Removal of emerging pathogenic bacteria using metal-exchanged natural zeolite bead filter

Tomislav Ivankovic, Jelena Dikic, Sabine Rolland du Roscoat, Svjetlana Dekic, Jasna Hrenovic and Marin Ganjto

ABSTRACT

Hospital wastewaters can become a route for dissemination of antibiotic-resistant bacteria to the environment if not properly treated. Some of these bacteria are able to survive conventional disinfection treatments (e.g. chlorination, UV irradiation), which evokes the need for novel disinfection methods. The metal-exchanged zeolites were tested as novel antibacterial agents for wastewater treatment. The natural zeolite – clinoptilolite enriched with silver (AgNZ) showed far better antibacterial activity towards hospital pathogenic bacteria *Acinetobacter baumannii* when compared to copper-exchanged zeolite (CuNZ), with minimal bactericidal concentration of 0.25–2 (AgNZ) compared to 32–64 mg L^{-1} (CuNZ) in batch system and respective log 5.6 reduction compared to log 0.5 reduction in flow system with pure bacterial culture. In the flow system with real effluent wastewater from the treatment plant, the removal of carbapenem-resistant bacteria using AgNZ was 90–100% during 4 days of experimental run. These results indicate that the AgNZ efficiently removes pathogenic bacteria from the wastewater, including *A. baumannii*, and is promising as a disinfectant material in a bead filter system.

Key words | *Acinetobacter baumannii*, column, disinfection, porous media, silver, wastewater

Tomislav Ivankovic (corresponding author)
Svjetlana Dekic
Jasna Hrenovic
Faculty of Science,
University of Zagreb,
Rooseveltov trg 6, 10000 Zagreb,
Croatia
E-mail: *tomislav.ivankovic@biol.pmf.hr*

Jelena Dikic
Innovation Center of the Faculty of Technology and Metallurgy,
University of Belgrade,
Karnegijeva 4, Belgrade,
Serbia

Sabine Rolland du Roscoat
Laboratoire 3SR,
Université Grenoble-Alpes/CNRS/G-INP,
UMR 5521, Grenoble,
France

Marin Ganjto
Zagreb Wastewater – Management and Operation Ltd.,
Culinecka cesta 287, Zagreb,
Croatia

INTRODUCTION

The bacterium *Acinetobacter baumannii* has emerged as a leading and critical nosocomial pathogen over the last decade (WHO 2017). The main reason is its extraordinary resistance to many classes of antibiotics, especially to carbapenems that were for long time the 'last resort' drug. In Croatia the resistance to carbapenems among clinical isolates of *A. baumannii* has increased from 10% in 2009 to 87% in 2017 (CAMS 2018). Nowadays, it's not rare to encounter multiple -, extensive- and even pan-drug resistant isolates of *A. baumannii* worldwide, both in hospital settings (Poulikakos *et al.* 2014; Asif *et al.* 2018), and in natural environments (Falagas & Karveli 2007; Goic-Barisic *et al.* 2017; Seruga-Music *et al.* 2017; Higgins *et al.* 2018). The community acquired infections involving *A. baumannii* are also being reported worldwide (Falagas & Karveli 2007; Dexter *et al.* 2015). The question is whether community-acquired *A. baumannii* originate from hospitals, *vice-versa*, or is it a closed circle?

The hospital wastewaters are usually collected in urban sewage system and undergo treatment in conventional wastewater treatment plants with activated sludge. However, there are evidence that isolates of *A. baumannii* originating from hospital wastewaters survive passage through sewage system and wastewater treatment and are being released to natural recipients (Goic-Barisic *et al.* 2016; Seruga-Music *et al.* 2017; Higgins *et al.* 2018). Multiple-drug resistant *A. baumannii* were isolated from hospital wastewaters both prior and after conventional disinfection by chlorination (Zhang *et al.* 2013). Karumathil *et al.* (2014) demonstrated how chlorine was not effective in destroying multidrug resistant *A. baumannii* isolates and that chlorine exposure increased the expression of genes conferring resistance to antibiotics. More generally, Jäger *et al.* (2018) and McKinney *et al.* (2012) showed limited efficacy of UV treatment in reduction of antibiotic-resistant bacteria and resistance genes. The ozonation was shown

doi: 10.2166/wst.2019.348

to be more effective in eliminating resistant bacteria (Jäger *et al.* 2018; Iakovides *et al.* 2019) but several reports pointed out *A. baumannii* as unusually resistant to ozonation in water media (Chamul *et al.* 2002; Allison *et al.* 2009). These findings suggest that unconventional disinfection techniques of wastewater may be a better approach for reducing the propagation of *A. baumannii* in the environment.

Disinfection techniques that employ metal-exchanged natural zeolites may be one of the novel approaches for reducing the numbers of antibiotic resistant bacteria in the environment. Zeolites are naturally occurring aluminosilicate minerals that show a high cation-exchange affinity due to their unique structural properties. Their open-framework lattice contains movable Mg, Ca, K and Na cations which can be readily replaced with different cations present in the water medium, yielding materials with antibacterial properties (Hrenovic *et al.* 2012, 2013). Natural zeolite – clinoptilolite which contains Ag^{+}, Zn^{2+}, or Cu^{2+} ions has been found to be antibacterial towards *Escherichia coli* (Milenkovic *et al.* 2017), *Staphylococcus aureus* (Hrenovic *et al.* 2012), *Salmonella typhii* (Lima *et al.* 2012) *Pseudomonas aeruginosa* (Kwakye-Awuah *et al.* 2008) and even *A. baumannii* (Hrenovic *et al.* 2013). If used as biofilter, the metal-exchanged natural zeolites are relatively cost-effective if compared to ozonation and chlorination because a complex technology and high operational costs are not required. Additionally, the production of dangerous by-products, characteristic for chlorination and ozonation (Gomes *et al.* 2019) was not shown in experiments with exchanged natural zeolites so far.

In here presented 'proof of concept' study, the metal-exchanged natural zeolites were tested as disinfection agents aimed specifically to remove clinically relevant pathogens from wastewaters. Experiments were designed as standard batch tests, but also as flow column system, where zeolite particles were used as porous media fill in bead filter. The experiments were intended to significantly expand current literature reports on the topic (Mpenyana-Monyatsi *et al.* 2012; El-Aassar *et al.* 2013; Lima *et al.* 2013; Fewtrell 2014), thus conducted through prolonged periods of time in column flow system (up to 14 days), using antibiotic-resistant environmental isolates and real effluent wastewater obtained from a large WWTP of the city of Zagreb (1.2 million population equivalent).

As a model organism in experiments with pure bacterial culture, several environmental isolates of *A. baumannii* were used, including one isolate from effluent wastewater that has been classified as pan-drug resistant. In experiments with real effluent wastewater the accent was on carbapenem-resistant bacteria (CRB) that pose the highest risk for human health (WHO 2017; Willyard 2017). The detection of presumably pathogenic CRB in environmental wastewater samples represents a practical problem since incubation at standard 22 or 37 °C allows for growth of native, intrinsically resistant and ubiquitous *Stenotrophomonas* sp. which, when cultivated on agar plates, shade most of the other CRB present in the sample. Therefore, a recently developed method of parallel incubation of environmental samples at different temperatures (37 and 42 °C) was used (Hrenovic *et al.* 2017; Higgins *et al.* 2018; Hrenovic *et al.* 2019). At 42 °C the growth of *Stenotrophomonas* sp. is suppressed (Denton & Kerr 1998) so presumably clinically relevant CRB of anthropogenic origin such as *Acinetobacter* sp., or Enterobacteriales, which are usually present in significantly smaller numbers, can be detected (Hrenovic *et al.* 2019).

MATERIALS AND METHODS

Metal-exchanged natural zeolites

The zeolitic tuff from Vranjska Banja deposit (Serbia) containing about 70 wt. % of natural zeolite – clinoptilolite and quartz and feldspar as major impurities, with particle size of 63–125 μm, was used in this work. Firstly, zeolitic tuff was converted into the Na-enriched form (NZ) in order to improve the clinoptilolite cation exchange capacity. The samples of metal-exchanged zeolite (Ag, Cu or Zn) were prepared by an ion exchange reaction following procedure published by Milenkovic *et al.* (2017). The AgNZ, CuNZ, ZnNZ contained 35.5 mg Ag^{+}, 16.2 mg Cu^{2+} or 18.7 mg Zn^{2+} per gram of metal-exchanged zeolite, respectively. The leaching of Ag^{+}, Cu^{2+} and Zn^{2+} from AgNZ, CuNZ and ZnNZ, respectively, was determined by atomic absorption spectrophotometer (AAS Varian, Spectra AA 55b).

