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Technical Note

Antimicrobial activity of metal oxide nanoparticles supported onto natural clinoptilolite

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HIGHLIGHTS

- Antibacterial activity of Cu_2O and ZnO nanoparticles in nonsterile secondary effluent.
- Antiprotozoan activity of Cu_2O and NiO nanoparticles.
- Nanoparticles are not affected by microorganisms and can be reused.
- Novel metal oxide/zeolite disinfectant for secondary effluent.

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ABSTRACT

The antimicrobial activity of Cu_2O, ZnO and NiO nanoparticles supported onto natural clinoptilolite was investigated in the secondary effluent under dark conditions. After 24 h of contact the Cu_2O and ZnO nanoparticles reduced the numbers of viable bacterial cells of *Escherichia coli* and *Staphylococcus aureus* in pure culture for four to six orders of magnitude and showed consistent 100% of antibacterial activity against native *E. coli* after 1 h of contact during 48 exposures. The antibacterial activity of NiO nanoparticles was less efficient. The Cu_2O and NiO nanoparticles showed 100% of antiprotozoan activity against *Paramecium caudatum* and *Euplotes affinis* after 1 h of contact, while ZnO nanoparticles were less efficient. The morphology and crystallinity of the nanoparticles were not affected by microorganisms. The metal oxide nanoparticles could find a novel application in the disinfection of secondary effluent and removal of pathogenic microorganisms in the tertiary stage of wastewater treatment.

1. Introduction

Water contaminated with certain bacteria and protozoan parasites provides an important route for human infections. The major pathogenic bacteria of concern in water are *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., *Vibrio cholerae* and enteropathogenic *Escherichia coli*. Some of waterborne protozoan pathogens include *Acanthamoeba* spp., *Cryptosporidium parvum*, *Entamoeba histolytica, Giardia duodenalis, Naegleria fowleri* (Marshall et al., 1997).

The use of metal oxide nanoparticles (MONPs) exhibiting the antimicrobial activity offers the possibility of an efficient removal of pathogens from wastewater. The MONP may not have the pronounced antimicrobial activity when compared to the bulk formulations of the metal oxide or solutions of metal salts (Heinlaan et al., 2008). But, the stability and slow release of metal ions from nanoparticles are main characteristics which give them the advantage in use.

The antimicrobial efficiency of MONP depends on the particle size, presence of light and composition of aqueous medium used in assay. The MONP tends to aggregate in aqueous media with true size in suspension differing significantly from that of dry powder (Adams et al., 2006). This prevents the effective interaction between particles and microorganisms and discerns the effect of particle size on the antimicrobial activity of MONP. In the presence of light the MONP produce reactive oxygen species that damage cells. In the literature light is usually provided by the specific wavelength high-intensity lamps and only in few studies sunlight as the source of illumination was used (Adams et al., 2006). The antimicrobial activity of MONP under dark conditions is due to yet undetermined mechanisms. The antimicrobial activity of MONP examined in media optimized for growth of microorganisms may not reflect the antimicrobial activity in natural water where the coagulation and precipitation of MONP might occur (Adams

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et al., 2006; Hrenovic et al., 2012). The studies on antimicrobial activity of MONP in real water are scarce. The short-term exposure to the ZnO nanoparticles induced the loss of biological nitrogen and phosphorus removal form wastewater (Zheng et al., 2011).

The aim of this study was to investigate the antibacterial and antiprotozoan activity of Cu_2O, ZnO and NiO nanoparticles supported onto natural clinoptilolite in the secondary effluent water under dark conditions.

2. Materials and methods

2.1. Preparation of MONP

The preparation of MONP supported onto natural clinoptilolite was previously described in details (Rajic et al., 2010, 2011; Stojakovic et al., 2011a, 2011b). In brief, the starting material was the natural zeolitized tuff containing 70 wt% of clinoptilolite (feldspar plagioclase and quartz were major impurities) from the sedimentary deposit Zlatokop, Serbia. The particle size of the sample was in the range 0.063–0.1 mm. Natural zeolite was firstly treated with a solution of NaCl to obtain Na-rich clinoptilolite and then by aqueous solution of MCl_2 (M = Cu, Zn or Ni) to obtain the metal-loaded zeolites. The metal-loaded zeolites containing in wt% 2.60 Cu^{2+}, 1.47 Zn^{2+} or 0.52 Ni^{2+} were dehydrated in the air at 550 °C for 1 h. The dehydration of metal-loaded zeolites resulted in the formation of Cu_2O, ZnO or NiO nanoparticles supported on the clinoptilolite lattice (subsequently named as Cu_2ONZ, ZnONZ and NiONZ, respectively). The particle size of MONP was 2–5 nm. Dry materials were sterilized by autoclaving (121 °C, 15 min) prior to the experiments.

