ORIGINAL ARTICLE

Comparison of *Escherichia coli* and *Klebsiella* Removal Efficiency in Aquatic Environments Using Silver and Copper Nanoparticles

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Received: 20 January 2021
Revised: 14 February 2021
Accepted: 28 March 2021

Abstract

Background: The main aspect of water purification to serve the human drinking purpose is the elimination of microbial agents and pathogens using the disinfectants. Although chemicals such as chlorine are the most common water disinfectants, the researchers have always sought to identify and introduce new disinfectants due to the formation of potentially carcinogenic byproducts. Owing to the high efficiency and lack of hazardous residues, nanoparticles have recently been used in many scientific activities. In this study conducted in summer 2018, the copper and silver nanoparticles were used to remove Escherichia coli (E. coli) and Klebsiella from the synthetic and real samples.

Methods: This experimental study was performed on Nano particles and by adding nanoparticles to samples (real and synthetic), the efficiency of removal of E. coli and Klebsiella was measured by MPN and pure plate methods.

Results: By optimizing the conditions, in 200 ppm concentration as 2ml with pH=7, it has the highest removal rate of 99.25% for E. coli, and in 250ppm concentration as 1.5 ml with pH=7, it has the removal rate of 81.25% for Klebsiella.

Conclusion: In this case study, we found that using Nano particles led to high level of efficiency at a short time; moreover, they were cost-effective and environmentally friendly.

Please cite this article as: Ghanavat Amani M, Jalilzadeh Yengejeh R. Comparison of *Escherichia coli* and *Klebsiella* Removal Efficiency in Aquatic Environments Using Silver and Copper Nanoparticles. J Health Sci Surveillance Sys. 2021;9(2):72-80.

Keywords: Water disinfection, Copper nanoparticles, Silver nanoparticles, Escherichia coli, Klebsiella

Introduction

Water is a limited vulnerable resource, which has been highly reduced by the the world population Explosion, increased use of drinking water, and the contamination of a great amount of water resources.[1-3] Although much improvement has been made today in the field of water remediation and treatment, contamination of water by microorganisms continues to be reported in drinking water resources.[4-8] Therefore, disinfection is one of the most important steps in water treatment to ensure that water is free from pathogenic bacteria.[9] The worldwide water contamination resulting from the presence of large amounts of microorganisms causes various water-borne diseases.[10] Every year, diarrhea leads to the death of more than 1.3 million children under 5 years of age across the world.[11, 12] Various bacteria account for the transmission of diseases through the contaminated water. Therefore, a major concern in different countries for the treatment of water is to supply pathogen-free, clean and safe drinking water.[13, 14] Bacteria are a group of prokaryotic microorganisms present in aquatic environments such as raw water and sewage. Gram-positive and gram-negative bacteria are responsible for a large number of human diseases such as diarrhea, hemolytic uremic syndrome, hemorrhagic colitis, etc.[15, 16] Therefore, the elimination of pathogenic agents is indispensable for the water treatment.[17] Recent advances in nanotechnology, especially the ability to produce nanoparticles in different shapes and sizes, have created a wide range of antimicrobial agents. Nano-materials have a higher area to volume ratio than larger particles with the same chemical composition, which makes them more biologically active.[18-20] Nanoparticles are of interest to researchers due to the unusual optical, chemical, photoelectrochemical, and electrical properties.[21] Copper is found to be a natural antimicrobial substance,[22] and silver is considered an effective antimicrobial substance used in surgery, advanced treatment, and pharmaceutical compositions.[19] The main mechanism for the effect of nanoparticles on bacteria is developed through the damage to protein DNA and destruction of cell wall.[15, 20, 23] Silver nanoparticles inhibit the respiratory system of bacteria without increasing the drug resistance. These elements have specific properties for microbial decontamination and are less expensive and easy to prepare.[13, 24, 25] Copper nanoparticles are not only potentially used in various medical, non-medical and hospital equipment combined with or coated on other materials, but also promising to replace conventional antibiotics.[19, 26] A lot of research has been conducted to examine the antimicrobial effects of silver and copper nanoparticles. Abu-Elala et al. (2018) evaluated the environmental risk of silver and copper nanoparticles as antimicrobial elements. This study investigated the effect of nanoparticles on the health of fish. The minimum inhibitory concentration (MIC) for each of the substances was considered for fish growth and analyzed for three weeks. Although low concentrations can improve antimicrobial performance, fish health is yet at risk in this situation.[1]

