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RESEARCH ARTICLE

Will the antimicrobial properties of ZnONPs turn it into a more suitable option than AgNPs for water filtration? Comparative study in the removal of fish pathogen, *Aeromonas hydrophila* from the culture of juvenile common carp (*Cyprinus carpio*)

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Abstract
The purpose of this study was to investigate the possibility of using zinc oxide nanoparticles (ZnONPs) instead of silver nanoparticles (AgNPs) for removing *Aeromonas hydrophila* from water used to culture *Cyprinus carpio* juvenile. Antibacterial materials as filter media were prepared by coating ZnONPs (two coating methods, referred as ZnA and ZnB) or AgNPs (referred as Ag) on the porous surfaces of zeolite beads. The characterization of coated samples was determined using FESEM, EDS, and GFAAS. The antibacterial activities of prepared samples were evaluated by the zone of inhibition test, tube test, and flow test. The diameter of inhibitory zones formed by ZnONP- and AgNP-coated zeolite beads was significantly higher than uncoated zeolite (control) ($P < 0.05$). Also, the tube test results revealed 100% killing of the bacterial cells after 24 h of contact to all coated materials. In the flow test (without fish), the antibacterial efficiency of filter columns that contained ZnA, ZnB, and Ag found to be 34.84, 23.77, and 100% after 96 h, respectively. The mortality rate of carp juveniles cultured in infected water treated with AgNP filters was significantly lower than those cultured in infected water or treated with ZnONPs filters ($P < 0.05$). The results indicated that although ZnONP filter media have somewhat antimicrobial properties (especially in vitro), their ability to complete removal of microorganisms from the water is not as high as AgNP filters. So, it still seems that zeolite coated with AgNPs has a higher potential for water disinfection in aquaculture.

Keywords Nanotechnology · Water treatment · Zinc oxide nanoparticles · Silver nanoparticles · Aquaculture · Disinfection

Introduction

Aquaculture industry is growing rapidly in the world and plays an important role in supplying human protein. In this regards, use of intensive and super-intensive aquaculture systems has been considered which in turn has accelerated the disease outbreaks and so huge economic losses (Reverter et al. 2014). *Aeromonas hydrophila* as a ubiquitous, gram-negative, and motile bacterium is abundant in aquatic habitats. This species is known as an opportunistic pathogen that causes mass mortality in farmed fish (Harikrishnan and Balasundaram 2005). Bacterial diseases such as hemorrhagic septicemia and epizootic ulcerative syndrome arising from *A. hydrophila* have been reported in freshwater and marine species (Shao et al. 2004; Lilley et al. 1997). It is known that *A. hydrophila* is one of the most possible cause of the disease in common carp (*Cyprinus carpio*) (Harikrishnan et al. 2003).

Water disinfection through many common methods such as chlorination, ozonation, and ultraviolet radiation is used to kill pathogens. The use of these methods is not wide spread because of some limitations such as resistance of some waterborne pathogens to chlorination, formation of carcinogenic compounds after adding chlorine to water, high price of ozonation, and low efficiency of UV treatment (Dimapilis et al. 2017). The application of other disinfectant chemicals may

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have disastrous effects on the organisms and environment (Johari et al. 2014). Therefore, new approaches need to be investigated to improve efficiency of water disinfection in aquaculture.

Nanotechnology is considered as a new way to synthesize more effectiveness and cost-effective materials for water disinfection (Dimapilis et al. 2017; Sarkheil et al. 2016). Nanomaterials (NMs) have special properties such as a larger surface area to volume compared to bulk particles, which enable them to interact directly with cell membranes and thus increase their antibacterial activity (Bryaskova et al. 2011; Heinlaan et al. 2008). The antibacterial properties of metal and metal oxide nanoparticles (NPs) such as AgNPs, CuONPs, TiO_2NPs, and ZnONPs against both gram-negative and positive bacteria have been reported in literature (Sarkar et al. 2012; Toodehzaeim et al. 2018; Zimbone et al. 2015; Agarwal et al. 2018). Nowadays, AgNPs are considered as one of the important alternatives to antibiotics in medicine field (Rai et al. 2012; Franci et al. 2015). ZnO also has gained considerable interest as an antibacterial agent due to higher stability under harsh processing conditions (Stoimenov et al. 2002), greater durability, and heat resistance compared to organic materials (Padmavathy and Vijayaraghavan 2008). ZnONPs exert their antibacterial activity through the production of reactive oxygen species (ROS) or the release of Zn^{2+} ions (Zhang et al. 2010). The tendency of NPs like AgNPs and ZnONPs to aggregate in aqueous environment and thus the reduction of their dispersion stability and efficiency limit their direct use as antibacterial agents for water disinfection (Park et al. 2017; Li et al. 2012). Moreover, it is known that these NPs can be released into water and have adverse effects on the aquatic organisms and environments (Baun et al. 2008; Chupani et al. 2017, 2018a,b; Dekani et al. 2019); therefore, direct use of NMs in aquaculture is not possible.

Immobilization or coating of the NPs in various porous substrates and filtration membranes can provide high-performance antibacterial agents for water disinfection (Agnihotri et al. 2013; Quang et al. 2013). Quang et al. (2013) developed AgNP-supported silica beads for removing *Escherichia coli* from water and showed that it can remove over 99% of the *E. coli* from the water in a short period. The results of another study also indicated that the thin ZnO film formed on a glass substrate effectively inactivated *Staphylococcus aureus* under UV-A light in continuous-flow reactors (Park et al. 2017). Although the antibacterial efficiency of different substrates that contained metal NPs has been evaluated in drinking water sources, but the information on the use of these novel antibacterial agents in aquaculture is limited.

There are a number of studies that have been shown high strength of AgNP-coated filter media for removal of pathogenic bacteria and fungi from water used in aquaculture (Johari et al. 2016; Sarkheil et al. 2016, 2017). Nevertheless, since there are some concerns about the toxic effects of Ag ions and NPs released from the surface of silver containing water filters, study of the possibility of using other less toxic antimicrobial NMs seems to be necessary. The goal of the present study was to investigate the possibility of using ZnONPs instead of AgNPs for removing *A. hydrophila* from water used to culture *Cyprinus carpio* juveniles. For this purpose, zinc oxide and silver nanoparticles were coated on the natural zeolite (clinoptilolite) beads. First, the antibacterial activities of prepared ZnONP- and AgNP-coated zeolite were investigated against *A. hydrophila* in vitro. Thereafter, the antibacterial efficiency of filter columns filled with coated zeolite samples was examined in removing *A. hydrophila* from water used for cultivation of carps in a water recirculating system. Release of total zinc and total silver from filter columns into water and their accumulation in fish tissues were also determined.

Materials and methods

Reagents

Natural zeolite (clinoptilolite) was purchased from Afrand Tooska Co., Tehran, Iran. Zinc acetate ($C_4H_6O_4Zn{\cdot}2H_2O$), sodium hydroxide (NaOH), silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), hydrochloric acid (HCl), and acetone (C_2H_6O) were purchased from Merck Millipore (Germany).