2.2. Secondary effluent water

The antimicrobial assay was carried out in the real effluent water from the secondary stage of the biological wastewater treatment plant at wastewater treatment plant in Zagreb, Croatia. The chemical composition of the effluent water was (in mg L^{-1}): chemical oxygen demand (COD) 54; total nitrogen (TN) 17.8; total phosphorus (TP) 2.02. The COD, TN and TP were measured spectrophotometrically (Hach, DR 2500) using the reactor digestion method (Hach method 8000), persulfate digestion method (Hach method 10072) and ascorbic acid method with acid persulfate digestion (Hach method 10127), respectively. In the examined secondary effluent water 4.50×10^4 colony forming units (CFU) mL^{-1} of heterotrophic bacteria, 4.88×10^3 mL^{-1} of total coliform bacteria, 1.35×10^3 mL^{-1} of faecal coliform bacteria and 2.10×10^2 mL^{-1} of faecal streptococci were measured. The number of these bacteria was determined by cultivation on nutrient agar at 22 °C/72 h, EC-X GLUC agar at 35 °C/48 h, EC-X GLUC agar at 44 °C/24 h and Slanetz–Bartley agar at 35 °C/48 h, respectively.

For the experiments with pure bacterial cultures and protozoa, the fresh sample of effluent water was filtered through a Buchner funnel with filter paper (blue band) and Sartorius nitrocellulose filters of pore diameter 0.45 μm. The pH of effluent water was adjusted (WTW, 330 pH-meter) to 7.0 ± 0.2 with 1 M NaOH or 1 M HCl. Effluent water was sterilized by autoclaving (121 °C, 15 min). For the experiments with native population of *E. coli*, the nonsterile effluent water of original pH of 7.9 was employed in experiments within 2 h after the sampling.

2.3. Antibacterial activity test

The antibacterial activity of Cu_2ONZ, ZnONZ and NiONZ was tested against pure bacterial cultures of Gram-negative bacteria *E. coli* (strain DSM no. 498) and Gram-positive bacteria *Staphylococcus aureus* (strain DSM no. 799), obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH. The bacteria *E. coli* and *S. aureus* were pre-grown on Luria Bertani (LB) agar for 16 h at 37.0 ± 0.1 °C. The bacterial biomass was then suspended in the sterile 0.05 M NaCl solution. One milliliter of the suspended biomass of *E. coli* or *S. aureus* was inoculated into Schott bottles which contained 100 mL of autoclaved effluent water, giving the initial number of 10^6–10^7 CFU mL^{-1}. To each of the bottles, 1.0 g of the autoclaved Cu_2ONZ, ZnONZ or NiONZ were added. The control bottles were left without addition of the zeolites. The bottles were sealed and incubated aerobically (concentration of dissolved oxygen 4.9–5.5 mg L^{-1}) in a dark for 24 h in a water bath (Memmert, WNB22) at 37.0 ± 0.5 °C with shaking at 70 rpm to assure the complete mixing. The experiments with native population of *E. coli* were performed in the same way, except that triplicate of fresh nonsterile effluent water was employed at 25.0 ± 0.5 °C. The antibacterial activity of 1.0 and 5.0 g of Cu_2ONZ, ZnONZ or NiONZ per 100 mL of nonsterile effluent water were tested.