Thamilselvi et al. (2017) studied the silver nanoparticles as absorbents for the treatment of agricultural wastewater. The results showed that COD reduction was variable (77-79%) and absorption isotherms were determined by Langmuir method, and the effect on E. coli by Homs model represented the removal rate above 80%.[9] Shimabuku et al. (2017) investigated the water treatment through the virus inactivation using the silver and copper nanoparticles. In this study, bacteriophage T4 was subjected to various concentrations of nanoparticles, and the characterization of nanoparticles was done by XRD method. Due to the use of modified granular activated carbon (GAC), a lower level of copper and silver nanoparticles was recorded than the minimum allowable one, and it can be concluded that the modified GAC along with copper and silver nanoparticles could be used as an appropriate filter bed to remove contamination from drinking water.[27] Rosbero et al. (2017) studied the catalytic degradation of chlorpyrifos by silver and copper nanoparticles, and it has been found that nanoparticles have a high potential to remove pesticide contamination from water.[28] Morsi et al. (2017) studied the antimicrobial properties of copper and silver nanoparticles in water, and it was observed that higher concentrations of nanoparticles represented less contact time against Escherichia coli and Staphylococcus aureus. Also, on average, 1% concentration of the nanoparticles in 10 minutes had significant effects on the removal of bacteria.[29] Smith et al. (2014) examined the effect of copper nanoparticle sheets on the point-of-use water treatment. At high concentrations of nanoparticles, the bacteria level was lowered by Log 8.8, and the level of residual copper in the environment based on the proposed limit was 1 ppm.[30] In a study by Hsieh et al., by producing a thin film of silver and copper nanocomposites, it was concluded that the simultaneous effect of silver and copper nanoparticles would have a better effect on the removal of both gram-positive and gram-negative bacteria, and each of the nanoparticles alone is more successful in removing a group of bacteria.[31] In the present study, the effect of silver and copper nanoparticles on E. coli and Klebsiella bacteria in aquatic environment was studied and the optimal concentration, volume of the superior nanoparticle, and the suitable pH for further removal of bacteria were determined.

Methods

Sampling

As the study was carried out in a synthetic and experimental manner, the samples were prepared from sterile distilled water and microbial strains containing pure Escherichia coli and Klebsiella in sterile laboratory conditions under a laminar flow hood. Therefore, for the preliminary studies, sampling from water resources was not performed. The real samples were designated as Wtp1, Wtp2, Wtp3, Wtp4, Wtp5, Wtp6, and Wtp7, which were taken from Ahwaz rural water treatment plants based on surface water sampling method 9060A, "Standard Method", edition 2012. Real samples were taken from the raw water Wharf of the rural water treatment plants. No treatment process was performed on the samples and they were transferred to the laboratory in less than 6 hours at a temperature below 8 °C. The considered points on the map are displayed in Figure 1.

Bacteria

The bacterial strains were Klebsiella ATCC10031 and Escherichia coli ATTC25922, which were prepared from the laboratory of Jundishapur University of Medical Sciences.

Nanoparticles

The nanoparticles used in this project were the product of US Research Nanomaterial Co. which were prepared from Nano Pishgaman Mavad Iranian Co. A 1000ppm solution of 20nm silver nanoparticle and a 20nm copper powder were used. The solubilization was performed with copper powder and more dilute 50, 150 and 300ppm solutions were prepared (Figure 2).

