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Effects of zirconium oxide nanoparticles on bacteria isolated from activate sludge detected by RAPD analysis

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ABSTRACT

This research has been conducted to determine the genotoxicity of nano-ZrO_2 towards *Pseudomonas putida* and *Aeromonas hydrophila* bacteria using randomly amplified polymorphic DNA (RAPD)-PCR method. Results obtained for the nanocompound were compared with those for ZrO_2 macro form. The nanocompound caused changes in the genetic material of bacteria. *P. putida* was more sensitive to nano-ZrO_2 than *A. hydrophila*. Degree of genetic similarity (*S*) of obtained profiles bands for primer OPA2 differed from the results obtained for the negative control by more than 30%, while from positive control – only by 12%. In turn, the largest decrease in genetic stability (GTS) was 75.1%. The results also showed that the nano-ZrO_2 can induce modifications of the genetic material to a greater extent than the same compounds in their macro form. The obtained data confirmed that RAPD-PCR can be successfully used to monitor potentially genotoxic effects of various nanoparticles.

Keywords: Nanoparticles; Zirconium oxide; RAPD assay; Genotoxicity

1. Introduction

Nanotechnology includes manufacturing of nanomaterials, which is due to their specific properties, can be widely used in all sectors of industry and medicine. Nanoparticles (NPs) are particles with dimensions less than 100 nm, often smaller than bacterial and eukaryotic cells. Increasing production and use of NPs contributes to their widespread dissemination in the environment and their unique physical and chemical properties lead to unlimited distribution in environmental compartments. In Sweden and Denmark, about 50 g of NPs (per person and day) is released to the environment with wastewater. The amount of these compounds can reach up to 50 t/d in the city of one million of inhabitants [1]. Particular attention is paid to the residues of these particles in bottom sediments of water reservoirs. Migration ability of NPs may have very dangerous consequences, as they can be transferred to potable water.

To date, most nanotoxicity assessments have been focused on phenotypic endpoint-based cytotoxicity [2,3]. Studies have demonstrated that low concentrations of NPs, typically detected in environmental compartments, may not trigger cytotoxic effects, but may result in effects at the molecular level [4,5]. For example, Lee et al. [4] noticed that the cytotoxic effects of titanium dioxide (nano-TiO_2), silicon dioxide or silica (nano-SiO_2) and cerium oxide (nano-CeO_2) NPs on daphnids and chironomids were not observed at the organism level for endpoints such as mortality, growth or reproduction, but adverse effects were clear at the genetic level.

Genotoxicity biomarkers are regarded as useful tools for the assessment of chemical hazards in aquatic ecosystems [6]. This is because chemicals which damage DNA, even at very low concentrations, can significantly alter the functioning of ecosystems [7]. Recent advances in molecular biology have led to the development of several PCR-based techniques, which can be used for DNA analysis in the field of

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genotoxicology. The randomly amplified polymorphic DNA (RAPD) method is a PCR-based technique that amplifies random DNA fragments with the use of single short primers of arbitrary nucleotide sequence under low annealing conditions [3]. RAPD assay has been applied successfully in order to detect genetic damage in animals and plants [8–12], however, the use of bacteria as bioindicators in RAPD method and investigation of the influence of oxide NPs on the genetic material of microorganisms are seldom known.

In the present study, the impact of zirconium oxide NPs (nano-ZrO_2 or ZrO_2 NPs) on *Pseudomonas putida* and *Aeromonas hydrophila* bacteria in terms of DNA damage was studied using RAPD analysis. The principal objectives were to assess the genotoxicity of nano-ZrO_2 on *P. putida* and *A. hydrophila*, and to explore the potential of RAPD technique in detection of genotoxicity towards microorganisms. The increased production and commercial use of 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. Hence, it was decided at work to investigate whether NPs can negatively influence the communities of microorganisms participating in biological processes of wastewater treatment. The choice of *P. putida* and *A. hydrophila* strains was dictated by the fact that bacteria of the genus *Pseudomonas* and *Aeromonas* belong to the most abundant microorganisms in activated sludge [13]. *P. putida* as well *A. hydrophila* are a gram-negative, rod-shaped and heterotrophic bacterium. These bacteria demonstrate a very diverse metabolism. *P. putida* has the ability to degrade organic solvents such as toluene. This ability has been put to use in bioremediation, or the use of microorganisms to biodegrade oil [14]. *A. hydrophila* can survive in aerobic and anaerobic environments, and can digest materials such as gelatin and hemoglobin [15].