The number of *E. coli* and *S. aureus* viable cells was determined at the beginning of experiment, after short-term exposure of 1 h (corresponding to the lag phase of bacterial growth) and long-term exposure of 24 h (corresponding to the stationary phase of bacterial growth). The Gram staining followed by light microscopy (Olympus, CX21) was performed in order to estimate the range of high or low bacterial numbers in the bottles and the immobilization of bacteria onto the zeolites. For the determination of high bacterial numbers, a 1 mL of the suspension was serially diluted (10^{-1}–10^{-9}) in triplicate in sterile 0.05 M NaCl and volumes of 0.1 mL were aseptically inoculated onto the LB agar plates (spread plate method). For the determination of low bacterial numbers, a 10, 20 and 30 mL of the suspension was filtered through 0.20 μm Sartorius sterile nitrocellulose filters and the filters were aseptically placed onto the LB agar. The LB agar plates were incubated at 37.0 ± 0.1 °C for 24 h. After the incubation period, the bacterial colonies were counted and the number of viable cells was reported as CFU mL^{-1}. In the experiments with NiONZ, except planktonic the number of immobilized bacteria onto NiONZ were determined as described previously (Stojakovic et al., 2011a), to determine the number of total cells in the bottles. The number of native *E. coli* in the nonsterile effluent water was determined at the beginning of experiment and after 1, 2, 3, 4, 5 and 24 h of exposure. CFU of *E. coli* was determined on EC-X GLUC agar (Bilolife, Italy) plates. After the incubation at 37.0 ± 0.1 °C for 24 h, a drop of Kovacs' reagent was putted onto blue colonies to confirm indole positive colonies of *E. coli*.

2.4. Antiprotozoan activity test

Ciliates were obtained from activated sludge. Following isolation of single ciliate with micropipette, specimens were repeatedly washed with sterile water. *P. caudatum* and *E. affinis* were cultured in boiled rice grain in filtered effluent water of the biological wastewater treatment plant in Zagreb, Croatia. Both species were maintained at room temperature.

For the experiments testing the sensitivity of protozoa to Cu_2ONZ, ZnONZ and NiONZ, 10 mL of ciliate cultures were suspended in 100 mL of autoclaved effluent water. Treatment bottles contained 1.0 g of the autoclaved Cu_2ONZ, ZnONZ or NiONZ, while control bottles were free of zeolite. Three replicates were run for each treatment. After sealing, the bottles were incubated in a water bath (Memmert, WNB22) at 25.0 ± 0.5 °C with shaking at 70 rpm to assure the complete mixing. The number of *P. caudatum* and *E. affinis* was determined under microscope (Jenaval) under magnification 50× at the beginning, after 1 and 24 h of contact. Only living ciliates were counted. Some dead ciliates did not lyse

immediately, however those were easily recognized by their immobility, lack of ciliary movement and changes in cell shape.

2.5. Analysis of MONP after contact with microorganisms

The leaching of Cu^{2+}, Zn^{2+} and Ni^{2+} from Cu_2ONZ, ZnONZ and NiONZ, respectively was determined after 24 h of contact of the pure cultures of bacteria with the effluent water. The suspension was filtered through 0.20 μm Sartorius syringe filters and the effluent water was analyzed by atomic absorption spectrophotometer (AAS Varian, Spectra AA 55B). TEM analysis of Cu_2ONZ, ZnONZ and NiONZ were performed using a 200-kV TEM (JEM-2100 UHR, Jeol, Japan) equipped with an ultra-high-resolution, objective-lens pole-piece having a point-to-point resolution of 0.19 nm, which is sufficient to resolve the lattice images of the MONP. The selected-area electron diffraction was performed over multiple nanocrystals to check the crystallinity of MONP.

2.6. Data analysis

The comparisons between the numbers of microorganisms were done using the ANOVA and subsequently the post-hoc Duncan test was performed for the calculations concerning pair-wise comparisons. The significantly different values were expressed as: ^{A} – indicates different numbers of microorganisms in the sample with respect to corresponding control, ^{B} – indicates different numbers of microorganisms in the sample with respect to Cu_2ONZ, ^{C} – indicates different numbers of microorganisms in the sample with respect to ZnONZ. The correlation between the leaching of metal ions from Cu_2ONZ, ZnONZ and NiONZ and percentage of bacterial reduction was estimated by Spearman correlation analysis. Statistical decisions were made at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Experiments with pure cultures of bacteria