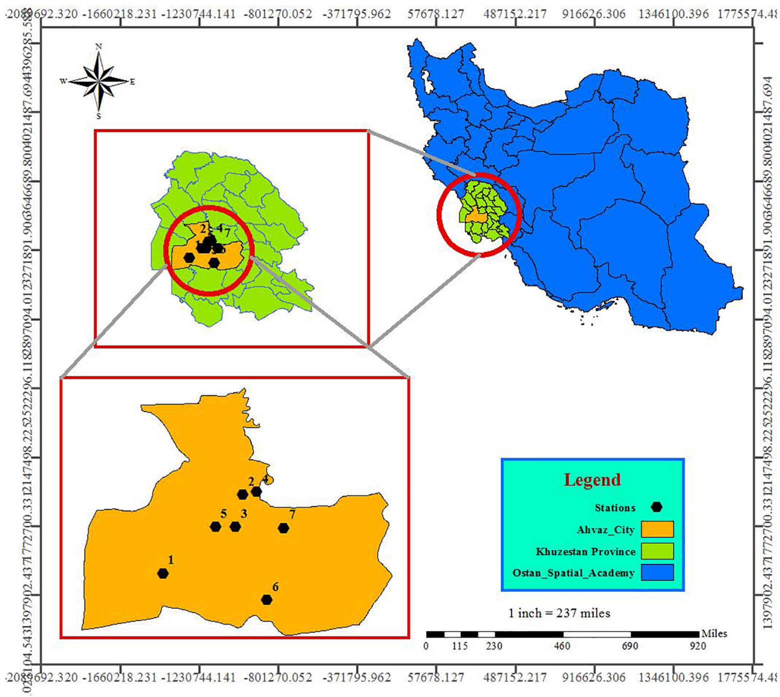

Figure 1: Sampling points

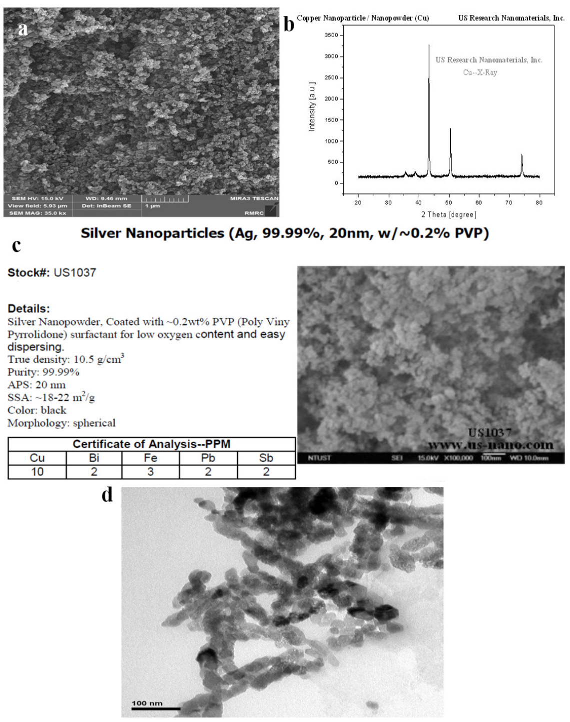

Figure 2: SEM images of (a) Cu nanoparticles and (b) XRD diagrams of Cu nanoparticles and, (c) and (d) SEM images OF Ag nanoparticles.

Test Method

The multiple-tube fermentation technique, section 9221: B was used with single- and double-strength concentration of lauryl sulfate broth (LSB), brilliant green lactose bile broth (BGB), and EC broth media to determine the level of Escherichia coli. The pure plate method was used according to section 9215: B (APHA) to determine the Klebsiella level. The sample taken from Escherichia coli strain was cultured using the 15-tube method and a single series of samples was considered as control, which was only in the vicinity of the culture medium. To investigate the effect of copper nanoparticles, 3 series of samples were placed in the vicinity of copper nanoparticles in concentrations of 50,150 and 300 ppm at 1 cc. To investigate the effect of silver nanoparticles, 3 series of samples were placed adjacent to silver nanoparticles in concentrations of 50, 150 and 300 ppm at 1 cc. At each stage, the E. coli level was read at the end of the culture. The synthetic samples prepared from Klebsiella strain were cultured in the bloodagar culture medium with sterile blood using the pure plate method. At each stage of the experiment, one plate was considered as control, consisting only of the sample and medium; in addition, 3 plates were placed adjacent to a sample of 1cc copper nanoparticles and 3 plates adjacent to 1cc silver nanoparticles. The number of Klebsiella colonies was read after the incubation for 24 hours. The Klebsiella colonies were mucous and silver-colored ones.[32] To evaluate the optimal concentration in the 100ml volumetric flasks, we prepared the solutions of 50-300 mg/L (ppm) from copper nanoparticles, and the samples were placed adjacent to different concentrations. The results are shown in Figure 3. Given that pH is an important factor in the optimization experiments, the copper solution was prepared as the superior nanoparticle with the optimized concentrations from the previous step for optimizing the pH parameter. After preparing the sample using the laboratory strains, it was divided into several parts and adjusted for the desired pH range using sodium hydroxide (NaOH) and hydrochloric acid (HCl). The 15-tube fermentation test was conducted for

