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Section A-Research paper

EVALUATION OF THE INHIBITORY EFFECT OF SILVER NANOPARTICLES AGAINST THE LARVAE OF *AMPHIBALANUS AMPHITRITE* (DARWIN, 1854) AS ANTIFOULING AGENT FROM VISAKHAPATNAM COAST

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Abstract
Since the early days of yachting, sea surface fouling has been a persistent issue. Seaweed and invertebrates' persistent clinging to man-made surfaces, particularly ship hulls, has resulted in unfavorable economic losses. Due to its exceptional ability to slow down the growth of barnacle larvae and serve as an antifouling agent, silver is highly regarded in the field of Nano materials. The morphological characters of the silver nanoparticles were investigated by UV-Vis Spectra analysis, X-ray spectroscopy (EDS) coupled to the Scanning Electron Microscope (SEM-EDS) imaging, X-ray diffraction (XRD) and FTIR analysis. Tests related to Mortality were also conducted. The larval populations LC_{50} were estimated by larval Toxicity Assay and were compared with copper in an attempt to assess the antifouling nature of the Silver Nano particles. The obtained results clearly showed that the LC_{50} on the silver Nanoparticles was almost 10 times less (1.0 µg/L), compared to the Copper sulphate (10 µg/L).
Keywords: Silver Nanoparticles, *Amphibalanus amphitrite*, Antifouling, SEM.

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Introduction

Amphibalanus amphitrite, a popular barnacle species of Balanidae family is considered to be an excellent model organism for studies related to antifouling compounds. The major reasons that attribute this fact are its rapid larval development, the ease with which it could be raised in contemporary mass cultures, and the most important one is its predictable settlement in static conditions. Since the adult animal is an important fouling species, the model is considerably relevant. None is comparable to *A. amphitrite* concerning ease of culture and laboratory experimentation. Hence, the current laboratory model is a representative of many, but not all barnacles. The fact that it is globally distributed and breeds all through the year, this organism is selected as study organism for toxicology assay to test the efficacy of silver nanoparticles as potential antifouling compound in this research study.

Ancient mariners were aware of the issues caused by boring and other fouling creatures. On the bottoms of their ships, the prehistoric Phoenicians and Carthaginians allegedly utilized pitch and possibly copper sheathing. The Romans utilized lead sheathing, which they fastened with copper nails, as did the Greeks. The British Navy has used copper on a regular basis since 1780. These humble beginnings led to the development of antifouling paints that include copper salts. When copper attaches to sulfur-containing cell components, it can cause a number of symptoms that are

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related to heavy metal toxicity. The three main categories of toxic anti-fouling paints currently in use are self-polishing systems, ablative paints, and soluble matrix paints, generally known as conventional paints. Most antifouling paints contain copper as a pigment, typically in the form of cuprous oxide (Cu2O) (Callow and Callow, 2002). The majority of efforts to develop new management strategies have been concentrated on finding substances that might deter or prevent the adhesion of fouling organisms. Therefore, copper is used in this study's toxicological assay as a control chemical.

The strength of laboratory assays lies in the quick, extremely sensitive evaluation of prospective antifouling agents for toxicity and antifouling efficacy. Assays have most recently been employed to explain the connection between the physicochemical characteristics of surfaces, microbial coatings, and barnacle settlement. Antifoulant chemicals identified to date lack an established mechanism of action. A first step in figuring out how to administer active agents is to conduct toxicology studies to examine the impact of antifouling chemical surface adsorption. Improved understanding of the compounds' mechanisms of action will make it possible to distribute active drugs more successfully. The development of a novel environmentally friendly (Silver nanoparticles) antifouling substance, however, now yields quick and measurable results from laboratory testing (Ahmed *et al.,* 2016; Zhang *et al.,* 2016).