Preparation of zinc oxide nanoparticle-coated zeolite

In the present study, ZnONPs were coated on zeolite based on two different methods and according to the modified method described by Sanatgar-Delshade et al. (2011). In the first method, zeolite beads were washed several times with distilled water and then dried at 70 °C. The zeolite surfaces were activated by adding the zeolite beads (100 g) and HCl (100 mL, 6 M) to a round bottom balloon and reflux for 24 h. The mixture was cooled, decanted, washed several times with distilled water and acetone, and dried overnight at 80 °C. In the next step (alkalization step), in order to alkalize zeolite, 50 mL of NaOH (3 M) was added to 100 g of zeolite beads in a round bottom balloon, and the mixture was stirred for 2 h at room temperature. Then, the mixture was washed several times with distilled water and dried overnight at 80 °C. Afterwards, zinc acetate ($C_4H_6O_4Zn{\cdot}2H_2O$) (10.975 g) was dissolved in 500 mL of double distilled water and poured onto 100 g of zeolite beads. This mixture was stirred for 24 h to enhance loading zinc ions on the pores of zeolite beads. In the following, a solution of sodium hydroxide (5 M) was added drop wise to the mixture to reach pH 13 and the mixture was stirred at room temperature for 4 h. The mixture was decanted, washed several times with distilled water (until pH

neutralizes), and dried at 80 °C. Finally, the zeolite beads were calcinated at 400 °C. The obtained ZnONP-coated zeolite bead by this method will be referred to as ZnA. In the second method, all the steps mentioned above except alkalization step were performed on zeolite beads and the synthesized sample based on this method will be referred to as ZnB.

Preparation of silver nanoparticle-coated zeolite

In this order, zeolite beads were washed, dried, and activated according to method described in section "Preparation of zinc oxide nanoparticle-coated zeolite". Then, 0.85 g silver nitrate ($AgNO_3$) was dissolved in 1 L of distilled water in a dark bottle and then added to 200 g zeolite and agitated in a rotary shaker for 24 h. The mixture was decanted and washed several times with distilled water. Thereafter, 0.75 g $NaBH_4$ dissolved in 25 mL of distilled water and added drop wise to a mixture of the zeolite beads and 1 L of distilled water. The mixture was stirred for 24 h at room temperature and after that decanted, washed with distilled water, and dried at 50 °C. The AgNP-coated zeolite bead will be referred to as Ag. Uncoated zeolite bead was considered as control media and will be referred to as Ze.

Characterization of prepared nanoparticle-coated zeolite

In order to determine the shapes, sizes, and elemental composition of ZnO and Ag particles coated on zeolite beads, field emission scanning electron microscopy (FE-SEM; MIRA3 TESCAN, Brno, Czech Republic) coupled with x-ray energy dispersive spectroscopy (EDS) was used. To estimate the mean size distribution of NPs, the diameter of 600 individual particles or their aggregates/agglomerates was randomly determined on SEM micrographs using AxioVision digital image processing software (Release 4.8.2.0, Carl Zeiss Micro Imaging GmbH, Germany). The total zinc and silver contents of prepared samples were measured after acid digestion of 0.2 of the samples in 5 mL of concentrated HNO_3 (Suprapur grade, Merck, Germany) using a graphite furnace flame atomic absorption spectrophotometer (GFAAS) (PinAAcle 900 T, PerkinElmer Inc., USA).

Antibacterial activity assay

Aeromonas hydrophila as a pathogenic bacterium in aquaculture (Noga 2010) was purchased freeze-dried from Pasteur Institute of Iran (collection No. ATCC: 7965). This bacterium species was cultured in tryptic soy broth (TSB) (Merck, Germany) and incubated at 28 °C for 48 h (Memmert, Model ICP 400). The bacterial cells were isolated from TSB medium by centrifugation at 6000 rpm for 10 min and then washed twice with sterile physiological saline solution (0.9%). The density of bacterial cell was determined at 600 nm using a UV–visible spectrophotometer (UNICO 2100 model) after appropriate dilution. The optical density (OD) of approximately 0.1 was considered as 10^8 CFU/mL.

Zone of inhibition test

The antibacterial activity of ZnA, ZnB, and Ag samples was determined by pour plate method in triplicate. For this purpose, 10 mL of bacterial suspension (10^5 CFU/mL) was transferred into sterilized Petri dishes, and then, tryptic soy agar (TSA) was poured onto them and allowed to solidify. Thereafter, 0.1 g of each NP-coated samples, uncoated zeolite (Ze), and ciprofloxacin (Cp) disk as controls were placed over solidified agar medium and incubated at 28 °C for 48 h. Subsequently, diameter of zone of inhibition formed around samples was measured using AxioVision software on the pictures taken from the cultured plates.

Tube test experiment

In the test tube experiment, 10 mL of bacterial suspension (10^5 CFU/mL) was poured into sterilized tubes. Then, 1 g of each zeolite samples (ZnA, ZnB, Ag, and Ze) was added to each tube test. The tubes were incubated in a shaking incubator at 28 °C for 24 h. Thereafter, a fraction of bacterial suspension was withdrawn from each tube test and inoculated onto TSA plates based on a serial dilution method. All plates were incubated at 28 °C and viable colonies were counted after 24 h (Jain and Pradeep 2005). This experiment was performed in three replicates.

Flow test (without fish)

In this experiment, antibacterial efficiency of the zeolite beads coated with ZnONPs and AgNPs as filter media was evaluated in a water recirculating system (Fig. 1). The components of this system consist of a glass aquarium, water pump (RS-4000 model, China), transparent plastic filter housing (height of 18 cm and diameter of 3.5 cm), and filter media. Each of the filter housings was filled with 400 g of filter media. The aquariums were filled with 30 L of infected water with *A. hydrophila* (1.5×10^6 CFU/mL). At the same time, the water was passed through the filter media at a flow rate of 2.7 L/min by a pump. The effluent water from bottom end of filter housing was returned into the aquariums. The systems that consisted of either filter filled with uncoated zeolite (Ze) or without filter media were considered as control. Each water recirculating system was launched in triplicate.

The efficiency of each filter in removing the pathogenic *A. hydrophila* from water was assayed by sampling the treated water from each aquarium after 0.5, 2, 5, 24, 48, and 96 h time intervals. The water samples were cultured on TSA plates

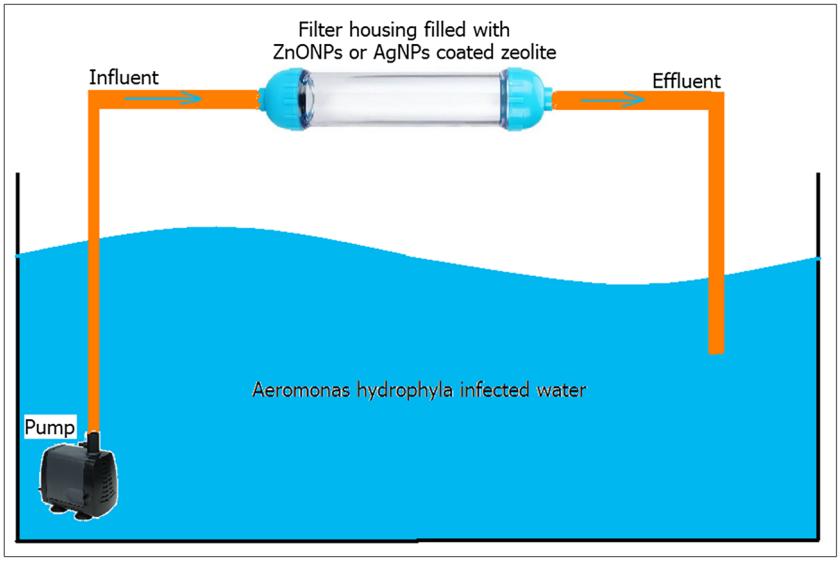

Fig. 1 Schematic diagram of a recirculating water system for evaluating antibacterial activity of ZnONP- and AgNP-coated zeolite beads as filter media

according to serial dilution method (Arkoosh et al. 2005) and incubated at 28 °C for 24 h (Rodríguez et al. 2008) before counting the bacterial cells. The bactericidal efficiency of filter was determined using following equation, where *R* is the bactericidal efficiency (%), *A* is the density of bacterial cells in initial time (CFU/mL), *B* is the density of bacterial cells in next time (CFU/mL).

