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Efficacy of some aquaculture disinfectants enhanced by silver nanoparticles against *F. enterococci, Ps. Putida* and *Flavobacterium columnare*

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Abbreviation:
AgNs: Silvernanoparticles, cfu\m: colony-forming units/ml, *O. niloticus: Oreochromus niloticus*

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Abstract
In this study, three aquaculture disinfectants were evaluated alone and when enhanced by silver nanoparticles as well as a novel disinfectant (Envirolyte-Anolyte) for their efficacy against three common species of aquatic bacteria, *F. enterococci, Ps. Putida* and *Flavobacterium columnare*. The results revealed that, Envirolyte-Anolyte (1\500) and other disinfectants containing Ag nanoparticles (Virkon-S, 1% and Glutaraldehyde, 2%) were very effective against *Fecal enterococci*, *Ps. Putida* and *Flavobacterium columnare*, killing all bacteria within 1 min (*Fecal enterococci*, *Ps. Putida*) and 5 min (*Flavobacterium columnare*) of contact time, respectively. Formalin containing Ag nanoparticles was effective after 5 min of contact time against *Fecal enterococci, Ps. Putida* and *Flavobacterium columnare*.

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1. Introduction

Diseases don't occur as a single caused event but are the result of interactions of the pathogens, fish, and the environment (Newaj-fyzol et al., 2008). It has been shown by several workers that the normal bacterial flora of fish is a direct reflection of bacterial population of the environment they inhabit (Bauman et al., 1971). The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture (Zorrilla, et. al., 2003).

Streptococcus sp. has recently created a major disease problem in cultured tilapia and considered of high importance in recent years because of the increased reports of infections and the high economic losses caused by gram-positive bacteria in both wild and cultured fish (Domenech et al., 1996; Torky et al., 2010)).

O. niloticus naturally infected with *Ps. putida* revealed that fish suffer externally from exophthalmia, eye cloudiness, dark pigmentation, ascites, frayed dorsal fin, tail rot,

ulceration on the dorsal surface of the fish, detached scales, excessive secreted mucus and presence of haemorragic lesions on the skin, fins and gills with signs of asphyxia in some fish. Postmortem examination revealed slight enlargement of liver, spleen and kidney while intestine was filled with yellowish fluid (Jeyasekaran et al. ,2006 ;Salama and Gharib, 2009).

Columnare disease is one of the main causes of mortality in tilapia rearing and is responsible for large economic losses worldwide (Sebastião et al., 2011). It affects many cool- and warm water fish species, typically in warm waters at 20–25° C and above; however, it is not unusual to diagnose column are disease in many cultured and free-ranging fish species are considered at risk for infection and possible disease (Austin and Austin, 2007; Starliper, 2010). *Flavobacterium columnare* is the causative agent of the columnare disease; it is a dermotropic disease that affects a wide range of cultured and wild ranging freshwater fishes around the globe.

The preferred methods for controlling infectious diseases in warm water aquaculture is by using ″best management practices″ that include improving and controlling the environment, handling and transporting fish properly, stocking at reasonable densities, using proper quantities of high quality feeds (Plumb, 1999).

Agents (substances that destroy harmful germs) which are applied externally on man, animals, water or inanimate objects to check the microbial population are grouped in the category - disinfectants and sanitizers. These terms are usually applied to the agents useful in destroying pathogens in the aquaculture pond water and hence improve the water quality. The improved water quality will provide healthy ecological conditions, ultimately promotes the survival and growth of the fish, prawn and shrimp in aquaculture ponds. It has been proved that whenever there is minimum stress on aquaculture organisms, there will be automatically increased weight gain by improved feed intake.

Disinfection is the process that eliminates many or all pathogenic microorganisms from inanimate objects (APIC 1996). There are three major methods of disinfection available for aquaculture: chemical, ultraviolet irradiation, and ozonation. However, there are only a few chemicals approved or registered in the USA for disinfection in food fish aquaculture. There has been considerable recent interest in bacterial adaptation and resistance to antiseptics and disinfectants (Russell, 2004).

Emerging infectious diseases and the increase in incidence of drug and antimicrobial resistance among pathogenic bacteria have made the search for new antimicrobials inevitable. In the current situation, one of the most promising is the nanoparticles (Rai and Bai, 2011).