Materials and methods

Characterization of Nanoparticles

The crystal phases were identified and the average crystallite size was calculated using X-ray diffraction (XRD). Utilising Cu-K radiation with a wavelength of 1.54056A and Ni-filtered, a PANalytical PW 3040/60 X'Pert PRO equipment was used to measure diffraction. A 45 kV accelerating voltage and a 40 mA emission current were used. A scintillation counter detector, a 2 scan range from 10 to 80, and a 0.01 scanning step size were employed. Philips X'Pert high score plus's proprietary software was used to do curve fitting and integration. Using Bragg's law, the size of the Ag NPs was determined from the PXRD peak locations. A minute amount of the material was dropped onto a copper grid that had been coated with carbon to create thin films. The Quanta 200 FEG-SEM machine (TEQIP, Centre for Nanotechnology A.U.) was used to measure the film on the FE-SEM grid after it had been dried under a mercury lamp for five minutes.

EDS provided proof that elemental Silver was present. EDS observations were made using the JOEL Model JSM-7100F in TEQIP and AUCEN. The EDS spectra captured in spot profile mode from a portion of the film’s surface that is heavily packed with silver nanoparticles. Quanta 200 FEG was used to analyze the nanocrystallites.

Silver nanoparticles (AgNPs) in aqueous solution were produced as samples by centrifugation at 10,000 rpm for 30 min. by employing the KBR pellet (FR-IR grade) procedure, the pellet was lyophilized and subjected to FT-IR analysis. The spectrum was acquired using a spectral range of 4000-500 cm^{-1}

Fourier transform infrared spectroscopy (FTIR) was used to analyse the interaction between *Senna siamea* extract and silver nanoparticles in a diffuse reflectance moderate solution of 4 cm^{-1} in KBR pellets. The spectra were obtained in the wavelength range of 4000 to 500 nm^{-1}. To pinpoint the biomolecules in charge of the acacia extract-produced bioreduced Ag NPs' capping and reduction of the Ag+ ions, FTIR measurements were made. The acacia filtrate was combined with KBR powder for a comparison analysis, dried, and then pelletized before measurement.

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Culturing and larval rearing of A. *amphitrite*

An artificial panel system (WIC) plates were employed to collect the adult *A. amphitrite* barnacle from the field and were brought to the laboratory. Fresh water was used to clean them and remove the debris. Scrapes were used to remove stressed-out dead shells and barnacles with loose operculum (Desai and Anil, 2005). To promote the release of nauplii, the brood stocks were regularly exposed to air. On the first day, the nauplii were collected and employed in a toxicity test (Desai *et al*., 2006).

Toxicity Assay LC_{50} of *A. Amphitrite* nauplii

By assessing the detrimental impact of different silver nanoparticle concentrations on *A. amphitrite* nauplii survival and mortality under intermittent flow-through conditions, the acute toxicity was ascertained. The study started with 24-hour-old nauplii and proceeded for 24 hours while being exposed. Twelve-well plates were used for the experiment. A total of 20 nauplii were put into each well, which contained 2 mL of 33 ppt saline water as a reference (without silver nanoparticles), after the addition of the required concentration of AgNPs, such as 0.1, 1, 10, and 100 ppm, respectively. Three replicates of each test concentration and the control should be run in a 12-well plate. The experimental setup was left in the dark for 24 hours, and after the incubation period, nauplii were counted. The LC_{50} value after 24 hours and percentages of mortality after 24 hours for various test concentrations of silver nanoparticles were determined and compared with the control. Results were tabulated and plotted as a graph.

The % mortality was determined using the formula as shown below
% Mortality = Number of dead *A. amphitrite* nauplii/Initial number of live *A. amphitrite* nauplii x 100

Adults released the stage II nauplii upon immersion in FSW after drying for 12 h (Chen *et al.*, 2008). Naupliar larvae actively swimming towards the light were collected using a pipette and were used in the toxicology assay.

Statistical analysis

Before choosing statistical tests for sample comparisons, data were first analyzed for the parametric assumptions of normality and equality of variances. All of the data met the aforementioned presumptions, and a one-way ANOVA was conducted using SPSS statistical software (SPSS Inc., Chicago IL), where $p<0.05$ was used for the multisampling hypothesis.

Table 1: The result for mortality rate (12/24 hours) of *A. amphitrite* nauplii treated with various concentrations of silver nanoparticles.

