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Use of silver nanoparticles to control *Vibrio fluvialis* in cultured angelfish *Pterophyllum scalare*

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ABSTRACT: Nanoparticles have multiple applications, among which is their use as antimicrobial agents in aquaculture. The objective of this work was to determine the antibacterial effect of silver nanoparticles (AgNPs) against *Vibrio fluvialis* in cultured angelfish *Pterophyllum scalare*. AgNPs were synthetized through chemical reduction and characterized by UV–visible and infrared spectroscopy. Particle size ranged from 60 to 170.8 nm, and scanning electron microscopy revealed cubic and spherical forms. A minimal inhibitory concentration of 222.5 ppm was determined, as well as inhibition halos between 8.66 and 14.3 mm. Inhibition of *V. fluvialis* growth was observed upon contact with AgNPs. An 88% survival of infected fish was obtained when treated with AgNPs, in contrast to 100% mortality of fish that were not treated. No damage to internal or external organs was observed in fish exposed to AgNPs. We conclude that AgNPs exert an antimicrobial effect against *V. fluvialis,* and thus represent a new alternative to control diseases caused by this microorganism in *P. scalare* culture.

KEY WORDS: Silver nanoparticles · *Vibrio fluvialis* · Antibacterial effect · *Pterophyllum scalare*

1. INTRODUCTION

Aquaculture encompasses all activities aimed at the production, development, and marketing of aquatic organisms in controlled ecosystems, such as ponds, or in open systems that include lakes, rivers, dams, lagoons, and the sea. This activity plays a pivotal role in the production of organisms with different objectives, from the production of food to ornamental species, and it has become an industry with a great economic potential for Mexico (Norzagaray Campos et al. 2012).

According to the National Commission of Aquaculture and Fishery (CONAPESCA) of Mexico, in 2018, the yearly production of ornamental fishes was around 60 million individuals. The state of Morelos is the main supplier, producing 30 million individuals of 100 different species, among which the angelfish *Pterophyllum scalare* stands out in terms of annual production. This species has been widely marketed due to its beauty and its popularity among aquarists around the world (CONAPESCA 2017, 2018). However, intensification in the production of this species and the lack of control of international marketing has

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led to the mobilization of large numbers of microorganisms, including pathogens that, under different environmental conditions, can cause infections, environmental impacts, and financial losses for the producers (Verschuere et al. 2000).

Among the most common diseases in fish is vibriosis, caused by members of the genus *Vibrio*, including *V. fluvialis* (Lee et al. 2002, Redondo et al. 2004). The disease typically presents as a generalized septicemia, in which hemorrhages can be observed at the base of the fins, in the eyes and gills, and in internal organs like the liver, spleen, and kidney.

For the treatment and prevention of diseases in fish, a wide range of chemical compounds and antibiotics are used, which can have a negative impact on environments and on the personnel handling the fish (Cabello 2004). Excessive and inadequate use of antibiotics fosters bacterial resistance that is transmitted in the progeny, either directly through genes or indirectly through the acquisition of plasmids that contain antibiotic resistance genes (R plasmids), and can spread among species. Therefore, alternatives for the control of diseases without the use of antibiotics are currently being investigated (FAO 2016). A novel technology that has come to the attention of the aquaculture sector in recent years is the use of metal nanoparticles. Positive effects have been reported for the protection of Asiatic carp using nano-encapsulated complexes against the bacterium *Vibrio anguillarum* (Rajesh Kumar et al. 2008); these compounds have also been used to control the white spot syndrome virus (WSSV) in shrimp (Ochoa-Meza et al. 2019).