Therefore, it is necessary to discover novel strategies and identify new antimicrobial agents to develop the next generation of agents to control microbial infections in aquaculture production.

The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials. The mechanism of antibacterial effect of silver nanoparticles has been reported in the literature (Sondi and Salopek-Sondi, 2004), which suggests that the particles are bactericidal.

The use of metal nanoparticles for water disinfection is relatively new (Stoimenov et al., 2002; Zhang et al., 2003) due to their high reactivity and the large surface to volume ratio (Ichinose, 1992).

In this study, three aquaculture disinfectants were evaluated alone and when enhanced by silver nanoparticles as well as a novel disinfectant (Envirolyte-Anolyte) for their efficacy against three common species of aquatic bacteria, F. enterococci, *Ps. Putida* and *Flavobacterium columnare*. Disinfectant was deemed effective if its use resulted in a3 log10 reduction of bacterial growth within 1 min of contact time (Best et al. 1990).

2. Experimental

2.1. Ag Nanoparticles

Silver oxide-nanoparticles - (AgNs) stock solution was prepared by adding 0.5 and 1 mg of 15 nanometer diameter AgNPs powder (IBU-tec / Nanotechnology, 15 nm in diameter)

2.2. Disinfectants

Disinfectants and their working concentrations used in this study are listed in Table 1.

Table 1: Disinfectants and the concentrations used in the efficacy assay against *F. enterococci Ps. Putida and Flavobacterium columnare*. All chemicals were mixed in sterile distilled water immediately prior to use.

Disinfectant	Concentration
Formalin (mg/L)	250
Glutaraldehyde	(2%)
Virkon-S	(1%)
Ag nanoparticles[1]	(1mg\L)
Disinfectants containing Ag nanoparticles[2]	(1mg\L)
Envirolyte-Anolyte[3]	(1/500)
Envirolyte-Anolyte	(1/1000)

1: Envirolyte-Anolyte [pH 2.5-3.5, ORP>1150mV, Cactive ~500mg/l]. It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite (HClO, ClO_2, $HClO_3$, $HClO_4$, H_2O_2, O_2, ClO^-, ClO_2^-, ClO_3^-, O^-, HO_2^-, OH^- working substances, 1\500 = 2 mg /L active chlorine, 1\1000 = 1mg /L active chlorine). 2: Formalin (250 mg/L), Glutaraldehyde (2%) and Virkon-S (1%); plus (1%) Ag nanoparticles.

2.3. Bacterial Cultures

Stock cultures of *F. enterococci, Ps. Putida* and *Flavobacterium columnare* were isolated from the Fish Health Research Laboratory (Department of Veterinary Hygiene and Environmental Pollution, Faculty of Veterinary Medicine, Cairo University, Egypt). Cultures used for the disinfection assays were initially subcultured : (*E. ictaluri*) brain heart infusion tryptic soya broth (TSB) then on KF streptococcus agar medium containing 2,3,5 triphenyltetrazolium chloride (TCC 1%) supplement (Kenner et al. 1961) , (*Ps. Putida*) onto tryptic soy agar, (Austin and Austin, 2007) (*Flavobacterium columnaris*) onto Hsu-Shotts agar medium (Bullock et al., 1986) and allowed to grow for 24-48 h, and were then cultured into tryptic soy broth (Fisher Scientific) and incubated at 28° C overnight before harvesting.

Bacteria were harvested by centrifugation at 1,900 3 gravity for 10 min and were washed twice with 10 ml of sterile distilled water (DW); the final pellet was then resuspended in 5 ml of sterile DW. A 1–2-ml quantity of this stock solution was added to 5 ml of sterile DW to attain a final working solution of 1-2.5 $x10^7$ colony-forming units/ml. The starting concentrations of bacteria were the same as the concentration in the working solution, which served as the control.

2.4. Test Tube Test

Experimental exposures were performed at room temperature (25° C) in sterile, 2.0-ml micro-centrifuge tubes (USA Scientific Co., Ocala, Florida) by using a modification of a previously described disinfection protocol (Best et al. 1990; Mainous and Smith 2005). Each disinfectant was tested in duplicate. Briefly, using a volumetric pipette, 100 µL of the working solution of bacterial suspension were added to the sterile micro-centrifuge tubes, followed by the addition of 900 µL of either sterile DW (control) or the working concentration of the disinfectant.