Escherichia coli and the pure plate test was done for Klebsiella. Then, 1 cc of nanoparticle was added with optimized concentrations for each bacterium. The results and the removal rate are shown in Figure 4. After selecting the superior nanoparticle and the optimized concentration of nanoparticle, the used rate or volume was optimized. At this stage, the solutions of optimized concentration were prepared from copper nanoparticles and the samples were tested. The results are shown in Figure 5. The variables considered in this study are Escherichia coli, Klebsiella, optimization of conditions such as the level of studied nanoparticles, concentration of nanoparticles, and pH. The experiments were carried out in a three-month period, and the samples were tested on a weekly basis. The experiments were carried out in the microbiological laboratory of Khuzestan rural water and sewage.

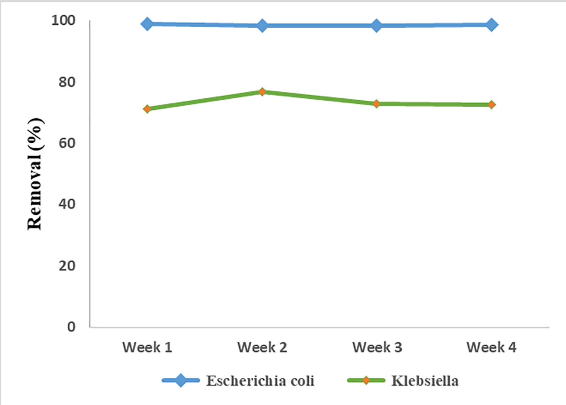

Figure 3: Results of the highest removal rate of bacteria in four consecutive weeks for 300ppm copper nanoparticles

Data Analysis

The results of each stage of the experiments were entered into Excel software. After collecting and finishing each step, the removal rate of each bacterium was compared with the control sample and the best nanoparticle with the highest removal rate was determined, and the removal rate diagrams were plotted. SPSS software was used for statistical analysis of data and results.

