Green synthesis of Silver nanoparticles through *Calotropis gigantea* leaf extracts and evaluation of antibacterial activity against *Vibrio alginolyticus*

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Abstract

Green synthesized silver nanoparticles by *Calotropis gigantea* leaf extract were used to study the inhibitory activity against pathogenic *Vibrio alginolyticus*, isolated from wild *Artemia franciscana* cysts. Silver nanoparticle synthesis was observed using UV-visible spectroscopy and the morphological characteristics were analyzed by atomic force microscope (AFM). In the present study, increasing concentrations of silver nanoparticles synthesized on LB agar plates effectively reduced the number of colonies of *V. alginolyticus*. A decrease in colonies (CFUs) was observed at 5 μg/mL of silver nanoparticle concentration and the complete inhibition of *V. alginolyticus* was observed at 20 μg/mL of silver nanoparticle concentration on LB agar plates. *In vivo* controlling efficiency of silver nanoparticles was tested in an *A. franciscana* hatching system. Effective control of *V. alginolyticus* in brine shrimp *A. franciscana* hatching units was achieved by experimental infection and treatment with silver nanoparticles. Experimental infection studies showed that *V. alginolyticus* infected *Artemia* nauplii treated with silver nanoparticles (10 μg/mL) had greater survival (>40%) than silver nanoparticles not treated with nauplii. Based on the findings of this study, it is recommended that low concentrations of green synthesized silver nanoparticles should be further investigated for other potential experimental models to control potential medical pathogens.

Introduction

The emergence of nanoscience and nanotechnology presents opportunities for exploring the bactericidal effect of metal nanoparticles. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes, and is not merely due to the release of metal ions in solution. Nanophasic and nanostructure materials are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications.[1] Technological development and applications are out-pacing research on health and environmental risks posed by nanoparticles. Like genetically modified organisms, the future of nanotechnology will depend on public acceptance of the risks versus benefits. However, the antimicrobial effects of silver nanoparticles (AgNps) have not been investigated in aquacultural practices. It is very important to note that opportunistic bacteria are one of the causative agents of disease in aquaculture, and effective, eco-friendly control methods are needed to avoid their outbreak.

Mass mortality due to infections by endemic microbes in the aquaculture industry causes severe economic loss.[2] In recent decades, the use of chemotherapeutic agents and antibiotics to control microbial disease outbreaks in aquaculture has increased dramatically, and this has given rise to antibiotic resistance to pathogenic bacteria.[3] *Vibrio* species naturally occur in culture systems and are resistant to various drugs, making these species difficult to control when outbreaks occur, as they adapt quickly to antibiotics. In this study, we have investigated the *in vivo* efficiency of control of AgNps against simple experimental system, *i.e. Artemia* hatching system. In aquaculture practice, live feeds, especially *Artemia* cysts, serve as the major carrier of *Vibrio* species in both larviculture and grow-out stages.[4] During the hatching process, these *Artemia* nauplii are heavily contaminated, mainly with *V. alginolyticus*,[5] an omnipresent organism in the marine environment. It is an opportunistic pathogen of vertebrates and invertebrates[6] causing septicemia, exophthalmia, corneal opaqueness, ascites, lethargy, melanosis and ulcers in finfish.[7] *V. alginolyticus* has been reported to cause diseases in the shrimp *Penaeus monodon* and necrosis in *Macrobrachium rosenbergii* larvae.[8-10] It has also been associated with diseases in sea bream (*Sparus aurata L.*), grouper (*Epinephelus malabaricus*), sea mullet (*Mugil cephalus*) and red sea bream (*Pagellus bogaraveo*).[5]

Recent studies using metallic nanoparticles have demonstrated a broad range of antimicrobial activity against several gram positive, pathogenic bacteria such as *Staphylococcus epidermis*, *S. aureus*, *Micrococcus luteus*,[11-13] gram negative pathogenic bacteria such as *Klebsiella pneumoniae*, *Salmonella typhi*, *V. cholera*, *Escherichia coli*, *Pseudomonas aeruginosa*,[14-16] viruses,[17] pathogenic fungi,[18] and eukaryotic microorganisms. However, there are still no available reports in scientific literature on antibacterial activity of AgNps against pathogenic bacteria causing disease in commercially important aquatic organisms. Therefore, the present study was undertaken to assess the inhibitory activity of AgNps synthesized by *Calotropis gigantea* leaf extract against pathogenic *V. alginolyticus*, and also to evaluate the *in vitro* inhibitory concentration of AgNps against *V. alginolyticus* blooms in *Artemia* hatching practices in aquaculture.