2.5 Disinfectants containing Ag nanoparticles

100 µL of the working solution of bacterial suspension were added to the sterile micro-centrifuge tubes, followed by the addition of 800 µL of either sterile DW (control) or the working concentration of the disinfectant and addition of 100 µL of the prepared Ag nanoparticl-solution.

After 1 min, 0.1 ml aliquots were taken from each micro-centrifuge tube, added to 9.9 ml of DW, and mixed well by inversion to inactivate the disinfectant (Best et al. 1990). Tenfold serial dilutions were then made, and five 10-µL replicates of each dilution were plated onto tryptic soy agar using a multichannel pipette. Plates were incubated overnight at 30 °C, and colonies were counted by hand and recorded the next day.

If a disinfectant was not effective in reducing bacterial growth to zero after the 1-min exposure, the experiment with that particular disinfectant was repeated the next day using exposure times of 5, 10, 20, 30, and 60 min.

2.6. Statistical analysis

Statistical analysis of the data was performed using the Statistical Analysis System version 9.2 (SAS Institute, Cary, North Carolina). A paired t-test was implemented, and differences between means were considered to be significant at P-values less than 0.05.

For tests, mean bacterial plate counts made at various times were compared with the initial counts (at time 0) to determine when these compounds had a significant effect on the respective microorganisms.

3. Results and Discussion

O. niloticus naturally infected with *Ps. putida* revealed that fish suffer externally from exophthalmia, eye cloudiness, dark pigmentation, ascites, frayed dorsal fin, tail rot and ulceration on the dorsal surface of the fish (Plate 1). Clinical signs of columnaris include frayed necrotic fins with grayish to white

margins and depigmented and necrotic skin. The bacteria attack the fins, skin, and gills of fish. *Streptococcus sp*. has recently created a major disease problem in cultured tilapia (Plate 2) and considered of high importance in recent years because of the increased reports of infections and the high economic losses caused by gram-positive bacteria in both wild and cultured fish (Domenech et al., 1996; Torky et al., 2010).

At 10 min of contact time, Ag nanoparticles and Envirolyte-Anolyte (1/1000) significantly reduced the population of *Fecal enterococci* with a complete reduction of the population.

Table 2: Mean bacterial count (colony-forming units/ml) of *Fecal enterococci*, exposed to disinfectants over time (na: no measurement). Significant differences at the given time compared with 0 min ($P < 0.05$) are denoted by asterisks

Disinfectant or chemical	*F. enterococci*, contact time (min)						
	0	1	5	10	20	30	60
Control	$2x10^7$	na	na	na	na	na	1.9×10^7
Glutaraldehyde (2%)	$1.5x10^7$	0*	0*	0*	0*	0*	0*
Virkon-S (1%)	$2x10^7$	0*	0*	0*	0*	0*	0*
Formalin (250 mg/L)	$1.5x10^7$	$1.6x10^7$	$2.6x10^7$	$1.6x10^7$	$5x10^6$	$9x10^6$	$4x10^6$
Ag nanoparticles (1%)	$1.5x10^7$	$1.6x10^4$	$2.6x10^2$	0*	0*	0*	0*
Envirolyte-Anolyte (1/500)	$2x10^7$	0*	0*	0*	0*	0*	0*
Envirolyte-Anolyte (1/1000)	$2x10^7$	$1.6x10^4$	$2.6x10^2$	0*	0*	0*	0*

Glutaraldehyde (2%), Virkon-S (1%) and Envirolyte-Anolyte (1/500) were very effective against *Fecal enterococci*, killed all bacteria population within 1 min of contact time

Table 3: Mean bacterial count (colony-forming units/ml) of *Ps. putida* exposed to disinfectants over time (na :no measurement). Significant differences at the given time compared with 0 min ($P < 0.05$) are denoted by asterisks.

