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RESEARCH ARTICLE

Bactericidal activity of Cu-, Zn-, and Ag-containing zeolites toward *Escherichia coli* isolates

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Abstract Two types of zeolites—natural clinoptilolite (NZ) and synthetic zeolite A (A)—were enriched with approx. 0.25 mmol of Cu(II), Zn(II), or Ag(I) ions, and the obtained materials (M-Z) were tested against three different isolates of *Escherichia coli*. Two isolates were environmental isolates from waters in Serbia whereas the third one was DSM 498. Antibacterial activity was studied in different water media—nutrient-rich media (peptone water), water from Sava Lake, and commercially available spring water. The Ag-containing zeolites showed bactericidal activity in the nutrient-rich peptone water after 1 h of contact. Cu- and Zn-containing zeolites showed bactericidal activity in real water samples. Antibacterial activity of the M-Z decreases in all three examined water media in the following order: Ag-NZ ≈ Ag-A > Cu-NZ ≈ Cu-A > Zn-NZ >>> Zn-A, suggesting that mainly the metal type and not the zeolite type have a role in the antibacterial activity. Leaching experiments showed small amounts of the leached Cu(II) and Zn(II) ions, indicating that the antibacterial activity is not due to the metal ions but should be attributed to the M-Z itself. However, leached amounts of Ag(I) from Ag-NZ and Ag-A in peptone water indicate that the released Ag(I) could be mainly responsible for the bactericidal effect of the Ag(I)-containing zeolites. Since no loss of cellular material was found, the antibacterial activity is not attributed to cytoplasmic membrane damage.

Keywords Clinoptilolite · Zeolite A · Copper · Zinc · Silver · *Escherichia coli* · Alternative disinfectants

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Introduction

Escherichia coli is a pathogen causing some infections in humans and animals. Disinfection based on chlorination and/or ozonation is frequently the applied method to control microbial pathogens and waterborne epidemics. However, the use of these strong oxidants has been under consideration, since they react with natural organic matter present in water producing carcinogenic by-products.

Development of safe, environment-friendly antimicrobial materials has been of a great interest. Recent studies have shown that natural inorganic materials such as zeolites or clay minerals could be good candidates for the design of antimicrobial agents because of their thermal stability, long-lasting action, and chemical resistance.

Metals and metal ions such as silver, zinc, copper, mercury, cadmium, chromium, and lead have been used in various forms as antimicrobials agents (Feng et al. 2000; Jung et al. 2008; Raghupathi et al. 2011; Hong et al. 2012; Pathak and Gopal 2012; Le Ouay and Stellacci 2015).

Several studies showed that zeolites exchanged by some of these metal cations (M-Z) exhibit also antimicrobial activity toward a broad range of microorganisms (Kawahara et al. 2000; Rivera-Garza et al. 2000; Top and Ulku 2004; De la Rosa Gomez et al. 2008a, b; Ferreira et al. 2012; Hrenovic et al. 2012; Guerra et al. 2012; Hrenovic et al. 2013; Akhigbe

et al. 2014; Demirci et al. 2014; Rossainz-Castro et al. 2016) although the non-modified zeolites (Z) are not active toward microorganisms (Hrenovic et al. 2012; Jiraroj et al. 2014). In comparison to other transition metal-containing zeolites (such as Cu- or Zn-Z), Ag-Z exhibits the most powerful antibacterial activity (Top and Ulku 2004; Hrenovic et al. 2013; Demirci et al. 2014). The porous zeolite lattice enables metal cations to move freely, and this seems to be responsible for their activity toward microorganisms (Kwakye-Awuah et al. 2008).

The antibacterial activity mechanism of heavy metal ions toward bacteria has been still long studied. Lemire et al. (2013) reported on mechanisms of metal toxicity highlighting the complexity of this issue. Growth inhibition and cell death seems to be the result of a combination of different mechanisms and also to be influenced by the metal chemistry. Thus, it has been proposed that Cr(VI), As(III), Fe(II), and Cu(II) increase amounts of short-lived reactive oxygen species (ROS) which can lead to both DNA damage and the inhibition of enzyme activities in *E. coli*. At least three mechanisms have been proposed for the increased ROS production: (1) catalysis by the transition metal cations of the Fenton reaction in vivo, (2) breaking of coordination bonds between Fe and the cellular donor atoms by transition metal cations, and (3) generation of ROS via thiol-mediated reduction. Moreover, protein dysfunction and loss of enzyme activity in *E. coli* has also been ascribed to an oxidation of the amino acid side chains caused by several transition metals (Ag, Zn, Co, Ni). For both Ag and Al, it has been found to impair the membrane function in *E. coli* by coordination to the membrane sites. Finally, lethal DNA damage in *E. coli* has been proposed to Fe-mediated Fenton chemistry (Lemire et al. 2013). The correlation between antimicrobial activity and DNA damage has not been evident for other metals. Thus, although copper produces hydroxyl radicals in vivo, copper was reported to protect *E. coli* from H_2O_2-mediated DNA damage (Macomber et al. 2007).

