Antibacterial activity of copper-chitosan complexes against zoonotic *Vibrio parahaemolyticus*

HARVEY C. LOQUIAS, REIGEN R. PLACIDO, and HAROLD P. MEDIODIA

Philippine Science High School - Western Visayas Campus, Brgy. Bito-on, Jaro, Iloilo City 5000, Department of Science and Technology, Philippines

Abstract

Copper-chitosan (Cu-Ch) complexes can be used as antibacterial agents against zoonotic microorganism strains. This study assessed the antibacterial activity of Cu-Ch complexes synthesized via a chemical method outlined by Usman et al. [7,8] against zoonotic *Vibrio parahaemolyticus* using disk diffusion assay. The complexes were characterized using Ultraviolet-Visible (UV-VIS) and Fourier Transform Infrared (FTIR) spectrophotometry. Characterization results indicate the formation of Cu-Ch complexes during synthesis, but not nanoparticles. The synthesized Cu-Ch complexes exhibited no antibacterial activity against *Vibrio parahaemolyticus*, suggesting that they are ineffective antimicrobials against zoonotic microorganisms. Further studies can look into their antibacterial activity against other types of microorganisms.

Keywords: *chitosan, copper, nanocomplexes, Vibrio parahaemolyticus, zoonotic*

Introduction. Metal nanoparticles and nanocomplexes have been synthesized from cost-efficient metals such as copper (Cu) and tested for antimicrobial activity [1,2,3,4]. However, copper rapidly oxidizes upon exposure to the atmosphere which can result in the formation of oxides, aggregation and decreased activity [5,6,7]. To counter the problem of agglomeration and rapid oxidation of copper, Usman et al. [8] synthesized pure copper nanoparticles in the presence of a chitosan stabilizer through a chemical process.

Chitosan (Ch), a modified carbohydrate polymer derived from chitin, has been used with metal nanoparticles as a chelating agent to retard oxidation and increase antimicrobial activity [9]. The presence of chitosan has been found to improve the antimicrobial activity of copper nanoparticles due to its stabilizing effect on copper [4, 10,11]. There are also no reports of bacteria resisting or developing resistance against this biopolymer [12].

Copper-Chitosan (Cu-Ch) nanocomplexes have possible applications for treatment of zoonotic and resistant organisms and biofilms. Tests by Usman et al. [7] and Syame et al. [13] have shown that Cu-Ch nanocomplexes can exhibit antibacterial activity on resistant gram-positive bacteria such as Methicillin-Resistant *Staphylococcus aureus*. Despite reports of Cu-Ch nanocomplexes exhibiting greater antimicrobial activity against gram-negative bacteria, a number of zoonotic gram-negative strains as well as other gram-positive strains have yet to be subjected to antimicrobial tests with Cu-Ch complexes.

Vibrio parahaemolyticus is a gram-negative target bacterial species to monitor according to the Philippine Antimicrobial Resistance Surveillance Plan for the Animal Health Sector (2018-2020). It has been found to cause gastroenteritis and septicemia on humans both directly and indirectly exposed *to V. parahaemolyticus* infected animals such as oysters [14] and shrimps [15]. It has also caused diseases in aquaculture organisms such as shrimp [16,17] and abalone [18].

Although there are a number of studies investigating the antimicrobial activity of copper-chitosan nanocomplexes [4,7,8,10] and a number of susceptibility studies conducted on *V. parahaemolyticus* [19,20,21,22], as far as can be ascertained, no research has yet been performed to test the antibacterial activity of copper-chitosan complexes against *V. parahaemolyticus*.