Antibacterial activity of Cu_2ONZ, ZnONZ or NiONZ against pure culture of *E. coli* is shown in Fig. 1a. All materials resulted in low reduction (<7%) of the bacterial numbers as compared to the control after 1 h of contact. The reduction in the bacterial numbers increased significantly after 24 h of contact in bottles containing the Cu_2ONZ and ZnONZ (87% and 54%, respectively) while stayed low (2%) in bottles containing the NiONZ. Antibacterial activity of Cu_2ONZ, ZnONZ or NiONZ against pure culture of *S. aureus* is shown in Fig. 1b. The Cu_2ONZ displayed significant reduction in the bacterial numbers (41%) after 1 h of contact, while low reduction of ZnONZ or NiONZ was comparable to those observed for *E. coli*. The reduction in the bacterial numbers increased after 24 h of contact in bottles containing the Cu_2ONZ and ZnONZ (94% and 68%, respectively) while stayed low (2%) in bottles containing the NiONZ. In effluent water the *E. coli* was more resistant to antibacterial activity of Cu_2ONZ and ZnONZ than *S. aureus* (Fig. 1). Except that NiONZ showed a negligible antibacterial activity against pure cultures of *E. coli* and *S. aureus*, the immobilized bacteria were found on the surface of this zeolite after 24 h of contact. There was $1.5 \pm 0.4 \times 10^7$ CFU per gram of dry NiONZ of immobilized *E. coli* and $2.0 \pm 0.1 \times 10^7$ CFU per gram of dry NiONZ of immobilized *S. aureus*. These numbers are similar to the numbers of *E. coli* and *S. aureus* immobilized onto Na form of natural zeolite from Serbia (Hrenovic et al., 2012), which confirms no antibacterial activity of zeolite used as support material or NiONZ.

The differences in final pH values among control and experimental bottles were not higher than 0.2 and 0.8 pH units in experiments with *E. coli* and *S. aureus*, respectively. Therefore, the changes in pH values were not the reason for the reduction in the bacterial numbers. The antibacterial activity of ZnONZ and NiONZ against *E. coli* and *S. aureus* was lower than those of Zn(II) and Ni(II)-loaded zeolites, but the antibacterial activity of Cu_2ONZ was comparable to those of Cu(II)-loaded zeolites (Hrenovic et al., 2012).

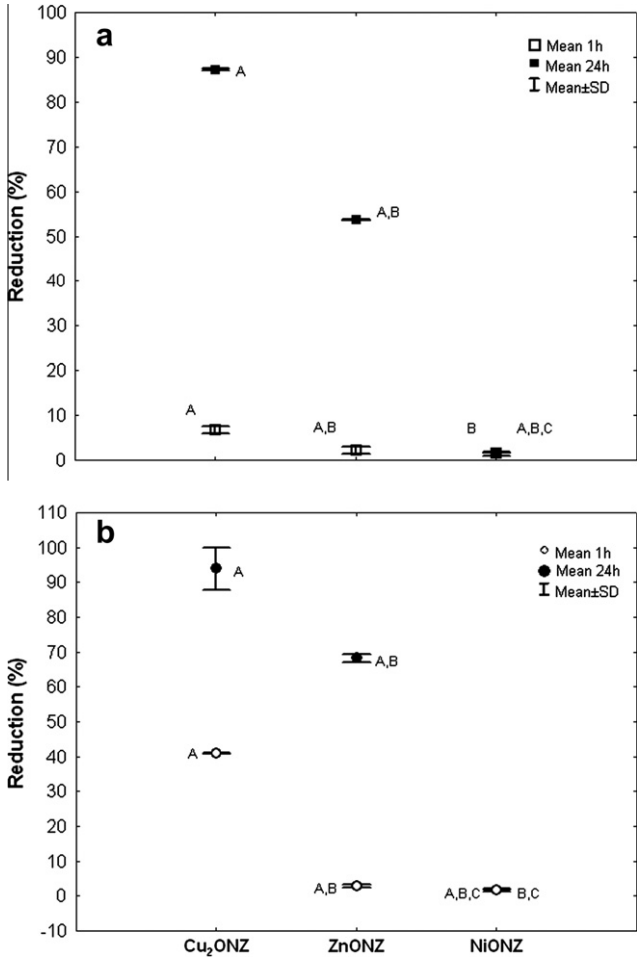

Fig. 1. Antibacterial activity of MONP supported onto clinoptilolite against pure culture of *E. coli* (a) and *S. aureus* (b) after 1 h and 24 h of contact in effluent water as compared to control. t_0 *E. coli* (10^6 CFU mL^{-1}) = 3.49 ± 1.02; t_0 *S. aureus* (10^7 CFU mL^{-1}) = 1.51 ± 0.26; significantly different as compared to: ^{A} – control, ^{B} – Cu_2ONZ, ^{C} – ZnONZ (left letters for 1 h of contact, right letters for 24 h of contact).