Parameter	Concentration Levels in (ppm)	Initial number of Amphitrite nauplii	Number of nauplii dead after 12 hours	Number of nauplii dead after 24 hours	% of mortality after 12 hours (mean±SD)	% of mortality after 24 hours (mean ± SD)
Mortality rate after 12 and 24 hours	Control	20	0	0	0	0
	0.1	20	0	01	1.67±0.58	1.67±0.58
	1	20	06	10	31.67±0.58	51.67±0.58
	10	20	09	12	46.67±0.58	61.67±0.58
	100	20	20	20	98.33±0.58	100

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The table represents the number of *A. Amphitrite* nauplii dead after 12 h and 24 h treated with various concentrations of silver nanoparticles. The table also indicates the percentage mortality ± standard deviation. The 24 h percentage mortality rate of the nauplii is expressed as LC_{50}.

Table 2: The result for mortality rate (12/24 hours) of *A. amphitrite* nauplii treated with various concentrations of $CuSo_4$ (Control).

Parameter	Concentration Levels in (ppm)	Initial number of Amphitrite nauplii	Number of nauplii dead after 12 hours	Number of nauplii dead after 24 hours	% of mortality after 12 hours (mean±SD)	% of mortality after 24 hours (mean ± SD)
Mortality rate after 12 and 24 hours	Control	20	0	0	0	0
	0.1	20	0	01	1.67±0.58	1.67± 0.58
	1	20	01	02	6.67±0.58	11.67±0.58
	10	20	11	14	56.67±0.58	71.67±0.58
	100	20	18	20	91.66±0.58	100

The table represents the number of *A.amphitrite* nauplii dead after 12 h and 24 h treated with various concentrations of copper sulfate. The table also indicates the percentage mortality ± standard deviation. The 24 h percentage mortality rate of the nauplii is expressed as LC_{50}.

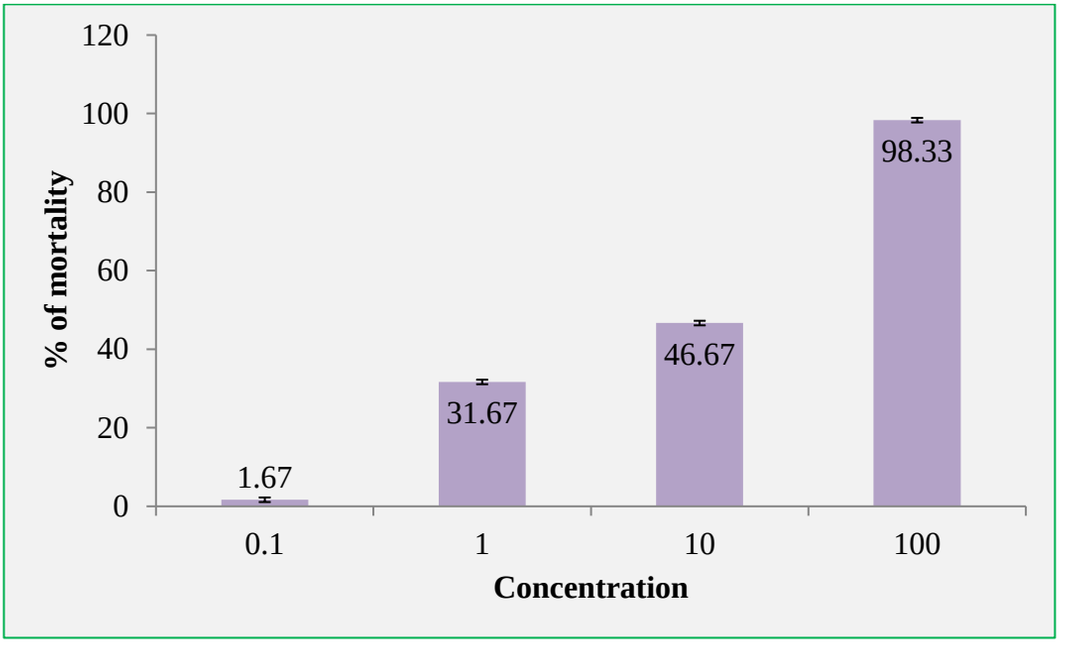

Figure 1: Percentage of mortality after 12 hours (mean±SD) by Silver nanoparticles

Graphical representation of rate of mortality of *A. amphitrite* nauplii by AgNP's at pH 7.0 Temperature 28ºC in time period of 12hrs, the graphs indicate the maximum rate of mortality occurred in 100ppm with 98.33%.