Metallic nanoparticles are generally spherical compounds, from 1 to 100 nm (equivalent spherical diameter), that can be synthetized from metals like gold, silver, platinum, copper, and palladium (Dananjaya et al. 2016, Hoseinzadeh et al. 2017). Recently, silver (Ag) has gained special interest due to its bactericidal effect in its ionic form (Ag^+) (Franci et al. 2015). Silver nanoparticles (AgNPs) permit the release of Ag^+ ions that are involved in several antimicrobial processes: they bind to and damage nucleic acids, which inhibits replication of microbial DNA, thereby inhibiting cell division; they interact with sulfur- and phosphorus-containing groups of proteins of the cell membrane causing ruptures; and Ag^+ causes the formation of reactive oxygen species, which are toxic to bacterial cells (Pelgrift & Friedman 2013). Therefore, this technology can be exploited for the control of diseases in aquaculture. In this work, we evaluated the antimicrobial effect of AgNPs against *V. fluvialis* in angelfish culture.

2. MATERIALS AND METHODS

2.1. Synthesis and characterization of AgNPs

AgNPs were prepared using the chemical reduction method according to Lee & Meisel (1982). $AgNO_3$ (Merck) was used as the precursor, and 1% sodium citrate (Merck) was used as the reducing agent. AgNPs were prepared at 2 concentrations: 90 and 180 ppm of $AgNO_3$. To characterize surface plasmon resonance of AgNPs, a UV–visible (UV–Vis) spectrophotometric scan of the solutions was made at a wavelength of 300–700 nm in a DU730 spectrophotometer (Beckman Coulter). Infrared (IR) spectroscopy was performed to determine vibrational spectra of metal citrate species present on the AgNP surfaces, using the IR Affinity-1S model 4350 (Shimadzu Scientific Instruments). The attenuated total reflection attachment was used for liquid samples, and transmittance was determined in the range of 400–4000 cm^{-1}, using 40 scans for each measurement. The size and morphology of AgNPs were analyzed with scanning electron microscopy (SEM) in a JSM-7401F microscope (JEOL). Particle sizes were determined from micrographs obtained by the diffraction of laser light by the AgNPs, using the program with which the equipment was preloaded and calibrated. Several random fields were searched for isolated AgNPs as well as AgNPs within agglomerates for measurement.

Distribution of particle sizes was also determined through laser light diffraction with a Partica LA 950 (Horiba). The scattered light collected on the detectors is used to calculate the particle size distribution of the sample analyzed using Mie theory (Niskanen et al. 2019). To determine the size of the AgNPs, 16 ml samples were taken (refractive index = 0.135, absorption = 3.99) and placed in a cell without any dilution. The experiments were performed in triplicate to obtain an average size distribution in volume.

The electrokinetic (zeta) potentials of the AgNPs samples were measured in water and cell media using a zeta sizer nano ZS particle-size analyzer (Malvern) in accordance with a certified method (Federal Register no. 1.34.2011.11076). The measurements were carried out in capillary cells at a temperature of the measuring cell of 25°C and without diluting the initial samples; the scattered light was collected at an angle of 90°. The zeta potential was obtained from the measured electrophoretic mobility values using the Smolukhovsky model.

2.2. *In vitro* antibacterial activity

Antibacterial activity was determined using the disk diffusion test by the Kirby-Bauer method, according to the Clinical and Laboratory Standards Institute (CLSI 2012a). *Vibrio fluvialis* strain QHL19 was previously obtained from kidney samples of diseased fish collected from a farm in Mexico City and identified by 16S rRNA gene sequencing (Monroy-Dosta et al. 2015). The bacterial inoculum was prepared adjusting to a concentration of 1×10^8 CFU ml^{-1} using a Densimat (bioMérieux) densitometer and sown on Müller-Hinton agar plates (Bioxon). AgNP and $AgNO_3$ solutions were prepared at concentrations of 90 and 180 ppm, and filter-paper disks of 0.6 cm in diameter were soaked with these solutions. The disks were then placed on the Müller-Hinton agar plates previously sown with *V. fluvialis* and incubated at 27°C for 24 h. The diameter of the inhibition halo was determined in triplicate.