Isolates of *A. baumannii*

Environmental isolates of *A. baumannii* were used in the experiments. The isolates were recovered in 2015 from influent and effluent of WWTP of the city of Zagreb and are described in (Higgins *et al.* 2018). Isolates were divided into three groups according to their antibiotic susceptibility profile: isolate EF 11 – susceptible to all antibiotics tested; isolates EF8 and IN 39 – resistant to carbapenems and fluoroquinolones; EF7 – resistant to carbapenems, fluoroquinolones and colistin (Pan-drug resistant). The isolates were kept as pure cultures in Microbank storage system at

−80 °C. Prior to experiments the isolates were inoculated on Nutrient agar plates (Biolife, Italy) and incubated at 37 °C, overnight, to obtain fresh biomass.

Antibacterial activity of zeolites in batch system

To test antibacterial activity in batch (stationary) conditions, NZ or CuNZ, ZnNZ and AgNZ in concentrations ranging from 1,024 to 0.062 mg L^{-1} were added to bacterial suspensions and minimal bactericidal concentration (MBC; concentration that kills all the bacterial cells in the suspension) was determined by the following procedure; fresh biomass was resuspended in 100× diluted Nutrient broth (Biolife, Italy). The diluted nutrient broth was chosen as testing media because, by it's nutrient content, it best simulates effluent wastewater (Dekic *et al.* 2018). Final concentration of bacterial suspensions was ~10^5 Colony Forming Units per mL (CFU mL^{-1}). Next, a slightly modified dilution-neutralisation method was performed (Ivankovic *et al.* 2017); for each isolate, a 5 mL of bacterial suspension was distributed to a series of sterile plastic tubes (Falcon type, 15 mL). First tube in a series contained 10 mL of suspension. A 10.24 mg of NZ or metal-exchanged zeolite was added to the first vial and then the concentration was halved to each following tube until the final concentration of 0.0625 mg L^{-1} was reached. Tubes were then incubated at room temperature (23–25 °C) on rotatory shaker (3 rpm, Biosan) to ensure maximal contact of zeolites and bacterial cells. After 1, 5, 7 and 24 h of contact, 10 µL of sample was inoculated on Nutrient agar plates (Biolife, Italy) and incubated for 24 h at 37 °C. The plates were examined, and the lowest zeolite concentration without bacterial growth was marked as MBC. The procedure for MBC determination was done in technical duplicate.

The same was done to test antibacterial activity of silver ions except $AgNO_3$ matching corresponding concentration of Ag^+ was added to bacterial suspension. Tested concentrations ranged from 1,000 to 0.0038 mg L^{-1}.

Antibacterial activity of zeolites in bead filter system

To test the antibacterial activity in a flow system, bacterial suspension was pumped through glass column filled with NZ (Run 1), CuNZ (Run 2) or AgNZ (Run 3 and 4). Each day an aliquot was collected at the column outlet, bacterial count was determined, and reduction was calculated in relation to the bacterial count at the column inlet. The schematic of the experimental setup is given in Figure 1. In detail; an autoclaved 5 L laboratory bottle was filled with commercial natural spring water (Jana™, Croatia) and inoculated with EF7 strain of *A. baumannii* in concentration of either 10^3 CFU mL^{-1} (Run 1, 2 and 3) or 10^5 CFU mL^{-1} (Run 4). The bottle was connected to autoclaved GE healthcare glass column (10 mm inner diameter, 100 mm height) filled with NZ, CuNZ or AgNZ (5 g, reaching approx. 50 mm height in the column, Figure 1) and suspension was pumped through the column using Gilson minipuls peristaltic pump at 30 mL h^{-1} flow rate. The retention time in the

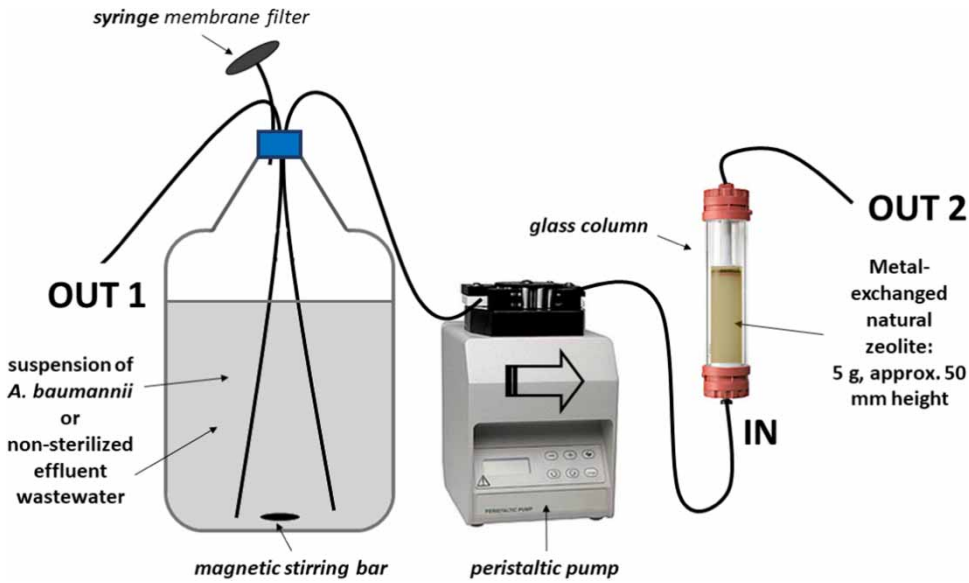

Figure 1 | Experimental setup for flow system experiments.

filled column was ~4 min. After each 24 h, a 50 mL aliquot was collected in sterile tube at the column outlet (OUT2, Figure 1) and number of bacteria was determined by inoculating TTC Tergitol agar plates (Biolife, Italy) and determining CFU after 24 h of incubation at 37 °C. An aliquot was also taken each day from Outlet 1 (Figure 1) to monitor the concentration of bacteria in the bottle, and this number was used as a starting number of bacteria for calculating reduction rate (Reduction = $\log CFU_{inlet} - \log CFU_{outlet}$). The EF7 isolate was chosen as the most resilient one from the experiments in batch system. The natural spring water was chosen media because it was shown that *A. baumannii* does not multiply nor die in such water, but cells remain in constant numbers and viable for prolonged period of time (Dekic *et al.* 2018). Regardless of the fact that spring water is nutrient-deprived (COD = 3 mg L^{-1}), such media was assumed to best mimic real WWTP effluent, where number of bacteria is constant through time (Hrenovic *et al.* 2017).

After the end of each experiment, the zeolites in the column were checked for immobilized bacteria by the following procedure: the whole content of the column was aseptically transferred to large Falcon type tube. Zeolite particles were gently washed three times with sterile saline solution to remove unattached cells. A 20 ml of sterile saline was then added to the tube and the whole content was vigorously shaken (Kartell mechanical shaker, 50 Hz/3 min) to detach cells immobilized on the zeolite particles which, after the shaking, remain as planktonic cells in the supernatant (Hrenovic *et al.* 2009). The supernatant was diluted and plated on TTC Tergitol agar plates, incubated for 24 h at 37 °C, and the colonies were counted. The remaining zeolite was dried (105 °C/6 h) and weighted. Number of immobilized cells was reported as CFU g^{-1} of zeolite.

Antibacterial activity of zeolites in bead filter system with real WWTP effluent

The final experiment was done in the same manner as described above, except real WWTP effluent was used instead of inoculated natural spring water. The NZ (Run 5) and AgNZ (Run 6 and 7) were used in these experiments. The effluent was collected from WWTP of the city of Zagreb on February 18th, March 4th and March 30th 2019 (Table 1), transported to the laboratory within 2 h and transferred to the autoclaved 5 L bottle. The wastewater was pumped through the column at the same conditions as described above, and analyzed every 24 h. The analysis included determination of three types of bacteria; total heterotrophs (He), carbapenem-resistant bacterial population incubated at 37 °C (CRB37) and carbapenem-resistant bacterial population incubated at 42 °C. The He were incubated (22 °C/72 h) and counted on Tryptic-glucose-yeast agar (Biolife, Italy) after serial dilution and plating. The CRB37 was incubated and counted on CHROMagar Acinetobacter™ plates with CR102 supplement (the supplement allows growth of carbapenem resistant bacteria) after incubation at 37 °C for 72 h. The CRB42 was incubated and counted on the same medium, but at 42 °C for 48 h. It was shown in earlier research (Hrenovic *et al.* 2017; Hrenovic *et al.* 2019) that *A. baumannii* carrying carbapenem-resistance is clearly distinguished from native bacterial population of wastewater when water samples are incubated at 42 °C on CHROMagar Acinetobacter™ plates with CR102 supplement. The assumption was that if no growth was observed at CR102 agar plates (42 °C), there is no carbapenem-resistant *A. baumannii* in the sample. Number of all three types of bacteria immobilized on the zeolite particles was determined as described in the previous section.