Zirconium oxide NPs are used to eliminate water pollutants (e.g., arsenic), and in bioengineering – in the production of prostheses and implants as well as the carriers of medicines (insulin) [16–18]. Widespread use of these NPs followed by their release to the environment may result in toxic reactions in the organisms of aquatic ecosystems. The interest in zirconium oxide NPs is due to the fact that their influence on genetic material of bacteria is practically unknown. In this study, the effect of activity of the nanoparticle form of ZrO_2 on bacteria was compared with their bulk counterparts (ZrO_2 in macro-form).

2. Materials and methods

2.1. Chemicals

Zirconium oxide NPs (nano-ZrO_2), nanopowder <100 nm with a specific surface area ≥25 m^2/g, zirconium oxide of purity over 98% and methyl methanesulfonate (MMS; positive control) was obtained from Sigma-Aldrich (Poland). CAS no. of compounds containing ZrO_2 is 1314-23-4 and MMS is 66-27-3. The stock solutions of chemicals of 500 mg/L were prepared in deionized water. Because nanocompounds are able to form aggregates, the stock dispersion was sonicated (0.4 kW, 20 kHz) for 30 min before being diluted to the exposure concentrations. The stock solutions were diluted (using the medium with respect to the procedures of tests) in descending order with a geometric series of quotient $q = 2$ to obtain final nominal concentrations of 500–0.19 mg/L.

2.2. Bacterial strains

Heterotrophic gram-negative rods of *P. putida* and *A. hydrophila* were isolated from the activated sludge working in laboratory conditions, in Department of Biology, Faculty of Building Services, Hydro and Environmental Engineering, Warsaw University of Technology. Bacteria were isolated from the activated sludge working in laboratory conditions. A lab-scale reactor with a working volume of 5 L was seeded with activated sludge from full-scale wastewater treatment plant located in Babice Stare near Warsaw (Poland). The plant had A2O configuration (anaerobic–anoxic–aerobic) with a predenitrification tank placed ahead of the anaerobic chamber without primary settling. The plant received typical domestic wastewater corresponding to 6,000 population equivalent (size designed 7,500).

API 20 NE bacterial identification was performed according to manufacturer's instruction (BioMérieux, Durham, NC, USA). The strains were multiplied in nutrient broth at a temperature of 26°C for 18 h until the commencement of the logarithmic growth phase. Cultures of bacteria of optical density of 0.2 at $\lambda = 610$ nm were added to a liquid medium containing defined concentrations of the tested compounds. Samples were incubated for 16 h at 26°C in the dark. Cultures obtained in this way were used for DNA isolation [19–21].

2.3. Extraction of genomic DNA

Genomic DNA was isolated from culture of *P. putida* and *A. hydrophila* using a "Genomic Mini" kit in accordance with the manual enclosed by the manufacturer (A&A Biotechnology, Poland). The isolation process was based on the ability of DNA binding to silica deposits in high concentrations of chaotropic salt. The amount and quality of DNA material, isolated from the bacterial cultures was checked by staining of 5 μL DNA sample resolving it in an electrophoresis system and observing the DNA bands under UV light. After the concentration and the index of purity (OD_{260}/OD_{280}) were measured via a "BioPhotometr" (Eppendorf, Poland). DNA samples were subpackaged and stored at 4°C until use. The conditions of DNA amplification were optimized by following the procedure of Conte et al. [22].