To address this, the study aimed to assess the antibacterial activity of researcher-synthesized copper-chitosan complexes against zoonotic *Vibrio parahaemolyticus* via disk diffusion assay and characterize them in terms of Ultraviolet-Visible Spectra (surface plasmon resonance peaks), absorbance spectra, and morphology for the complexes exhibiting the highest antimicrobial activity. It specifically aimed to:

(i) measure the zone of inhibition of researcher-synthesized Cu-Ch complexes against zoonotic *Vibrio parahaemolyticus* using disk diffusion assay;

(ii) characterize researcher-synthesized Cu-Ch complexes in terms of:
- (a) surface plasmon resonance peaks;
- (b) FTIR / Transmittance spectra; and
- (c) morphology (size, shape, agglomeration) for the Cu-Ch complex treatment exhibiting the highest antibacterial activity; and

(iii) determine the viability of researcher-synthesized Cu-Ch complexes as nanoparticles in comparison with
- (a) surface plasmon resonance peaks in the range 500-600 nm as outlined by Mallick et al. [23] and Usman et al. [7];
- (b) blue shifts and decreased intensity peaks in FTIR Spectra as outlined by Usman et al. [7,8]; and
- (c) diameter size in the range of 50-270nm for the 0.1% Cu-Ch NPs, in the range of 5-50nm for the 0.2% Cu-Ch NPs, and in the range of ~2-50nm for the 0.5% Cu-Ch

How to cite this article:
CSE: Loquias HC, Placido RR, Mediodia HP. 2020. Antibacterial activity of copper-chitosan complexes against zoonotic *Vibrio parahaemolyticus.* Publiscience. 3(1):27-31.
APA: Loquias, H.C., Placido, R.R., & Mediodia, H.P. (2020). Antibacterial activity of copper-chitosan complexes against zoonotic *Vibrio parahaemolyticus. Publiscience*, 3(1):27-31.

NPs; predominantly spherical in shape; and polydispersed in agglomeration, as outlined by Usman et al. [7,8] for the Cu-Ch complex treatment exhibiting the highest antibacterial activity.

Methods. Copper-chitosan complexes were synthesized via a chemical method outlined by Usman et al. [7,8] and subjected to both antimicrobial tests using the standard disk diffusion method as outlined by the Clinical and Laboratory Standards Institute (CLSI, Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard 11th Edition, 2012) and characterization using UV-VIS and FTIR Spectrophotometry.

Materials. Laboratory grade copper (II) sulfate ($CuSO_4$), acetic acid, ascorbic acid, barium chloride ($BaCl_2$), sulfuric acid (H_2SO_4), and sodium hydroxide (NaOH) were provided by the Science Research Assistant in Philippine Science High School-Western Visayas Campus (PSHS-WVC). Analytical grade hydrazine (N_2H_4) was purchased from Sigma-Aldrich Pte Ltd. Laboratory grade chitosan was purchased from KAN Phytochemicals Pvt. Ltd. *Vibrio parahaemolyticus* was sourced from the Philippine National Collection of Microorganisms (PNCM) at the University of the Philippines - Los Baños College, Los Baños, Laguna.

Chemical Synthesis. Ten (10) mL of $CuSO_4 \cdot 5H_2O$ (0.05 M) was added to 40 mL of acetic acid solution (0.1 M) containing chitosan (0.1, 0.2, and 0.5 wt%). After constant stirring and refluxing at around 100°C-140°C for 20 minutes, 0.5 mL of ascorbic acid (0.05 M) was added, and the solution was stirred for 20 minutes at room temperature. Two (2) mL of NaOH (0.6 M) was then added, obtaining a darker blue-green solution after stirring for another 20 minutes. Then 0.5 mL of N_2H_4 (0.05M) was added and the solution was stirred for five (5) minutes. The pH was kept at an average of 8.0 throughout the process. The solution was centrifuged at 10,000 G for 10 minutes and washed with acetone (90%, v/v). The precipitate was vacuum dried at 50°C for 18 hours.