The minimal inhibitory concentration (MIC) of AgNPs and $AgNO_3$ was determined through the microdilution method according to CLSI guidelines (CLSI 2012b). A *V. fluvialis* inoculum was prepared adjusting to a concentration of 1×10^8 CFU ml^{-1} using a Densimat, and then diluted to 1×10^6 CFU ml^{-1}. Serial 1:2 dilutions were made of the AgNPs or $AgNO_3$ solutions in tubes with 1 ml of Müller-Hinton broth; 8 dilutions were tested. Each tube was supplemented with 1 ml of the bacterial inoculum (1×10^6 CFU ml^{-1}). Tubes were incubated at 27°C for 24 h, and determinations were performed in triplicate.

The growth kinetics of *V. fluvialis* were determined in the presence of AgNPs or $AgNO_3$. For this, 50 µl of *V. fluvialis* at a concentration of 1×10^8 CFU ml^{-1} were supplemented with 50 µl of the AgNPs or $AgNO_3$ solutions at 90 and 180 ppm concentrations, and incubated at 27°C. Growth of the bacterium was measured in a Densimat, every hour for 8 h, and a final determination was made at 24 h.

2.3. *In vivo* antibacterial activity

The fish used in this research were obtained from an ornamental-fish-producing center in Mexico City. The study was approved by the Ethics Committee of the División de Ciencias Biológicas y de la Salud de la Universidad Autónoma Metropolitana-Xochimilco. *Pterophyllum scalare* juveniles (n = 150) with an average weight of 0.50 ± 0.05 g and a standard length of 2.5 ± 0.06 cm were maintained for a 15 d acclimation period at an average temperature of 28°C, under the following conditions of water quality: pH 7; dissolved oxygen, 5 mg ml^{-1}; and 0.3 ppm of nitrates and nitrites. Fish were fed daily with a species-specific commercial diet at 10% of their weight, and distributed randomly in 40 l aquaria (8 fish per aquarium). Treatments included 1 control (without nanoparticles or bacteria), 1 infection control group (only with bacterial inoculum), 1 infected group treated with ciprofloxacin (500 mg per 40 l), and 2 infected groups treated with AgNPs, one at a 90 ppm concentration and the other at 180 ppm. The experiment was performed in triplicate. Experimental infection was induced in the fish by direct inoculation of the pathogen into the water; therefore, the route of entry was respiratory and digestive, at a final concentration of 1.25×10^7 CFU ml^{-1}. Ciprofloxacin and AgNPs were supplemented in the water 24 h after the infection, with AgNPs at 90 ppm to reach a final concentration of 1.125 ppm, and at 180 ppm to reach a final concentration of 2.25 ppm. Water was not changed throughout the experiment. After the pathogen was introduced, we monitored and characterized the clinical condition of the fish over 7 d, observing the development of signs and lesions typical of the disease, including erratic swimming, a distended abdomen, anorexia, bleeding of fins and eyes, torn fins, furuncles, and scale injuries, following previously described methodology (Fuentes Rodríguez & Pérez Hernández 1998, Carnevia et al. 2010, Vásquez-Piñeiros et al. 2010). To fulfill Koch's postulates and ensure that *V. fluvialis* was the causal agent of the infection and/or mortality, samples were taken from the injuries and kidneys of the diseased animals and inoculated on agar plates with TCBS medium, and were isolated afterwards on BHI medium. The presence of *V. fluvialis* was confirmed using API identification strips (bioMérieux).

2.4. Toxicity of AgNPs to fish

To determine whether exposure to AgNPs causes toxic effects in fish, a toxicity assay was performed in which 60 juvenile fish were exposed to AgNPs at 90 and 180 ppm concentrations for 96 h. Starting at the exposure, fish were monitored to observe any changes in their behavior, or damage at the external level or in internal organs, following the methodology described in the previous section.

2.5. Statistical analysis

Data obtained for *in vitro* experiments were analyzed with an ANOVA followed by the Dunnett test.

All data obtained for *in vivo* experiments, including signs and injury data, were deposited in a database in Excel 2010 and transferred to a statistical program (Systat 13.0) to determine significant differences by 1-way ANOVA ($p < 0.05$). When significant differences were obtained, multiple mean comparisons were made with a Tukey test to determine significant differences between treatments.