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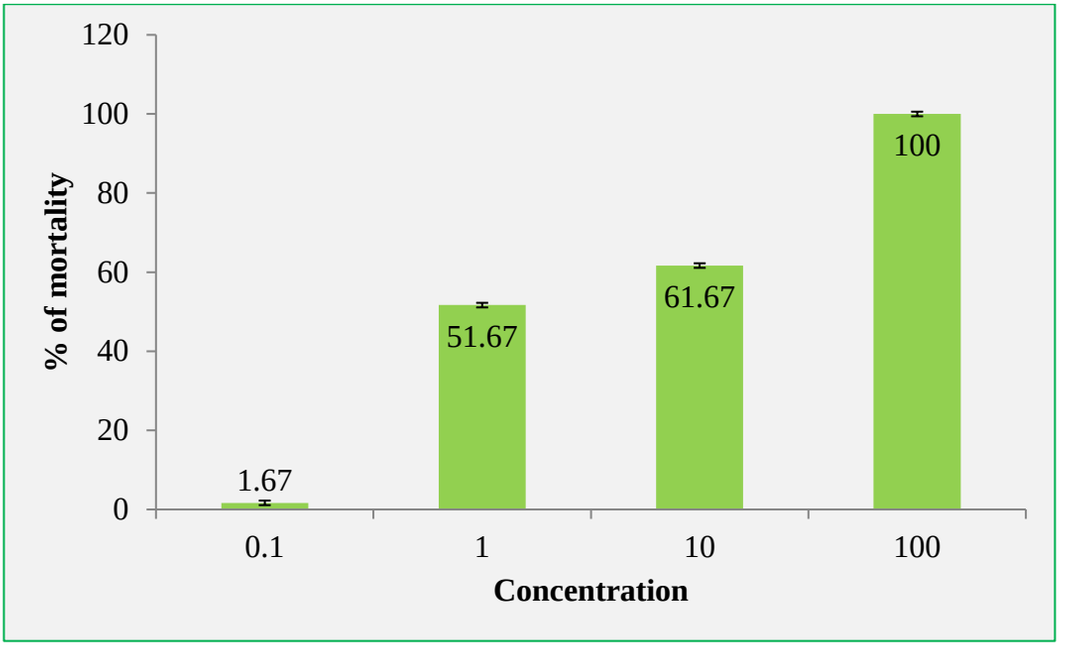

Figure 2: Percentage of mortality after 24 hours (mean±SD) by Silver nanoparticles

Graphical representation of rate of mortality of *A. amphitrite* nauplii by AgNP'sat pH 7.0 Temperature 28ºC in time period of 24hrs, the graphs indicate the maximum rate of mortality occurred in 1 ppm with 51.67±0.58%.