Antibacterial Assay. Mueller-Hinton (MH) Agar was utilized as culture media. The copper-chitosan complexes (0.1, 0.2, 0.5 wt% chitosan content) were suspended in distilled water and loaded onto blank sterilized Whatman No. 1 filter paper disks. Ciprofloxacin-loaded antibacterial discs (5 ug) and distilled water loaded onto blank sterilized Whatman No. 1 filter paper disks served as the positive and negative controls, respectively. Chitosan was also loaded onto blank sterilized Whatman No. 1 filter paper disks, totaling 6 treatments. The experiment was carried out in triplicate and the diameters of the zones of inhibition (in mm) were measured after incubating for 16-18 hours at 35±2°C in ambient air.

Characterization. The synthesized Cu-Ch complexes were characterized in terms of Ultraviolet-Visible Spectra (surface plasmon resonance) and Absorbance Spectra using a UV-1800 Shimadzu UV Spectrophotometer (Ultraviolet-Visible Spectrophotometry) and an IRAffinity-1S Shimadzu Fourier Transform Infrared (FTIR) Spectrophotometer.

Data analysis. The susceptibility of *V. parahaemolyticus* to the complexes was determined based on the CLSI test interpretation document M45-Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. Results of the characterization of the synthesized complexes were analyzed by comparison to previously published articles, specifically on the UV-Vis and FTIR results of Mallick et al. [23], Zhou et al. [24], Huang et al. [25], Shameli at al. [26], Usman et al. [7], and Sportelli et al. [27].

Safety procedure. The handling of *V. parahaemolyticus* was performed under a biosafety cabinet in the Department of Science and Technology VI (DOST VI) Microbiology laboratory. Organic and inorganic chemical waste were disposed separately in designated containers found in PSHS-WVC. Biohazard waste from the antibacterial testing phase was properly disposed with the aid of laboratory personnel from DOST VI, following their protocol for disposal.

Results and Discussion. The complexes (0.1 wt%, 0.2 wt%, 0.5 wt%) and the pure chitosan solution (50% w/v) did not exhibit bacterial inhibition. As shown in Table 1, the negative and positive controls resulted in 6mm and 30mm diameter zones of inhibition, respectively. Non-uniform radii of inhibition around the 0.2% discs (2.45mm, 3.10mm, and 1.35mm) were observed but were not interpreted as zones of inhibition as stated in the CLSI M45 guide document. *V. parahaemolyticus* is resistant to the Cu-Ch complexes and pure chitosan, and susceptible to ciprofloxacin (5ug). Spectrophotometric analysis of the researcher-synthesized Cu-Ch complexes suggests formation of Cu-Ch complexes and non-formation of Cu-Ch nanoparticles. The Cu-Ch complexes were not subjected to morphological characterization as they did not exhibit antibacterial activity against the test organism.

Table 1. Summary of the antibacterial and characterization results of the study.

Treatment	Antibacterial activity (Zone of inhibition in mm)	Copper Complex formation	NP formation
Ciprofloxacin (+)	Susceptible (30 mm)	-	-
Distilled Water (-)	Resistant (6 mm)	-	-
Pure Ch	Resistant (6 mm)	-	-
0.1 wt% Ch content	Resistant (6 mm)	Y	N
0.2 wt% Ch content	Resistant (6 mm)	Y	N
0.5 wt% Ch content	Resistant (6 mm)	Y	N

Legend: Y = formation; N = non-formation

The synthesized Cu-Ch complexes are not identified to be nanoparticles due to the lack of a surface plasmon resonance (SPR) peak in the

500-600 nm range of the UV-Vis absorbance spectra of the complexes (Figure 1), as well as a lack of decreased intensity peaks and a significant peak in the 600-700 cm^{-1} range of the FTIR spectra (Figure 2) [7,26,27]. Cu-Ch nanoparticles are known to show absorbance in the range of 500–600 nm of their UV-Vis spectra [7]. Copper-polymer nanoparticles by Mallick et al. [23] also showed UV-VIS absorbance peaks at the 500-600 nm range.