Disinfectant or chemical	contact time (min)						
	0	1	5	10	20	30	60
Control	$2.4x10^7$	Na	na	na	na	na	na
Glutaraldehyde (2%)	$2.5x10^7$	$2.5x10^7$	$1.5x10^7$	$1.5x10^6$	$5x10^5$	$1.5x10^4$	$1.9x10^4$
Virkon-S (1%)	$2.5x10^7$	$1.8x10^7$	$1.8x10^7$	$1.9x10^6$	$5x10^6$	0*	0*
Formalin (250 mg/L)	$2.5x10^7$	$2.5x10^7$	$2. x10^7$	$1.9x10^7$	$1.5x10^6$	$9x10^6$	$4x10^6$
Ag nanoparticles (1%)	$1.5x10^7$	$1.6x10^5$	$2.6x10^2$	0*	0*	0*	0*
Envirolyte-Anolyte (1/500)	$1.5x10^7$	0*	0*	0*	0*	0*	0*
Envirolyte-Anolyte (1/1000)	$1.5x10^7$	$1.6x10^4$	$2.4x10^2$	0*	0*	0*	0*

Glutaraldehyde (2%), Virkon-S (1%) and Envirolyte-Anolyte (1/500) were very effective against *Fecal enterococci*, killing all bacteria within 1 min of contact time.

At 1 min of contact time, Ag nanoparticles and Envirolyte-Anolyte (1/1000) significantly reduced the population of *Fecal enterococci* (produce a 3 log10 reduction in bacterial growth) (Table 2). Formalin was ineffective no differences were detected in control counts at time 0 versus 60 min.

The present results concerning *Fecal enterococci*, are similar to those of Mainous and Smith (2010) who found that glutaraldehyde (2%), and potassium peroxymonosulfate/sodium chloride (1%) were effective disinfectants, as each reduced or eliminated the number of detectable *E. ictaluri* and E. tarda within 1 min of contact time but formalin (250 mg/L) did not reduced bacterial counts even after 60 min of contact time.

Table 4: Mean bacterial count (colony-forming units/ml) of *Flavobacterium columnare* exposed to disinfectants over time (na: no measurement). Significant differences at the given time compared with 0 min ($P < 0.05$) are denoted by asterisks

Disinfectant or chemical	*Flavobacterium columnare* contact time (min)						
	0	1	5	10	20	30	60
Control	$2x10^7$	na	na	na	na	na	na
Glutaraldehyde (2%)	$2x10^7$	$1.5x10^7$	$1.5x10^6$	$5x10^2$	$1. 5x10^=$	$1. 5x10^3$	$1. 5x10^3$
Virkon-S (1%)	$2x10^7$	$2x10^7$	$1. 5x10^2$	0*	0*	0*	0*
Formalin (250 mg/L)	$1.9x10^7$	$1.6x10^7$	$2.6x10^5$	$2.6x10^4$	0*	0*	0*

Ag nanoparticles (1%)	$1.5x10^{7}$	$1.6x10^{3}$	$2.6x10^{2}$	0*	0*	0*	0*
Envirolyte-Anolyte (1/500)	$2x10^{7}$	0*	0*	0*	0*	0*	0*
Envirolyte-Anolyte (1/1000)	$2x10^{7}$	$2.6x10^{2}$	0*	0*	0*	0*	0*

Table 5: Mean bacterial count (colony-forming units/ml) of *Fecal enterococci*, *Ps. Putida* and *Flavobacterium columnare* exposed to disinfectants plus Ag nanoparticles, ($P < 0.05$)

Disinfectant or chemical / Contact time (min)	*F. enterococci,*			*Ps. Putida*			*F. columnare*		
	0	1	5	0	1	5	0	1	5
Glutaraldehyde (2%)	$2.5x10^{6}$	0*	0*	$1.5x10^{2}$	0*	0*	$2x10^{6}$	$2x10^{3}$	0*
Virkon-S (1%)	$2x10^{6}$	0*	0*	$1.9x10^{2}$	0*	0*	$2.1x10^{6}$	$1.51x10^{2}$	0*
Formalin (250 mg/L)	$1.5x10^{6}$	$1.6x10^{2}$	0*	$9x10^{6}$	$4x10^{2}$	0*	$1.9x10^{6}$	$1.51x10^{2}$	0*