$$R\ (\%) = (\mathrm{A–B})/\mathrm{A} \times 100$$

Release of silver and zinc from filter media into the water

Water samples (20 mL) were collected from each aquarium after 96 h of passage of infected water through filter column to measure the released silver and zinc into the water. The concentrations of total silver and total zinc were determined after acid digestion using a GFAAS (PinAAcle 900 T, PerkinElmer Inc., USA).

Experimental challenge of fish with bacterial pathogen

In this experiment, the antibacterial efficiency of filter columns contained different filter media was examined in a system (described in section "Flow test (without fish)") used to culture common carp (*C. carpio*) juveniles. The experimental treatments were (1) negative control (without filter media + without infected water), (2) positive control (without filter media + infected water), (3) Ze + infected water, (4) ZnA + infected water, (5) ZnB + infected water, and (6) Ag + infected water. A total of 200 carp juveniles (weight of 12.94 ± 2.90 g and length of 10.43 ± 0.78 cm) were purchased from a fish supplier in north of Iran and transferred to the Aquatic Nanobiotechnology Lab. (University of Kurdistan, Iran) and stocked into three 500-L fiberglass tanks. Fish were acclimatized to laboratory conditions and fed with commercial diet for 2 weeks. Then, 10 fish were randomly stocked into each glass aquarium that filled with 100 L of freshwater and aerated for 48 h. After system start up as described in section "Flow test (without fish)", the water of each aquarium was infected with *A. hydrophila* at density of 1.5 ± 10^6 CFU/mL on days of 1, 14, and 24 of experiment. Fish were fed to satiation every 2 days during 44 days of experimental period and any unusual symptoms and mortality of fish were recorded. Water temperature, dissolved oxygen, pH, and total ammonia nitrogen (TAN) were recorded as 19.25 ± 1.53 °C, 6.9 ± 0.52 mg/L, 8.05 ± 0.14, and 1.34 ± 0.08 mg/L, respectively.

Measurement of silver and zinc in fish tissues

At the end of bacterial challenge experiment, three fish were randomly sampled from each glass aquarium to measure the accumulation of silver and zinc released from filter media in fish tissues. The gills and liver of sampled fish were removed and stored at − 24 °C for 24 h. The samples were dried at − 40 °C under vacuum condition in a freeze dryer and then grounded into powder. The total silver and zinc contents were determined according to acid digestion method. Briefly, 0.1 g of each sample was transferred to a 50-mL Teflon tube, and 2.5 mL of concentrated HNO_3 (Suprapur grade, Merck, Germany) was added and then stored at room temperature for 24 h. Thereafter, the samples were heated on a steam bath at 100 °C for 2 h and diluted to volume of 5 mL. The total concentrations of silver and zinc were analyzed using a GFAAS (PinAAcle 900 T, PerkinElmer Inc., USA).

Statistical analysis

The data were recorded as mean ± SD. The statistical analysis was performed using SPSS software (Version, 19, IBM SPSS, Armonk, NY, USA). Normality assumption of data was investigated using the Kolmogrov-Smirnov test. The significant differences between means were determined using one-way analysis of variance (ANOVA) following Duncan's new multiple range test. The significant differences between two independent samples were analyzed using the independent sample *t* test. The accepted level of statistical significance was $P < 0.05$.

Results

Characterization of filter media

The FESEM micrographs of Ze, ZnA, ZnB, and Ag samples are shown in Fig. 2a–d. These micrographs showed the distribution of spherical ZnONPs with a mean diameter of 114.7 ± 58.5 nm and a size distribution from 20.5 to 452.9 nm on the surfaces of ZnA samples. The ZnONPs had spherical and oval shapes with a mean diameter of 80.8 ± 4.8 nm and a size distribution from 12.8 to 388.9 nm on the surfaces of ZnB samples. The spherical shape of AgNPs with a mean diameter of 113.6 ± 3.0 nm and a size distribution that ranged from 23.3 to 401.7 nm was determined on the surfaces of Ag samples.

The EDS elemental analyses of samples are shown in Fig. 3a–d and Table 1. The EDS spectra confirmed the presence of Zn, O, and Ag as elemental forms attached to the surfaces of zeolite beads.

The zinc and silver contents of synthesized samples measured using GFAAS are shown in Table 2. The results showed that amounts of zinc coated on ZnA and ZnB were significantly higher than Ze samples (uncoated zeolite) ($P < 0.05$). A much higher amount of silver was also detected in Ag sample compared to control ($P < 0.05$).

Antibacterial activity of filter media

Zone of inhibition

The results of inhibitory zone formed by ZnONP- and AgNP-coated zeolite, uncoated zeolite, and ciprofloxacin disk for *A. hydrophila* in antibiogram test are shown in Figs. 4 and 5. The highest mean diameter of inhibitory zone was observed around ciprofloxacin disk ($P < 0.05$). The Ag samples formed higher inhibitory zone than ZnA and ZnB samples ($P < 0.05$). No inhibitory zone was observed around uncoated zeolite (Ze).