2.4. RAPD procedures

The PCR was performed in a final volume of 25 μL using 25 ng of genomic DNA under the following conditions: and 1U or 2U of Taq DNA polymerase (A&A Biotechnology, Poland), 10× reaction buffer (A&A Biotechnology, Poland), 100 mM dNTPs (25 mM each; A&A Biotechnology, Poland), 10 μM primer (Laboratory of DNA Sequencing and Oligonucleotide Synthesis, IBB PAS, Poland). The sequences of four primers utilized in this study were shown in Table 1. Amplifications were implemented in a DNA thermocycler (Mastercycler pro, Eppendorf, Poland) programmed for 2 min at 94°C (initial denaturing step), 35 consecutive cycles each consisting of 1 min at 94°C (denaturing), 1 min

Table 1
Sequences of four primers used in this experiment

Primer	Sequence (5′ → 3′)
OPA2	TGCCGAGCTG
OPA9	GTGATCGCAG
OPA10	GGGTAACGCC
OPA18	AGGTGACCGT

at 31°C or 33°C (annealing), 2 min at 72°C (extension) and followed by 1 min at 72°C for the final extension. Control PCRs lacking genomic DNA were run with every set of samples. After amplification, RAPD reaction products were analyzed by electrophoresis on 1% agarose gels in 1× Tris-Borate-EDTA buffer (45 mM Tris base, 20 mM boric acid and 1 mM ethylenediaminetetraacetic acid) at 100 V for about 30–50 min. GeneRuler 1kb DNA Ladder (Fermentas, USA) was used as molecular weight DNA standard. DNA bands were stained with ethidium bromide, visualized and photographed under UV light. Then, the electrophorograms were photographed under a GelDoc-It Imaging System (Ultra-Violet Products Ltd). Digital processing of the images and computational analysis were performed using Gelix One 1-D Analysis Software (Biostep, Germany). All amplifications were repeated twice in order to confirm their reproducibility of RAPD patterns. Only repeatable and clear amplification bands were scored for the construction of the data matrix.

2.5. Data analysis and processing

Genetic similarity index (*S*, %) between treated samples and control bacteria was calculated as the proportion of amplification products which were not polymorphic with respect to the total number of amplified products, 2× number of shared fragments/total number of fragments [22,23].

Genomic template stability (GTS, %) was calculated for each primer as the formula: 100 – (100*a*/*n*) where *a* was RAPD polymorphic profiles detected in each samples treated and *n* the number of total bands in the control. Polymorphism observed in RAPD profiles included disappearance of a normal band and appearance of a new band in comparison with controls (negative and positive) RAPD profiles [24,25], and the mean values and standard deviations were then calculated for each experimental group exposed to different nano-ZrO_2 and ZrO_2 treatment. Changes in these values were calculated as a percentage of their control (set to 100%). Analysis was performed using Gelix One 1-D Analysis Software (Biostep, Germany). Negative control in the work was DNA isolated from *P. putida* and *A. hydrophila*, which were not treated with NPs. In contrast, the positive control was DNA isolated from *P. putida* and *A. hydrophila*, which were exposed to MMS at a concentration of 10 mg/L, the mutagenic activity of which proved earlier [26,27].

3. Results

Changes in the genetic material caused by the impact of zirconium oxide NPs and zirconium oxide on DNA isolated from bacteria *P. putida* and *A. hydrophila* were evaluated using the RAPD-PCR technique. The PCR reaction involved primers: OPA2, OPA10, OPA9 and OPA18. Photos of RAPD bands profiles are shown in Figs. 1 and 2. The obtained bands were used for the analysis of degree of similarity between bands profile (*S*, %) and genetic stability of DNA (GTS, %) with relation to negative control and the results are shown in Tables 2–5 and Figs. 3 and 4.

On the basis of RAPD profiles obtained for the PCR, it was found that the zirconium oxide NPs can be genotoxic to *P. putida* and to a lesser extent to *A. hydrophila*. The most mutagenic action of ZrO_2 NPs was indicated by the PCR products obtained with the use of primer OPA2 in case of *P. putida*. The obtained bands profiles were different from the negative control from over 33.3% (concentration 1,000 mg/L) to 7.7% (concentration of 0.1 mg/L) (Table 2). The obtained bands profile in concentration 1,000 mg/L differed from the result obtained for the positive control only by 12.2% (Table 2). Differences were much greater in case of other tested concentrations, within 31.2%–37.8%. For the PCR products obtained with the use of primer OPA9, the values of degree of similarity of RAPD bands profiles in relation to negative control were 88.8% in all concentrations tested (Table 3). In the case of primer OPA10 and OPA18 the mutagenic effect of zirconium oxide NPs was the lowest among all studied (Tables 4 and 5). The degrees of similarity (*S*) of the obtained bands profiles for primers OPA9, OPA10 and OPA18 were different from the values obtained for the positive control. The differences ranged from 32.4% for OPA10 and 38.8% for OPA9 to 50% for OPA18 at all concentrations tested (Tables 3–5).