In our previous works, we have found good antibacterial activity for Cu- and Zn-exchanged natural clinoptilolite toward *E. coli* and *Staphylococcus aureus* (Hrenovic et al. 2012). Good antibacterial activity was also found for the Ag-, Cu-, and benzalkonium-containing clinoptilolite toward *Acinetobacter baumannii* (Hrenovic et al. 2013). In this paper, we report on the bactericidal activity of the Cu-, Ag-, and Zn-exchanged clinoptilolite and zeolite A toward three different isolates of *E. coli.*

Material and methods

Preparation of zeolite samples

Zeolitic tuff (NZ) provided by Iran (Semnan deposit) with the grain size 63–125 μm and powder of synthetic zeolite A (A) purchased from Ventron (Lynde, 4A, 600 mesh) were used in this work. The zeolites were enriched with ions of Cu(II), Zn(II), and Ag(I) by ion exchange procedure. The ion exchange was performed by mixing zeolite with the corresponding metal solution in a weight ratio of 1:100 and shaking the suspension at 25–45 °C in a thermostatic water bath (Memmert WNB22, Germany). After 24 h, the suspensions were separated by filtration and products were dried at the 60 °C overnight. The experimental conditions and the content of metal in the obtained products are given in Table 1.

Before testing the antibacterial activity, all dry products, except Ag-containing zeolites, were sterilized by autoclaving at 121 °C, 20 min. Ag-A and Ag-NZ were sterilized at 70 °C for 2 h in a dry sterilizer (Binder B28, Germany). No microbial contamination of the prepared samples was found.

Identification and antibiotic resistance profile of the *E. coli* isolates

Two isolates were environmental isolates from water in Serbia. *E. coli* DSM 498 was purchased from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH. Identification of the bacterial isolates was carried out using VITEK MS (BioMerieux, France) with a standard procedure given by manufacturer. The spectra obtained for the examined strains were then correlated with the database, and for both isolates, excellent matching for *E. coli* was found (99.9%).

The bacterial isolates were pre-grown on the MacConkey agar overnight, and the antibiotic profile was determined based on minimal inhibitory concentration (MIC) values by VITEK 2 Compact 15 automated system using AST-N204 cards in accordance with the manufacturer's instructions.

E. coli DSM 498 and environmental isolate 2 were sensitive to all 16 examined antibiotics and belong to the wild type of microorganism, while environmental isolate 1 showed resistance to four antibiotics (ampicillin, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole) (Table 2). MIC values were interpreted according to EUCAST criteria (EUCAST 2014).

Table 1 Experimental conditions and metal content after ion exchange

Zeolite type	Metal (M)	Concentration of metal solution (mg M dm^{-3})	Temperature (°C)	Products	M content (mmol M/1 g zeolite)
NZ	Cu	400	45	Cu-NZ	0.28
	Zn	600	45	Zn-NZ	0.24
	Ag	400	25	Ag-NZ	0.24
A	Cu	200	45	Cu-A	0.27
	Zn	200	45	Zn-A	0.28
	Ag	300	25	Ag-A	0.27

Table 2 MIC values (mg dm^{-3}) of tested antibiotics toward *E. coli* isolates

Antibiotic	DSM 498		Isolate 1		Isolate 2	
	MIC	Profile	MIC	Profile	MIC	Profile
Ampicillin	4	S	>16	R	≤ 2	S
Amoxicillin/clavulanic acid	4	S	8	S	≤2	S
Piperacillin/tazobactam	≤4	S	≤4	S	≤4	S
Cefotaxime	≤1	S	≤1	S	≤1	S
Ceftazidime	≤1	S	≤1	S	≤1	S
Cefepime	≤1	S	≤1	S	≤1	S
Ertapenem	≤0.5	S	≤0.5	S	≤0.5	S
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S
Meropenem	≤0.25	S	≤0.25	S	≤0.25	S
Amikacin	≤2	S	≤2	S	≤2	S
Gentamicin	≤1	S	≤1	S	≤1	S
Ciprofloxacin	≤0.25	S	>2	R	≤0.25	S
Norfloxacin	≤0.5	S	>8	R	≤0.5	S
Fosfomycin	≤16	S	≤16	S	≤16	S
Nitrofurantoin	≤16	S	≤16	S	≤16	S
Trimethoprim/sulfamethoxazole	≤20	S	≤160	R	≤20	S

S sensitive, *R* resistant

Antibacterial activity tests

Bacterial biomasses were pre-grown on a nutrient agar (Torlak, Serbia) for 16 h at 37 ± 0.1 °C to obtain cultures in a log phase of growth. Antibacterial activity was investigated in three different water media: peptone, real water (Sava Lake, Belgrade, Serbia), and real, commercially available spring water (Jana, Croatia). The peptone water (PW) was prepared by dissolving the 10.0 g of Pepton-4 (Torlak, Serbia), 5.0 g of NaCl (p.a., Lachner, Czech Republic) in 1 dm^3 of distillate water (pH = 7.0). The chemical oxygen demand (COD) was measured spectrophotometrically (Hach, DR 2500) using a reactor digestion method (Spectroquant CR420, Merck). COD of PW was 17,900 mg O_2 dm^{-3}. The sample of fresh, lake water from the Sava Lake (SW) was firstly filtrated through a Buchner funnel with filter paper (blue band) and then through a glass funnel B4 (COD 2.270 mg O_2 dm^{-3}). Jana water (JW) was used as received. All three water samples were autoclaved (121 °C, 20 min) before antibacterial tests.