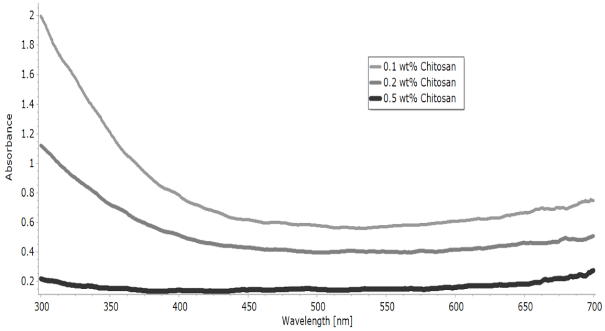

Figure 1. UV-VIS Absorbance spectra of copper-chitosan complexes (0.5 wt%, 0.2 wt%, 0.1 wt% chitosan content).

Decreasing intensity peaks and peaks in the 600-700 cm^{-1} range are also characteristic in the FTIR spectra of Cu-Ch nanoparticles [32]. The presence of peaks at 3310 cm^{-1} , 3317 cm^{-1}, and 3315 cm^{-1} indicate N-H and O-H bonds, both of which are present due to the characteristic structure of chitosan [28]. Stretches observed at 2360 cm^{-1} indicate the presence of C-N and C-C triple bonds which were found to be present in the complexes [7,24]. The 2000-1450 cm^{-1} region contains peaks corresponding to double bond stretching vibrations. The presence of blueshifts indicates that ligand groups of chitosan are combined with the surface of Cu NPs [28]. Furthermore, no color change was observed after the addition of the reducing agent (N_2H_4), indicating that no nanocomplexes were formed [7,8]. Li et al. [28] states this is possibly due to the concentration of the reducing agent that was unable to reduce the synthesized complexes into nanoparticles.

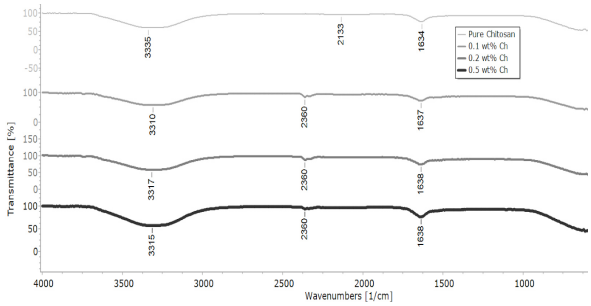

Figure 2. Processed fourier transform infrared spectra of Ch and Cu-Ch complexes (0.5 wt%, 0.2 wt%, 0.1 wt% Ch content).

Factors such as pH, metal concentration, and metal/ligand ratio influence the complexation of metal species and polymers [29]. While chitosan has been observed to exhibit good metal ion uptake, in crosslinked and uncrosslinked forms [30], it is also known to form a complex with copper in conditions below pH 6.1 due to its poor solubility in alkaline media [10]. However, complexation in acidic conditions results in chitosan protonation which significantly reduces the affinity of the sorbent for the uptake of metal cations [31]. Incomplete capping or instability of the synthesized complexes can be attributed to the pH 8.0 level maintained in the synthesis process modeled after the chemical procedure of Usman et al. [7,8].

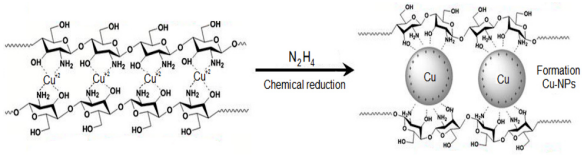

Figure 3. Molecular structure of Cu-Ch complexes (left) and Cu-Ch nanocomplexes (right) after chemical reduction presented by Usman et al. [7].

Pure chitosan and the Cu-Ch complexes exhibited no antibacterial activity against *V. parahaemolyticus* that was susceptible to ciprofloxacin (5ug), which exhibited activity resulting in an inhibition zone of 30 mm. This coincides with previous literature stating pure chitosan does not have antimicrobial activity in alkaline media due to its poor solubility above pH 6.5 [7]. The inhibitory activity of pure chitosan can be linked to changing the bacterial cell membrane permeability, and poorly soluble chitosan is unable to penetrate outer bacterial membranes as a macromolecule [29,32].