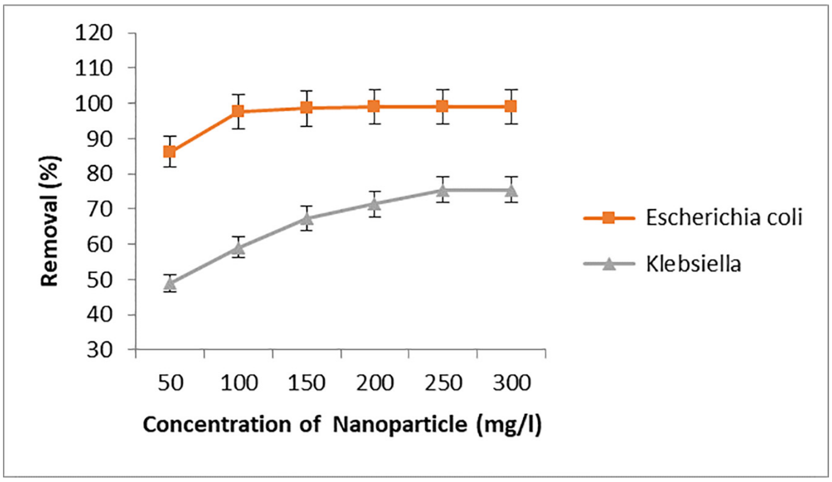

Figure 4: Removal rate based on optimization of nanoparticle concentration

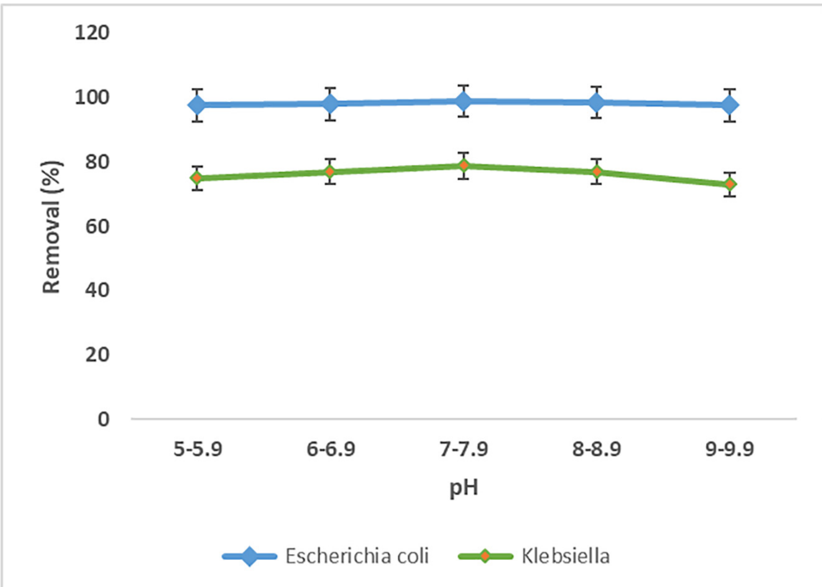

Figure 5: Removal rate based on pH optimization

Results

According to the objectives of the study and the methodology mentioned above, the results are as follows.

First-stage Results of Escherichia Coli Removal

At this stage, it was observed that the copper nanoparticles with 1 ml of nanoparticle of 300ppm concentration exhibited the highest rate of E. coli removal as 98.93% (Table 1).

First-stage Results of Klebsiella Removal

At this stage, it was observed that copper nanoparticles with with 1 ml of nanoparticle of 300ppm concentration had the highest rate of Klebsiella removal as 71.11% (Table 2). The same steps were performed for three consecutive weeks to determine the highest removal rate of Escherichia coli and Klebsiella bacteria using silver and copper nanoparticles. The results for each week are presented in the following section.

Based on the results obtained from the four-stage experiments, it was found that copper nanoparticles with 1 ml of used nanoparticle, and concentration of 300 ppm had a higher potential to remove Escherichia coli and Klebsiella bacteria compared with silver nanoparticles under similar conditions in aquatic environment. Therefore, in the next step, the copper nanoparticles as the superior ones were further studied by making changes in the optimization conditions. In the studies conducted on the antibacterial effects of copper and silver oxides on Escherichia coli and Bacillus subtilis, it was found that copper oxide nanoparticles exhibited more antibacterial activity than the silver nanoparticles.[33]

Nanoparticle Concentration Effect

The results showed that for removing Escherichia coli by copper nanoparticles, the maximum removal rate of 98.93% was obtained at concentrations of 200, 250 and 300 ppm. According to the optimization discussion, the concentration of 200 ppm was determined as the lowest concentration with the highest removal rate. In order to remove Klebsiella by copper nanoparticles, the highest removal rate of 75.51% was obtained at concentrations of 250 and 300 ppm; according to the optimization conditions, the concentration of 250 ppm was determined as the lowest concentration with the highest removal rate.

Effect of Solution pH

Based on the results, it was found that the highest removal rate of E. coli and Klebsiella was achieved by copper nanoparticles at concentrations of 200 and 250 ppm in the range of pH=7-7.9.

Study on the Effect of Nanoparticle Content

As to optimization of the contents of the nanoparticles for the removal of Escherichia coli based on the results, it was found that the highest removal rate of 99.25% by copper nanoparticles at concentration of 200 ppm in the range of pH=7-7.9 was obtained using 2 ml of nanoparticle (Figure 6). The highest removal rate of Klebsiella was obtained by copper nanoparticles at the optimized concentration of 250 ppm for pH=7-7.9 using 1.5 ml of nanoparticles.

Table 1: First-stage results of Escherichia coli removal in presence of silver and copper nanoparticles

Week 1	E. Coli										
Sample	pH	Method	Nano-particle	Nano-particle (ml)	Nano-particle Concentration (ppm)	Positive Tube Results			Contaminated Tubes	MPN/100 ml	Removal Rate
						0.1 ml	1 ml	10 ml			MPN/100ml
Control	7.17	15-tube	-	-	-	5	5	5	15	1600	0
Sample	7.17	15-tube	Copper	1	50	2	4	4	10	47	96.06
	7.17	15-tube	Copper	1	150	2	3	3	8	21	98.68
	7.17	15-tube	Copper	1	300	1	2	3	7	17	98.93
	7.17	15-tube	Silver	1	50	4	4	5	13	350	78.12
	7.17	15-tube	Silver	1	150	2	4	4	10	47	97.06
	7.17	15-tube	Silver	1	300	2	3	4	9	39	97.56