The kinetics of bacterial growth in PW in the presence of metal-containing zeolites (M-Z) was examined in 96-well plates by measuring the optical density (OD) at 630 nm. Bacterial suspensions of approximately 10^5–10^6 CFU cm^{-3} were prepared using the overnight broth culture of tested strains. The wells of the microtiter plates, filled with 50 μL of M-Z in PW, were then inoculated with 50 μL of the bacterial suspension. A final concentration of M-Z was 0.1 g of M-Z/100 cm^3. Negative controls were wells with the growth medium, while PW with the tested bacteria served as positive controls. The microplate reader (ELx808, BioTek Instruments, Inc., USA) controlled by Gen5™ software was used to monitor the cell growth by measuring the turbidity OD_{630} at 1-h intervals for 24 h. Plate was shaken for 10 s before each reading. The results are expressed as the correlation between OD_{630} and time. The experiments were done in triplicate.

Antibacterial tests in the SW and JW samples were carried out as follows: 1 cm^3 of the prepared biomass suspension of approximately 10^7 CFU cm^{-3} was inoculated into the Schott's bottles with 100 cm^3 of autoclaved SW/JW samples, and M-Z in a concentration of 0.1 g/100 cm^3 were added. The bottles were incubated in a thermostatic water bath for 24 h at 37 ± 0.1 °C with shaking at 105 rpm. As a control system, the Schott's bottles with parent zeolites at a concentration of 0.1 g/100 cm^3 were set up. The number of viable cells was determined at the beginning of the experiment and after 1, 3, 6, and 24 h. Optical microscope (Olympus CX23) was used to define the dilution ratio. The sample (0.1 cm^3) was plated by a spread plate method directly on a nutrient agar, and then, another amount of the sample (1 cm^3) was serially diluted (10^{-1}–10^{-7}). Diluted samples have also been plated onto nutrient agar and incubated (Colo Lab Experts, IN1017, Slovenia) for 24 h at 37 ± 0.1 °C, and then, the bacterial colonies were counted. All experiments were done in triplicate. The number of bacteria was reported as CFU cm^{-3} which was logarithmically transformed, and the antibacterial activity was expressed as the percent of reduction of the log of CFU as compared to positive control. The results were statistically processed using Statistica Software 10.0 (StatSoft, Tulsa, USA). Numbers of CFU were firstly logarithmically