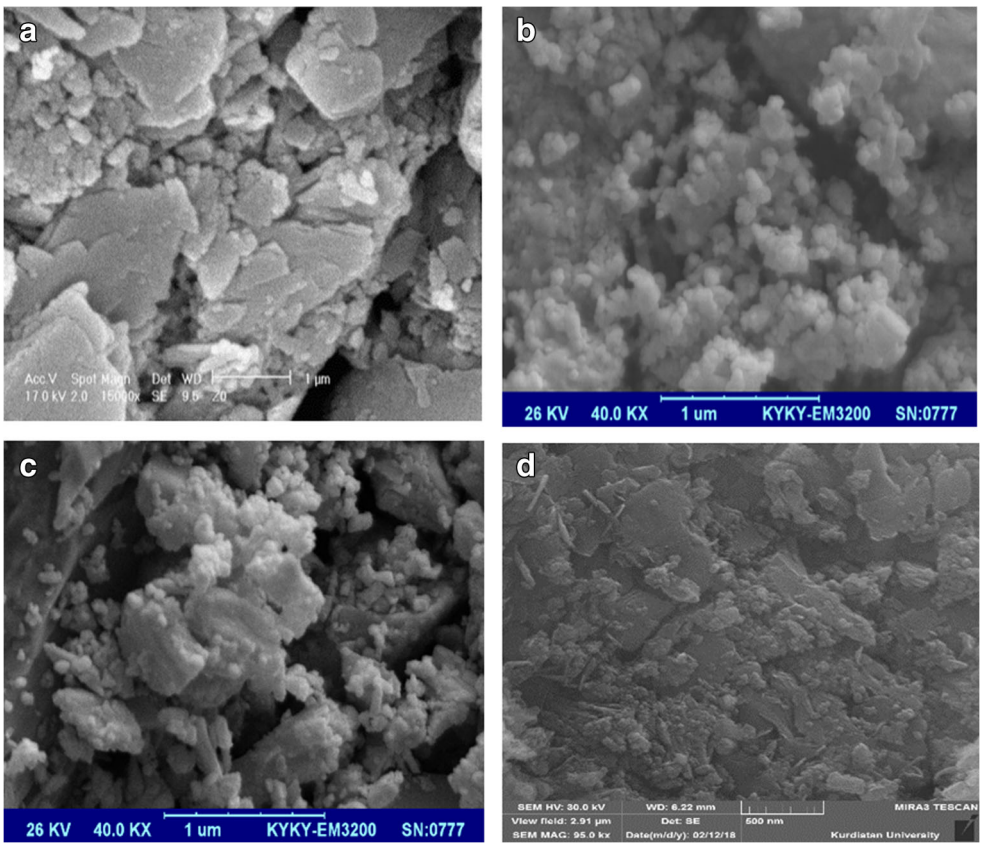

Fig. 2 FESEM micrographs of zeolite beads: Ze (**a**), ZnA (**b**), ZnB (**c**), and Ag (**d**) samples (Ze: uncoated zeolite, ZnA and ZnB: ZnONP-coated zeolite, Ag: AgNP-coated zeolite)

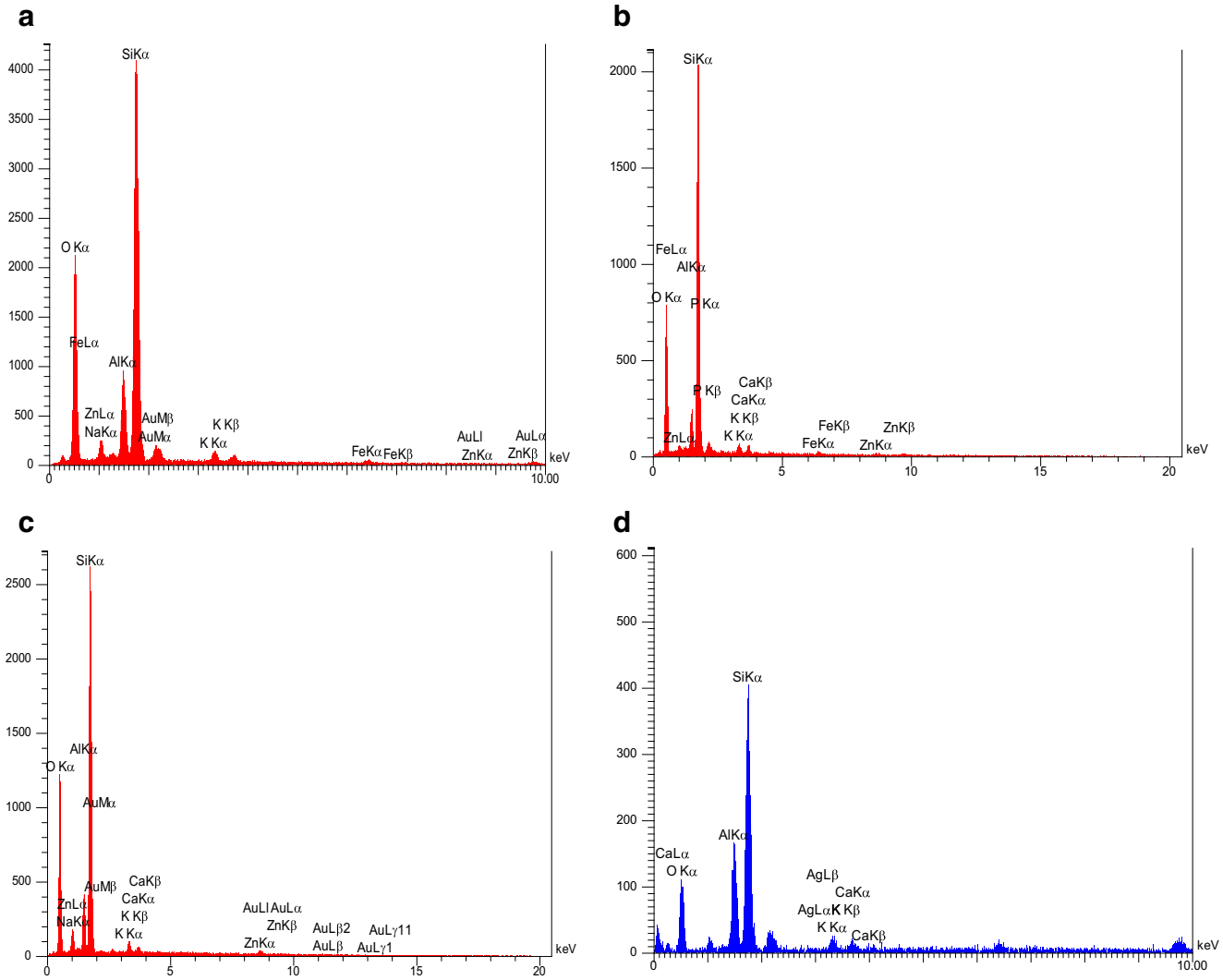

Fig. 3 @EDS spectrum of zeolite beads: Ze (**a**), ZnA (**b**), ZnB (**c**), and Ag (**d**) samples (Ze: uncoated zeolite, ZnA and ZnB: ZnONP-coated zeolite, Ag: AgNP-coated zeolite)

Tube test

The results of tube test for *A. hydrophila* showed that ZnONP- and AgNP-coated zeolite were able to inactivate 100% of bacterial cells after 24 h of contact time. In the contrast, the density of bacterial cells was counted approximately $(4.76 \pm 38.01) \times 10^4$ CFU/mL in tube that contained uncoated zeolite (Ze) after 24 of contact period.