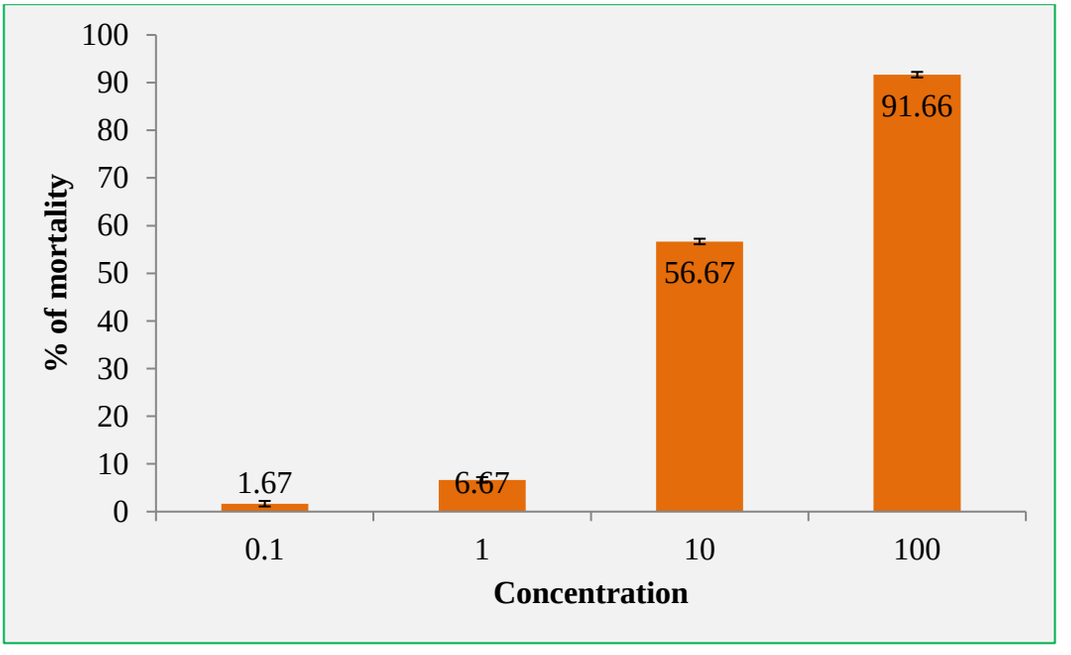

Figure 3: Percentage of mortality after 12 hours (mean±SD) by Copper sulfate

Graphical representation of rate of mortality of *A. amphitrite* nauplii by Copper sulfate at pH 7.0 Temperature 28ºC in time period of 12hrs, the graphs indicate the maximum rate of mortality occurred in 10ppm with 56.67±0.58%.

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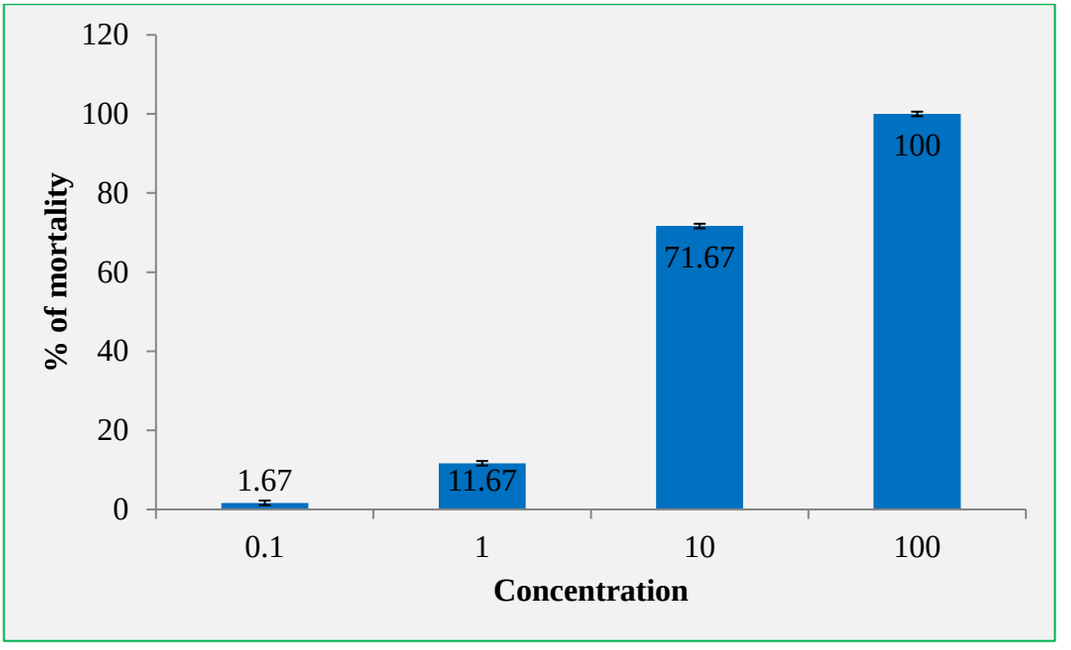

Figure 4: Percentage of mortality after 24 hours (mean±SD) Copper sulfate

Graphical representation of rate of mortality of *A. amphitrite* nauplii by $CuSo_4$at pH 7.0 Temperature 28ºC in time period of 24hrs, the graphs indicate the maximum rate of mortality occurred in 10 ppm with 71.67±0.58%.