X-Ray Microtomography imaging

To visualize the distribution of zeolite particles in the column, laboratory X-ray Nanotomography was used. The columns were filled with the AgNZ and experiment was run under same conditions as described above but without the bacteria. When the preferential pathway was observed, the column was subjected to scanning on a laboratory nanotomograph manufactured by RX Solutions (Annecy, France) with a Hamamatsu X-Ray source (Hamamatsu City, Japan)

Table 1 | Parameters of effluent wastewater used in the experiments. The number of heterotrophic (He) and carbapenem-resistant bacteria cultivated at 37 °C (CRB37) and 42 °C (CRB42) are expressed as logCFU/100 mL

Date (dd/mm/yy)	COD (mg L^{-1})	Total P (mg L^{-1})	Total N (mg L^{-1})	O_2 (mg L^{-1})	T (°C)	He	CRB37	CRB42
18/02/19 (Run 6)	41	2.2	16.4	7.9	13.3	6.7 ± 0.2	3.7 ± 0.3	2.3 ± 0.2
04/03/19 (Run 7)	37	2.8	22.8	9.5	14.4	6.7 ± 0.2	4.0 ± 0.1	2.2 ± 0.1
29/04/19 (Run 5)	32	2.9	24.7	9.0	16.7	7.1 ± 0.1	4.7 ± 0.5	2.7 ± 0.1

and a Varian flat panel detector (Varian Medical Systems, Salt Lake City, UT, USA). The column was irradiated with an X-ray beam (generated with a 110 kV 90 μA electron beam on a tungsten target) for 1,200 angular projections equally spaced over 360°, and the chosen pixel size was set to 4 μm px^{-1}. The 2-D radiographs were converted into 3-D datasets using a filtered backprojection algorithm and analyzed using Fiji software.

RESULTS AND DISCUSSION

Antibacterial activity of metal-exchanged zeolites in batch system with *A. baumannii*

In the batch system ZnNZ did not show antibacterial activity, expressed as minimal bactericidal concentration (MBC), during 24 h of contact with *A. baumannii* isolates (Table 2). The CuNZ showed moderate antibacterial activity which was increasing with contact time (Table 2). After 24 h the MBC's were 32 and 64 mg L^{-1} for EF8, IN39 and EF7, EF11, respectively. The AgNZ exhibited superior antibacterial activity when compared to other materials with MBC's after 24 h of contact of 0.25 (IN39), 0.5 (EF8, EF11) and 2 mg L^{-1} for EF7, which seemed to be the most resistant isolate.

All the tested isolates were isolated from WWTP and are of clinical significance (Higgins *et al.* 2018). The EF7 is classified as pandrug-resistant (resistant to carbapenems, fluoroquinolones and colistin) and it can be discussed whether antibiotic resistance and highest resilience to AgNZ are linked. It is well-known that many metal resistance genes are often associated with antibiotic resistance gene cassettes on the same mobile genetic elements (Hobman & Crossman 2014). The problem of metal and antibiotic cross-resistance in WWTPs is also debated in detail (Davies & Davies 2010; Barancheshme & Munir 2018). However, one of our tested isolates, EF11, was classified as susceptible to commonly applied antibiotics and was same/more resilient to bactericidal action of AgNZ than multidrug-resistant isolates (EF8 and IN39), not indicating any relationship between antibiotic susceptibility profile and biocidal action of AgNZ. Both EF11 and EF7 were shown to endure extreme pH values and temperatures (Dekic *et al.* 2018) and together with EF8 were classified as strong biofilm formers (Dekic *et al.* 2017). Highest resistance of EF7 towards antibacterial efficacy of AgNZ can thus be linked to any or all of the above factors and no clear connection between antibiotic susceptibility profile or phenotypic characteristics of *A. baumannii* and biocidal action of AgNZ were established.

Susceptibility of EF7 to Ag^+ ion was also determined and MBC after 24 h of contact was 0.015 mg L^{-1} (Table 2). The leaching measurements (Table 3) showed higher leaching of Cu^{2+} ions from the CuNZ when compared to leaching of Ag^+ from AgNZ. The percentage of leached ions was around 1 wt.% of the metal cations loaded onto NZ for Ag^+ and in the range of 8–48 wt.% for Cu^{2+} (Table 3).

The leaching measurements confirm already reported statement (Hrenovic *et al.* 2013; Milenkovic *et al.* 2017) that bactericidal activity of AgNZ cannot be ascribed solely to the leaching of Ag^+ ions, but to cell/AgNZ contact as well. The MBC of Ag^+ ions towards EF7 was 0.015 mg L^{-1}, but the MBC of AgNZ was 2 mg L^{-1}, and at determined 1 wt.% rate of leaching (Table 3), the theoretical concentration of leached Ag^+ could have been ~0.0007 mg L^{-1}. Additionally, the concentrations of leached Ag^+ lower than the determined MBC of 0.015 mg Ag^+ L^{-1} were measured in vials containing ≤64 mg L^{-1} of AgNZ. The MBC's of AgNZ were much lower than 64 mg L^{-1} (Table 2) again confirming that cell/AgNZ contact was

Table 2 | MBC (mg L^{-1}) against tested isolates of *A. baumannii* after 1, 3, 5, 7 and 24 h of contact with metal-exchanged zeolites and Ag^+

	CuNZ					ZnNZ					AgNZ				
	1 h	3 h	5 h	7 h	24 h	1 h	3 h	5 h	7 h	24 h	1 h	3 h	5 h	7 h	24 h
EF7	/	512	256	256	64	/	/	/	/	/	256	32	16	4	2
EF8	/	256	128	64	32	/	/	/	/	/	256	16	4	2	0.5
EF11	/	1,024	512	256	64	/	/	/	/	/	128	8	8	4	0.5
IN39	/	512	256	64	32	/	/	/	/	/	64	16	8	2	0.25
											Ag^+				
EF7											/	0.06	0.06	0.03	0.015

The '/' marks concentration >1,024 mg L^{-1}.

Table 3 | Leaching of Cu and Ag determined after 24 h of contact with *A. baumannii* isolates in diluted nutrient media at 22 °C

c (mg L^{-1})		1	2	4	8	16	32	64	128	256	512	1,024
CuNZ	Mean	<0.02	<0.02	<0.02	0.041	0.119	0.24-	0.35-	0.456	0. – 01	1.090	1.291
	±SD	–	–	–	0.010	0.043	0.124	0.089	0.064	0.052	0.050	0.190
	%	–	–	–	32	46	48	34	22	1-	13	8
AgNZ	mean	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.024	0.040	0.0-6	0.145	0.223
	±SD	–	–	–	–	–	–	0.011	0.018	0.018	0.021	0.038
	%	–	–	–	–	–	–	1	0.9	0.8	0.8	0.6

The c (mg L^{-1}) is the concentration of metal-exchanged-zeolite in the experimental vial and (%) is the percentage of leached metal from the total metal load on the zeolite. c_0 (*A. baumannii*) = 10^5 CFU ml^{-1}. The value of <0.02 mg L^{-1} designates measurement below the detection limit.
SD, standard deviation.

responsible for bactericidal activity of AgNZ. The cell/AgNZ particle contact also explains increasing of antibacterial activity with increasing time of contact (1–24 h, Table 2).

Antibacterial activity of metal-exchanged zeolites in bead filter system with *A. baumannii*

The focus of our research was on the bead filter system, with assumption that cell/zeolite contact is maximized in a porous media flow system. The ZnNZ was not used as it showed no antibacterial activity in batch experiments. In the first experiment (Run 1, pure culture of *A. baumannii*), the column was filled with non-modified NZ to test if bacterial immobilization on the zeolite particles causes cell count reduction while suspension passes the column. Indeed, during the first 8 hours of run, the cell counts on the column outlet were reduced for ~99.5% when compared to inlet (Table supplemental). However, after 24 h, there was no reduction, cell counts were even significantly higher on the outlet ($p < 0.05$, t-test) and remain constant during four days of run (Run 1, Figure 2). The explanation would be that bacteria were being adsorbed onto NZ particles during first hours of suspension passage. After 24 h, in system where bacteria do not multiply, the equilibrium was achieved and, in the bead filter, the bacteria were probably attaching and detaching to/from zeolite particles in equal amount, thus exiting the column. The number of