Results of the analysis of the genetic stability of DNA (GTS, %) in the case of *P. putida*, which is a measure reflecting changes in RAPD profiles, showed that the average value of GTS in all concentrations tested was lower than negative control (Fig. 3). The largest decrease in genetic stability was found in the three highest concentrations tested. GTS value was 75.1% and 78.1%, while at the concentration of 1 and 0.1 mg/L – 82.3%. The data indicate that GTS in the three highest concentrations tested differed from the value obtained for the positive control by 33.4% and 36.4%, while in other concentrations by as much as 40.6% (Fig. 3).

In the case of *A. hydrophila* for all tested concentrations and all primers, the RAPD bands profiles did not differ from the profiles obtained for the negative control, whereas differed from the profiles obtained for the positive control by 50% (Tables 2–5).

The results of the GTS analysis indicated that in the case of nano-ZrO_2 in relation to *A. hydrophila*, the mean GTS value in all studied concentration was equal to the values obtained for the negative control (Fig. 4).

The results also indicate that zirconium oxide in bulk counterparts is less mutagenic than the compound in the nanoparticle form (Tables 2–5, Figs. 3 and 4). RAPD profiles obtained for samples of ZrO_2 showed greater similarity compared with negative control than the profiles for samples with nano-ZrO_2. In the presence of zirconium oxide a smaller decrease in genetic stability in all concentrations tested as compared with the zirconium oxide NPs was observed.

4. Discussion

The analysis of profiles obtained in RAPD assay showed that zirconium oxide NPs have the potential to cause changes in the genetic material of bacteria. In case of using nano-ZrO_2