3. RESULTS

3.1. Synthesis and characterization of AgNPs

AgNPs were obtained by chemical reduction at 2 concentrations: 90 and 180 ppm. Fig. 1 depicts the plasmon peaks of the UV–Vis spectrum of the AgNPs. For AgNPs with a 90 ppm concentration, the plasmon peak was obtained at 411 nm, and for AgNPs at 180 ppm, the peak was obtained at 427 nm.

The IR spectra of AgNPs compared to the sodium citrate spectrum are shown in Fig. 2. Sodium citrate shows 2 high-intensity peaks at 1581 and 1387 cm^{-1}, which are drastically reduced with AgNPs.

Characterization by laser light dispersion indicates that the size distribution of the AgNP population varies between 60.7 and 155.8 nm at a concentration of 90 ppm and between 63.3 and 170.8 nm at a concentration of 180 ppm. SEM revealed that AgNPs at both concentrations are mostly spherical in shape, although rod-shaped (Fig. 3A) and cubic particles were also observed (Fig. 3B), with an average size of 60 nm; formation of conglomerates was also evident (Fig. 3A).

Zeta potentials were measured in water and cell media; values were −18.15 and −19.86 mV for 90 ppm AgNPs in water and cell media, respectively. For AgNPs of 180 ppm, values were−26.66 and −10.6 mV, respectively.

3.2. *In vitro* activity of AgNPs against *Vibrio fluvialis*

Antibacterial activity of AgNPs on *V. fluvialis* determined by disk diffusion presented inhibition halos of 8.66 ± 0.57 mm (mean ± SD) with AgNPs at 90 ppm concentration and of 14.3 ± 1.15 mm at 180 ppm concentration. MICs of 22.5 and 11.25 ppm were determined for AgNPs at 90 and 180 ppm, respectively, and MICs of 45 and 22.5 ppm were determined for $AgNO_3$ at 90 and 180 ppm, respectively.

The growth kinetics of *V. fluvialis* in contact with AgNPs or $AgNO_3$ showed better antibacterial activity when AgNPs were used at both concentrations ($p < 0.01$), than when $AgNO_3$ was used (Fig. 4), demonstrating that AgNPs exert a bactericidal effect by inhibiting *V. fluvialis* growth.

3.3. *In vivo* antibacterial activity of AgNPs against *V. fluvialis*

Results of the *in vivo* antibacterial activity show that starting at 48 h after inoculating the pathogen, 100% of fish in the infection control group presented

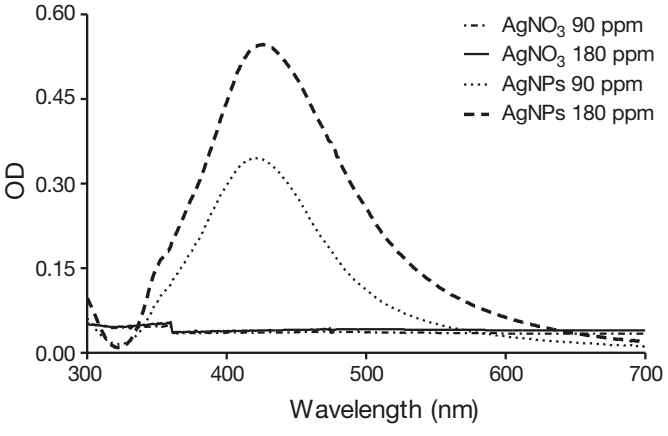

Fig. 1. UV–visible spectrum of $AgNO_3$ solutions and silver nanoparticles (AgNPs) at concentrations of 90 and 180 ppm
OD: optical density