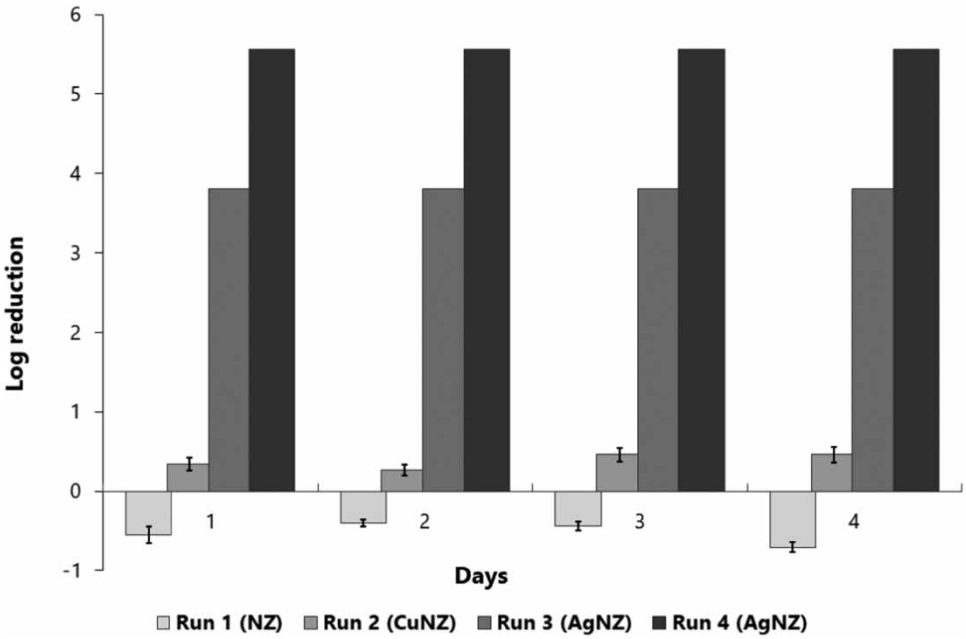

Figure 2 | Antibacterial activity of natural zeolite (NZ), copper (CuNZ), and silver (AgNZ) exchanged zeolite in flow column system with *A. baumannii*. The log reduction was calculated as $\log CFU_{inlet} - \log CFU_{outlet}$. The $\log CFU_{inlet}$ were 3.8 ± 0.3 (Run 1, 2 and 3) and 5.6 ± 0.1 for Run 4.

viable immobilized bacteria on the zeolite particles after 4-day run was measured and amounted 9.19 ± 1.75 log CFU g^{-1}, confirming that non-modified zeolite did adsorb bacterial cells.

The CuNZ showed slight antibacterial activity: complete log reduction at 3 and 5 h time points (Table supplemental) and 0.38 ± 0.09 log reduction during the 4-day run (Run 2, Figure 2). Initial reduction (up to 24 h) was most probably due to adsorption and latter due to antibacterial activity of CuNZ demonstrated in batch experiments or perhaps due to leaching of Cu ions. However, since bacterial reduction was minimal during the entire run, the activity of CuNZ was not investigated further.

The AgNZ showed excellent antibacterial activity with 100% reduction of bacterial counts during the 4-day run, in experiments with starting log CFU/mL of 3.8 (Run 3, Figure 2) and 5.6 (Run 4, Figure 2), the same. The experiments with AgNZ were continued for additional 10 days (Figure 3) and significant antibacterial activity was maintained through the entire 14 days run. This means that 5 g of AgNZ effectively reduced bacterial counts in 10.1 L of bacterial suspension.

After 14 days there were no viable bacterial cells adsorbed on the AgNZ (detection limit <10 CFU g^{-1}) so the observed reduction was not caused by adsorption to zeolite particles. However, dead (non-viable) cells could have adsorb onto AgNZ particles which probably explains the drop in antibacterial activity in all subsequent days after the 4th day (Figure 3); the dead biomass was covering the surface of AgNZ, thus limiting the cell/AgNZ contact. It is safe to presume that eventually all the active sites on AgNZ particles would become covered with dead biomass, completely reducing the antibacterial activity in flow system.

There were two unexpected events in which the log reduction was measured as none (6th day, Run 3, Figure 3) and 0.57 (8th day, Run 4, Figure 3), which was substantially lower when compared to other measured time points. The reason for this failure of antibacterial effectiveness was

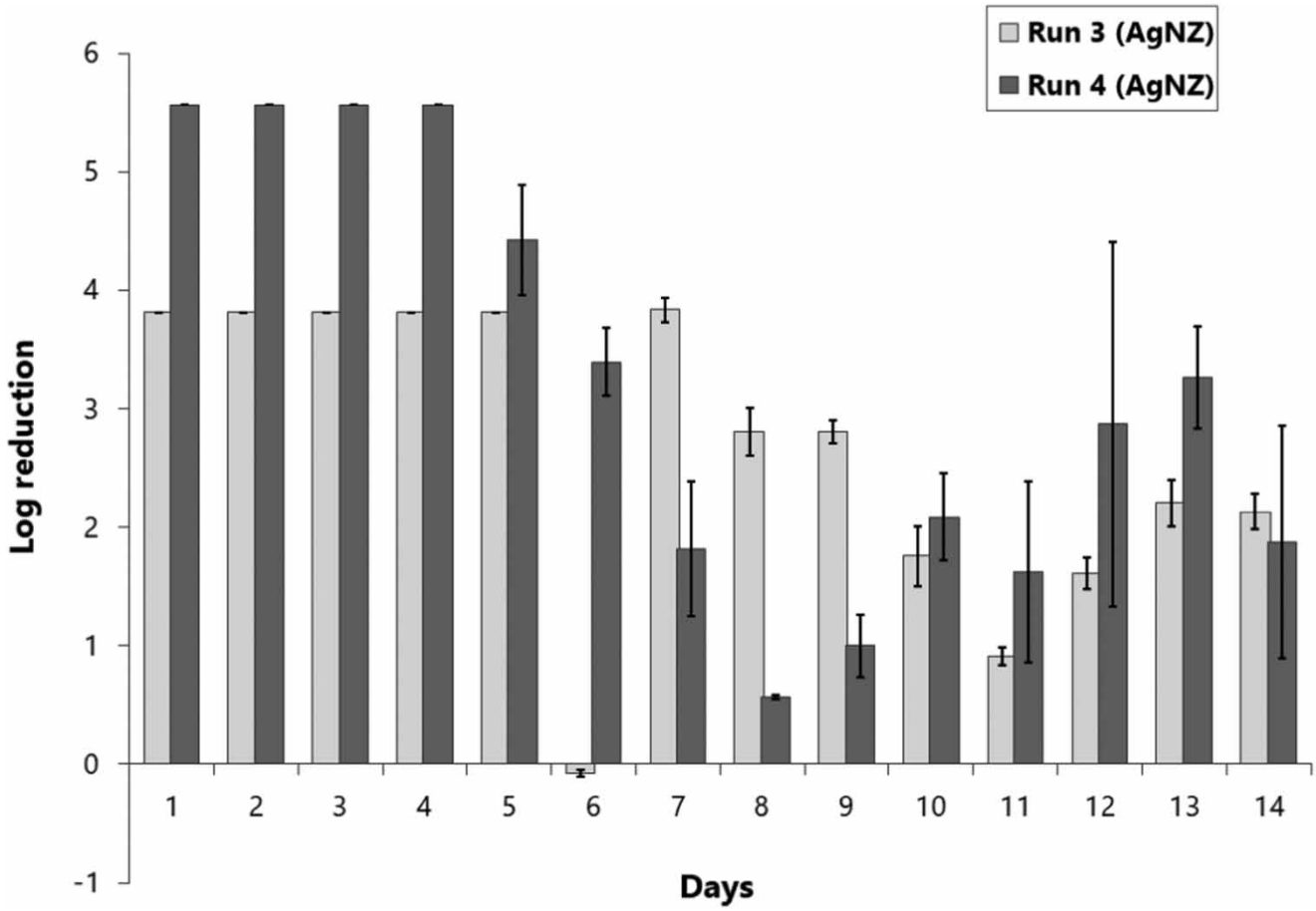

Figure 3 | Antibacterial activity of silver-exchanged zeolite (AgNZ) in flow column system with *A. baumannii* during 14 days of experimental run. The log reduction was calculated as $\log CFU_{inlet} - \log CFU_{outlet}$. The $\log CFU_{inlet}$ were 3.8 ± 0.3 (Run 3) and 5.6 ± 0.3 (Run 4).