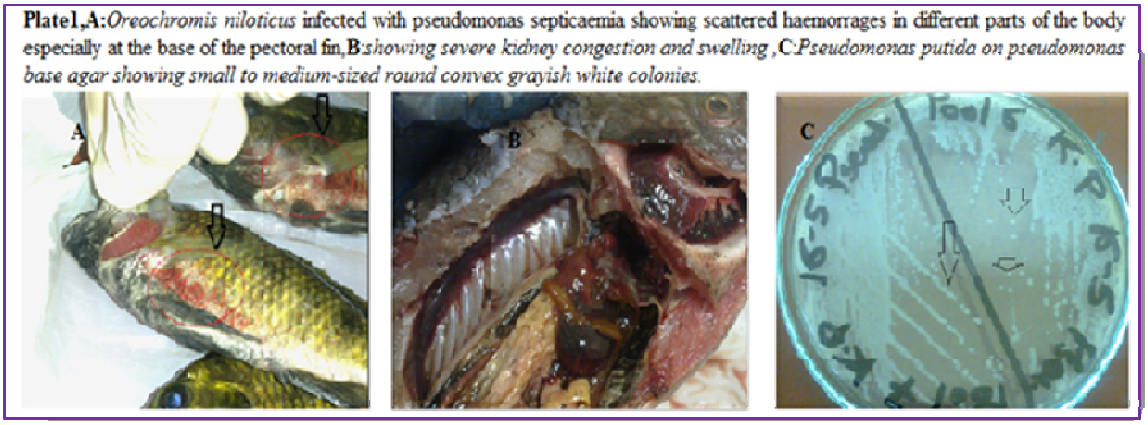

Plate1,A:*Oreochromis niloticus* infected with pseudomonas septicaemia showing scattered haemorrages in different parts of the body especially at the base of the pectoral fin,**B:***showing severe kidney congestion and swelling ,C:Pseudomonas putida on pseudomonas base agar showing small to medium-sized round convex grayish white colonies.*

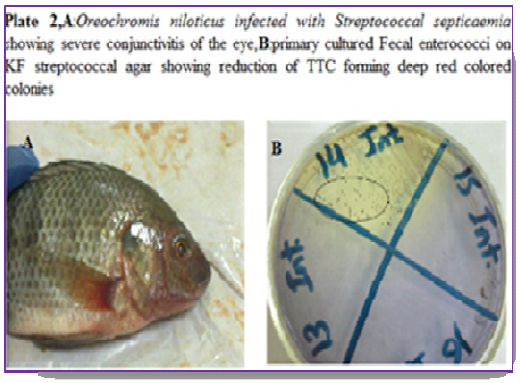

Plate 2,A:*Oreochromis niloticus infected with Streptococcal septicaemia* showing severe conjunctivitis of the eye,**B:**primary cultured Fecal enterococci on KF streptococcal agar showing reduction of TTC forming deep red colored colonies

After 1 min of contact time, Envirolyte-Anolyte (1/500) significantly reduced the population of *Ps. putida* with a complete reduction of the population.But,after 10 m exposure Envirolyte-Anolyte (1/1000) and Ag nanoparticles (1%) were completely destroyed *Ps. Putida* Virkon-S (1%) was ineffective against Ps. putida; within 20 min of contact time (did not produce a 3 $\log_{10}$ reduction in bacterial growth). However, at 30 min of contact time Virkon-S (1%) killed all bacteria, Table, 3.

Envirolyte-Anolyte (1/500) and (1/1000) was very effective against *Flavobacterium columnare*, killing all bacteria within 1 min and 5 min of contact time, respectively (Table 4). The bactericidal effect of the supper-oxide water (Envirolyte-Anolyte) against bacteria was due to the combined action of hydrogen ion concentration, oxidation-reduction potential and dissolved chlorine. Envirolyte-Anolyte is a strong acid, but it is different to hydrochloric acid or sulphuric acid. These acids have a strong degree of ionization, and when oxidation occurs, H^{+} is used and new H^{+} is generated (Iwasawa et al., 1993).

Ag nanoparticles (1%) was effective against *Flavobacterium columnare*, within 5min of contact time (produced a 4 $\log_{10}$ reduction in bacterial growth). However, AgNPs at10 min of contact time killed all bacterial population (Table 4).

Virkon-S (1%) and Glutaraldehyde (2%) were effective against *Flavobacterium columnare*, killing all bacteria within 10 min and 20 min of contact time, respectively (Table 3). Formalin (250 mg/L) was ineffective except after 30 min of contact time (i.e., produced a 4 log10 reduction in bacterial growth) (Table 4). However, after 60 min of contact time formalin was unable to produce complete reduction in the population of *Flavobacterium columnare* ($P < 0.05$).