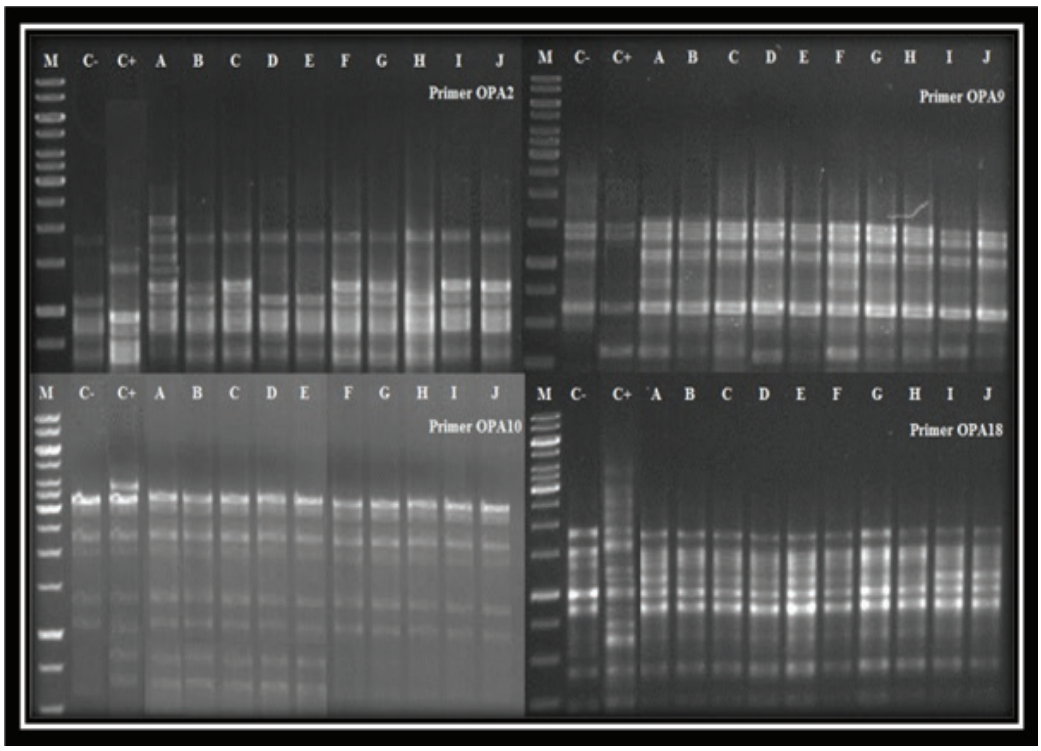

Fig. 1. RAPD profiles generated using primers OPA2, OPA9, OPA10 and OPA18 for genomic DNAs extracted from bacteria *P. putida* exposed to negative control (C–), positive control (C+) and various concentrations (1,000, 100, 10, 1 and 0.1 mg/L) of nano-ZrO_2 (A, B, C, D and E) and ZrO_2 (F, G, H, I and J) for 16 h. M: GeneRuler 1kb DNA Ladder (10,000–250 kb).

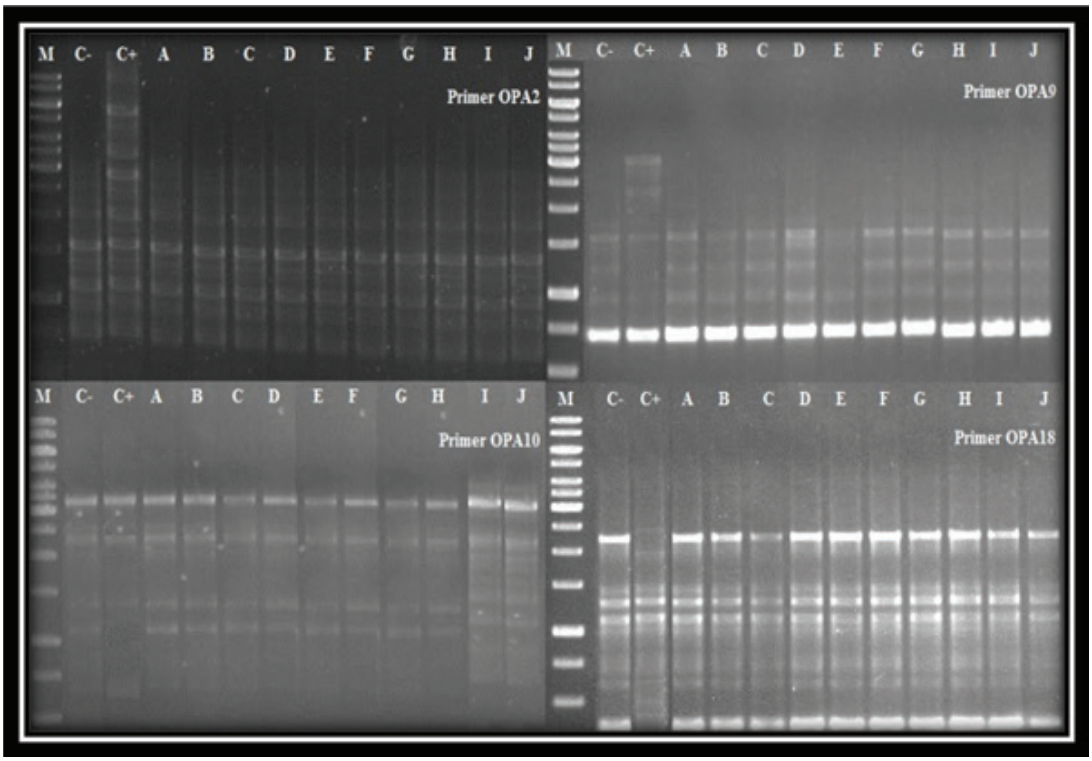

Fig. 2. RAPD profiles generated using primers OPA2, OPA9, OPA10 and OPA18 for genomic DNAs extracted from bacteria *A. hydrophila* exposed to negative control (C–), positive control (C+) and various concentrations (1,000, 100, 10, 1 and 0.1 mg/L) of nano-ZrO_2 (A, B, C, D and E) and ZrO_2 (F, G, H, I and J) for 16 h. M: GeneRuler 1kb DNA Ladder (10,000–250 kb).