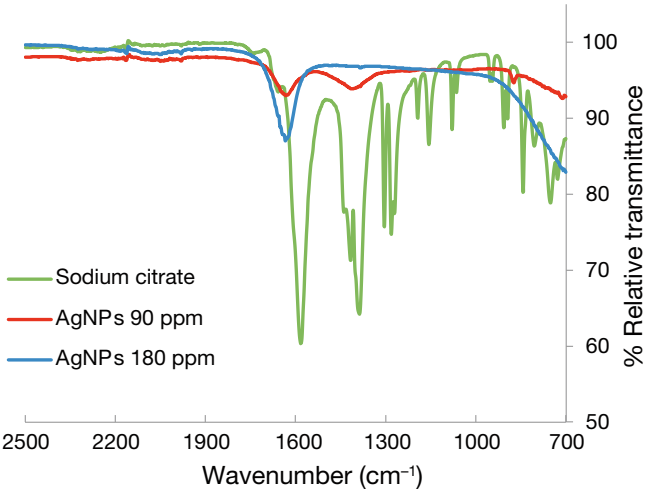

Fig. 2. Infrared spectrum of silver nanoparticles (AgNPs) at concentrations of 90 and 180 ppm compared to sodium citrate

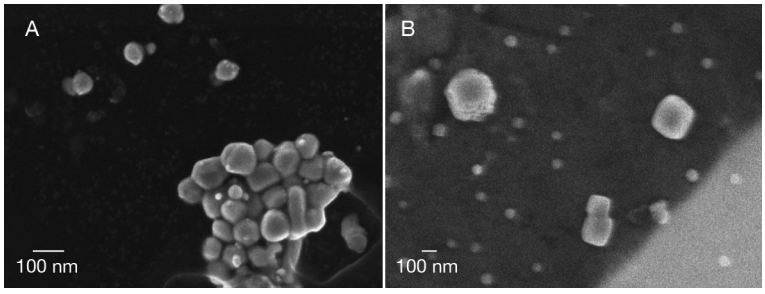

Fig. 3. Scanning electron micrographs of silver nanoparticles (AgNPs) at a concentration of 180 ppm: (A) spherical and rod-shaped and (B) cubic particles

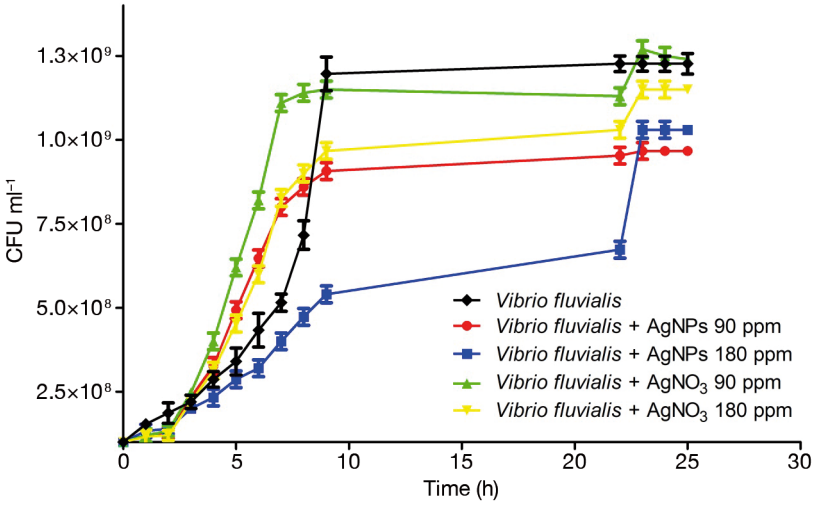

Fig. 4. Growth kinetics of *Vibrio fluvialis* in contact with silver nanoparticles (AgNPs) and $AgNO_3$. The black line shows data for *V. fluvialis* alone, whereas the other lines show the bacterium plus the indicated AgNP or $AgNO_3$ concentration. Data are means ± SD (n = 3)

all the signs and injuries characteristic of vibriosis in *Pterophyllum scalare* (Fig. 5), such as hemorrhages in fins, erratic swimming, gasping for air, tail hemorrhage, and anorexia (Fig. 5). All fish died on the third day after being exposed to the bacterium. In the groups treated with either nanoparticles or ciprofloxacin, although 100% of fish presented signs and injuries on the second day of exposure, they showed improvement starting at 72 h (Fig. 5), eliminating the injuries or signs completely on the fifth day. Treatments with AgNPs and ciprofloxacin resulted in 88 and 100% survival of the fish, respectively (Fig. 5).