At 10 min of contact time, Ag nanoparticles significantly reduced the population of *Fecal*

enterococci, *Ps. putida* and *Flavobacterium columnare*, with a complete reduction of the population.

In one hand, the three different species of bacteria used in this study, *Fecal enterococci, Ps. Putida* and *Flavobacterium columnare*, are distinguished from each other in their susceptibilities to disinfection.

On the hand, *Fecal enterococci*, *Ps. Putida* and *Flavobacterium columnare*, were responded similarly to Formalin (250 mg/L) after 1 min of contact time when they contained Ag nanoparticles (i.e., produced a 4 log10 reduction in bacterial growth) (Table 4).

After 5 min of contact time when it contained Ag nanoparticles (all bacteria were killed within 5 min).

Glutaraldehyde (2%) and Virkon-S (1%), their disinfectant efficacy were increased against *Ps. Putida* (killing all bacteria within 1 min) and *Flavobacterium columnare* (killing all bacteria within 5 min) by mixing Ag nanoparticles.

Because of their small size, nano-Ag particles have greater specific surface area when compared to the same mass of material in larger particles (Luoma, 2008) and have a greater surface area-to-volume ratio (Auffan et al., 2009). Auffan et al. estimate that a 10-nm particle has approximately 35-40% of its atoms on the surface compared to 15-20% of the atoms on a particle larger than 30 nm in diameter. This large surface area of nanoparticles relative to their mass or volume increases the reactivity and sorption behavior of the particle (Auffan et al., 2009; Tiede et al., 2008; U.S. EPA, 2010). Several possible modes of action are discussed in the literature on nano-Ag effects on bacteria and fungi. These are (1) membrane disruption through direct attachment of the nanoparticle to the bacterial membrane, (2) cellular invasion and enzyme disruption by nanoparticles, (3) changes in cell membrane permeability (4) interference with cellular S-containing compounds, and (5) intracellular ROS accumulation (Hwang et al., 2008; Kim et al., 2009; Lok et al., 2006; Morones et al., 2005; Pal et al., 2007; Panáček et al., 2006; Sondi and Salopek-Sondi, 2004). That several of these events might act together to result in cell death is probable, but the specific processes and interactions required for toxicity have not been fully confirmed.

There are many factors that can affect the efficacy of disinfectants, including time of contact, temperature, pH, concentration, and the presence of suspended solids and organic and inorganic constituents (Danner and Merrill 2006). The contact time required for greatest efficacy of a chemical disinfectant is variable and may be affected by factors such as the presence of organic and inorganic matter and the concentration of the chemical (Rutala et al. 2008). Generally, a higher concentration of a chemical will allow for a shorter contact time. In addition, as the temperature increases, the activity of most disinfectants also increases, thus shortening the contact time. The pH can also influence the efficacy of a disinfectant, possibly by altering the chemical or the surface of the cell, and it has been shown that quaternary ammonium compounds can be inactivated by high water hardness (Rutala et al. 2008). The present study evaluated the disinfectants under what were considered suitable conditions for bacterial survival. The experimental design did not take into account (1) the organic loads that are commonly present in aquaculture tanks or aquaria or (2) the ability of target organisms to be incorporated into biofilms; both of these factors would presumably reduce the efficacy of the disinfection process. Although there is only one report of biofilm formation by *Edwardsiella* (Gunduz and Tuncel 2006), a thorough cleaning, a longer contact time, or both may be required in actual aquarium or aquaculture situations to eliminate species of *Edwardsiella*.

Conclusion

Envirolyte-Anolyte (1\500) and other disinfectants containing Ag nanoparticles (Virkon-S, 1% and Glutaraldehyde, 2%) were very effective against *Fecal enterococci, Ps. Putida* and *Flavobacterium columnare*, killing all bacteria within 1 min (*Fecal enterococci*, *Ps. Putida*) and 5 min (*Flavobacterium columnare*) of contact time, respectively. Formalin containing Ag nanoparticles was effective after 5 min of contact time against *Fecal enterococci, Ps. Putida* and *Flavobacterium columnare*.

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