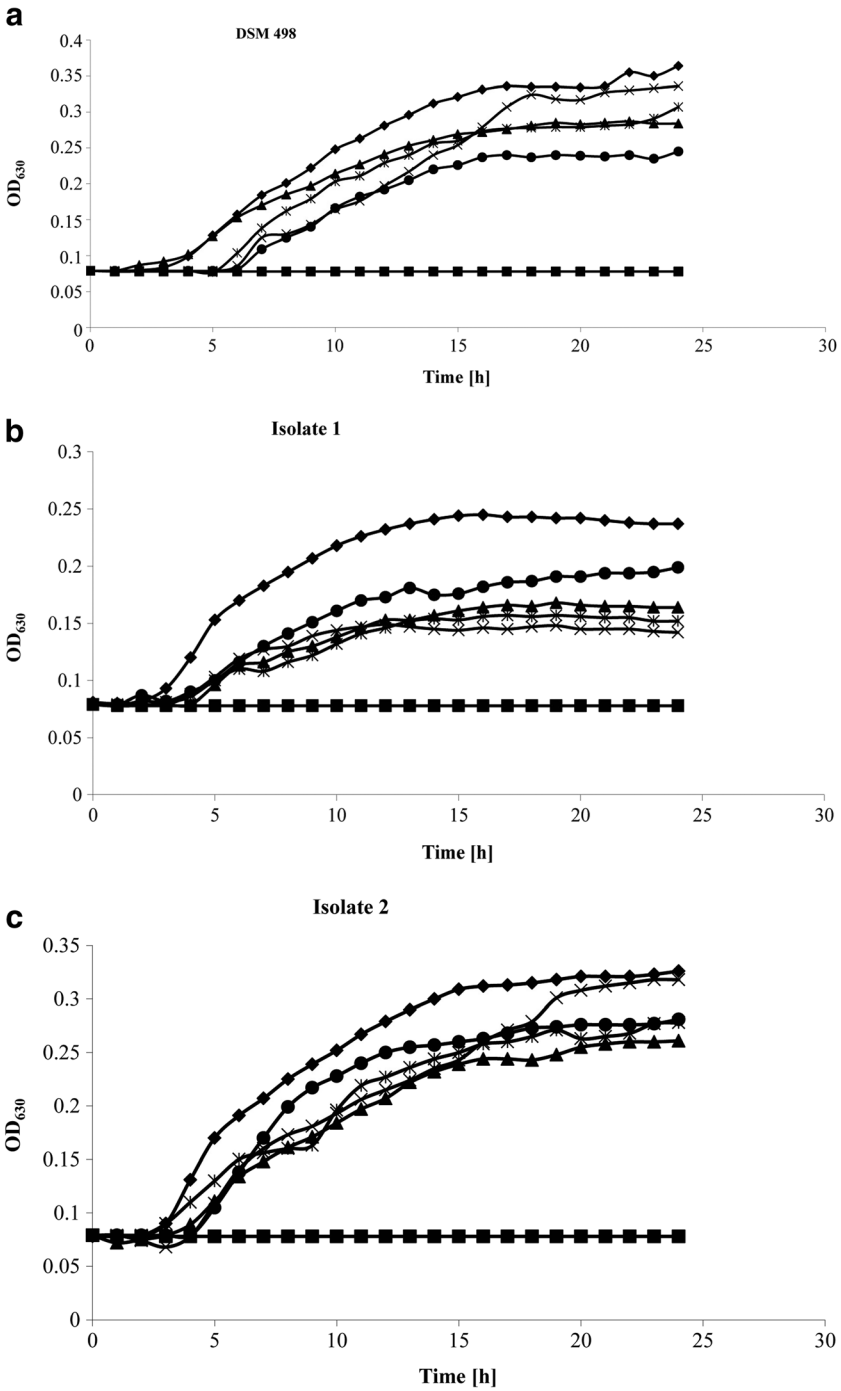

Fig. 1 Antibacterial activity of M- Z in PW toward *E. coli*. **a** DSM 498. **b** Isolate 1. **c** Isolate 2. Positive control; negative control; Cu-NZ; Cu-A; Zn-NZ; Zn-A; Ag-NZ; Ag-A (lines Ag-NZ and Ag-A are parallel with negative control)

transformed in order to normalize distribution and to equalize variances of the measured parameters. For the comparison, the one-way analysis of variance (ANOVA) was used, and at the end, the post hoc Duncan test was performed for the calculations concerning pairwise comparisons. Statistical decisions were made at a significance level of $p < 0.05$.

The leaching of Ag(I) ions from Ag-containing zeolites in PW after 1 h was measured by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 7500ce spectrometer. The concentration of Cu(II) and Zn(II) leached from Cu-Z and Zn-Z suspended in SW or JW was measured after 24 h by an atomic absorption spectrophotometer (Varian Spectra 55B, Australia).

Release of cellular material

The extracellular 260-nm-absorbing material released by the bacterial cells was determined using slightly modified method described by Carson et al. (2002). Bacterial suspensions of approximately 10^5–10^6 CFU cm^{-3} were taken, diluted in a

ratio 1:100, and filtered through a 0.22-μm pore size filter (Sartorius, Germany). M-Z was added into 1 cm^3 of bacterial suspensions to reach a final concentration of 0.1 g M-Z/100 cm^3. Bacterial suspensions without M-Z were used as a control. All the samples were incubated at 37 °C, and additional aliquots of the suspensions were taken after 1, 3, and 6 h for Cu- and Zn-Z, or after 10, 20, 30, and 60 min for Ag-Z, diluted and filtered as described above. The release of UV-absorbing material was measured using a Shimadzu UV-1800 UV-VIS Spectrophotometer.

The obtained results of the measurements of absorbance of the obtained supernatant at 260 nm at each time were expressed as a proportion of the initial OD_{260} value. The assay was carried out in triplicate.

Results and discussion

Antibacterial activity of metal-loaded zeolites

Growth curves with and without the M-Z in PW are shown in Fig. 1.

The COD of PW was extremely high showing high amount of organic compounds. Both Ag-NZ and Ag-A shows the bactericidal effect (100% reduction) in PW after 1 h of contact toward all the examined bacteria strains. The antibacterial activity of Ag-NZ and Ag-A in real water media was not examined because of low COD values.

Cu- and Zn-containing zeolites do not show bactericidal effect in PW after 24 h (Fig. 1). Cu-NZ after 24 h of contact exhibits antibacterial activity toward DSM 498 and isolate 2 around 27% and around 46% toward isolate 2. Antibacterial activity of Cu-A after 24 h is lower toward DSM 498 and isolate 2 (being around 10 and 3% toward DSM 498 and isolate 2, respectively) than toward isolate 1 (around 60%). After 24 h, the antibacterial activity of DSM 498 and isolate 2 by Zn-NZ is around 20%, and toward isolate 1 is 53%. Antibacterial activity of Zn-A after 24 h is evident toward all three isolates: 42, 24, and 18% for DSM 498, isolate 1 and isolate 2, respectively.

Antibacterial activity toward *E. coli* DSM 498 decreases in the order Zn-A > Cu-NZ > Zn-NZ > Cu-A, and an opposite trend appears toward isolate 1. Antibacterial activity toward isolate 2 increases as follows: Cu-NZ < Zn-NZ < Zn-A < Cu-A. It is evident that no clear trend of the antibacterial activity of Cu- and Zn-Z in PW can be seen. These results should be taken with a reserve because the main aim of our work has been to use the metal-containing zeolites as antimicrobial agents in real water, which are not the nutrient-rich media.

Results of the antibacterial activity of Cu-Z and Zn-Z toward all three bacteria strains in SW and JW are given in Tables 3, 4, and 5.