Regarding hemorrhages in fins and tails, erratic swimming, gasping for air, and anorexia, ANOVAs showed significant differences between the infection control group and treatments with AgNPs and ciprofloxacin ($p < 0.001$), but treatments with both AgNP concentrations showed no significant differences ($p > 0.05$). Survival tests on the fifth day showed significant differences between the infection control group and treatments with AgNPs and ciprofloxacin ($p < 0.001$), but no significant differences were found between the control group and the group treated with ciprofloxacin, nor between the 2 concentrations of AgNPs ($p > 0.05$).

3.4. Toxicity of AgNPs to fish

The toxicity test did not reveal any negative effects on contact with AgNPs at both concentrations used in this study. We observed no damage to fins, eyes, gills, scales, or internal organs; dissection of the internal organs revealed that they were intact and in normal condition. No behavioral alterations were observed, and survival was 100%.

4. DISCUSSION

In this study, a chemical synthesis of nanoparticles was performed, which is the most common method to obtain AgNPs (Natsuki et al. 2015). We used sodium citrate as the reducing agent, following Pacioni et al. (2015), who indicated that one of the advantages of using this salt in the synthesis of AgNPs is that it acts both to reduce the metallic action and to stabilize the resulting nanoparticles.

Characterization of AgNPs is necessary because the physicochemical properties of the particles could have a significant impact on their biological effect (Lin et al. 2014). UV-Vis absorption spectroscopy allows detecting the absorption bands and showed plasmon peaks at 422 and 427 nm in the AgNPs at concentrations of 90 and 180 ppm, respectively (Fig. 1). Pillai & Kamat (2004) reported that when using the boiling method with different citrate concentrations, the plasmon of AgNPs shows a maximal absorbance starting at 420 nm, which agrees with our results. The IR results indicate that a union can be formed between citrate and AgNPs, which can be reasonably explained by the conformation/coordination model, as indicated by Wulandari et al. (2015). Other characteristics include the size and form of the particles, which we determined by laser light diffraction and SEM studies to be spherical, cubic, and rod-shaped at both concentrations, with an average size of 60 nm (Fig. 3).

The stability of AgNPs, as evaluated by the zeta potential, is maintained in the culture medium. AgNPs at 90 ppm had similar zeta potential in water and cell media, although the value of AgNPs at 180 ppm in culture medium decreased; however, the biological activity was maintained.

An issue to be considered is the formation of conglomerates, which was also reported by Šileikaitė et al. (2009). By evaluating the synthetized nanomaterials and considering the report by Sapsford et al. (2011), these AgNPs lie within the nanometric range, hence they can be used to evaluate their antimicrobial effect. The antimicrobial potential of nanoparticles depends on how they were obtained, their shape, size, and structure, as pointed out by He et al. (2010) and Hajipour et al. (2012). The size increases the contact surface, which allows them to destroy the bacterial membrane and cross inside the microorganism, causing intracellular damage (Knetsch & Koole 2011).

The results from the *in vitro* antibacterial activity test against *Vibrio fluvialis* through the plate diffusion method revealed that the 180 ppm concentration had a greater antimicrobial affect against *V. fluvialis* with an inhibition halo of 14.3 mm as compared to the 90 ppm AgNP concentration. Sivaramasamy et al. (2016) evaluated the antimicrobial activity of AgNPs obtained through biological synthesis for the inhibition of *V. parahaemolyticus* and observed inhibition halos of 19.27 mm. Wang et al. (2016) used AgNPs obtained through green synthesis for the control of *Vibrio* spp. and obtained inhibition halos of 16.4 and 13.6 mm for *V. anguillarum* and *V. alginolyticus*, respectively. Our results with *V. fluvialis* agree with these studies.