Table 2
Degree of similarity of obtained profiles (%) of *P. putida* and *A. hydrophila* RAPD bands exposed to nano-ZrO_2 and ZrO_2 for OPA2 primer

Genetic similarity index (*S*, %) after 16 h (standard deviation, SD)				
Primer OPA2				
Concentration (mg/L)	*P. putida*		*A. hydrophila*	
	Nano-ZrO_2	ZrO_2	Nano-ZrO_2	ZrO_2
1,000	66.7 (3.6–2.1)	92.3 (2.8–1.3)	100 (1.0–1.1)	100 (1.0–0.1)
100	85.7 (4.6–3.7)	92.3 (3.5–1.9)	100 (1.0–0.1)	100 (1.0–1.4)
10	85.7 (2.4–3.4)	98.4 (2.4–2.8)	100 (1.0–1.4)	100 (1.0–0.1)
1	92.3 (2.3–4.3)	98.4 (2.6–2.8)	100 (1.0–0.1)	100 (1.0–0.1)
0.1	92.3 (2.4–3.8)	98.4 (3.6–2.1)	100 (1.0–0.1)	100 (1.0–0.1)
C(+)	54.5 (1.4–2.2)		57.1 (0.7–1.3)	

Table 3
Degree of similarity of obtained profiles (%) of *P. putida* and *A. hydrophila* RAPD bands exposed to nano-ZrO_2 and ZrO_2 for OPA9 primer

Genetic similarity index (*S*, %) after 16 h (standard deviation, SD)				
Primer OPA9				
Concentration (mg/L)	*P. putida*		*A. hydrophila*	
	Nano-ZrO_2	ZrO_2	Nano-ZrO_2	ZrO_2
1,000	88.8 (1.5–1.7)	88.8 (1.6–1.1)	100 (1.0–0.1)	100 (1.0–0.1)
100	88.8 (1.7–1.2)	88.8 (1.1–2.2)	100 (1.0–0.1)	100 (1.4–0.1)
10	88.8 (1.0–1.1)	88.8 (1.4–2.3)	100 (1.0–0.1)	100 (1.0–0.1)
1	88.8 (1.8–1.7)	88.8 (1.3–1.4)	100 (1.0–0.4)	100 (1.0–0.1)
0.1	88.8 (1.4–1.1)	88.8 (2.0–1.8)	100 (1.0–0.1)	100 (1.0–0.4)
C(+)	50.0 (1.0–2.3)		60.0 (2.1–1.6)	

Table 4
Degree of similarity of obtained profiles (%) of *P. putida* and *A. hydrophila* RAPD exposed to nano-ZrO_2 and ZrO_2 for OPA10 primer

Genetic similarity index (*S*, %) after 16 h (standard deviation, SD)				
Primer OPA10				
Concentration (mg/L)	*P. putida*		*A. hydrophila*	
	Nano-ZrO_2	ZrO_2	Nano-ZrO_2	ZrO_2
1,000	85.7 (1.6–0.4)	100 (0.2–0.1)	100 (1.1–0.1)	100 (1.0–0.1)
100	85.7 (2.1–1.3)	100 (1.4–0.2)	100 (0.0–0.4)	100 (0.4–0.1)
10	85.7 (1.2–2.3)	100 (1.4–1.1)	100 (0.1–1.1)	100 (1.0–0.1)
1	85.7 (1.1–0.1)	100 (1.1–0.2)	100 (1.0–0.1)	100 (1.0–1.1)
0.1	85.7 (1.0–12)	100 (1.2–1.2)	100 (1.0–0.1)	100 (0.1–0.1)
C(+)	53.3 (1.2–2.7)		54.5 (2.1–1.6)	

against *P. putida* with primer OPA2, the changes in the genetic material increased with the increasing concentration. The lowest values of the degree of genetic similarity of bands as well as the greatest decrease in DNA genetic stability, as compared with negative control, was obtained at the concentration of 1,000 mg/L of nano-ZrO_2, which indicates high mutagenic potency of the nanocompound in this concentration. A similar effect was observed in our earlier studies [12], in which it has been shown that DNA damage of *P. putida* increased with an increase in concentrations of nano-Al_2O_3. The test compound generated a similar number of mutations as MMS (positive control), which mutagenic action was confirmed in numerous publications [26–28].