Table 2: First-stage results of Klebsiella removal in presence of silver and copper nanoparticles

Week 1	Klebsiella						
Sample	pH	Medium	Nano-particle	Used Nano-particle	Nano-particle Concentration	Number of Colonies	Removal Rate
				ml	ppm		
Control plate	7.22	Blood agar	-	-	-	45	0
Sample plate	7.22	Blood agar	Copper	1	50	23	48.88
	7.22	Blood agar	Copper	1	150	16	64.44
	7.22	Blood agar	Copper	1	300	13	71.11
	7.22	Blood agar	Silver	1	50	32	28.88
	7.22	Blood agar	Silver	1	150	28	37.77
	7.22	Blood agar	Silver	1	300	20	55.55

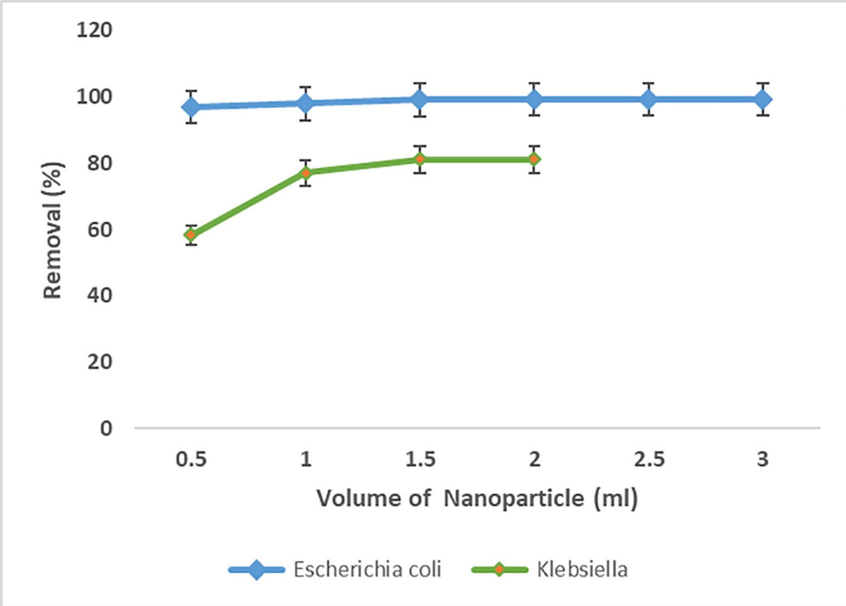

Figure 6: Removal rate based on nano-particle content optimization

After selecting the best nanoparticles and optimizing all conditions, the samples were taken from the Khuzestan rural water and sewage treatment plants to work on the real samples.

Based on the results regarding the removal rate of Escherichia coli in real samples taken from 7 points that supply water to rural water supply facilities and industries such as steel and rolling, it was observed that at concentration of 200 ppm for copper nanoparticle in the range of pH=7-7.9 using 2 ml of nanoparticle, the removal rate of E. coli was always above 98%. In the case of the real samples used for Klebsiella test, none of the colonies was associated with Klebsiella bacteria (Tables 3, 4).

Statistical Software Results

Study of Simultaneous Effect of Copper Nanoparticle Concentration and pH

By increasing the concentration of nanoparticles, the removal rate was expected to be increased, and it was observed that due to the increase in the surface caused by the increase in the concentration of nanoparticles, the removal rate was improved. Based on Figure 7, it is clear that the simultaneous effect of nanoparticle concentration and solution pH on the removal rate is positive.

Table 3: Test results for Escherichia coli removal in real samples

Sampling Point	pH	Nano-particle	Nano-particle Concentration (ppm)	Nano-particle (ml)	Number MPN/100ml Control	Number MPN/100ml Sample	Removal Rate MPN/100ml
Wtp1	7.89	Copper	200	2	1600	6	99.62
Wtp2	7.65	Copper	200	2	430	3.6	99.16
Wtp3	7.84	Copper	200	2	280	1.8	99.35
Wtp4	7.61	Copper	200	2	210	2	99.04
Wtp5	7.72	Copper	200	2	220	3.6	98.36
Wtp6	7.24	Copper	200	2	280	2	99.28
Wtp7	7.91	Copper	200	2	430	2	99.53