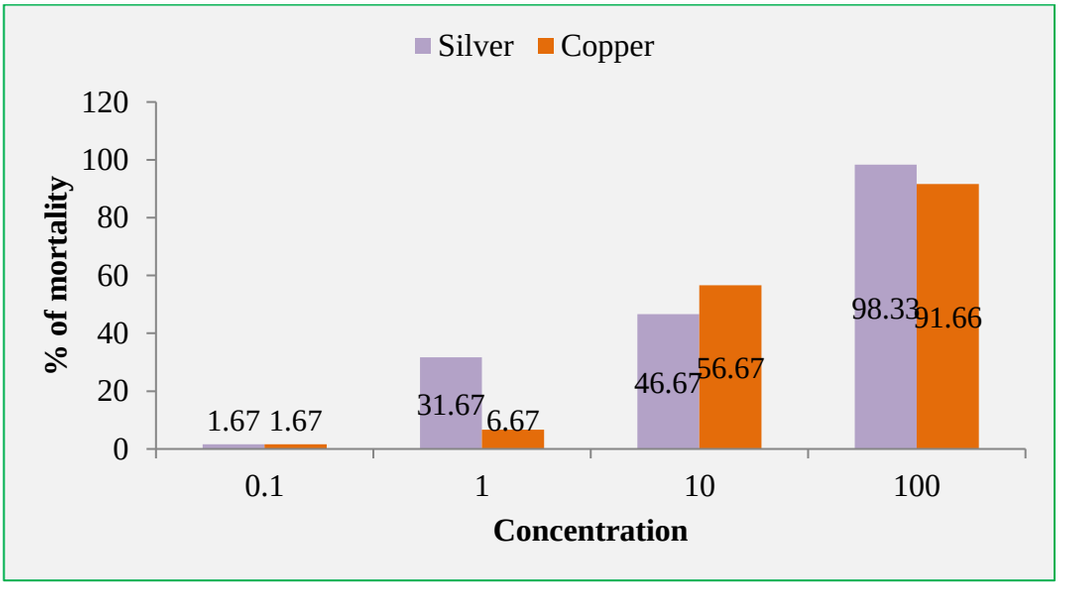

Figure 5: Comparison of percentage of mortality after 12 hours (mean ± SD) of Silver nanoparticles and Copper sulfate

Graphical representation Comparison of rate of mortality of *A. amphitrite* nauplii by AgNP's and $CuSo_4$at pH 7.0 Temperature 28ºC in time period of 12hrs, the graphs indicate the maximum rate of mortality occurred in 10 ppm with 46.67±0.58 and 56.67±0.58% respectively.

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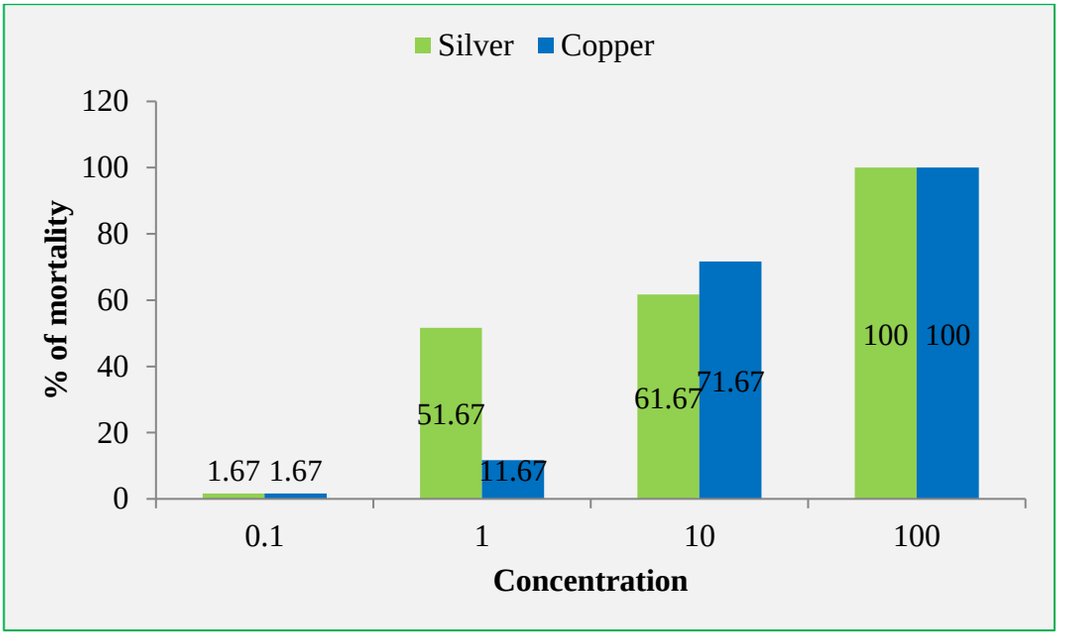