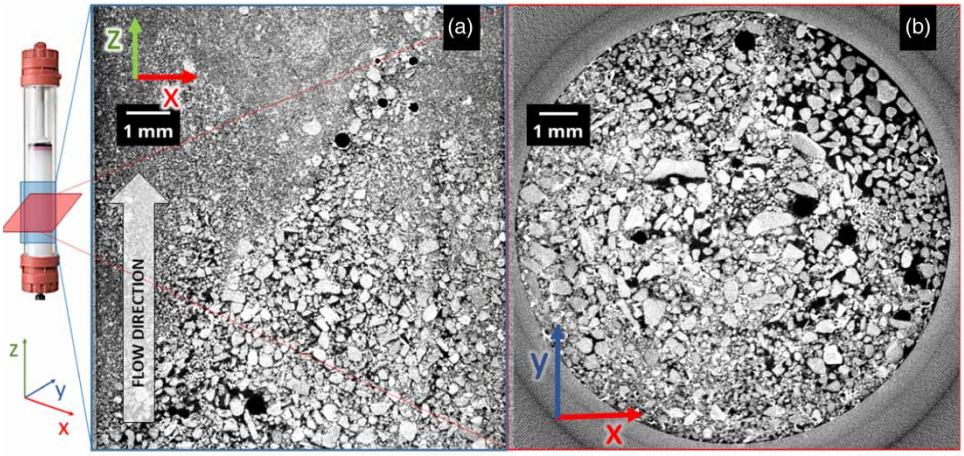

Figure 4 | The appearance of preferential pathways in column filled with AgNZ imaged using X-Ray Nanotomography, vertical (a) and horizontal (b) cross-section.

initially suggested by visual observation: it seemed that water found preferential pathway through the column so contact with AgNZ was limited and the antibacterial effect diminished. At this point, the column filling was stirred with sterilized metal rod, after which antibacterial efficacy was regenerated. To confirm appearance of preferential pathways, the column packed with AgNZ was scanned using X-ray nanotomography. The images clearly show preferential flow pathways, heterogeneous distribution of finer particles and creation of larger aggregates (Figure 4). Stirring of filling removed preferential flow pathways restoring homogeneity of column packing.

The preferential flow occurred inside the biofilter due to non-homogenous porosity, both vertical and horizontal, caused by ununiformed particle distribution. In preferential pathways bacterial suspension runs at a higher velocity (Nimmo 2012) which minimizes the contact between particles of porous media (AgNZ) and the colloids (bacterial cells). The preferential flow occurs mostly in saturated or unsaturated porous medium in the absence of hydraulic equilibrium and arises from contrast in conductance between different types of flow paths (Nimmo 2012). It is a well-known phenomenon described for natural porous media such as soils or wetlands (Steenhuis *et al.* 2005; Nimmo 2009; Heeren *et al.* 2010) as well as in designed biofilters (Le Coustumer *et al.* 2007; Flanagan *et al.* 2019). In our experiments the preferential pathways happened most likely due to small size of AgNZ particles (63–125 μm) since similar effect was not observed in additional experiment using larger fraction of particles (125–250 μm, Figure 5). This emphasized the need for a careful selection of particle size for experimental setup. For future investigation a special emphasis will

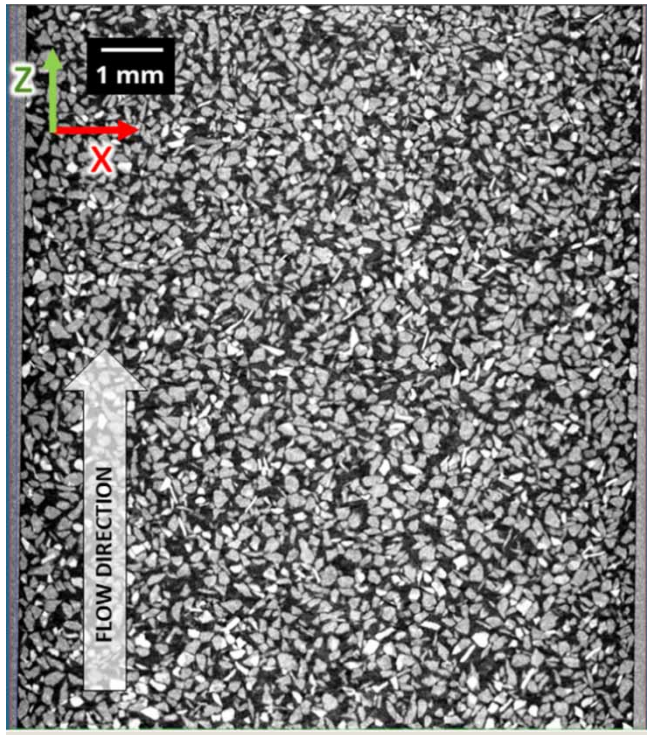

Figure 5 | Vertical cross-section of column filled with natural zeolite (NZ) of particle size ranging from 125 to 250 μm, imaged using X-Ray Nanotomography.

be given to filter preparation in order to obtain more compact filter.

Antibacterial activity of AgNZ in bead filter system with effluent wastewater

The log reduction is a clear and common presentation of disinfectant efficacy when starting number of bacteria is the

same in all the compared samples. But when the starting number of bacteria (i.e. log CFU) is different, the percent reduction is a better choice for comparison of various samples. Since effluent wastewater was a real environmental sample, not confined with controlled laboratory conditions and pure bacterial culture, the cell counts of He, CRB37 and CRB42 at the column inlet differed significantly ($p < 0.05$, *t*-test) from day to day and between the experimental runs (Table 1). Therefore, the efficacy of AgNZ was expressed as percentage of bacterial reduction (outlet to inlet ratio).

The biofilter filled with non-modified zeolite (NZ) reduced bacterial numbers of all types of monitored bacteria to certain extent (Figure 6). The most probable reason is the bacterial adsorption onto NZ particles in the column, which was confirmed by the numbers of immobilized bacteria after 4 day run: 7.12 ± 0.04 logCFU g^{-1} of He, 5.07 ± 0.21 logCFU g^{-1} of CRB37 and 2.39 ± 0.58 logCFU g^{-1} of CRB42. Such effect was expected as removal of indicator organisms such as *E. coli* (Nair & Ahammed 2014), faecal coliforms and faecal streptococci (Filipkovska & Krzemieniewski 1998) by adsorption is a normal process associated to trickling and bead filters (Sisson *et al.* 2013; O'Connell *et al.* 2017). As to why there is significant adsorption to NZ in experiments with real wastewater, but only for 24 h in experiments with pure culture of *A. baumannii*, the best assumption is that the equilibrium (when bacteria attach and detach to NZ in the same amount and exit the column) was quickly achieved in the case of pure culture, but not in experiments with real wastewater (at least not for 4 days of experimental run). Such assumption was supported by the fact that *A. baumannii* has a high capacity for adsorption and formation of biofilm and NZ is shown to facilitate bacterial immobilization of *A. baumannii* (Dekic *et al.* 2017) and *Acinetobacter junii* (Hrenovic *et al.* 2009) which is probably not the case with native wastewater population.

The biofilter filled with AgNZ reduced all of CRB42 (100%) and over 90% of CRB37 during 4 days of experimental run (Figure 7). Since we consider that *A. baumannii* is a part of CRB42 population (Hrenovic *et al.* 2017; Hrenovic *et al.* 2019) it is valid to assume that AgNZ effectively removed *A. baumannii* from effluent wastewater and thus is an effective disinfectant aimed specifically at clinically-relevant pathogens. In our system, 5 g of AgNZ completely reduced carbapenem-resistant bacteria in 2.9 L of real effluent wastewater.

Leaching of Ag^+ from AgNZ was most prominent during the first 24 h and left constant during the remaining three days of the experiment (Table 4). The concentration of leached Ag^+ was higher than the determined MBC (0.015 mg L^{-1}) for EF7 isolate, indicating that leaching of Ag^+ is a significant factor which contributes to the antibacterial efficacy of AgNZ in the bead filter system during the first two days of the experimental run.

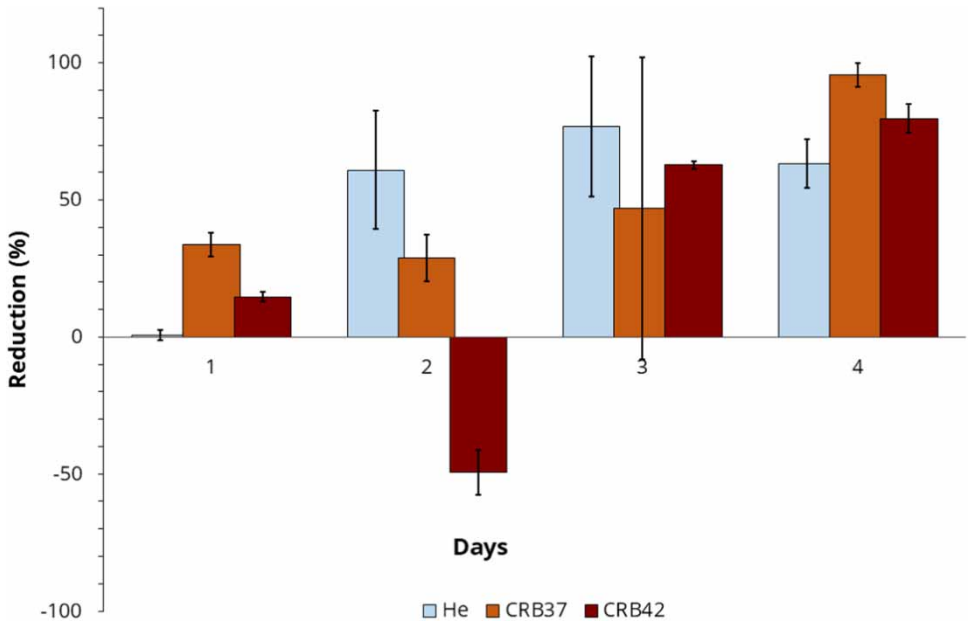

Figure 6 | Reduction (% of total counts$_{outlet}$/total counts$_{inlet}$) of heterotrophic (He) and carbapenem-resistant bacteria incubated at 37 (CRB37) or 42 °C (CRB42) in flow system with real effluent wastewater and natural zeolite (NZ). Shown are mean ± SD values of technical triplicates from single experiment.