Wtp: Sample codes from 1 to 7

Table 4: Test results for Klebsiella removal in real samples

Sampling Point	pH	Nano-particle	Nano-particle Cocentration (ppm)	Nano-particle (ml)	Number of Conotrol Colony	Number of Sample Colony	Removal Rate
Wtp1	7.89	Copper	250	1.5	0	0	0
Wtp2	7.65	Copper	250	1.5	0	0	0
Wtp3	7.84	Copper	250	1.5	0	0	0
Wtp4	7.61	Copper	250	1.5	0	0	0
Wtp5	7.72	Copper	250	1.5	0	0	0
Wtp6	7.24	Copper	250	1.5	0	0	0
Wtp7	7.91	Copper	200	1.5	0	0	0

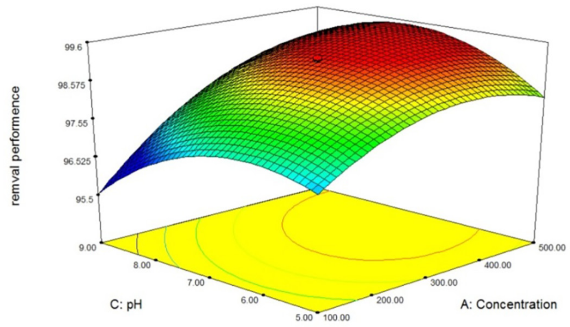

Figure 7: Simultaneous effect of nanoparticle concentration and solution pH on removal rate

Simultaneous Effect of the Used Volume of Copper Nanoparticles and pH

The volume of nanoparticle solution has a positive and incremental effect on the removal rate. Thus, it is clear that with the increase in the volume of the nanoparticle solution, the removal rate is also increased. Based on Figure 8, it is clear that the simultaneous effect of the used volume of nanoparticle and solution pH on the removal rate is positive.

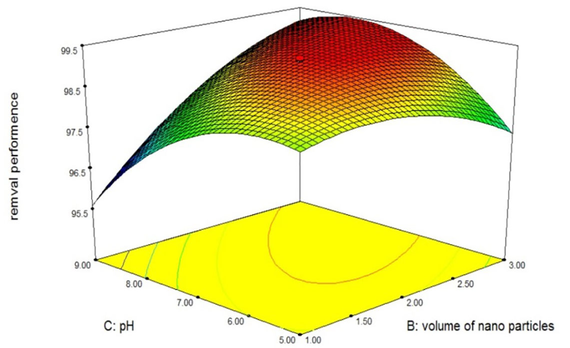

Figure 8: Simultaneous effect of nanoparticle volume and solution pH on removal rate

Simultaneous Effect of the Used Volume of Copper Nanoparticles and Nanoparticle Concentration

Based on the diagram, it is clear that the simultaneous effect of the used volume of nanoparticle and nanoparticle concentration on the removal rate is negative (Figure 9).

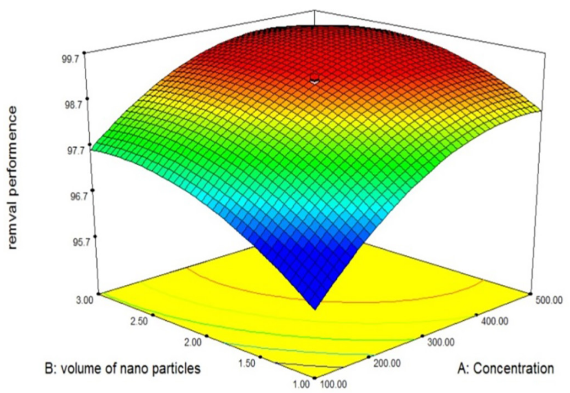

Figure 9: Simultaneous effect of nanoparticle volume and nanoparticle concentration on removal rate

$$Y=63.183 + 1.088A + 0.66B + (-0.233)C + (-0.258AB) + 0.558AC + 1.11BC$$

A: Nanoparticle concentration
B: Used volume of nanoparticle
C: solution pH
Y: Removal rate

By placing the optimal points of the removal rate according to the ANOVA table, the result was equal to 98.74%. According to the results, the R^2 value was 0.98, which indicates a significant relationship between the parameters affecting the removal rate. The average removal rate of all software blocks is 99.33% and the desirability level of the process is 90.31%. The optimal point for the model is defined on the basis of conditions that are operationally desirable. In this case, if the lowest concentration of nanoparticles, appropriate pH range, and lowest content of used nanoparticle solution are considered as optimization priorities, and on the other hand, if we expect to consider the maximum response or removal rate at the optimum point, then the removal rate of our research would be 98.74%.

