion

Genotoxicity tests conducted using nano-Al_2O_3 revealed induction of genetic damage in *E. coli* bacteria and erythrocytes from *C. carpio*. This type of research is becoming increasingly popular, but there is still little data available on the genotoxic effect of NPs on aquatic organisms. Environmental monitoring requires large numbers of samples to be processed in a relatively short period of time, and a test that allows this type of assessment is the SOS Chromotest. This test is the most rapid and simplest short-term test for genotoxicity, meaning it can be used as a screening test for many potentially genotoxic compounds. Advantages of the test include the fact that survival of the tester strain is not required, the results can be obtained in a single working day, sample sterility is not usually required, the organism responds to a wide range of DNA damage scenarios, and the test can easily accept biological samples such as tissue extracts and biological fluids. Despite these advantages the test is plagued with the same interpretation problems as other microbioassays [53]. Furthermore, the assay is limited to one specific test species, the *E. coli PQ37* strain.

The comet assay, although used in *in vivo* and *in vitro* toxicology, still has many constraints. They are in large part due to difficulties in obtaining conclusive cause and effect relationships from complex environments. Indeed, interpreting DNA damage data is particularly challenging due to its ability to adapt to continuous environmental stressors, including toxicants.

So far, none of the molecular tests applied have met the criterion of an ideal tool by which to assess genotoxicity and mutagenicity, and they are frequently characterized by poor reproducibility and accuracy. Nevertheless, these challenges have not hindered efforts to establish standardize protocols and general guidelines on the interpretation of SOS Chromotest and comet assay bioindicator data. Results obtained indicate that the basis for ecotoxicological assessment of the effect of NPs on organisms in aquatic ecosystems should not only be conventional toxicology tests commonly used in ecological risk assessment. Instead, a set of molecular tests evaluating genotoxic effects (such as DNA adducts, DNA lesion, DNA repair or oxidative stress) of NPs on various test species would be more effective for the detection of mutagens and would lead to more reliable risk assessment in aquatic ecosystems. Various hypotheses still need to be confirmed, however many perspectives for future research but have been opened up.

Authors' contributions

Conceptualization, N.D and M.Z-R..; methodology, N.D.; validation, M.Z.-R.; investigation, N.D.; resources, N.D.; data curation, N.D.; writing—original draft preparation, N.D.; writing—review and editing, N.D. and M.Z.-R.; visualization, N.D.; supervision, M.Z.-R.; project administration, N.D.; funding acquisition, N.D. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

References

[1] L. Benameur, L. Wei, A. Botta, In: B. Bhushan, Ed., Encyclopedia of Nanotechnology, Springer, Dordrecht, 2012, pp. 952–962.
[2] I. Lopes, R. Ribeiro, F.E. Antunes, T.A.P. Rocha-Santos, M.G. Rasteiro, A.M.V.M. Soares, F. Gonçalves, R. Pereira, Toxicity and genotoxicity of organic and inorganic nanoparticles to the bacteria *Vibrio fischeri* and *Salmonella typhimurium*, Ecotoxicology, 21 (2012) 637–648.
[3] J.P. Wise, B.C. Goodale, S.S. Wise, G.A. Craig, A.F. Pongan, R.B. Walter, W.D. Thompson, A. Ng, A. Aboueissa, H. Mitani, M.J. Spalding, M.D. Mason, Silver nanospheres are cytotoxic and genotoxic to fish cells, Aquat. Toxicol., 97 (2010) 34–41.
[4] N. Nikdehghan, H. Kashiri, A.H. Ali, CuO nanoparticles-induced micronuclei and DNA damage in *Cyprinus carpio*, AACL Bioflux, 11 (2018) 925–936.
[5] E. Prato, A. Fabbrocini, G. Libralato, L. Migliore, I. Parlapiano, R. D'Adamo, A. Rotini, L. Manfra, G. Lofrano, F. Carraturo, M. Trifuoggi, F. Biandolino, Comparative toxicity of ionic and nanoparticulate zinc in the species *Cymodoce truncata*, *Gammarus aequicauda* and *Paracentrotus lividus*, Environ. Sci. Pollut. Res., 28 (2021) 42891–42900.
[6] S. Zhang, K. Yi, A. Chen, J. Shao, L. Peng, S. Luo, Toxicity of zero-valent iron nanoparticles to soil organisms and the associated defense mechanisms: a review, Ecotoxicology, 31 (2022) 873–883.
[7] Z. Liu, C.R. Malinowski, M.S. Sepúlveda, Emerging trends in nanoparticle toxicity and the significance of using *Daphnia* as a model organism, Chemosphere, 291 (2021) 132941, doi: 10.1016/j.chemosphere.2021.132941.
[8] H.-J. Ahn, Y. Ahn, M.B. Kurade, S.M. Patil, G.-S. Ha, P.O. Bankole, M.A. Khan, S.W. Chang, M.H. Abdellattif, K.K. Yadav, B.-H. Jeon, The comprehensive effects of aluminum oxide nanoparticles on the physiology of freshwater microalga *Scenedesmus obliquus* and it's phycoremediation performance for the removal of sulfacetamide, Environ. Res., 215 (2022) 114314, doi: 10.1016/j.envres.2022.114314.
[9] M. Załęska-Radziwiłł, N. Doskocz, K. Affek, A. Muszyński, Effect of aluminum oxide nanoparticles on aquatic organisms – a microcosm, study, Desal. Water Treat., 195 (2020) 286–296.
[10] N. Doskocz, M. Załęska-Radziwiłł, K. Affek, Ecotoxicity of selected nanoparticles in relation to micro-organisms in the water ecosystem, Desal. Water Treat., 186 (2020) 50–55.
[11] N. Doskocz. M. Załęska-Radziwiłł., K. Affek., M. Łebkowska, Effects of selected nanoparticles on aquatic plants, Desal. Water Treat., 117 (2018) 42–48.
[12] M.T. Botelho, M.J. de A.R. Campos Passos, T.H. Trevizani, R.C. Lopes Figueira, G. de A. Umbuzeiro, V. Gomes, Genotoxic effects of silver nanoparticles on a tropical marine amphipod via feeding exposure, Mutat. Res. Genet. Toxicol. Environ. Mutagen., 8 (2022) 503527, doi: 10.1016/j.mrgentox.2022.503527.