Figure 6: Comparison of percentage of mortality after 24 hours (mean ± SD) of Silver nanoparticles and Copper sulfate

Graphical representation Comparison of rate of mortality of *A. amphitrite* nauplii by AgNP's and $CuSo_4$at pH 7.0 Temperature 28^oC in time period of 24 hrs, the graphs indicate the maximum rate of mortality (LC_{50}) occurred in 10 ppm with 51.67±0.58 and 11.67±0.58% respectively.

Results and Discussion

Currently, we have looked into how Ag NPs affect Amphitrite nauplii developed in a lab, but thorough research on barnacle larvae and the impact of various environmental factors on the uptake and interaction of Ag NPs is lacking. The model organisms used, *A. amphitrite*, were shown to be suitable for examining the biological impacts and interactions of Ag NPs in preliminary tests. The effect of different concentrations of Ag NPs to nauplii is summarized in (**Figures 1 to6**). **Table 1 &2** shows the results obtained with a toxicology assay of silver and copper respectively. Exposure for 24 h to a concentration of 1 ppm of Ag NPs showed a significant effect on *amphitrite* nauplii. Results are expressed as percentage mortality (±SD). Data were analyzed using one-way ANOVA, where $P < 0.01$.

The 24 h LC_{50} values (Toxicity assay) of silver nanoparticles and copper have been reported as 1 and 10 mg L^{-1}, respectively. Our data (**Table 1 & 2**) are consistent with the reported values. These results suggest that the action of the silver nanoparticles have a higher nonspecific toxic action than the traditional antifouling compound (copper sulfate) when tested with the larval Toxicity Assay. In the current study, the synthesized silver nanoparticles were tested as potential antifouling compound using copper as a control. AgNP's showed maximum affects with a significant percentage of mortality (LC_{50}) of **51.67±0.58** and were proved to be potential antifouling compound when compared to copper sulfate (**11.67±0.58**) after 24 hr respectively. The experiment is done in triplicate, the results show significant with p value <0.01. The AgNP's at 1 ppm (which is equivalent to 1 mg L^{-1}) is used to test the evaluation of toxicity, this concentration did not significantly affect the larvae of *A. amphitrite*, at 12 h, however the following 24 h of exposure to AgNP's as low as 1 ppm experienced a decrease in survival to 50%.

Significantly, the tested compound (Silver nanoparticles) is toxic to *A. amphitrite* than the copper sulfate. Hence this study suggests that the silver nanoparticles were promising antifouling compound which is less toxic to the environment compared to copper sulfate. After a 24-hour incubation period at 28°C, the number of swimming and dead nauplii was counted. Larvae that weren't swimming were thought to be dead (Rittschof *et al.,* 1992). (Hellio *et al.,* 2004) Toxicity results are shown as 24 h LC_{50} with 95% confidence intervals. It is common knowledge that marine fouling organisms settle on man-made surfaces submerged in seawaters and frequently cause technical and financial issues (Richmond and Seed, 1991).

The International Maritime Organization's Marine Environment Protection Committee has proposed a ban on the application of TBT-based antifouling paints as of January 1, 2008 (Yebra *et al*., 2004), despite the fact that these compounds, such as tributyltin (TBT) and tributyltin oxide (TBTO), are very effective at controlling these fouling organisms (Ellis, 1991). Antifouling paint formulas currently use certain booster biocides, but they may also contaminate aquatic habitats (Thomas, 2001). New antifouling chemicals that are effective and environmentally friendly are therefore urgently needed.

This study clearly shows that the larvae of *A. amphitrite*, is most sensitive to silver nanoparticles than copper in toxicity testing, not only because its surface adsorption, but also the systemic integration of silver due to its nano size. Previous toxicity studies of AgNPs (Kvitek *et al.,* 2009, Panacek *et al.,* 2009, and Allen *et al.,* 2010) stated that higher toxicity of AgNPs were possibly due to smaller aggregation size, which increased the surface area to volume ratio and enhance the uptake of AgNPs by the test organism, hence had a higher toxicity. Similar results were observed in this study stating that silver nanoparticles were more effective than the copper.