The MIC obtained in this study was 11.25 and 22.5 ppm (equivalent to 11.25 and 22.5 µg ml^{-1}) for 90 and 180 ppm AgNPs, respectively, which are lower

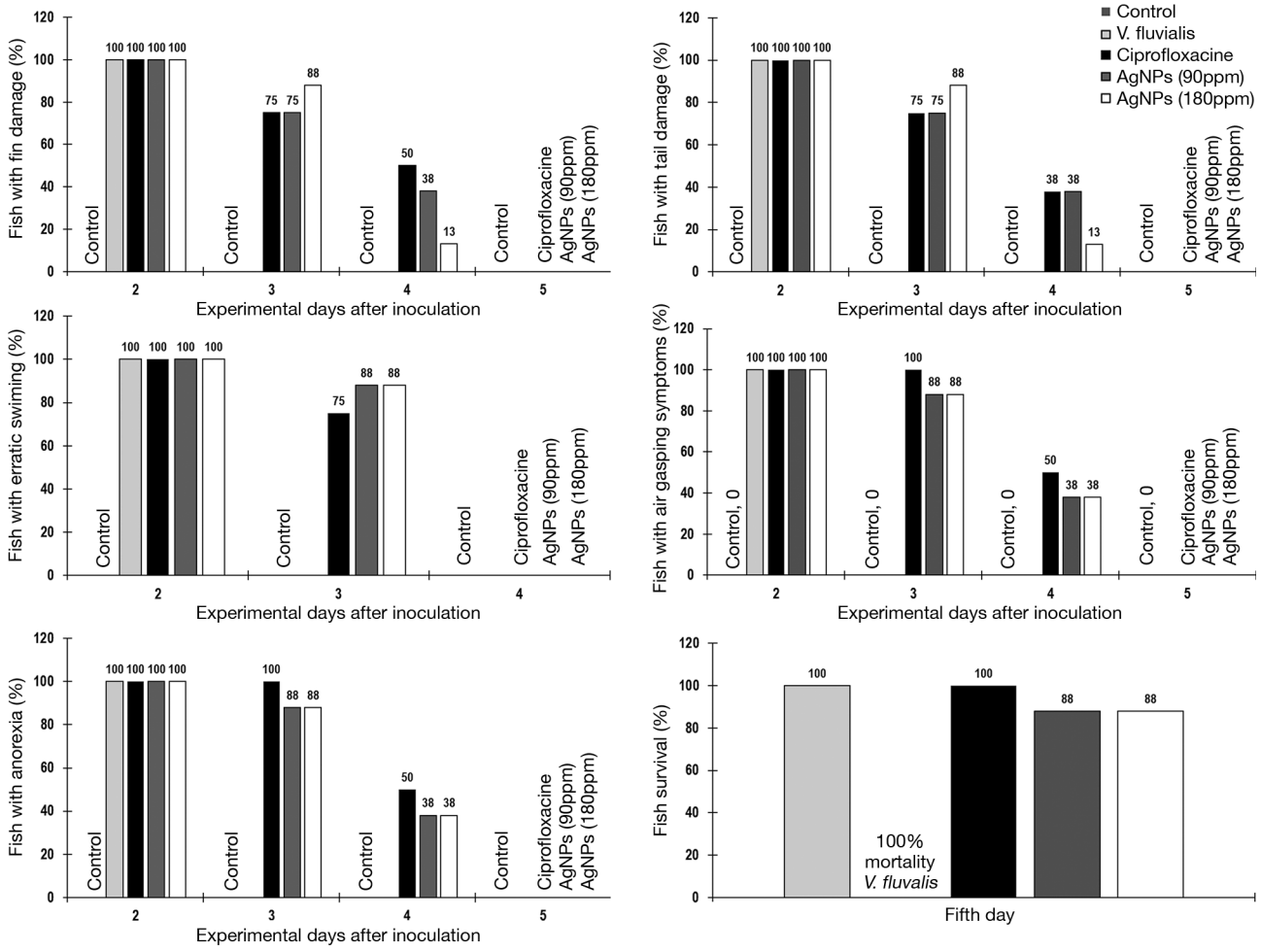

Fig. 5. *In vivo* antibacterial activity of silver nanoparticles (AgNPs) at concentrations of 90 and 180 ppm in *Pterophyllum scalare* infected with *Vibrio fluvialis*. Percentage of fish with hemorrhages in fins and tails, erratic swimming, gasping for air, and anorexia, as well as surviving fish are shown. Different letters above bars indicate significant differences ($p < 0.05$) among treatments at each time point

than those of $AgNO_3$, and lower than the values reported by Dananjaya et al. (2014), who found a MIC of 50 µg ml^{-1} for both *V. tapetis* and *Aliivibrio salmonicida*.

In vitro experiments involving disk diffusion, MIC, and the growth curve showed that the efficiency of AgNPs is greater than that of $AgNO_3$, possibly because AgNPs can act as a reservoir to release Ag^+ ions more efficiently within bacterial cells. Additionally, they can control the rate of Ag^+ release to adjust antimicrobial activity. AgNPs allow Ag^+ to be less susceptible to binding to natural ligands that can reduce their efficiency (Durán et al. 2016, Zheng et al. 2018).

Results of *in vivo* antibacterial activity revealed a positive response when using AgNPs to control *V. fluvialis*, obtaining 88% survival with the 90 and 180 ppm concentrations (Fig. 5); these results exceed those obtained by Sivaramasamy et al. (2016), who used AgNPs in shrimp and obtained 40.6% survival with 0.1 mg AgNPs in 60 d, using 10^6 CFU ml^{-1} of *V. parahaemolyticus*, as well as those reported by Vaseeharan et al. (2010), who obtained 71% survival with a 10 µg AgNP treatment and a concentration of 10^4 CFU ml^{-1} of *V. harveyi* over 14 d. It must be noted that we used only a single dose of 90 or 180 ppm of AgNPs, and obtained positive results after 4 d of treatment, including an improvement in the condition of the fish, gradually reduced bleeding, and tissue recovery. The hemorrhage disappeared on the fifth day, which we think is due to the elimination of the pathogen and the immune response of the fish.