Cu-NZ exhibits bactericidal effect (100% reduction) toward examined bacteria after 6 h in SW (except for the isolate 1—bactericidal activity was achieved after 3 h toward this isolate), and after 3 h in JW. The strain DSM 498 is more resistant than both isolates, and the reduction of bacterial cells of DSM 498 in SW is 43% after 1 h and 82% after 3 h. The bactericidal effect was achieved after 6 h. For JW, the reduction of DSM 498 after 1 h was 67% and the bactericidal effect

Table 3 Antibacterial activity expressed as percent of reduction of DSM 498 in real water media by (a) NZ, Cu-NZ, and Zn-NZ and (b) zeolite A, Cu-A, and Zn-A

Zeolite	1 h	3 h	6 h	24 h
(a)				
Lake water (SW)				
NZ	0.3 ± 1.7	−1.2 ± 0.7	−0.3 ± 0.4	−1.1 ± 0.4
Cu-NZ	43.0 ± 1.4[a]	82.4 ± 2.4[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-NZ	13.5 ± 3.5[a]	69.5 ± 1.2[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Commercial spring water (JW)				
NZ	0.6 ± 0.7	0.2 ± 0.0	2.2 ± 1.3	−0.4 ± 1.6
Cu-NZ	67.5 ± 0.9[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-NZ	10.9 ± 0.0[a]	49.7 ± 0.2[a]	55.2 ± 0.6[a]	59.1 ± 0.4[a]
c_0 (log CFU cm^{-3}) = 7.1 ± 0.3				
(b)				
Lake water (SW)				
A	0.6 ± 1.5	0.9 ± 1.0	2.0 ± 1.7	1.8 ± 0.9
Cu-A	49.2 ± 4.7[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-A	2.5 ± 0.9	0.9 ± 1.0	0.7 ± 0.4	1.6 ± 0.5
Commercial spring water (JW)				
A	1.3 ± 0.0	−0.1 ± 0.1	1.7 ± 0.7	1.9 ± 1.0
Cu-A	62.7 ± 7.0[a]	72.3 ± 0.1[a]	86.1 ± 0.2[a]	100.0 ± 0.0[a]
Zn-A	−0.6 ± 0.7	0.1 ± 0.6	1.9 ± 0.8	−0.2 ± 0.1
c_0 (log CFU cm^{-3}) = 7.3 ± 0.2				

Mean values of triplicate measurements and standard deviations are presented. The lowest limit of detection was 10 CFU cm^{-3}

[a] Significantly lower than positive control without addition of zeolite

Table 4 Antibacterial activity expressed as percent of reduction of *E. coli* isolate 1 in real water media by (a) original and Cu- and Zn-containing natural zeolite; (b) original and Cu- and Zn-containing zeolite A

Zeolite	1 h	3 h	6 h	24 h
(a)				
Lake water (SW)				
NZ	−0.2 ± 0.4	−1.3 ± 2.1	0.8 ± 0.1	−1.4 ± 0.8
Cu-NZ	73.6 ± 0.3[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-NZ	40.5 ± 2.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Commercial spring water (JW)				
NZ	0.3 ± 0.3	2.1 ± 3.0	−2.3 ± 0.2	−1.9 ± 0.7
Cu-NZ	84.5 ± 0.2[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-NZ	82.1 ± 0.1[a]	95.3 ± 4.4[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
c_0 (log CFU cm^{-3}) = 7.2 ± 0.1				
(b)				
Lake water (SW)				
A	0.7 ± 1.1	−0.6 ± 0.9	0.8 ± 0.5	−1.4 ± 0.9
Cu-A	60.7 ± 0.1[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-A	1.4 ± 1.0	−1.4 ± 2.1	0.8 ± 0.5	1.8 ± 1.2
Commercial spring water (JW)				
A	0.5 ± 0.5	4.7 ± 0.6[a]	2.6 ± 0.0[a]	9.6 ± 0.2[a]
Cu-A	83.6 ± 0.2[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-A	−0.2 ± 1.1	4.2 ± 0.8[a]	3.1 ± 0.4[a]	26.5 ± 1.3[a]
c_0 (log CFU cm^{-3}) = 7.2 ± 0.1				

Mean values of triplicate measurements and standard deviations are presented. The lowest limit of detection was 10 CFU cm^{-3}

[a] Significantly lower than positive control without addition of zeolite

was achieved after 3 h (Table 3). Isolate 1 is the most sensitive on Cu-NZ showing after 1 h of contact the reduction rate of 74 and 84% for SW and JW, respectively (Table 4). For the isolate 2 (Table 5), the reduction was around 60% for 1 and 3 h in SW, and 65% for 1 h in JW, the bactericidal effect being achieved after 6 h (SW) and after 3 h (JW).