The lack of activity of the synthesized Cu-Ch complexes is in contrast with the synthesized nanocomplexes of Usman et al. [7] which exhibited antibacterial activity. Huang et al. [32] showed that despite a synergistic effect on the antibacterial activity of copper and chitosan, Cu-Ch complexes exhibit less antibacterial activity compared to Cu-Ch nanoparticles. Mekhalia and Bouzid [29] posits that chitosan, as a macromolecule, is unable to pass the outer membrane of bacteria which functions as a permeability barrier against macromolecules. Unlike their macromolecule counterparts, smaller chitosan molecules such as nanocomplexes can diffuse into the bacterial cells through pervasion and disrupt normal physiological activity, resulting in cell death. The coordination bonding between the functional groups of the chitosan molecules and copper ions may weaken a bond near the coordinating site and cause some weak points on the chitosan chain, resulting in smaller chitosan molecules [10]. This coincides with how the antibacterial activity of previously synthesized Cu-Ch nanocomplexes [7,8,27] has been attributed to morphology, specifically decreased size and increased surface area. The UV-VIS and FTIR spectra of the synthesized complexes in this study both indicate non-formation of nanoparticles. Improvement of coordination bonding between the chitosan polymer and the copper ions, and thus decreasing complex sizes, may be achieved with higher concentrations of the reducing agent [28].

On the resistance exhibited by the test organism, a previous study by Gordon et al. [33] showed that *V. parahaemolyticus* can recover from stress induced by copper. The same study also found proteins in copper-induced *V. parahaemolyticus* cultures similar to extracellular copper-binding proteins found in *Vibrio alginolyticus,* which may be one of the defense mechanisms of *V. parahaemolyticus* against copper. This coincides with the results of the study of Chari

et al. [34] where copper nanoparticles tested against *V. parahaemolyticus* exhibited no antibacterial activity against the test organism.

Limitations. The results only reflected the activity of Cu-Ch as complexes and not as nanoparticles. Furthermore, results only reflected the activity of the complexes against *V. parahaemolyticus*. Only the Kirby-bauer method was used to test for antibacterial activity, which investigated activity at one concentration.

Conclusion. Results indicated that Cu-Ch complexes, in contrast to Cu-Ch nanoparticles synthesized by Usman et al. [8,7] exhibit no antibacterial activity against zoonotic *Vibrio parahaemolyticus*.

Recommendations. Due to the observed non-activity of the complexes against *V. parahaemolyticus*, further tests may consider other compounds exhibiting different modes of action compared to Cu-Ch complexes. Antibacterial activity of the complexes against other types of microorganisms may be investigated, especially against Gram-positive bacteria. An assay investigating the minimum inhibitory concentration (MIC) of the complexes may be utilized to investigate the activity of the complexes at other concentrations and in direct contact with the test organism. Morphological testing of Cu-Ch complexes and further tests comparing the antibacterial activity of Cu-Ch coordination complexes and Cu-Ch nanoparticles are also recommended for further studies. Further studies are suggested to employ measures to monitor the temperature of the reflux setup at 120 ˚C during the synthesis process.

Acknowledgement. The researchers would like to extend their gratitude to the following: to Dr. Czarina Nobleza and Dr. Stephen Sabinay for their assistance in conceptualizing and conducting this research, to the Department of Health and Department of Agriculture for providing data for conceptualization, to the Department of Science and Technology- Region VI and the University of the Philippines- Visayas for allowing the researchers to work in their laboratories, to Mr. Joeben Gabutanga for his assistance the procurement of their test organism, and to everyone who supported this research.

References

[1] Yoon KY, Byeon JH, Park JH, Hwang J. 2007. Susceptibility constants of *E. coli* and *B. subtilis* to silver and copper nanoparticles. Sci Total Environ. 373(2007):*572-575*.