[13] E. Demir, G. Vales, B. Kaya, A. Creus, R. Marcos, Genotoxic analysis of silver nanoparticles in *Drosophila*, Nanotoxicology, 5 (2011) 417–424.
[14] H. Barabadi, M. Najafi, H. Samadian, A. Azarnezhad, H. Vahidi, M.A. Mahjoub, M. Koohiyan, A. Ahmadi, A systematic review of the genotoxicity and antigenotoxicity of biologically synthesized metallic nanomaterials: are green nanoparticles safe enough for clinical marketing?, Medicina (Kaunas), 55 (2019) 439, doi: 10.3390/medicina55080439.
[15] M. Załęska-Radziwiłł, K. Affek, N. Doskocz, A. Affek, *In vitro* and *in vivo* genotoxicity assessment of selected pharmaceuticals in relation to *Escherichia coli* and *Cyprinus carpio*, J. Environ. Sci. Health A, 51 (2016) 1053–1061.
[16] T. Ohe, T. Watanabe, K. Wakabayashi, Mutagens in surface waters: a review, Mutat. Res., 567 (2004) 109–149.
[17] L. Xie, Y. Sapozhnikova, O. Bawardi, D. Schlenk, Evaluation of wetland and tertiary wastewater treatments for estrogenicity using *in vivo* and *in vitro* assays, Arch. Environ. Contam. Toxicol., 48 (2004) 81–86.
[18] L. Li, Y. Deng, X. Meng, H. Chang, C. Ling, D. Li, Q. Wang, T. Lu, Y. Yang, G. Song, Y. Hu, Genotoxicity evaluation of silica nanoparticles in murine: a systematic review and meta-analysis, Toxicol. Mech. Methods, 32 (2022) 1–17.
[19] Y. Kohl, E. Rundén-Pran, E. Mariussen, M. Hesler, N. El Yamani, E.M. Longhin, M. Dusinska, Genotoxicity of nanomaterials: advanced *in vitro* models and high throughput methods for human hazard assessment-a review, Nanomaterials (Basel), 10 (2020) 1911, doi: 10.3390/nano10101911.
[20] X. Guo, Y. Li, J. Yan, T. Ingle, M.Y. Jones, N. Mei, M.D. Boudreau, C.K. Cunningham, M. Abbas, A.M. Paredes, T. Zhou, M.M. Moore, P.C. Howard, T. Chen, Size- and coating-dependent cytotoxicity and genotoxicity of silver nanoparticles evaluated using *in vitro* standard assays, Nanotoxicology, 10 (2016) 1373–1384.
[21] M. Ghosh, M. Bandyopadhyay, A. Mukherjee, Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes, Chemosphere, 81 (2010) 1253–1262.
[22] M. Güneş, B. Yalçın, M. Muddassir Ali, İ.H. Ciğerci, B. Kaya, Affiliations expand genotoxic assessment of cerium and magnesium nanoparticles and their ionic forms in *Eisenia hortensis* coelomocytes by alkaline comet assay, Microsc. Res. Tech., 85 (2022) 3095–3103.
[23] J. Gopalraaj, M. Pappuswamy, M. Arun, B. Balamuralikrishnan, A.V. Anand, Toxic effects of nanoparticles on fish embryos, Res. J. Biotechnol., 16 (2021) 140–149.
[24] M. Auffan, J. Rose, M.R. Wiesner, J.Y. Bottero, Chemical stability of metallic nanoparticles: a parameter controlling their potential cellular toxicity *in vitro*, Environ. Pollut., 157 (2009) 1127–1133.
[25] A. Scherzad, T. Meyer, N. Kleinsasser, S. Hackenberg, Molecular mechanisms of zinc oxide nanoparticle-induced genotoxicity short running title: genotoxicity of ZnO NPs, Materials (Basel), 10 (2017) 1427, doi: 10.3390/ma10121427.