Modifications of the RAPD patterns are likely due to one or a combination of the following events: DNA adducts, DNA breakage and mutation (point mutations and large rearrangements) [10,24,26,28–32]. In case of *P. putida* (primers OPA9, OPA10 and OPA18) and *A. hydrophila* (all primers),

Table 5
Degree of similarity of obtained profiles (%) of *P. putida* and *A. hydrophila* RAPD bands exposed to nano-ZrO_2 and ZrO_2 for OPA18 primer

Genetic similarity index (*S*, %) after 16 h (standard deviation, SD)				
Primer OPA18				
Concentration (mg/L)	*P. putida*		*A. hydrophila*	
	Nano-ZrO_2	ZrO_2	Nano-ZrO_2	ZrO_2
1,000	100 (1.1–2.0)	100 (1.1–0.2)	100 (0.1–1.1)	100 (0.1–1.1)
100	100 (0.1–1.1)	100 (0.1–1.1)	100 (0.1–1.0)	100 (1.2–1.0)
10	100 (0.1–1.2)	100 (0.1–0.1)	100 (0.1–1.1)	100 (1.1–1.2)
1	100 (0.3–1.1)	100 (0.1–1.2)	100 (0.1–1.1)	100 (1.1–1.1)
0.1	100 (1.1–1.1)	100 (0.3–1.1)	100 (1.1–0.1)	100 (0.1–1.2)
C(+)	50.0 (0.2–1.2)		61.5 (1.1–0.3)	

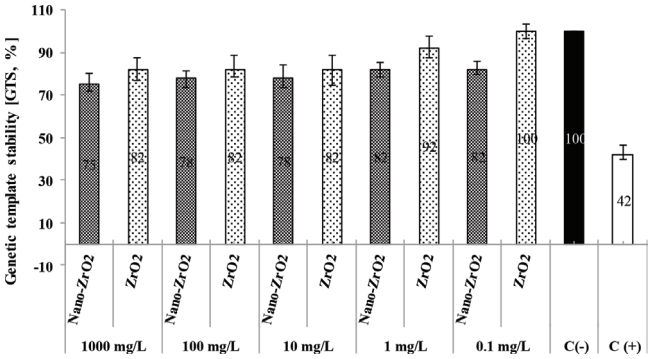

Fig. 3. Genetic stability (GTS, %) of *P. putida* exposed to various concentrations of nano-ZrO_2 and ZrO_2.

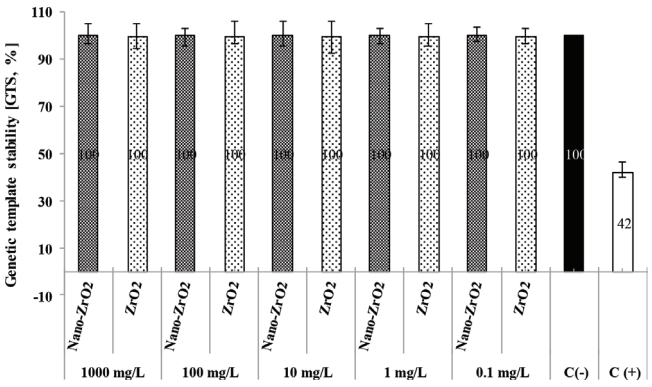

Fig. 4. Genetic stability (GTS, %) of *A. hydrophila* exposed to various concentrations of nano-ZrO_2 and ZrO_2.

it was found, that the *S* and GTS do not differ much from the results obtained for the negative control. Minor changes in the genome may indicate that the presence of zirconium oxide NPs may lead to changes of a random nature [33]. Moreover, RAPD profiles with a similar degree of genetic similarity to negative control and a slight decrease in the genomic template stability may indicate the induction of mechanisms responsible for DNA repair beyond a certain critical level of damage, which, for ZrO_2 NPs, may be even less than the lowest concentration used (0.1 mg/L) [10,28].