[2] Ruparelia JP, Chatterjee AK, Duttagupta SP, Mukherji S. 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomaterialia. 4(3):707-716.

[3] Ramyadevi J, Jeyasubramanian K, Marikani A, Rajakumar G, Rahuman AA. 2012. Synthesis and antimicrobial activity of copper nanoparticles. Mater lett. 71: 114-116.

[4] Du WL, Niu SS, Xu YL, Xu ZR, Fan CL. 2009. Antibacterial activity of chitosan tripolyphosphate nanoparticles loaded with various metal ions. Carbohydr Polym. 75(3): 385-389.

[5] Balela MDL, Amores KLS. 2015. Formation of highly antimicrobial copper nanoparticles by electroless deposition in water. Sci Diliman. 27(2):10-20.

[6] DeAlba-Montero I, Guajardo-Pacheco J, Morales-Sánchez E, Araujo-Martínez R, Loredo-Becerra GM, Martínez-Castañon GA, Ruiz F, Compeán-Jasso ME. 2017. Antimicrobial properties of Copper nanoparticles and amino acid chelated Copper nanoparticles produced by using a soya extract.

[7] Usman MS, El Zowalaty ME, Shameli K, Zainuddin N, Salama M, Ibrahim NA. 2013. Synthesis, characterization, and antimicrobial properties of copper nanoparticles. Int J Nanomed. 2013(8):4467-4479.

[8] Usman MS, Ibrahim NA, Shameli K, Zainuddin N, and Yunus WM. 2012. Copper nanoparticles mediated by chitosan: synthesis and characterization via chemical methods. Molecules. 2012(17): 14928-14936.

[9] Al-Dhabaan FA, Shoala T, Ali AA, Alaa M, Abd-Elsalam K. 2017. Chemically-produced copper, zinc nanoparticles and chitosan–bimetallic nanocomposites and their antifungal activity against three phytopathogenic fungi. IJAT. 13(5):753- 769.

[10] Qi L, Xu Z, Jiang X, Hu C, Zou X. 2004. Preparation and antibacterial activity of chitosan nanoparticles. Carbohyd Res. 339(16): 2693-2700.

[11] Manikandan A, Sathiyabama M. 2015. Green synthesis of copper-chitosan nanoparticles and study of its antibacterial activity. J Nanomed Nanotechnol. 6(1): 1-5.

[12] Divya K, Vijayan S, George TK, Jisha MS. 2017. Antimicrobial properties of chitosan nanoparticles: mode of action and factors affecting activity. Fiber Polym. 18(2): 221-230.

[13] Syame OM , Mohamed WS, Mahmoud RK and Omara ST. 2017. Synthesis of copper-chitosan nanocomposites and their applications in treatment of local pathogenic isolates bacteria. Orient J Che. 33(6):2959-2969.

[14] McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napolilli NP, Allison CG, Murray SA, Thompson EC, Bird MM, Middaugh JP. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N Engl J Med. 353(14): 1463-1470.

[15] Daniels NA, MacKinnon L, Bishop R, Altekruse S, Ray B, Hammond RM, Thompson S, Wilson S, Bean NH, Griffin PM, Slutsker L. 2000. *Vibrio parahaemolyticus* infections in the United States, 1973–1998. J Infect Dis. 181(5): 1661-1666.

[16] Alapide-Tendencia EV, Dureza LA. 1997. Isolation of *Vibrio spp.* from Penaeus monodon (Fabricius) with red disease syndrome. Aquaculture. 154(2): 107-114.

[17] Joshi J, Srisala J, Truong VH, Chen IT, Nuangsaeng B, Suthienkul O, Lo CF, Flegel TW, Sritunyalucksana K, Thitamadee S. 2014. Variation in *V. parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND). Aquaculture. 428(2014): 297-302.