Cu-A exhibits bactericidal effect toward all examined strains. The strain DSM 498 is more sensitive in SW than in JW. The reduction rate after 1 h was around 50 and 63% for SW and JW, respectively. The bactericidal effect was achieved after 3 h in SW and after 24 h in JW (Table 3). Both examined isolates were more sensitive toward Cu-A than DSM 498

Table 5 Antibacterial activity expressed as percent of reduction of *E. coli* isolate 2 in real water media by (a) original and Cu- and Zn-containing natural zeolite and (b) original and Cu- and Zn-containing zeolite A

Zeolite	1 h	3 h	6 h	24 h
(a)				
Lake water (SW)				
NZ	−1.5 ± 1.5	1.4 ± 0.6	3.0 ± 0.1	1.8 ± 0.6
Cu-NZ	60.4 ± 1.6[a]	59.5 ± 0.2[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-NZ	10.2 ± 2.3[a]	49.8 ± 1.0[a]	77.8 ± 0.3[a]	100.0 ± 0.0[a]
Commercial spring water (JW)				
NZ	1.0 ± 0.5	0.5 ± 1.2	−2.8 ± 2.4	−0.2 ± 0.6
Cu-NZ	65.5 ± 5.5[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-NZ	26.9 ± 1.8[a]	52.3 ± 0.3[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
c_0 (log CFU cm^{-3}) = 7.2 ± 0.2				
(b)				
Lake water (SW)				
A	0.3 ± 0.5	0.7 ± 0.7	1.7 ± 1.5	4.1 ± 0.2[a]
Cu-A	52.6 ± 5.5[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-A	3.1 ± 0.6[a]	9.7 ± 2.1[a]	7.0 ± 0.4[a]	6.9 ± 0.5[a]
Commercial spring water (JW)				
A	1.9 ± 1.4	1.3 ± 0.4	0.1 ± 0.8	6.0 ± 1.0[a]
Cu-A	64.1 ± 6.7[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Zn-A	2.2 ± 1.8	2.7 ± 0.4[a]	5.3 ± 1.6[a]	14.1 ± 1.1[a]
c_0 (log CFU cm^{-3}) = 7.2 ± 0.2				

Mean values of triplicate measurements and standard deviations are presented. The lowest limit of detection was 10 CFU cm^{-3}

[a] Significantly lower than positive control without addition of zeolite

(Tables 4 and 5). The bactericidal activity was achieved in both water media after 3 h. Isolate 1 is more sensitive than isolate 2 toward Cu-A, and activity of Cu-A is more evident in the JW than in SW medium.

Antibacterial activity of Zn-NZ is slightly lower than that of Cu-NZ and Cu-A. DSM 498 is the most resistant strain toward Zn-NZ (Table 3). The reduction rate of DSM 498 is around 14 and 70% after 1 and 3 h in SW, respectively, and the bactericidal effect was achieved after 6 h. Zn-NZ does not exhibit bactericidal effect toward DSM 498 in JW after 24 h. Isolate 1 is more sensitive toward Zn-NZ than isolate 2 (Tables 4 and 5). Bactericidal effect was achieved for isolate 1 after 3 and 6 h for SW and JW, respectively. Antibacterial activity of Zn-NZ in SW toward isolate 2 increases with time, and after 24 h, a bactericidal effect was achieved. The isolate 2 is also more sensitive in JW, and antibacterial activity increases from 27 to 52% for 1 and 3 h, respectively, and after 6 h, Zn-NZ exhibits a bactericidal effect toward this isolate.

Zn-A does not exhibit antibacterial activity toward DSM 498 (Table 3). Low activity was detected after 24 h for isolate 1 (26%) and isolate 2 (14%) in JW.

Antibacterial activity of the M-Z decreases in all three water media in the following order: Ag-NZ ≈ Ag-A > Cu-NZ ≈ Cu-A >Zn-NZ >>> Zn-A. The fact that Ag-NZ shows similar activity as Ag-A, and Cu-NZ similar to Cu-A indicates that the antibacterial activity depends mainly on the metal but not on the zeolite type.

Leaching of the metal ions

In order to check if the antibacterial activity could be ascribed to metal ions leaching the amounts of Ag(I) in PW after 1 h, and Cu(II) and Zn(II) ions in SW and JW were measured after 24 h. It was found that 4.17 and 0.81 mg Ag dm^{-3} leached from Ag-A and Ag-NZ, respectively. The concentration of the leached Ag(I) from Ag-A is somewhat higher than MIC for silver ions toward *E. coli* (3.996 mg Ag dm^{-3}; Mulley et al. 2014) but from Ag-NZ is significant lower than the MIC. This indicates that bactericidal effect of these zeolites could be ascribed not only to released Ag(I) ions but also to Ag-Z itself .

The leached amounts of Cu(II) and Zn(II) from the zeolites are lower than 0.47 mg M dm^{-3} and negligible for Zn-A (Table 6). It is important to notice that the amount of the leached Cu(II) and Zn(II) ions is in the range of the maximum allowable concentrations in drinking water (WHO 2003a, b). According to the literature data, the MIC of Cu(II) and Zn(II) ions toward *E. coli* is 1 mM (Navarro et al. 2013). The MIC values are significantly higher than the concentrations of metal ions leached from M-Z. This indicates that the antibacterial activity of M-Z could be ascribed to the M-Z itself and not to the leached metal ions.