The results also showed that not every primer used in RAPD method is sensitive enough to provide reliable information. The lowest degrees of genetic similarity were observed in RAPD profiles obtained for *P. putida* and *A. hydrophila*, which used primer OPA2. This allows to conclude that this primer, because of the sensitivity of the detection, is the most effective of all the primers used, as it captures the changes in the genetic material in a wide range of concentrations. The use of a higher number of primers or their combinations increases the chance of detecting mutations [28]. Results of this work are consistent with the observations of Zhou et al. [34], Nan et al. [35] and Zhang et al. [36] who have also demonstrated concentration-relationships between DNA changes and genotoxicant stress with the use of RAPD analyses. Previous studies have also confirmed that GTS is a sensitive parameter to reflect the changes in RAPD profiles induced by toxic pollutants. Data obtained in this work proved the potential of RAPD assay in the detection of bacterial DNA damage induced by toxic pollutants [12,34,37].

The research also revealed that zirconium oxide leads to smaller changes in genetic material than zirconium oxide NPs (Tables 2–5). Similar conclusions were obtained by Ghosh et al. [38], who demonstrated that the extent of DNA damage in cells of *Allium cepa* induced by ZnO NPs was significantly higher than that induced by bulk particles. Our previous research also demonstrated that the aluminum oxide have much smaller impact on DNA of *P. putida* than the nanoparticle form of these compounds [12].

The obtained results as well as data from literature confirm that RAPD assay has been successfully used to monitor the potentially genotoxic effects of various NPs [38–41]. Although RAPD still requires reaction optimization in terms of primer concentration, concentration of DNA template, polymerase and magnesium ions, and the parameters of the thermal cycler, or electrophoresis, it has been shown to be a reliable, easy, quick, sensitive and reproducible assay and therefore may be used to detect a wide range of DNA damage. In addition, studies have reported that RAPD assay is more sensitive than classic tests such as the comet and micronucleus assay, because RAPD analysis is capable of detecting temporary DNA changes at lower concentrations of pollutants [10,34,36]. One should remember that RAPD-PCR remains only a qualitative method. Effect of each category of

DNA damage (e.g., strand breakage, modified bases, abasic sites, oxidized bases and bulky adducts) on RAPD profiles can only be speculated unless amplicons are analyzed (e.g., sequencing) and more specific methods are needed to obtain quantitative data [26,29,30,32,42].

In addition, it must be noted that there is no data in the literature about the concentration of zirconium oxide NPs in the environment. Generally, in the literature for most of the NPs there is no data about predicted environmental concentration (PEC) in the environment. The problem with determining their amount in the environment results from: (1) low concentration of some NPs in the environment (1 ng/L or lower), (2) the relatively high concentration (mg/L) of natural NPs (i.e., iron oxide and carbon monoxide) and (3) the lack of reliable methods for their detection and determination of contents in environmental components [43–45]. So far, modeling methods based on the production and lives of nanoproducts have been used to estimate the predicted PEC concentration [44,45]. The research carried out in this work was preliminary/cognitive, related to a compound for which there is little ecotoxicological data, so the choice of concentrations of nano-ZrO_2 used in the study was directed to the possibility of capturing all potential changes in the genetic material, which is possible in quite wide range concentrations. Moreover, single literature data shows that NPs may be present in the environment at high concentrations reaching values in milligrams. Sun et al. [46] showed that PEC for zinc oxide NPs was very high and even up to 200 mg/kg (bottom sediment). Based on all this information, a fairly wide range of concentrations of this compound was used at work.

5. Conclusions

In conclusion, the results suggested that zirconium oxide NPs (nano-ZrO_2) had genotoxicity effects on *P. putida* and to a lesser extent in relation to *A. hydrophila*. The study also showed that the currently available ecotoxicity data about compounds in bulk counterparts cannot be used to assess the harmfulness of their nano-form. Meanwhile, RAPD analysis proved to be a highly sensitive method for the detection of DNA damage induced by emerging environmental pollutants such as NPs. The results obtained and data from subject-related literature indicate that NPs can induce modifications of genetic material, which are often irreversible. This type of impact may be a threat not only to microorganisms, but also for entire ecosystems. Negative consequences may concern disturbances in element cycling (carbon, sulfur, nitrogen, etc.), pollutants' degradation and plant growth promotion. There is a constant need for further studies, including not only conventional, but also multispecies and molecular tests – such as molecular cloning and sequencing to search for specific genomic targets affected by NPs and functional analysis of target genes, in order to explain the genotoxic mechanisms of NPs' effects on microorganisms and to ensure safety of beneficial microorganisms in the environment.

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