However, data on the physiological effects of AgNPs on marine organisms, especially invertebrates, remain very insufficient. This paper provides one of the first evidence that AgNPs may have a significant effect on the larvae of *A. amphitrite* a major biofouling organism. It is demonstrated that the low lethal concentrations of AgNPs can affect larval survival compared to copper (**Figure 4**). Results of our toxicity tests (24 h LC_{50}) demonstrated that AgNPs could cause mortality in *A. amphitrite* larvae at the concentration 1ppm, i.e., almost 10 times more effective than the copper sulfate. (**Figure 1 & 2**), it has been reviewed that AgNPs were most toxic to crustaceans and algae followed by fish, bacteria and ciliates (Kahru and Dubourguier, 2010).

In the present study, the mortality rate was observed in larvae of *A. amphitrite*, at different concentrations (0.1 ppm, 1 ppm, 10 ppm and 100 ppm) exposure of AgNPs for 24 hours. Nevertheless, the physiological mechanisms of AgNPs toxicity in marine invertebrates remain completely unknown in aquatic species. Previous studies showed that the toxic effect of silver on aquatic species involves disruption of osmoregulation (Wood *et al.,* 1999, Grosell *et al.,* 2002, Bianchini *et al.,* 2005).

In the present study, we did not distinguish the effect of AgNPs on *A. amphitrite* larvae via waterborne and dietary exposure, but the AgNPs exposure through the latter route has been reported to exert deleterious effects on various aquatic invertebrates (Hook and Fisher, 2001; Huang *et al.,* 2010, Brix *et al.,* 2012). AgNPs may be ingested by the larvae, possibly leading to greater bioaccumulation of AgNPs, and thus higher toxicity. Concerns have been raised about the microscopic size of NPs that potentially allows them to penetrate cells and cell organelles and further disrupt the normal cellular functions (Buffet *et al.,*2013).

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Several *in vitro* studies have shown that AgNPs were introduced in the cytoplasm of human mesenchymal stem cells, hepatoma cells, rat primary neural cells, and rainbow trout gill cells (Farkas *et al.,* 2011; Greulich *et al.,* 2011; Haase *et al.,* 2012; Yu *et al.,* 2013), and this was further supported by *in vivo* studies employing terrestrial plant, freshwater algae and fish (Ma *et al.,* 2010; Miao *et al.,* 2010 and Scown *et al.,* 2010). It is suggested that the general route of uptake of particles or molecules ranging up to 100 nm is endocytosis (Luoma, 2008, Iversen *et al.,* 2011).

Extensive *in vitro* and *in vivo* studies reported that AgNPs evoked increase in reactive oxygen species (ROS) level, abnormal accumulation of ROS refers to oxidative stress that damages DNA, cellular proteins, and cell membrane (Apel and Hirt, 2004, Wei *et al.,* 2010). It is possible for silver to mediate DNA damage and apoptosis by inducing ROS production (Kim and Ryu, 2012) and the positive relationship between AgNP induced ROS and resulting oxidative stress and apoptosis has been supported by several *in vitro* studies (Hsin *et al.,* 2008, Foldbjerg *et al.,* 2009, Eom and Choi, 2010, Liu *et al.,* 2010). Given the rapid development of nanotechnology industry, contamination of the coastal marine environment with toxic chemicals like copper, mercury, TBT etc, there is an urgent need to replace these chemicals, hence the emerging nanomaterials like silver nanoparticles have become a growing concern. The outcome of this work will provide a better answer to antifouling technologies with the integration of silver nanoparticles.

Conclusion

Numerous kinds of barnacles are gregarious, and their cypris larvae are remarkably adept at exploring surfaces before deciding to establish a permanent connection. Therefore, in this study, we focused on nauplii of the barnacle sp. to assess the potential toxicity of silver nanoparticles as antifouling agents. The outcomes demonstrated that synthesized silver nanoparticles were 10 times more efficient than copper. Hence the study suggests a new compound as antifouling agent.

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