3.2. Experiments with native E. coli

Antibacterial activity of Cu_2ONZ, ZnONZ or NiONZ at mass concentration of 1.0 and 5.0 g 100 mL^{-1} during 24 h of contact against native population of *E. coli* in nonsterile effluent water is shown in Table 1. The Cu_2ONZ and ZnONZ reached 100% of reduction of *E. coli* within 1 h of contact without any variation of results. The NiONZ at dosage of 1.0 g 100 mL^{-1} showed significantly lower reduction of *E. coli*. At NiONZ dosage of 5.0 g 100 mL^{-1} high reduction decreased after 4 h of contact, which correspond to the beginning of the log phase of bacterial growth in the control bottles (data not shown). The final antibacterial activity of NiONZ after 24 h of contact at both dosages was not significantly different. The antibacterial activity of silver nanoparticles supported onto clinoptilolite was determined by the amount of silver and better reduction of *E. coli* and *Salmonella typhi* was observed by higher dosage of material (Guerra et al., 2012). It seems that the antibacterial activity of examined MONP supported onto clinoptilolite was depended on the type of metal and the increased antibacterial

Table 1
Antibacterial activity of MONP supported onto clinoptilolite at mass concentration of 1.0 and 5.0 g 100 mL^{-1} against native *E. coli* during 24 h of contact in effluent water as compared to control. t_0 *E. coli* (10^2 CFU mL^{-1}) = 1.55 ± 0.22.

	Reduction (%) at 1.0 g 100 mL^{-1}			Reduction (%) at 5.0 g 100 mL^{-1}		
Time (h)	Cu_2ONZ	ZnONZ	NiONZ	Cu_2ONZ	ZnONZ	NiONZ
1	100 ± 0[a]	100 ± 0[a]	16 ± 0[a,b,c]	100 ± 0[a]	100 ± 0[a]	95 ± 5[a,b,c]
2	100 ± 0[a]	100 ± 0[a]	37 ± 4[a,b,c]	100 ± 0[a]	100 ± 0[a]	92 ± 4[a,b,c]
3	100 ± 0[a]	100 ± 0[a]	31 ± 2[a,b,c]	100 ± 0[a]	100 ± 0[a]	88 ± 5[a,b,c]
4	100 ± 0[a]	100 ± 0[a]	26 ± 0[a,b,c]	100 ± 0[a]	100 ± 0[a]	32 ± 1[a,b,c]
5	100 ± 0[a]	100 ± 0[a]	4 ± 1[a,b,c]	100 ± 0[a]	100 ± 0[a]	46 ± 1[a,b,c]
24	100 ± 0[a]	100 ± 0[a]	54 ± 7[a,b,c]	100 ± 0[a]	100 ± 0[a]	57 ± 1[a,b,c]

[a] Significantly different as compared to control.
[b] Significantly different as compared to Cu_2ONZ.
[c] Significantly different as compared to ZnONZ.

Table 2
Antiprotozoan activity of MONP supported onto clinoptilolite against *P. caudatum* and *E. affinis* after 1 h and 24 h of contact in effluent water as compared to control. t_0 *P. caudatum* (mL^{-1}) = 485 ± 23; t_0 *E. affinis* (mL^{-1}) = 26 ± 11.

Time (h)	Reduction (%)					
	Paramecium caudatum			*Euplotes affinis*		
	Cu_2ONZ	ZnONZ	NiONZ	Cu_2ONZ	ZnONZ	NiONZ
1	100 ± 0[a,c]	8 ± 3[a,b]	100 ± 0[a,c]	100 ± 0[a,c]	40 ± 5[a,b]	100 ± 0[a,c]
24	100 ± 0[a,c]	19 ± 5[a,b]	100 ± 0[a,c]	100 ± 0[a,c]	21 ± 4[a,b]	100 ± 0[a,c]

[a] Significantly different as compared to control.
[b] Significantly different as compared to Cu_2ONZ.
[c] Significantly different as compared to ZnONZ.

activity of NiONZ could not be obtained by the use of higher mass concentration of material.