Table 1 The EDS elemental composition of uncoated zeolite (Ze) and coated zeolite with ZnONPs (ZnA and ZnB) and AgNPs (Ag)

Samples	Zinc (%)	Ag (%)
Ze	0.26	ND
ZnA	1.07	ND
ZnB	1.38	ND
Ag	ND	1.13

Flow test

The antibacterial activity of filter columns filled with ZnONP- and AgNP-coated zeolite as filter media is shown in Fig. 6. Based on the results, the bacterial count decreased

Table 2 Percentage of zinc and silver content in zeolite beads in uncoated zeolite (C) and coated zeolite with ZnONPs (ZnA and ZnB) and AgNPs (Ag) (mean ± SD, $n = 3$)

Samples	Zinc (%)	Ag (%)
Ze	0.0009 ± 0.00005^a	0.00004 ± 0.00001^a
ZnA	1.68 ± 0.13^c	ND
ZnB	0.61 ± 0.07^b	ND
Ag	ND	0.12 ± 0.02^b

Values with different letters in each column are significantly different (ANOVA, $P < 0.05$)

ND not detected

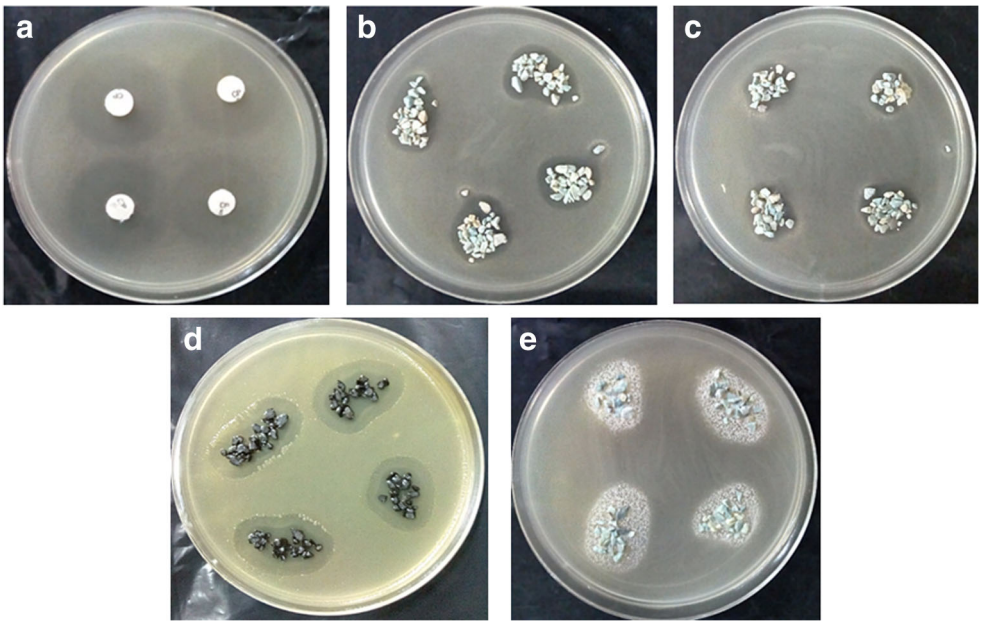

Fig. 4 The inhibitory zones formed by Cp (**a**), ZnA (**b**), ZnB (**c**), Ag (**d**), and Ze (**e**) samples for *A. hydrophila* on agar plate (Cp: ciprofloxacin disk, Ze: uncoated zeolite, ZnA and ZnB: ZnONP-coated zeolite, Ag: AgNP-coated zeolite)

significantly in treatment with infected water and without filter media during 96 h of filter run ($P < 0.05$). In uncoated zeolite treatment (Ze + infected water), the bacterial cells increased after 2 h of water passage through filter column but a decreasing trend was observed then up to 96 h ($P < 0.05$). The water passage through filter column filled with ZnA treatment (ZnA + infected water) for 5 h led to increased bacterial density but a decreasing trend was recorded then up to 96 h ($P < 0.05$). The bacterial density increased up to 5 h of water passing through filter column filled with ZnB sample (ZnB + infected water), while a significant reduction in bacterial density was recorded up to 24 h ($P < 0.05$) and this trend continued up to 96 h ($P > 0.05$).

The antibacterial efficiency of filter columns filled with ZnONP- and AgNP-coated zeolite as filter media against *A. hydrophila* during 96 h of water passage through filter columns is shown in Table 3. The efficiency of filter column filled with ZnA sample increased significantly from 3.32 to 9.22% during 24 h and this value increased to 34.84% after 96 h ($P < 0.05$). The efficiency of filter column that contained ZnB sample was recorded as 21.45% and 23.77% in 24 and 96 h, respectively ($P > 0.05$). The Ag media increased the efficiency of filter to 100% in 24 h and this trend was recorded up to 96 h ($P < 0.05$). At all times, the highest antibacterial efficiency was observed in filter column filled with Ag media ($P < 0.05$). The efficiency of filter that contained ZnB samples was significantly higher than filter of ZnA up to 24 h but this value decreased significantly at 96 h ($P < 0.05$).

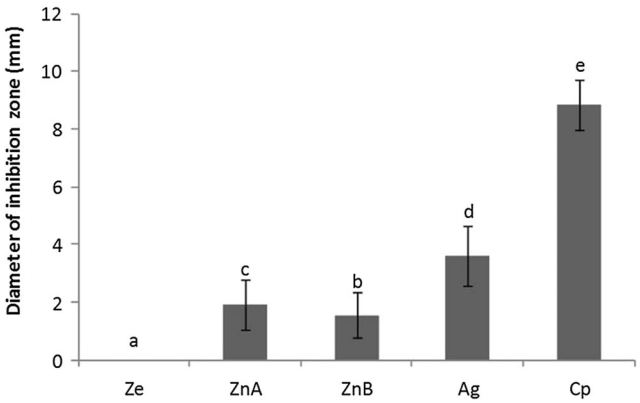

Fig. 5 Diameter of inhibition zone formed by different samples for *A. hydrophila* on agar plate (mean ± SD, $P < 0.05$). Ze: uncoated zeolite, ZnA and ZnB: ZnONP-coated zeolite, Ag: AgNP-coated zeolite, Cp: ciprofloxacin. The data with different letters are significantly different (ANOVA, $P < 0.05$)

Release of zinc and silver from filter columns into water

The amount of total zinc released from one kind of filter column that contained ZnONPs (type ZnA) samples was significantly higher than other filter columns ($P < 0.05$). There was no significant difference between total zinc released from filter columns filled with uncoated zeolite (Ze) and other kind of filter media coated with ZnONPs (type ZnB) ($P > 0.05$). The concentration of released total silver into water from filter column that contained Ag media was 36.38 ± 5.71 μg/L after 96 h (Table 4).

Response of fish to *A. hydrophila* challenge

The mortality rate of common carp (*C. carpio*) juveniles cultured in different treatments for 44 days is shown in Fig. 7. The mortality rate of fish increased significantly in treatments II, III, IV, and V compared to treatment I (negative control) ($P < 0.05$), whereas no significant difference was observed in treatments I and VI ($P > 0.05$).

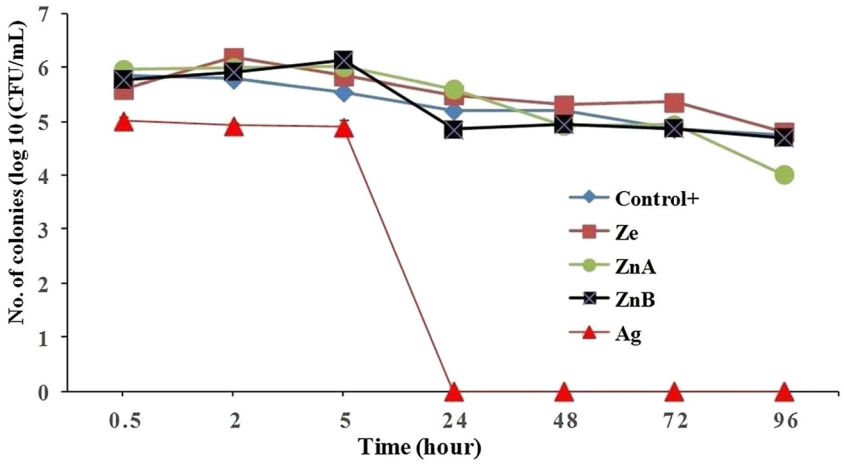

Fig. 6 Antibacterial activity of filter columns filled with uncoated zeolite (Ze), ZnONP-coated zeolite (ZnA and ZnB), and AgNP-coated zeolite (Ag) as filter media against *A. hydrophila* in flow test during 96 h (mean ± SD, $P < 0.05$) (control+: without any filter media)

Symptoms of *A. hydrophila* infection in fish cultured in challenged treatments (II, III, IV, and V) for 44 days are shown in Fig. 8. The fish infected with *A. hydrophila* after challenge with the density of 1.5 ± 10^6 CFU/mL showed signs of skin darkness, exophthalmia, eyes and fins bleeding, swollen abdomen, and erratic swimming. The signs of infection were much less and only observed in dead fish in treatment VI (cultured with water treated with AgNP-coated zeolite).