In aquaculture, when the dose is high and the treatment is prolonged, there is some risk of toxicity to the fish, frequently causing damage in the liver, kidney, or some other organ, generally resulting in irreversible damage. On the other hand, if the antibiotic dose is very low and the treatment is short, the bacterium will not be eliminated and the risk of developing antibiotic resistance is increased (Yanong 2013). For this reason, the doses of AgNPs and the exposure period must be further studied in other ornamental fishes.

Regarding the toxicity test, no adverse effects on fish were observed with any of the AgNP concentrations used, since no signs or injuries were observed in the skin, tissues, and internal organs, nor were there changes in the behavior of the fish. These results agree with the report by Juarez-Moreno et al. (2017), who evaluated the effect of nanoparticles on *Litopenaeus vannamei* during 96 h of exposure; survival of the shrimp was >90% with all treatments. In the present study, dissection of internal organs revealed that they were intact and in normal condition, and we observed no changes in behavior. Similarly, Scown et al. (2010) evaluated the effect of nanoparticle exposure in rainbow trout *Oncorhynchus mykiss* at AgNP concentrations of 10 and 100 µg l^{-1} over a 10 d treatment, and observed no adverse effects or toxic damage. It must be pointed out that exposure periods and administered doses can vary, and outcomes depend on the species being studied; thus, constantly assessing the toxicity of AgNPs must be considered.

Our results show that the AgNPs used in this study exerted antimicrobial activity against *V. fluvialis*, without showing apparent toxicity signs; hence, they could be used as a novel alternative antimicrobial agent to improve the health of cultured angelfish.

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