Table 6 Amounts of the leached metals from M-Z in the studied real water media

Sample	Amount of leached metal (mg M dm^{-3})		
	DSM 498	Isolate 1	Isolate 2
Lake water (SW)			
Cu-A	0.17	0.06	0.33
Cu-NZ	0.19	0.30	0.08
Zn-A	0.01	0.01	0.01
Zn-NZ	0.39	0.36	0.47
Commercial spring water (JW)			
Cu-A	0.22	0.24	0.17
Cu-NZ	0.30	0.25	0.33
Zn-A	0.01	0.01	0.07
Zn-NZ	0.28	0.26	0.17

Release of cellular material

The increase of absorbance at 260 nm (OD_{260}) has been taken as an indicator that the cytoplasmic membrane of bacteria is damaged due to leakage of genetic material (Cox et al. 2001; Carson et al. 2002; de Souza et al. 2010). In this work, the OD_{260} does not change (results not shown) indicating that the activity of M-Z does not correlate with a damage of the cytoplasmatic membrane of *E. coli* cells. This suggests that the primary mechanism of antibacterial activity of M-Z does not include the release of macromolecular cytosolic constituents. At this moment, it is not possible to offer a reasonable explanation for the bactericidal effect of the metal-containing zeolites without additional detailed analyses (such as EXAFS and XANES). Our preliminary results based on EXAFS/XANES measurements (research is in progress) indicate that the bactericidal effect of metal-containing zeolites could be ascribed to the ability of movable metal cations inside the zeolite lattice to make donor-acceptor bonds with enzymes, thus causing disruption of enzyme-catalyzed reactions and the electron transport chain within the cell. A similar observation has been reported for the interaction of silver ions with *S. aureus*, *Listeria monocytogenes*, and *E. coli* (Bovenkamp et al. 2013).

Conclusions

The antibacterial activity of Ag-, Cu-, and Zn-containing natural and synthetic zeolite A against *E. coli* DSM 498 and two isolates of *E. coli* in three different water media decreases in the following order: Ag-NZ ≈ Ag-A > Cu-NZ ≈ Cu-A > Zn-NZ. Zn-A is not significantly active in real water media against the studied isolates.

The concentrations of the leached Ag(I) from Ag-A and Ag-NZ indicates that bactericidal effect of these zeolites could be mainly ascribed to released Ag(I) ions. The leached amounts of Cu(II) and Zn(II) were significantly lower than those of MIC of these metal ions, suggesting that the antibacterial activity of Cu- and Zn-Z could be attributed to the (Cu,Zn)-Z itself.

Since no loss of cellular material was found, it is concluded that the antibacterial activity is not due cytoplasmic membrane damage.

Taking all into accounts, Cu-NZ, Cu-A, and Zn-NZ could be considered as promising materials against pathogenic bacteria present in water media.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Akhigbe L, Ouki S, Saroj D, Min Lim X (2014) Silver-modified clinoptilolite for the removal of *Escherichia coli* and heavy metals from aqueous solutions. Environ Sci Pollut R 21:10940–10948. doi:10.1007/s11356-014-2888-6

Bovenkamp GL, Zanzen U, Krishna KS, Hormes J, Prange A (2013) X-ray absorption near-edge structure (XANES) spectroscopy study of the interaction of silver ions with *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*. Appl Environ Microbiol 79: 6385–6390. doi:10.1128/AEM.01688-13

Carson C, Mee B, Riley T (2002) Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage, and salt tolerance assays and electron microscopy. Antimicrob Agents Ch 46:1914–1920. doi:10.1128/AAC.46.6.1914-1920.2002

Cox SD, Mann CM, Markham JL, Gustafson JE, Warmington JR, Wyllie SG (2001) Determining the antimicrobial actions of tea tree oil. Molecules 6:87–91. doi:10.3390/60100087

De la Rosa-Gomez I, Olguin MT, Alcantara D (2008a) Bactericides of coliform microorganisms from wastewater using silver-clinoptilolite rich tuffs. Appl Clay Sci 40:45–53. doi:10.1016/j.clay.2007.07.001

De la Rosa-Gomez I, Olguin MT, Garcia-Sosa I, Alcantara D, Rodriguez-Fuentes G (2008b) Silver supported on natural Mexican zeolite as an antibacterial material. Micropor Mesopor Mater 39:431–444. doi:10.1016/S1387-1811(00)00217-1

Demirci S, Ustaoğlu Z, Yılmazer GA, Sahin F, Baç N (2014) Antimicrobial properties of zeolite-X and zeolite-A ion-exchanged with silver, copper and zinc against a broad range of microorganisms. Appl Biochem Biotech 172:1652–1662. doi:10.1007/s12010-013-0647-7

de Souza EL, de Barros JC, Eduardo C, de Oliveira V, da Conceição ML (2010) Influence of *Origanum vulgare* L. essential oil on enterotoxin production, membrane permeability and surface characteristics of *Staphylococcus aureus*. Int J Food Microbiol 137:308–311. doi:10.1016/j.ijfoodmicro.2009.11.025

EUCAST (2014) European Committee on Antimicrobial Susceptibility Testing. Reading guide. Version 4.0 available at http://www.Eucast.Org/clinical_breakpoints/ (online December 2, 2016)