Accumulation of zinc and silver in fish tissues

The zinc and silver contents in gill and liver of fish after 44-day culture under different treatment are shown in Figs. 9a, b and 10a, b, respectively. There was no significant difference between the amount of accumulated zinc in gill of fish in treatment I (control) and treatments II and III ($P > 0.05$). The accumulation of zinc in liver tissue was significantly higher in treatment III than treatments I (control) and II ($P < 0.05$). The silver content increased significantly in gill and liver of fish cultured in treatment II compared to treatment I (control) ($P < 0.05$).

Discussion

This study was conducted to compare ZnONP- and AgNP-coated zeolite beads as antibacterial filter media for removing pathogenic bacterium (*A. hydrophila*) from water used in cultivation of common carp (*C. carpio*) juveniles. According to results obtained by characterization methods of prepared samples, spherical AgNPs and spherical and oval ZnONPs were formed correctly on the surfaces of porous zeolite beads. The results of a study showed that the mean diameter of ZnONPs coated on the surfaces of tanks used to culture larvae and fry of Indian major carp, rohu, *Labeo rohita* was about 122.4 nm (Swain et al. 2016). The mean diameter of ZnONPs in ZnB and ZnA samples was also measured as 80.8 and 114.7 nm, respectively. Mpenyana-Monyatsi et al. (2012) tested zeolite beads coated with AgNPs ranged from 40 to 90 nm for removal of *E. coli* from synthetic water. Silica beads that contained AgNPs with sizes ranging from 0.5 to 1 mm were also utilized for water disinfection (Quang et al. 2013). In the present study, Ag sample containing AgNPs with the mean diameter of 113.6 nm was synthesized.

The antibacterial activity of ZnONPs has been referred to several mechanisms such as direct contact with cell membrane (Brayner et al. 2006; Zhang et al. 2007), release of Zn^{2+} ions (Kasemets et al. 2009; Li et al. 2011), and formation of reactive oxygen species (ROS) (Jalal et al. 2010; Park et al. 2017). AgNPs also exert their antibacterial activity through attachment to cell wall (Lv et al. 2009; Morones et al. 2005), the ROS formation and thus causing oxidative stress (Carlson et al. 2008; Feng et al. 2000) as well as antibacterial silver ions released from AgNPs (Marambio-Jones and Hoek 2010; Sotiriou and Pratsinis 2010). The results of antibacterial assays of prepared media revealed that the ZnA samples formed the higher inhibitory zone for *A. hydrophila* than ZnB sample. This result is most likely due to higher zinc content of ZnA sample (Table 2) which allows the higher release of zinc ions (Table 4) with antibacterial properties (Kasemets et al. 2009; Li et al. 2011). Chakra et al. (2017) based on zone inhibitory tests showed that ZnONPs inhibited the growth of two gram-positive and two gram-negative bacteria. In the present study, the quantified value of the inhibitory zone obtained by Ag sample was significantly higher than ZnA and ZnB samples. This result indicates the higher antibacterial activity of AgNPs compared to ZnONPs. This may be attributed to the direct contact mechanism of AgNPs to destroy cell membranes of bacteria (Agnihotri et al. 2013). The antibacterial test results of chitosan-Ag/PVP nanocomposite film against *Staphylococcus aureus* exhibited a clear zone of 3–4 mm forming around the film (Wang et al. 2012a). Lv et al. (2009) also reported that inhibition zone of 10 mm for *E. coli* was formed around porous ceramic decorated with AgNPs. In the present study, the growth of *A. hydrophila* was inhibited by AgNP-coated zeolite and an inhibitory zone of 3.61 mm was recorded. The

Table 3 Comparison of antibacterial efficiency (%) of filter columns filled with uncoated zeolite (Ze), ZnONP-coated zeolite (ZnA and ZnB), and AgNP-coated zeolite (Ag) as filter media against *A. hydrophila* for 96 h in flow test (mean ± SD)

Time (h)	Filter media			
	Ze	ZnA	ZnB	Ag
0.5	$9.32 \pm 0.69^{b,D}$	$3.32 \pm 0.17^{d,D}$	$6.39 \pm 1.03^{c,C}$	$18.79 \pm 1.08^{a,C}$
2	$-0.25 \pm 1.79^{c,F}$	$2.74 \pm 1.46^{b,D}$	$4.10 \pm 0.17^{b,C}$	$20.27 \pm 0.86^{a,BC}$
5	$5.24 \pm 0.9^{b,E}$	$2.57 \pm 1.75^{b,D}$	$4.57 \pm 2.46^{b,C}$	$20.68 \pm 2.28^{a,B}$
24	$10.94 \pm 1.28^{c,CD}$	$9.22 \pm 1.32^{c,C}$	$21.45 \pm 0.38^{b,AB}$	$100 \pm 0.00^{a,A}$
48	$13.94 \pm 0.22^{c,B}$	$20.14 \pm 1.37^{b,B}$	$19.78 \pm 2.26^{b,B}$	$100 \pm 0.00^{a,A}$
72	$13.23 \pm 0.31^{d,BC}$	$19.9 \pm 0.79^{c,B}$	$21.02 \pm 0.35^{b,AB}$	$100 \pm 0.00^{a,A}$
96	$22.05 \pm 2.98^{c,A}$	$34.84 \pm 2.81^{b,A}$	$23.77 \pm 2.62^{c,A}$	$100 \pm 0.00^{a,A}$

Values with different lowercase letters in each row and capital letters in each column are significantly different (ANOVA, $P < 0.05$)

results of tube test also proved the antibacterial activity of both ZnONP- and AgNP-coated zeolite against *A. hydrophila*, so that 100% of bacterial cells were killed within 24 h of contact period. Schwartz et al. (2012) showed that the growth of *E. coli* suspension incubated on ZnONPs embedded in hydrogel film decreased with increasing NP content from 1 to 10 wt%. In the present study, efficient antibacterial activity against *A. hydrophila* was observed in ZnB and ZnA samples that contained 0.61% and 1.68% of zinc, respectively. AgNPs synthesized chemically with average size of 20 nm exhibited their antibacterial efficiency against *A. hydrophila* at concentrations of 153.6 μg/mL, 76.8 μg/mL, and 30.7 μg/mL (Sarkar et al. 2012). Mahanty et al. (2013) revealed that AgNPs synthesized using the papaya (*Carica papaya*) leaf extract with size ranged from 20 to 40 nm and round shape had the highest antibacterial activity against *A. hydrophila*, but this efficiency decreased with increasing size of oval NPs synthesized from eucalyptus (*Eucalyptus terticornis*) leaves to 60–150 nm. In current study, AgNPs coated on the natural zeolite surface with mean diameter of 113.6 nm showed very high antibacterial activity.