[26] C.M. Sayes, J.D. Fortner, W. Guo, D. Lyon, A.M. Boyd, K.D. Ausman, Y.J. Tao, B. Sitharaman, L.J. Wilson, J.B. Hughes, J.L. West, V.L. Colvin, The differential cytotoxicity of water-soluble fullerenes, Nano Lett., 4 (2004) 1881–1887.
[27] C.M. Goodman, C.D. McCusker, T. Yilmaz. V.M. Rotello, Toxicity of gold nanoparticles functionalized with cationic and anionic side chains, Bioconjugate Chem., 15 (2004) 897–900.
[28] V. Kononenko, N. Repar, N. Marušič, B. Drašler, T. Romih, S. Hočevar, D. Drobne, Comparative *in vitro* genotoxicity study of ZnO nanoparticles, ZnO macroparticles and $ZnCl_2$ to MDCK kidney cells: size matters, Toxicol. in Vitro, 40 (2017) 256–263.
[29] J.A. Rodríguez, M. Fernández-García, Eds., Synthesis, Properties, and Applications of Oxide Nanomaterials, John Wiley and Sons, New Jersey, 2008.
[30] EBPI (Environmental Bio Detection Products Inc.), Instructions for Use, The SOS-Chromotest Kit Version 6.0, Ontario, Canada, 1992.
[31] R. Legault, C. Blaise, D. Rokosk. R. Chong-Kit, Comparative assessment of the SOS Chromotest kit and the Mutatox test with the *Salmonella* plate incorporation (Ames test) and fluctuation tests for screening genotoxic agents, Environ. Toxicol. Water, 9 (1994) 45–57.
[32] G.H.S. Strauss, Non-random cell killing in cryopreservation: implications for performance of the battery of leukocyte tests (BLT), I. Toxic and immunotoxic effects, Mutat. Res., 252 (1991) 1–15.
[33] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell. Res., 175 (1988) 184–191.
[34] R. Liman, B. Başbuğ, M.M. Ali, Y. Acikbas, H. Ciğerci, Cytotoxic and genotoxic assessment of tungsten oxide nanoparticles in *Allium cepa* cells by Allium ana-telophase and comet assays, J. Appl. Genet., 62 (2021) 85–92.
[35] S. Aziz, S. Abdullah, H. Anwar, F. Latif, DNA damage and oxidative stress in economically important fish, bighead Carp (*Hypophthalmichthys nobilis*) exposed to engineered copper oxide nanoparticles, Pak. Vet. J., 42 (2022) 1–8.
[36] N.A.L. Flower, B. Brabu, M. Revathy, C. Gopalakrishnan, S.V.K. Raja, S.S. Murugan, T.S. Kumaravel, Characterization of synthesized silver nanoparticles and assessment of its genotoxicity potentials using the alkaline comet assay, Mutat. Res., 742 (2012) 61–65.
[37] Resolution European Parliament on Regulatory Aspects of Nanomaterials (2008/2208(INI)), European Parliament, Brussels, Belgium, 2009.
[38] M. Martins, P.M. Costa, In: M.L. Larramendy, Ed., Ecotoxicology and Genotoxicology: Non-Traditional Aquatic Models, The Royal Society of Chemistry, Cambridge, 2017, pp. 1–32.
[39] J. Catalán, S. Suhonen, A. Huk, M. Dusinska, In: L.M. Sierra, L. Gaivão, Eds., Genotoxicity and DNA Repair. Methods in Pharmacology and Toxicology, Humana Press, New York, 2014, pp. 241–268.
[40] Z.A. Hassan, H.H. Obaid, M.N. al-Darraji, *In vivo* genotoxicity assessment of gold nanoparticles of different doses by comet assays, Indian J. Forensic Med. Toxicol., 14 (2020) 2414–2420.