[18] Liu PC, Chen YC, Huang CY, Lee KK. 2000. Virulence of *Vibrio parahaemolyticus* isolated from cultured small abalone, *Haliotis diversicolor supertexta*, with withering syndrome. Lett Appl Microbiol. 31(6): 433-437.

[19] Han F, Walker RD, Janes ME, Prinyawiwatkul W, Ge B. 2007. Antimicrobial susceptibilities of *V. parahaemolyticus* and *V. vulnificus* isolates from Louisiana Gulf and retail raw oysters. Appl Environ Microbiol. 73(21): 7096-7098.

[20] Shaw KS, Goldstein RER, He X, Jacobs JM, Crump BC, Sapkota AR. 2014. Antimicrobial susceptibility of *Vibrio vulnificus* and *Vibrio parahaemolyticus* recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. PLoS One. 9(2): e89616.

[21] Letchumanan, V, Yin WF, Lee LH, Chan KG. 2015. Prevalence and antimicrobial susceptibility of *V. parahaemolyticus* isolated from retail shrimps in Malaysia. Front Microbiol. 6(33).

[22] Stevens D, Higbee J, Oberhofer T, Everett D. 1979. Antibiotic susceptibilities of human isolates of *V. parahaemolyticus*. Antimicrob Agents Chemother. 16(3):322-324.

[23] Mallick K, Witcomb MJ, Scurrell MS. 2006. In situ synthesis of copper nanoparticles and poly (o-toluidine): a metal–polymer composite material. Eur Polym J. 42(3): 670-675.

[24] Zhou YG, Yang YD, Guo XM, Chen GR. 2003. Effect of molecular weight and degree of deacetylation of chitosan on urea adsorption properties of copper chitosan. J App Polym Sci. 89(6); 1520-15.

[25] Huang H, Yuan Q, Yang X. 2004. Preparation and characterization of metal–chitosan nanocomposites. Colloids Surf B. 39(1-2); 31-37.

[26] Shameli K, Ahmad MB, Yunus WMZ., Rustaiyan A, Ibrahim NA. Zargar M, Abdollahi Y. 2010. Green synthesis of silver/montmorillonite/ chitosan bionano-composites using the UV irradiation method and evaluation of antibacterial activity. Int J Nanomed. 5(2010): 875887.

[27] Sportelli M, Volpe A, Picca R, Trapani A, Palazzo C, Ancona A, Lugarà P, Trapani G, Cioffi N. 2016. Spectroscopic character-rization of copper-chitosan nano-antimicrobials prepared by laser ablation synthesis in aqueous solutions. Nanomaterials. 7(1):6.

[28] Li Y, Li B, Wu Y, Zhao Y, Sun L. 2013. Preparation of carboxymethyl chitosan/copper composites and their antibacterial properties. Mater Res Bull. 48(2013).

[29] Mekhalia S, Bouzid B. 2009. Chitosan-Copper (II) complex as antibacterial agent: synthesis, characterization and coordinating bond-activity correlation study. Physcs Proc. 2(3): 1045-1053.

[30] Sobahi TR, Abdelaal MY, Makki MS. 2014. Chemical modification of chitosan for metal ion removal. Arab J Chem. 7(5): 741-746.

[31] Guibal E. 2004. Interactions of metal ions with chitosan-based sorbents: a review. Sep Purif Technol. 38(1): 43-74.

[32] Huang Y, Huang J, Cai J, Lin W, Lin Q, Wu F, Luo J. 2015. Carboxymethyl chitosan/clay nanocomposites and their copper complexes: fabrication and property. Carbohydr Polym. 134(2015).

[33] Gordon AS, Howell LD, Harwood V. 1994. Responses of diverse heterotrophic bacteria to elevated copper concentrations. Can J Microbiol. 40(5): 408–411.

[34] Chari N, Felix L, Davoodbasha M, Ali AS, Nooruddin T. 2017. In vitro and in vivo antibiofilm effect of copper nanoparticles against aquaculture pathogens. Biocatal Agric Biotechnol. 10: 336-341.