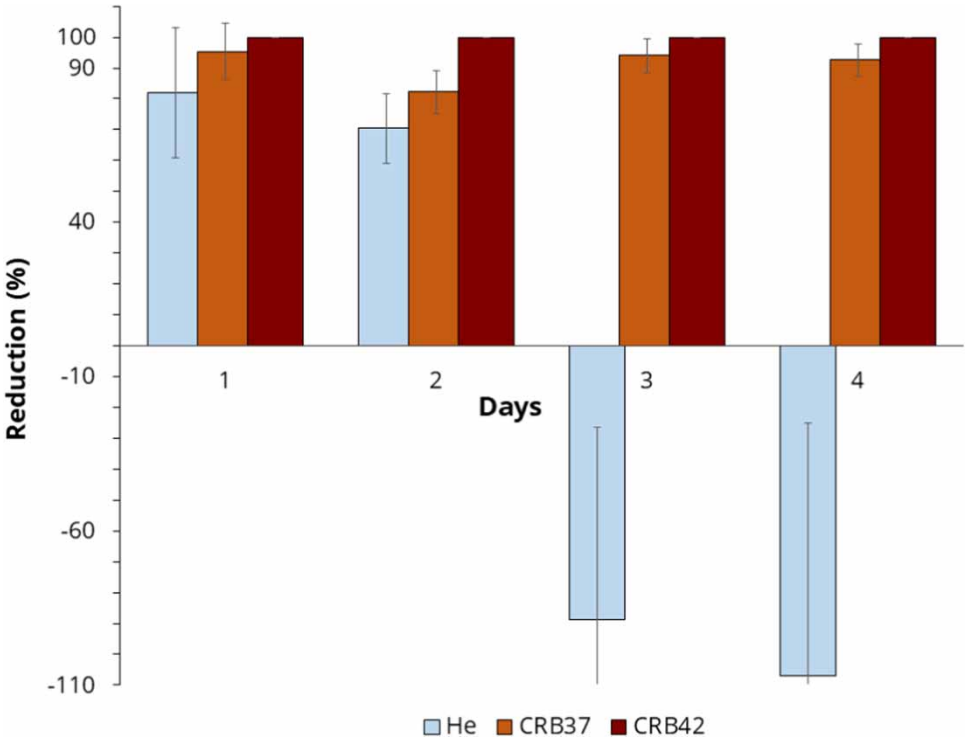

Figure 7 | Reduction (% of total counts$_{outlet}$/total counts$_{inlet}$) of heterotrophic (He) and carbapenem-resistant bacteria incubated at 37 (CRB37) or 42 °C (CRB42) in flow system with real effluent wastewater and silver-exchanged zeolite (AgNZ). Shown are mean ± SD values of technical triplicates from two separate experiments each with different wastewater sample.

Table 4 | Leaching of Ag^+ (mg L^{-1}) in the bead filter system with effluent wastewater

	Days			
	1	2	3	4
Run 6	1.258	0.034	0.025	<0.02
Run 7	0.026	<0.02	<0.02	<0.02

The value of <0.02 mg L^{-1} designates measurement below the detection limit. Shown are the measurements from two separate experiments, marked as Run 6 and 7.

Similar system was operated by El-Aassar *et al.* (2013) where column was filled with activated carbon coated with Ag nanoparticles and was effective in reducing *E. coli* and faecal coliforms in pure culture and from raw wastewater respectively, although details about wastewater volume and operation time were not provided. Mpenyana-Monyatsi *et al.* (2012) demonstrated moderate efficacy of zeolite coated with silver nanoparticles in comparison to coated resin towards pure culture of *E. coli* in column filter system. Both materials were nevertheless effective in removing pathogenic bacteria from groundwater during 120 min run; meaning 0.24 L of purified water in the experimental run in the column containing 125 cm^3 of silver coated material. The leaching of silver from zeolite was high in the first 10 min of the experiment but was reduced below 0.1 mg L^{-1} after 90 min (Mpenyana-Monyatsi *et al.* 2013). The 0.1 mg L^{-1} is the recommended limit of silver in drinking water set by World Health Organisation and US Environmental Protection Agency (Mpenyana-Monyatsi *et al.* 2013; NSDWR 2019). Our experiments showed similar leaching dynamics; the amount of leached silver was high during first 24 h of the experiment (1.2 mg L^{-1}, Table 4 but dropped below 0.1 mg L^{-1} on the second day, and below detection limit of the measuring instrument (<0.02) after 4 days of the experimental run. Such concentrations comply with Croatian regulations for drinking water (NN 2017) where allowed concentration of silver is set to 0.01 mg L^{-1}, with exception when silver is used for disinfection and in that case the limit is 0.1 mg L^{-1}.

Leaching dynamics could explain the measurements of heterotrophic bacteria (He) obtained in experiments with AgNZ; while there was substantial leaching the reduction of He was evident (first two days, Figure 7) and later on there was no reduction. Some He bacteria were probably unaffected by cell/AgNZ contact and were exiting the column. The carbapenem resistant bacteria (CRB37, CRB42) seem to be more susceptible to action of AgNZ so their reduction is evident through the entire 4 days. To confirm the latter, the numbers of immobilized bacteria on the

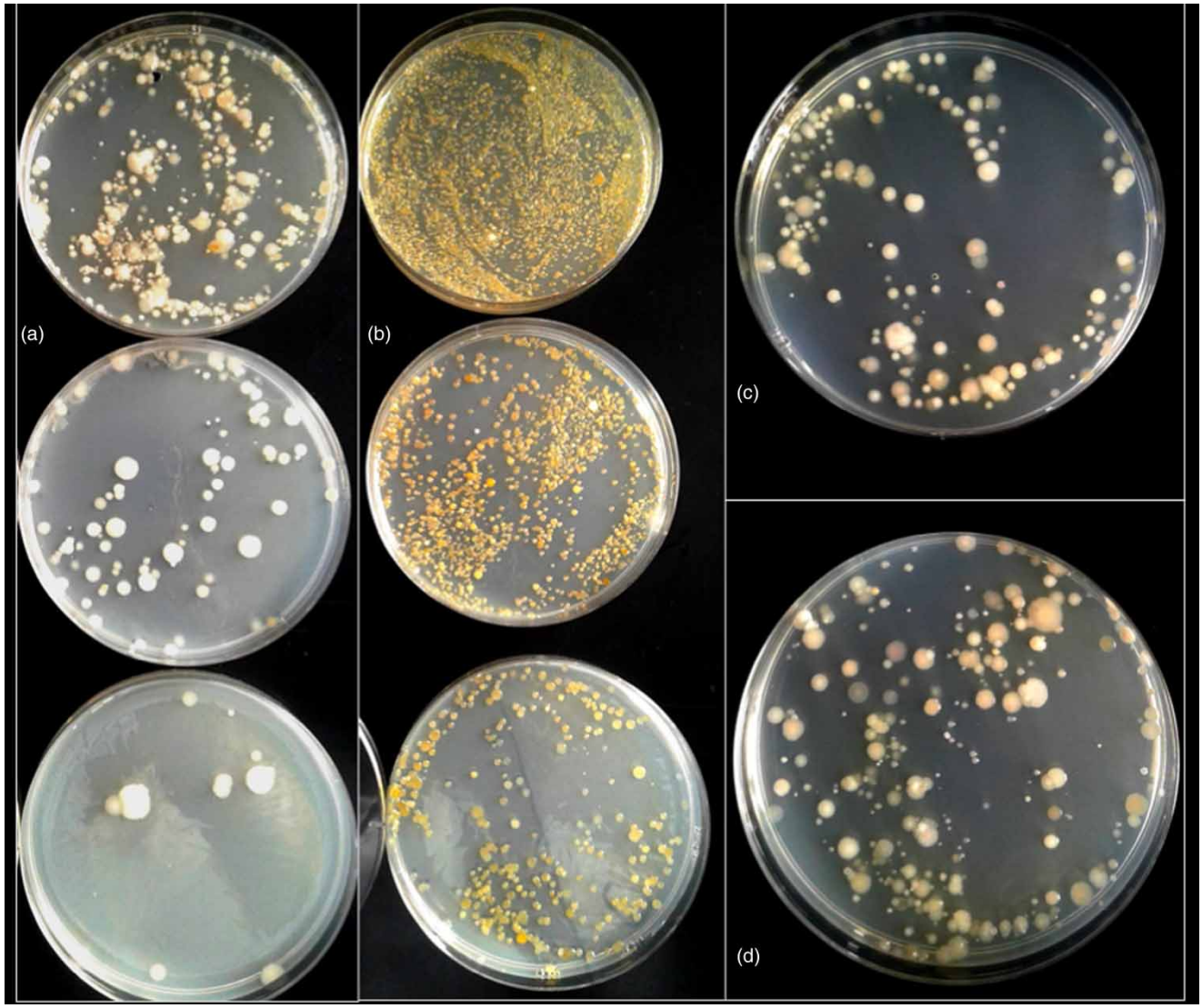

Figure 8 | Colonies of heterotrophic bacteria grown on nutrient agar plates (25 °C/72 h) from flow experiments with real effluent wastewater and silver-exchanged zeolite (AgNZ) after 4 days of experimental run: (a) – inlet, (b) – bacteria adsorbed onto AgNZ. On the far right is the same, but in experiment with natural zeolite (NZ) at inlet (c) and bacteria adsorbed onto NZ (d).