The differences in final pH values among control and experimental bottles were not higher than 0.3 and 0.6 pH units at material dosage of 1.0 and 5.0 g 100 mL^{-1}, respectively. The higher antibacterial activity of Cu_2ONZ, ZnONZ or NiONZ against native population of *E. coli* as compared to results with pure culture of *E. coli* (Fig. 1a) is explained by the lower bacterial numbers subjected in experiment. The other bacteria present in nonsterile raw effluent water may increase the solubilization and bioavailability of metal cations from metal oxides (Ivask et al., 2002).

3.3. Experiments with protozoa

Effects of Cu_2ONZ, ZnONZ and NiONZ against *P. caudatum* and *E. affinis* are shown in Table 2. After 1 h of contact the Cu_2ONZ and NiONZ resulted in 100% reduction of both ciliate species, while ZnONZ resulted in reduction between 8% and 40%. The ZnONZ showed stronger effect against *E. affinis* than against *P. caudatum*. Decrease in reduction of *E. affinis* after 24 h of contact could indicate certain level of adaptation to ZnONZ. The adaptation of the protozoan cells to ZnO nanoparticles was observed by Mortimer et al. (2010) suggesting that certain cell mechanisms could lead to metal sequestration. Opposite to *E. affinis*, the reduction of *P. caudatum* by ZnONZ significantly increased after 24 h of contact. Stronger toxic effect of Cu^{2+} compared to Zn^{2+} was observed toward *Euplotes* sp. (Martin-Gonzalez et al., 2006). However, Mortimer et al. (2010) reported ZnO nanoparticles to be more toxic than CuO nanoparticles for ciliate species *Tetrahymena thermophila*. Interspecific variations in metal toxicity (Martin-Gonzalez et al., 2006) could account for this reverse response to metals between studies. Our results are in accordance with the order of heavy metal toxicity for ciliates: Cu > Hg > Cd > Ni > Pb > Cr > Zn (Madoni, 2000).

3.4. Stability of MONP after contact with microorganisms

After 24 h of contact of pure bacterial cultures with Cu_2ONZ, ZnONZ or NiONZ in the effluent water, there was no detectable

Table 3
Leaching of metal ions (wt% of the metal loaded onto clinoptilolite) from Cu_2ONZ, ZnONZ and NiONZ after 24 h of contact in effluent water in experiments with pure cultures of *E. coli* and *S. aureus*.