[41] D. Ritter, J. Knebel, Genotoxicity testing *in vitro* – development of a higher throughput analysis method based on the comet assay, Toxicol. in Vitro, 23 (2009) 1570–1575.
[42] Y. Lu, Y. Liu, C. Yang, Evaluating *in vitro* DNA damage using comet assay, J. Vis. Exp., 11 (2007) 56450, doi: 10.3791/56450.
[43] K. Sunjog, S. Kolarević, K. Héberger, Z. Gačić, J. Knežević-Vukčević, B. Vuković-Gačić, M. Lenhardt, Comparison of comet assay parameters for estimation of genotoxicity by sum of ranking differences, Anal. Bioanal. Chem., 405 (2013) 4879–4885.
[44] T.S. Kumaravel, B. Vilhar, S.P. Faux, A.N. Jha, Comet assay measurements: a perspective, Cell Biol. Toxicol., 25 (2009) 53–64.
[45] L.P. Zakharenko, I.K. Zakharov, E.A. Vasiunina, T.V. Karamysheva, A.M. Danilenko, A.A. Nikiforov, Determination of the genotoxicity of fullerene C60 and fullerol using the method of somatic mosaics on cells of *Drosophila melanogaster* wing and SOS-chromotest, Genetika, 33 (1997) 405–409.
[46] S.H. Nam, S.W. Kim, Y.J. An, No evidence of the genotoxic potential of gold, silver, zinc oxide and titanium dioxide nanoparticles in the SOS Chromotest, J. Appl. Toxicol., 33 (2012) 1061–1069.
[47] H.A. Alhadrami, R.A.M. Shoudri, Titanium oxide (TiO_2) nanoparticles for treatment of wound infection, J. Pure Appl. Microbiol., 15 (2021) 437–451.
[48] P.V. Vidya, K.C. Chitra, Evaluation of genetic damage in *Oreochromis mossambicus* exposed to selected nanoparticles by using micronucleus and comet bioassays, Croat. J. Fish., 76 (2018) 115–124.
[49] W.F. Vevers, A.N. Jha, Genotoxic and cytotoxic potential of titanium dioxide (TiO_2) nanoparticles on fish cells *in vitro*, Ecotoxicology, 7 (2008) 410–420.
[50] M. Naguib, I.A. Mekkawy, U.M. Mahmoud, A.E.H. Sayed, Genotoxic evaluation of silver nanoparticles in catfish *Clarias gariepinus* erythrocytes; DNA strand breakage using comet

assay, Sci. Afr., 16 (2022) e01260, doi: 10.1016/j.sciaf.2022.e01260.
[51] K. Shahzad, M.N. Khan, F. Jabeen, N. Kosour, A.S. Chaudhry, M. Sohail, N. Ahmad, Toxicity of zinc oxide nanoparticles (ZnO-NPs) in tilapia (*Oreochromis mossambicus*): tissue accumulation, oxidative stress, histopathology and genotoxicity, Int. J. Environ. Sci. Technol., 16 (2019) 1973–1984.
[52] I. Nabiev, S. Mitchell, A. Davies, Y. Williams, D. Kelleher, R. Moore, Y.K. Gun'ko, S. Byrne, Y.P. Rakovich, J.F. Donegan, A. Sukhanova, J. Conroy, D. Cottell, N. Gaponik, A. Rogach, Y. Volkov, Non-functionalized nanocrystals can exploit a cell's active transport machinery delivering them to specific nuclear and cytoplasmic compartments, Nano Lett., 7 (2007) 3452–3461.
[53] P.A. White, J.B. Rasmussen, SOS Chromotest results in a broader context: empirical relationships between genotoxic potency, mutagenic potency, and carcinogenic potency, Environ. Mol. Mutagen., 27 (1996) 270–305.