AgNZ after 4 day run were determined and amounted 8.98 ± 0.41 logCFU g^{-1} of He, but none (<1 CFU 50 ml^{-1}) of CRB37 and CRB42, which confirms antibacterial activity of AgNZ against CRB and not potential adsorption.

An interesting observation in the experiments with AgNZ was a change in heterotrophic bacterial community composition between the column inlet and outlet. The observations were from three technical replicates per measure, and two separate experiments with two different samples of wastewater, collected on February 18th (Run 6, Table 1) and March 4th 2019 (Run 7, Table 1). Therefore, these measurements can be considered replicable. In both experiments, the numbers of heterotrophs exiting the column filled with AgNZ increased on the third day, and visual inspection showed shift in community composition (Figure 8); yellow bacterial colonies dominated the water exiting the column and the community of bacteria immobilized on the AgNZ after 4 days (Figure 8(a) and 8(b)) which was not the case with NZ (Figure 8(c) and 8(d)). It seems that this bacterium predominately colonized the AgNZ particles and was able to suppress the antibacterial activity of silver. The bacteria was isolated in pure culture and characterized as Gram-negative, straight rods, capsulated, non-spore forming, oxidase negative, catalase positive, producing yellow pigmented colonies on nutrient agar. Detail characterization of here obtained isolate is ongoing and

why is this particular bacteria dominating the columns filled with AgNZ is at this point unknown. The community shift upon wastewater disinfection is, however, not an unknown event and was shown to occur during chlorine (Pang *et al.* 2016; Li *et al.* 2017), UV (Kauser *et al.* 2019) and ozone (Li *et al.* 2017; Chen *et al.* 2019) treatment. Similar to experiments with pure culture, it is safe to presume that more resilient He bacteria would eventually cover all the active sites of AgNZ and diminish the antibacterial activity.

CONCLUSION

The silver-exchanged natural zeolite – clinoptilolite (AgNZ) acted as an efficient antibacterial agent aimed specifically at *A. baumannii*. The bead filter filled with AgNZ completely removed pathogenic carbapenem-resistant bacteria from real effluent wastewater. Such efficiency indicates that the AgNZ is promising as an alternative material for wastewater disinfection and invokes further research that will surpass limitations of here presented study, mainly the maximum effective duration of biofilter filled with AgNZ. In addition, technological aspects of such biofilter must be detailly considered taking into the account the AgNZ particle size, flow rates, hydraulic retention time, cost-effectiveness, equipment maintenance etc. Next step should be the monitoring of the removal of clinically relevant pathogens in a pilot-scale biofilter, with experiments preferably incorporated into a tertiary stage of WWTP.

Here presented study can be considered as a contribution to the 'One-Health' approach which recognizes that human health, animal health and environmental sciences are all innately interrelated. The disinfection methods aimed specifically at antibiotic-resistant bacteria is a step forward in combating the worldwide expansion of drug resistance.

SUPPLEMENTARY DATA

The Supplementary Data for this paper is available online at http://dx.doi.org/10.2166/wst.2019.348.

ACKNOWLEDGEMENTS

This research was supported by the Croatian Science Foundation project no. IP-2014-09-5656 and partially by 'COGITO' Hubert-Curien Partnership program, in collaboration with Universite Grenoble-Alpes. There are no conflicts of interests to declare.

REFERENCES

Allison, K., Hook, J., Cardis, D. & Rice, R. G. 2009 Quantification of the bactericidal, fungicidal, and sporicidal efficacy of the JLA Ltd. Ozone laundering system. *Ozone Sci. Eng.* **31**, 369–378.

Anstey, N. M., Currie, B. J. & Withnall, K. M. 1992 Community acquired *Acinetobacter* pneumonia in the Northern Territory of Australia. *Clin. Infect. Dis.* **14**, 83–91.

Asif, M., Alvi, I. A. & Rehman, S. U. 2018 Insight into *Acinetobacter baumannii*: pathogenesis, global resistance, mechanisms of resistance, treatment options, and alternative modalities. *Infect. Drug. Resist.* **11**, 1249–1260.

Barancheshme, F. & Munir, M. 2018 Strategies to combat antibiotic resistance in the wastewater treatment plants. *Front. Microbiol.* **8**, 2603.

Chamul, R. S., Reed, M. & Silva, J. L. 2002 Chiller water treatment from channel catfish (Ictalurus punctatus) processing plants with ultrasound, ozone, and pulsed-light. *J. Miss. Acad. Sci.* **47**, 158.

Chen, J., Yang, Y., Liu, Y., Tang, M., Wang, R., Zhang, C., Jiang, J. & Jia, C. 2019 Bacterial community shift in response to a deep municipal tail wastewater treatment system. *Bioresour. Technol.* **281**, 195–201.

Croatian Academy of Medical Sciences. 2018 *Antibiotic Resistance in Croatia, 2017*. CAMS, Zagreb.

Davies, J. & Davies, D. 2010 Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* **74**, 417–433.

Dekic, S., Hrenovic, J., Hunjak, B., Kazazic, S., Tibljas, D. & Ivankovic, T. 2017 Virulence factors of *Acinetobacter baumannii* environmental isolates and their inhibition by natural zeolite. *Int. J. Curr. Microbiol. App. Sci.* **6**, 1697–1709.

Dekic, S., Hrenovic, J., Ivankovic, T. & Wilpe, E. 2018 Survival of ESKAPE pathogen *Acinetobacter baumannii* in water of different temperatures and pH. *Water Sci. Technol.* **78**, 1370–1376.

Dexter, C., Murray, G. L., Paulsen, I. T. & Peleg, A. Y. 2015 Community-acquired *Acinetobacter baumannii*: clinical characteristics, epidemiology and pathogenesis. *Expert Rev. Anti-Infect. Ther.* **13**, 567–573.

El-Aassar, A. H. M., Said, M. M., Abdel-Gawad, A. M. & Shawky, H. A. 2013 Using silver nanoparticles coated on activated carbon granules in columns for microbiological pollutants water disinfection in Abu Rawash area, Great Cairo, Egypt. *Aust. J. Basic Appl. Sci.* **7**, 422–432.

Falagas, M. E. & Karveli, E. A. 2007 The changing global epidemiology of *Acinetobacter baumannii* infections: a development with major public health implications. *Clin. Microbiol. Infect.* **13**, 117–119.

Fewtrell, L. 2014 *Silver: Water Disinfection and Toxicity*. Centre for Research into Environment and Health, World Health Organization, Geneva.

Filipkovska, Z. & Krzemieniewski, M. 1998 Effectiveness of indicatory microorganism removal on trickling filter with biofilm in magnetic field. *Pol. J. Environ. Stud.* **7**, 201–205.

Flanagan, K., Branchu, P., Boudahmane, L., Caupos, E., Demare, D., Deshayes, S., Dubois, P., Meffray, L., Partibane, C., Saad, M. & Gromaire, M. C. 2019 Retention and transport processes of particulate and dissolved micropollutants in stormwater biofilters treating road runoff. *Sci. Total Environ.* **656**, 1178–1190.

Goic-Barisic, I., Hrenovic, J., Kovacic, A. & Seruga-Music, M. 2016 Emergence of oxacillinases in environmental carbapenem resistant *Acinetobacter baumannii* associated with clinical isolates. *Microb. Drug Resist.* **22**, 559–563.

Goic-Barisic, I., Seruga-Music, M., Kovacic, A., Tonkic, M. & Hrenovic, J. 2017 Pan drug-resistant environmental isolate of *Acinetobacter baumannii* from Croatia. *Microb. Drug Resist.* **23**, 494–496.