It is essential to coat nanomaterials with antibacterial performance on substrate for the application in water disinfection especially in aquaculture. To the best of our knowledge, there is no enough information on indirect usage of ZnONPs as filter media for removing pathogen microorganism from water. In the present study, ZnONP-coated zeolite was used as filter media for removing *A. hydrophila* from water in a recirculating system. The result indicated that the ZnONP-coated zeolite (ZnA and ZnB) were unable to remove the bacteria from water in the first 5 h of water passage through filter column but a significant removing of the bacterial cells was recorded after 24 h. The antibacterial efficiency of ZnA and ZnB samples increased to 34.84% and 23.77% after 96 h, respectively. The higher antibacterial activity of ZnA samples was also confirmed in zone of inhibition test. The antibacterial activity of ZnONPs can be verified under UV light, ambient light, and dark conditions. The findings of a study revealed that UV-A irradiation significantly influenced on the antibacterial activity of ZnONPs towards *E. coli* and *S. aureus* compared to unirradiated ZnO (Sirelkhatim et al. 2015). Park et al. (2017) confirmed that ZnONPs effectively can absorb UV light below 380 nm to produce ROS. They also found that the antibacterial result of these NPs against *S. aureus* was related to ROS generation rather than dissolved zinc ions and ZnONPs themselves. In the present study, the antibacterial results of ZnA and ZnB samples were achieved under ambient light condition and most probably due to release of total zinc or direct contact of ZnONPs with bacteria membrane. The higher antibacterial efficiency of ZnA sample can be attributed

Table 4 The concentrations of total zinc and total silver released from filter column filled with different filter media into the water after 96 h (mean ± SD)

Filter media	Concentrations of total zinc and silver released into the water (μg/L)	
	Zinc	Silver
Ze	179.33 ± 4.70^{a}	ND
ZnA	251.82 ± 5.07^{b}	ND
ZnB	180.28 ± 8.48^{a}	ND
Ag	ND	36.38 ± 5.71

Values with different letters in each column are significantly different (ANOVA, $P < 0.05$)

ND not detected

Ze uncoated zeolite, *ZnA and ZnB* ZnONP-coated zeolite, *Ag* AgNP-coated zeolite

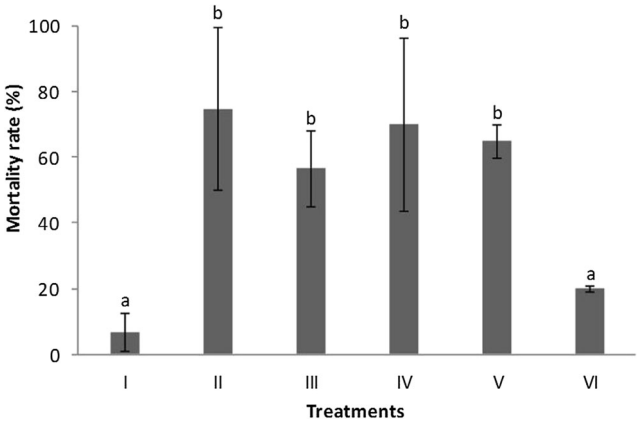

Fig. 7 Mortality rate of common carp (*C. carpio*) juveniles cultured under different treatments for 44 days. (I) Without filter media + without infected water (negative control). (II) Without filter media + infected water (positive control). (III) Uncoated zeolite (Ze) + infected water. (IV) ZnONP-coated zeolite (ZnA method) + infected water. (V) ZnONP-coated zeolite (ZnB method) + infected water. (VI) AgNP-coated zeolite (Ag) + infected water. The values with different letters are significantly different (mean ± SD, $P < 0.05$)

to the release of more total zinc (251.82 ± 5.07 μg/L) from filter column that contained ZnA sample. The application of filters that contained AgNPs for drinking water disinfection has already been reported (Gunawan et al. 2011; Lv et al. 2009; Mpenyana-Monyatsi et al. 2012; Quang et al. 2013). The ability of AgNP-coated zeolite as filter media for preventing fungal infection (*Saprolegnia* sp.) of rainbow trout (*Oncorhynchus mykiss*) eggs (Johari et al. 2016) and immobilized AgNPs on TEPA-Den-SiO_2 for bacterial disinfection in culture of *Penaeus vannamei* post-larvae (Sarkheil et al. 2016) have also been examined in aquaculture. The findings of the present study showed that the filter column filled with AgNP-coated zeolite had capability to remove the bacteria completely from water after 24 h of filter run. The concentration of total silver released into the water was 36.38 μg/L after 96 h of water treatment. Sarkheil et al. (2016) also reported that the concentration of total silver released from filter column filled with AgNP/TEPA-Den-SiO_2 reached to maximum level of 33 μg/L after 96 h. According to the result of another study, the released silver from filter column that contained AgNPs coated on zeolite was detected as 1800 μg/L after treating 10 L of infected water with *E. coli* for 10 min (Mpenyana-Monyatsi et al. 2012).

The release of NPs from water filters into the water affects not only the antibacterial efficiency and life span of these materials but also their bioaccumulation in aquatic organisms. The ecotoxicological effects of ZnONPs and AgNPs on different aquatic animals have been evaluated in many studies (Cong et al. 2017; Hao and Chen 2012; Johari et al. 2018; Khan et al. 2015; Salari-Joo et al. 2012; Yue et al. 2017). Also, previous studies indicated that silver ions (Wang et al. 2012b) and zinc ions (Ye et al. 2018), respectively, released from AgNPs and ZnONPs can contribute to their overall toxicity to aquatic organism. Subashkumar and Selvanayagam (2014) reported that the median lethal concentration (LC_{50}) of ZnONPs for common carp (*C. carpio*) juveniles was 4.89 mg/L. It is well known that liver is main organ participating in metabolism and gill as respiratory tissue is the first organ that contact with ambient water (Hao et al. 2013). Exposure of juvenile common carp (*C. carpio*) to ZnONPs (50 mg/L) for 30 days resulted in the bioaccumulation of 4000 mg/kg and 6000 mg/kg of NPs in gill and liver tissues, respectively (Hao et al. 2013). Khosravi-Katuli et al. (2018) found that the 96-h LC_{50} value of AgNPs for common carp (*C. carpio*) juveniles was 0.29 ± 0.02 mg/L. They also reported that exposure of fish to 0.4 and 0.8 mg/L of AgNPs for 21 days led to the accumulation of total silver in tissues in order of liver > gill > kidney. In the present study, 44-day treatment of water with ZnA and ZnB media did not result in significant increase in total zinc content of gill. The accumulation of total zinc in liver was significantly higher in treated water with ZnB sample than control. It may be due to the