	E. coli	*S. aureus*
Cu_2ONZ	0.00	0.00
ZnONZ	0.25	0.95
NiONZ	0.51	0.65

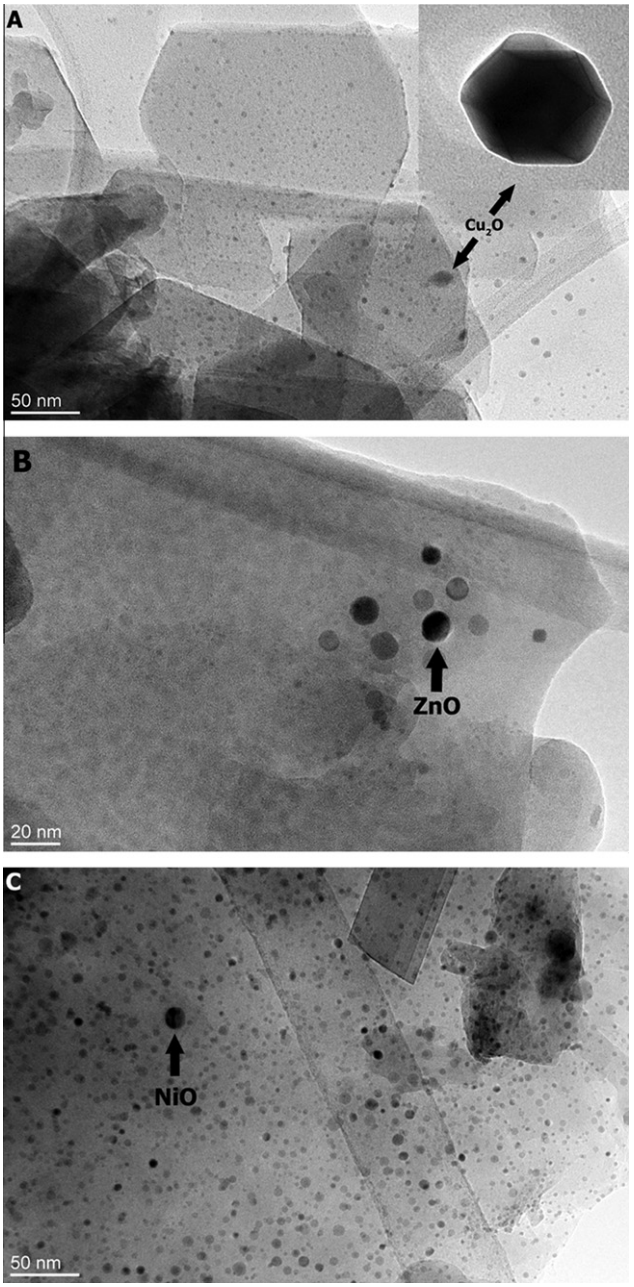

Fig. 2. TEM images of Cu_2ONZ (A) and single Cu_2O nanoparticle (right corner), ZnONZ (B) and NiONZ (C) after 24 h of contact with pure culture of *E. coli* in effluent water.

Cu^{2+} leached from Cu_2ONZ while less than 1 wt% of Zn^{2+} and Ni^{2+} was leached from ZnONZ and NiONZ, respectively (Table 3). The leaching of metal cations from ZnONZ and NiONZ was much lower than reported leaching of Zn^{2+} (1.07–1.61 wt%) and Ni^{2+} (3.44–9.13 wt%) from Zn(II) and Ni(II)-containing clinoptilolite, respectively (Hrenovic et al., 2012). This suggests higher stability of MONP supported onto clinoptilolite as compared to the metal ions sorbed onto natural zeolite. The leaching of metal ions from Cu_2ONZ, ZnONZ and NiONZ did not show the significant correlation with the percent of bacterial reduction ($R = -0.446$). The best antibacterial activity was observed with Cu_2ONZ for which no leaching of Cu^{2+} was detected, which suggests that the leaching of metal cations was not responsible for the antibacterial activity. Since the antibacterial activity of Cu_2ONZ and ZnONZ was observed in the dark, the role of reactive oxygen species produced form MONP (Adams et al., 2006) in the mechanism of toxicity can be excluded. The entry of MONP which are supported onto clinoptilolite into bacterial cells is far to occur, because bacteria do not have the transport mechanism for colloidal particles. It can be presumed that the MONP act bactericidal itself, where bacteria come into contact with MONP and active take up metal ions (while not the whole MONP), which consequently damage the cells.

TEM analyses of Cu_2ONZ, ZnONZ and NiONZ after 24 h of contact with the microorganisms in the effluent water showed nanoparticles of Cu_2O (cuprite), ZnO of wurtzite structure and NiO of cubic structure, respectively which are randomly dispersed on the surface of clinoptilolite crystals (Fig. 2). The contact with the microorganisms did not affect the crystallinity nor morphology of the nanoparticles. The low leaching and stability of MONP after 24 h of contact with microorganisms suggest the possibility of reuse of the MONP in disinfection of secondary effluent. No changes of the antibacterial activity of Cu_2ONZ and ZnONZ against native *E. coli* in the effluent water was observed during 48 exposures of 1 h (data not shown). The loss of antibacterial activity was evident trough the increase of the contact time up to 3 h to obtain the 100% of bacterial reduction when used for 49–96 exposures of 1 h. The Cu_2ONZ retained the 100% of antibacterial activity after 3 h of contact up to 100 exposures, while 24 h of contact was needed to obtain the 100% of antibacterial activity of ZnONZ during 97th exposure.

4. Conclusions

The antimicrobial activity of MONP supported onto clinoptilolite was dependent on the type of MONP and on the species of microorganism. Excellent antibacterial activity was obtained for Cu_2ONZ and ZnONZ, while antiprotozoan activity for Cu_2ONZ and NiONZ after 1 h of contact in the secondary effluent water. The MONP were stabile during the contact with microorganisms. The natural zeolite after being used for the sorption of Cu(II), Zn(II) and Ni(II) from wastewater could find the subsequent application in the disinfection of secondary effluent water and removal of pathogenic microorganisms in the tertiary stage of wastewater treatment.

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