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO (2000) A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J Biomed Mater Res 52:662–668. doi:10.1002/1097-4636(20001215)52:4<662::AID-JBM10>3.0.CO;2-3

Ferreira L, Fonseca AM, Botelho G, Almeida-Aguiar C, Neves IC (2012) Antimicrobial activity of faujasite zeolites doped with silver. Micropor Mesopor Mater 160:126–132. doi:10.1016/j.micromeso.2012.05.006

Guerra R, Lima E, Viniegra M, Guzman A, Lara V (2012) Growth of *Escherichia coli* and S*almonella typhi* inhibited by fractal silver nanoparticles supported on zeolite. Micropor Mesopor Mater 147: 267–273. doi:10.1016/j.micromeso.2011.06.031

Hong R, Kang TY, Michels CA, Gadura N (2012) Membrane lipid peroxidation in copper alloy-mediated contact killing of *Escherichia coli*. Appl Environ Microbiol 78:1776–1784. doi:10.1128/AEM.07068-11

Hrenovic J, Milenkovic J, Ivankovic T, Rajic N (2012) Antibacterial activity of heavy metal-loaded natural zeolite. J Hazard Mater 201-202:260–264. doi:10.1016/j.jhazmat.2011.11.079

Hrenovic J, Milenkovic J, Goic-Barisic I, Rajic N (2013) Antibacterial activity of containing natural zeolite against clinical isolates of *Acinetobacter baumannii*. Micropor Mesopor Mater 169:148–152. doi:10.1016/j.micromeso.2012.10.026

Jiraroj D, Tungasmita S, Tungasmita DN (2014) Silver ions and silver nanoparticles in zeolite A composites for antibacterial activity. Powder Technol 264:418–422. doi:10.1016/j.powtec.2014.05.049

Jung WK, Koo HC, Kim KW, Shin S, Kim SH, Park YH (2008) Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*. Appl Environ Microbiol 74:2171–2178. doi:10.1128/AEM.02001-07

Kawahara K, Tsuruda K, Morishita M, Uchida M (2000) Antibacterial effect of silver-zeolite on oral bateria under anaerobic conditions. Dent Mater 16:452–455. doi:10.1016/S0109-5641(00)00050-6

Kwakye-Awuah B, Williams C, Kenward MA, Radecka I (2008) Antimicrobial action and efficiency of silver-loaded zeolite X. J Appl Microbiol 104:1516–1524. doi:10.1111/j.1365-2672.2007.03673.x

Lemire J, Harrison J, Turner R (2013) Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nat Rev Microbiol 11:371–384. doi:10.1038/nrmicro3028

Le Ouay B, Stellacci F (2015) Antibacterial activity of silver nanoparticles: a surface science insight. Nano Today 10:339–354. doi:10.1016/j.nantod.2015.04.002

Macomber L, Rensing C, Imlay JA (2007) Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. J Bacteriol 189:1616–1626. doi:10.1128/JB.01357-06

Mulley G, Jenkins AT, Waterfield N (2014) Inactivation of the antibacterial and cytotoxic properties of silver ions by biologically relevant compounds. PLoS One 9(4):e94409. doi:10.1371/journal.pone.0094409

Navarro CA, von Bernath D, Jerez CA (2013) Heavy metal resistance strategies of acidophilic bacteria and their acquisition: Importance for biomining and bioremediation. Biol Res 46:363–371. doi:10.4067/S0716-97602013000400008

Pathak SP, Gopal K (2012) Evaluation of bactericidal efficacy of silver ions on *Escherichia coli* for drinking water disinfection. Environ Sci Pollut R 19:2285–2290. doi:10.1007/s11356-011-0735-6

Raghupathi KR, Koodali RT, Manna AC (2011) Size-dependent bacterial growth inhibition and mechanism of antibacterial activity of zinc oxide nanoparticles. Langmuir 27:4020–4028. doi:10.1021/la104825u

Rivera-Garza M, Olguin MT, Garcia-Sosa I, Alcantara D, Rodriguez-Fuentes G (2000) Silver supported on natural Mexican zeolite as an antibacterial material. Micropor Mesopor Mater 39:431–444. doi:10.1016/S1387-1811(00)00217-1

Rossainz-Castro LG, De la Rosa-Gomez I, Olguín MT, Alcantara-Díaz D (2016) Comparison between silver- and copper-modified zeolite-rich tuffs as microbicidal agents for *Escherichia coli* and *Candida albicans*. J Environ Manag 183:763–770. doi:10.1016/j.jenvman.2016.09.034

Top A, Ulku S (2004) Silver, zinc, and copper exchange in Na-clinoptilolite and resulting effect on antibacterial activity. Appl Clay Sci 27:13–19. doi:10.1016/j.clay.2003.12.002

WHO (2003a) Zinc in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality. Geneva, World Health Organization (WHO/SDE/WSH/03.04/17) Available at http://www.who.int/water_sanitation_health/dwq/chemicals/zinc.pdf (Online December 2, 2016)

WHO (2003b) Copper in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality. Geneva, World Health Organization (WHO/SDE/WSH/03.04/88) Available at http://www.who.int/water_sanitation_health/dwq/chemicals/copper.pdf (Online December 2, 2016)