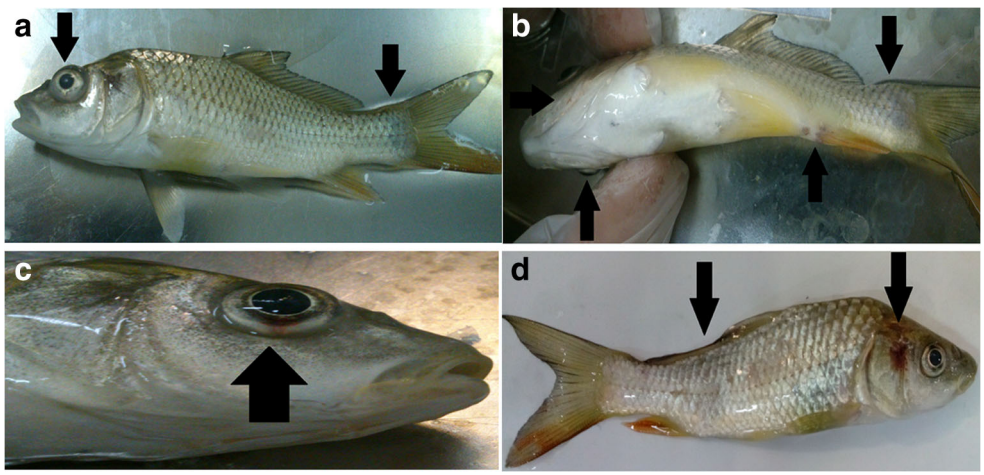

Fig. 8 Symptoms of *A. hydrophila* infection including exophthalmia (**a**), swollen abdomen (**b**), eyes and fins bleeding (**c**, **d**) in fish cultured in treatments II, III, IV, and V, respectively. II: Without filter media + infected water (positive control), III: Ze + infected water, IV: ZnA + infected water, V: ZnB + infected water

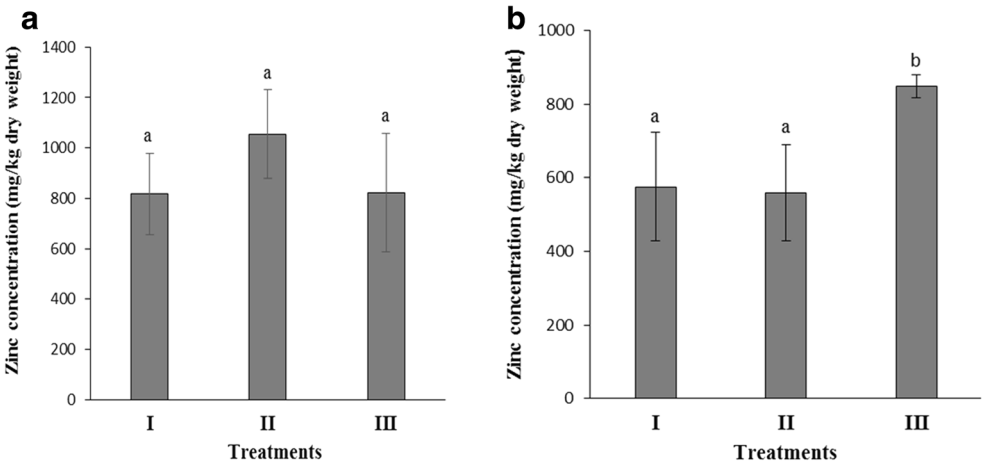

Fig. 9 Zinc concentration in gills (**a**) and liver (**b**) of fish cultured in different treatments. I: Ze + infected water (control), II: ZnA + infected water, III: ZnB + infected water. The values with different letters are significantly different (mean ± SD, $P < 0.05$)

higher release of total zinc from filter that contained ZnB sample after 44 days of filter run, although this amount was less than ZnA sample in flow test. The accumulation of total silver in the gill and liver tissues of fish cultured in water treated with filter contained AgNP-coated zeolite increased significantly compared to the control (untreated zeolite). Total silver accumulated in the liver also was higher than gill. However, the mortality rate of fish cultured under AgNP-coated zeolite filter did not increase significantly compared to the control (non-infected water). The mortality rate of cultured fish under infected water with *A. hydrophila* at density of 1.5 ± 10^6 CFU/mL and without filter treatment (treatment II) increased to 70%. The survival rate of fish cultured in infected water treated with ZnA and ZnB samples did not improve compared to fish cultured in infected water. In fact, the lower antibacterial efficiency of filters filled with ZnONP-coated zeolite resulted in occurrence of disease and thus fish death. These results revealed that the usage of AgNP-coated zeolite as filter media could effectively inactivate the *A. hydrophila* and prevent their infection in common carp juveniles.

Conclusion

In the present study, we synthesized zinc oxide and silver nanoparticle-coated zeolite beads in different methods as filter media for water disinfection in aquaculture. The strong antibacterial activity of synthesized samples against *A. hydrophila* was proven by the test tube and zone of inhibition tests. The efficiency of filters made of ZnONPs (ZnA and ZnB methods) and Ag samples in removing the bacterial cells from water after 96 h was 34.84%, 23.77%, and 100%, respectively. The survival rate of juvenile common carps (*C. carpio*) cultured in infected water treated with filter that contained AgNPs improved significantly compared to fish cultured in infected water. Although these results suggest that ZnONP-coated zeolite cannot be used as an effective antibacterial agent for water disinfection in aquaculture industry, it seems that further research is needed to improve the antibacterial efficiency of ZnONP-coated zeolite (e.g., under UV light condition).

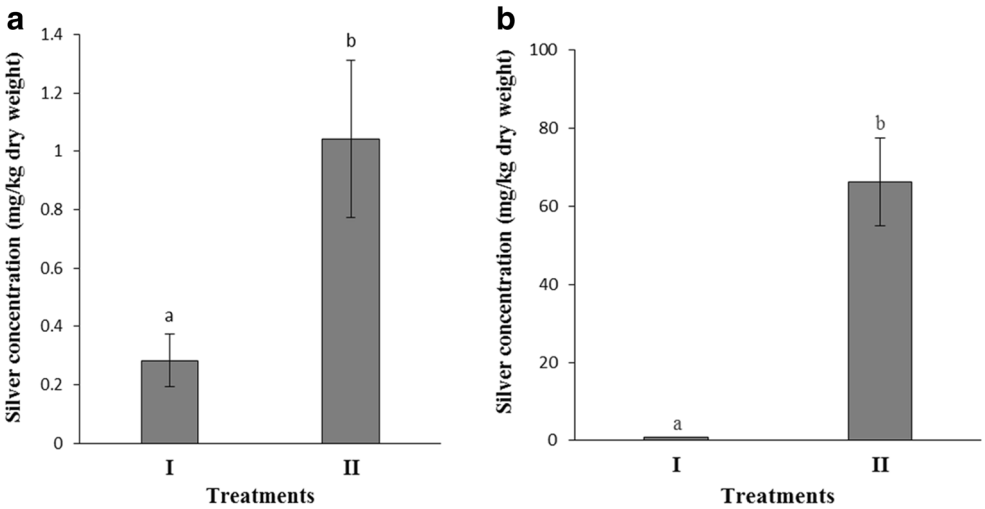

Fig. 10 Silver concentration in gill (**a**) and liver (**b**) of fish cultured in different treatments. I: Ze + infected water (control). II: Ag + infected water. The values with different letters are significantly different (mean ± SD, $P < 0.05$)

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Compliance with ethical standards

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

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