Discussion

The summary of the optimization test results for copper nanoparticles in the laboratory conditions is displayed in Table 5.

Regarding the real samples taken for removing Escherichia coli at concentration of 200 ppm of copper nanoparticles in the pH range of 7-7.9 used as 2 ml, the removal rate above 98% was always achieved. It was not possible to study the real Klebsiella samples as they were not recognized. Based on the SPSS software analysis, it was found that the highest removal rate was obtained by increasing the nanoparticle concentration, increasing the volume of nanoparticle and maintaining pH in the neutral range. The simultaneous interaction of nanoparticle concentration and pH as well as the volume of nanoparticles had a positive effect on the removal rate. The R^2 value of the process according to the ANOVA table was 0.98, indicating that there was a significant relationship between the parameters affecting the removal rate. Based on the obtained equation and by replacing the related values, the best computational removal rate was 98.74%, which is close to the best removal rate from the laboratory optimization equal to 99.25%. In a study on the removal of Klebsiella bacteria using nanoparticles, it was found that Klebsiella had a higher resistance to the nanoparticles relative to E. coli due to having a protective protein capsule, and that is why more nanoparticles are needed to inactivate Klebsiella.[15, 33] To achieve the highest removal rate of Escherichia coli and Klebsiella, the parameters of the effect of nanoparticle concentration, pH and nanoparticle level was examined in the lab. In all cases, the

Table 5: Results for optimization of conditions

Parameter	E. coli	Klebsiella
Nanoparticle type	Copper	Copper
Concentration of used nanoparticle	200 ppm	250 ppm
pH	7-7.9	7-7.9
Content of used nanoparticle	2 cc	1.5 cc

one-variable-at-a-time (OVAT) method was used, in which all parameters are kept constant and only one parameter is changed. In previous studies, it was found that at high concentrations of nanoparticles, the reduction of bacteria was Log 8.8 and the residual copper content in the environment was 1 ppm based on the proposed limit.[16, 17, 30] Morsi et al. (2017) studied the antimicrobial properties of copper and silver nanoparticles in water. The high concentration of nano-compounds represents less contact times against gram-positive and gram-negative bacteria such as Escherichia coli and Staphylococcus aureus, and on average, 1% concentration of these nanoparticles has a significant effect on the removal of bacteria within 10 minutes.

Conclusion

In this study, the four-stage study of the samples prepared in laboratory conditions revealed that using 1ml copper nanoparticles at a concentration of 300 ppm had the ability to remove Escherichia coli and Klebsiella bacteria compared with silver nanoparticles under the same conditions in aquatic environments. By optimizing the conditions, the concentration of copper nanoparticles to remove E. coli was determined as 200 ppm and was 250 ppm to remove Klebsiella. By optimizing the conditions for the removal of both Escherichia coli and Klebsiella bacteria, the appropriate range for the best removal was achieved as pH=7-7.9. By optimizing the conditions, the content of copper nanoparticles for the removal of Escherichia coli was 2 cc and that for removing the Klebsiella bacterium was 1.5 cc. By optimizing the conditions, the removal rates for E. coli and Klebsiella were 99.25% and 81.25%. In this case study, using Nano particles, led to a high efficiency at a short time; also, it was cost-effective and environmentally friendly. It is suggested that an economic comparison should be made for the disinfection of water with silver, copper and chlorine nanoparticles and their derivatives. Furthermore, the efficiency of copper and silver nanoparticles in the elimination of secondary contaminations of rural water distribution networks should be compared with the capability of chlorine and its derivatives, and the final destiny of nanoparticles in high volumes and the environmental effects should be analyzed.

ACKCOWLEDGMENT

This article was extracted from the MSc degree thesis of Miss Masoomeh GhanavatAmani. The authors of this paper are sincerely grateful to the Islamic Azad University of Ahvaz, Iran for providing the financial and academic support to the project.

Conflict of Interest: None declared.

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