Gomes, J., Matos, A., Gmurek, M., Quinta-Ferreira, R. M. & Martins, R. C. 2019 Ozone and photocatalytic processes for pathogens removal from water: a review. *Catalysts* **9**, 46.

Heeren, D. M., Miller, R. B., Fox, G. A., Storm, D. E., Mittelstet, A. R. & Penn, C. J. 2010 Impact of preferential flow paths on alluvial groundwater flow patterns and phosphorus transport. In *Biological Systems Engineering: Papers and Publications*. Paper 373.

Higgins, P. G., Hrenovic, J., Seifert, H. & Dekic, S. 2018 Characterization of *Acinetobacter baumannii* from water and sludge line of secondary wastewater treatment plant. *Water Res.* **14**, 261–267.

Hobman, J. L. & Crossman, L. C. 2014 Bacterial antimicrobial metal ion resistance. *J. Med. Microbiol.* **64**, 471–497.

Hrenovic, J., Ivankovic, T. & Tibljas, D. 2009 The effect of mineral carrier composition on phosphate-accumulating bacteria immobilization. *J. Hazard Mater.* **166**, 1377–1382.

Hrenovic, J., Milenkovic, J., Ivankovic, T. & Rajic, N. 2012 Antibacterial activity of heavy metal-loaded natural zeolite. *J. Hazard. Mater.* **201–202**, 260–264.

Hrenovic, J., Milenkovic, J., Goic-Barisic, I. & Rajic, N. 2013 Antibacterial activity of modified natural clinoptilolite against clinical isolates of *Acinetobacter baumannii*. *Micropor. Mesopor. Mater.* **169**, 148–152.

Hrenovic, J., Ivankovic, T., Ivekovic, D., Repes, S., Stipanicev, D. & Ganjto, M. 2017 The fate of carbapenem-resistant bacteria in a wastewater treatment plant. *Water Res.* **126**, 232–239.

Hrenovic, J., Ivankovic, T., Durn, G., Dekic, S., Kazazic, S. & Kisic, I. 2019 Presence of carbapenem-resistant bacteria in soils affected by illegal waste dumps. *Int. J. Environ. Health Res.* **29**, 154–163.

Iakovides, I. C., Michael-Kordatou, I., Moreira, N. F. F., Ribeiro, A. R., Fernandes, T., Pereira, M. F. R., Nunes, O. C., Manaia, C. M., Silva, A. M. T. & Fatta-Kassinos, D. 2019 Continuous ozonation of urban wastewater: removal of antibiotics, antibiotic-resistant Escherichia coli and antibiotic resistance genes and phytotoxicity. *Water Res.* **159**, 333–347.

Ivankovic, T., Goic-Barisic, I. & Hrenovic, J. 2017 Reduced susceptibility to disinfectants of *Acinetobacter baumannii* biofilms on glass and ceramic. *Arh. Hig. Rada Toksikol.* **68**, 99–108.

Jäger, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G. & Schwartz, T. 2018 Reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment, and the disseminated loads released to the environment. *Front. Microbiol.* **9**, 2599.

Kauser, I., Clesielski, M. & Poretsky, R. S. 2019 Ultraviolet disinfection impacts the microbial community composition and function of treated wastewater effluent and the receiving urban river. *PeerJ Preprints*. https://doi.org/10.7287/peerj.preprints.27605v1.

Kwakye-Awuah, B., Williams, C., Kenward, M. A. & Radecka, I. 2008 Antimicrobial action and efficiency of silver-loaded zeolite X. *J. Appl. Microbiol.* **104**, 1516–1524.

Le Coustumer, S., Fletcher, T. D., Deletic, A. & Barraud, S. 2007 Hydraulic performance of biofilters: first lessons from both laboratory and field studies. *Water Sci. Technol.* **56**, 93–100.

Li, Q., Yu, S., Li, L., Liu, G., Gu, Z., Liu, M., Liu, Z., Ye, Y., Xia, Q. & Ren, L. 2017 Microbial communities shaped by treatment processes in a drinking water treatment plant and their contribution and threat to drinking water safety. *Front. Microbiol.* **8**, 2465.

Lima, E., Guerra, R., Vinierga, M., Guzman, A. & Lara, V. 2012 Gold nanoparticles as efficient antimicrobial agents for Escherichia coli and *Salmonella typhi*. *Micropor. Mesopor. Mater.* **147**, 267.

Lima, E., Guerra, R., Lara, V. & Guzmán, A. 2013 Gold nanoparticles as efficient antimicrobial agents for *Escherichia coli* and *Salmonella typhi*. *Chem. Cent. J.* **7** (11).

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T. & Monnet, D. L. 2012 Multidrug-resistant, extensively drug resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **18**, 268–281.

Milenkovic, J., Hrenovic, J., Matijasevic, D., Niksic, M. & Rajic, N. 2017 Bactericidal activity of Cu-, Zn-, and Ag-containing zeolites toward *Escherichia coli* isolates. *Environ. Sci. Pollut. Res.* **24**, 20273–20281.

Mpenyana-Monyatsi, L., Mthombeni, N. H., Onyango, M. S. & Momba, M. N. B. 2012 Cost-effective filter materials coated with silver nanoparticles for the removal of pathogenic bacteria in groundwater. *Int. J. Environ. Res. Public Health.* **9**, 244–271.

Nair, A. T. & Ahammed, M. M. 2014 Biosand filtration: a sustainable option for household treatment of drinking water. In: *Symposium on Integrated Water Resources Management (IWRM–2014)*, February 19–21, 2014. CWRDM, Kozhikode, Kerala, India.

Nimmo, J. R. 2009 Vadose water. In: *Chapter in: Encyclopedia of Inland Waters* (G. E. Likens ed.). Academic Press, pp. 766–777.

Nimmo, J. R. 2012 Preferential flow occurs in unsaturated conditions. *Hydrol. Process.* **26**, 786–789.

NN 125/2017 – Narodne Novine 2017 *Pravilnik o parametrima sukladnosti, metodama analize, monitoringu i planovima sigurnosti vode za ljudsku potrošnju*. Available from: https://narodne-novine.nn.hr/clanci/sluzbeni/full/2017_12_125_2848.html (accessed 16 June 2019).

NSDWR 2019 *National Secondary Drinking Water Regulations*. United States Environmental Protection Agency. Available from: https://www.epa.gov/dwregdev/drinking-water-regulations-and-contaminants (accessed 16 June 2019).

O'Connell, B. J., Slawson, D., Quinn, M., Scheuerman, P. & Ogunleye, O. O. 2017 Review of biosand water filters. *Waterlines.* **36**, 1–10.

Pang, Y. C., Xi, J. Y., Xu, Y., Huo, Z. Y. & Hu, H. Y. 2016 Shifts of live bacterial community in secondary effluent by chlorine disinfection revealed by Miseq high-throughput sequencing combined with propidium monoazide treatment. *Appl. Microbiol. Biotechnol.* **100**, 6435–6446.

Poulikakos, P., Tansarli, G. & Falagas, M. 2014 Combination antibiotic treatment versus monotherapy for multidrug-resistant, extensively drug-resistant, and pandrug-resistant *Acinetobacter* infections: a systematic review. *Eur. J. Clin. Microbiol. Infect. Dis.* **33**, 1675–1685.

Seruga-Music, M., Hrenovic, J., Goic-Barisic, I., Hunjak, B., Skoric, D. & Ivankovic, T. 2017 Emission of extensively-drug resistant *Acinetobacter baumannii* from hospital settings to the natural environment. *J. Hosp. Infect.* **96**, 323–327.

Sisson, A. J., Wampler, P. J., Rediske, R. R., McNair, J. N. & Frobish, D. J. 2013 Long-term field performance of biosand filters in the Artibonite valley, Haiti. *Am. J. Trop. Med. Hyg.* **88**, 862–867.

Steenhuis, T. S., Parlange, Y., Kim, J., DiCarlo, D. A., Selker, J. S., Nektarios, P. A., Barry, D. A. & Stagnitti, F. 2005 Unstable flow. In: *Encyclopedia of Soils in the Environment* (D. Hillel ed.). Academic Press, New York, pp. 197–201.

WHO – World Health Organization 2017 Available from: http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1 (accessed 07 July 2019).

Willyard, C. 2017 Drug-resistant bacteria ranked. *Nature* **543**, 15.

Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., Liu, X., Shi, Y., Hu, X., An, D., Li, Z., Li, P., Wang, L., Cui, J., Wang, P., Huang, L., Klena, J. D. & Song, H. 2013 Higher isolation of NDM-1 producing Acinetobacter baumannii from the sewage of the hospitals in Beijing. *PLoS One.* **8**, e64857.

First received 22 July 2019; accepted in revised form 11 October 2019. Available online 22 October 2019