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Materials and Methods

Synthesis of AgNps from *C. gigantea* leaf extracts

Calotropis gigantea mediated synthesis of AgNps was carried out based on the green synthesis procedure with slight modifications.[19,20] *C. gigantea* leaves (6.5 g) were washed thoroughly with sterile distilled water for 10 min, air dried, finely cut and boiled for 15 min with 350 mL of sterile, distilled water. After boiling, the broth was cooled at room temperature and

filtered through Whatman filter paper to obtain a clear, leaf broth. Silver nitrate ($AgNO_3$) (purchased from Sigma Aldrich company, product n. 204390; St Louis, MO, USA) was used for the synthesis of AgNps. In a typical synthesis, 75 mL of 0.01 M $AgNO_3$ was added to 10 mL of leaf extract with continuous stirring, at 40ºC. Within 30 min, a yellow colouration appeared, indicating the onset of AgNps formation. The synthesis of AgNps was monitored by UV-visible spectroscopy operating at the wavelength of 280-700 nm (ELICO SL 159, India) at time intervals of 1, 5, 10, 15, 20, 25 and 30 days. The synthesized AgNps were collected from Day 10 onwards and centrifuged at 5000 g at room temperature for 10 min. The pellets were dried at 45°C for 24 h and the powder was dispensed in sterile, distilled water to obtain the required experimental concentration for the experiments. The size and the morphology of the AgNps were examined by Atomic Force Microscope (Veeco di CP-II, USA).

Collection and identification of *V. alginolyticus*

The experimental *V. alginolyticus* was isolated from the wild *Artemia franciscana* cysts (Veppalodai solar salt works, Tuticorin, India).[21] Briefly, the total cyst microflora was counted using serial dilution method followed by a quadrant streaking method. Further pure cultures grown in TCBS agar (Himedia, Bombay, India) supplemented with 1.5% NaCl, were chosen and identified using standard biochemical methods.[22] The *V. alginolyticus* (isolate code DHMVA07) strain was selected and confirmed by PCR and 16S rDNA typing methods.[23] The pathogenicity of the strain was evaluated using experimental infection in *A. franciscana* nauplii (LD_{50} value of 10^5 CFU mL^{-1} in experimental infection of *Artemia* nauplii). The colony counts reported in the present study were estimated through serial dilution, plating and counting methods.

Inhibitory activity against *V. alginolyticus*

Green synthesized AgNps were tested for antibacterial activity against pathogenic *V. alginolyticus* using the well-diffusion method. *V. alginolyticus* (10^4 CFU) was inoculated on Muller-Hinton agar plates. 100µL samples of synthesized AgNps (experimental), crude *C. gigantea* leaf broth and $AgNO_3$ (0.75mM final concentration) solution were added to individual wells in separate plates. After incubation at 37°C for 24 h, the zone of inhibition was measured. The minimum inhibitory concentration of the synthesized AgNps was determined using 10^4 colony forming units (CFU) of *V. alginolyticus* on Luria-Bertani (LB) agar plates supplemented with AgNps at concentrations of 5, 10, 15, and 20 µg mL^{-1} (in triplicate). The plates were incubated for 24 h at 37°C and the numbers of colonies were counted. If the number of CFU on an agar plate was greater than 300 colonies, then the result was interpreted as being too numerous to count (TNTC).[24]

Artemia nauplii hatching test

The hatchability of *A. franciscana* (San Francisco Bay, USA) was evaluated following the procedure of Lavens and Sorgeloos.[25] Prehydrated (160 mg) cysts were hatched in cylindroconical tubes containing 80 mL of pre-aerated filter sterilized sea water under constant illumination and aeration at 28°C. Three experimental tubes in triplicate (control, *V. algynolyticus*, and AgNps+*V. alginolyticus*), were left for 24 h to assess the hatching percentage. *V. alginolyticus* (4×10^5 CFU) were used in this experiment with the concentration of 5µg/mL AgNps (based on the effective minimum inhibitory concentration (MIC) determined through the previous experiment) in seawater. The hatching percentage was calculated with the following formula:

$$\%H=\frac{N}{N+C+U}\times100$$

where N=Number of nauplii; C=Number of unhatched cysts; U=Number of umbrella stage nauplii.

Artemia nauplii challenge infections

Three test groups were set up in triplicate: Control (C), *V. alginolyticus* inoculated (V) and *V. alginolyticus*+5 µg/ml AgNps (VN). Each group contained 100 *Artemia* nauplii (Instar I stage) inoculated in pre-aerated seawater (10 mL), and was challenged with *V. alginolyticus* (5×10^4 CFU). The active napulii were considered live and were counted under a light microscope.

Survival rate was determined at 2 h intervals over the period of 12 h. The survival percentage was calculated using the formula: survival rate (%)=[number of live nauplii at the 2 h intervals/number of nauplii at the time of inoculation]×100.

Statistical analysis

Experimental groups, percentage of hatched cysts and survival rates were analyzed by a one-way ANOVA followed by Tukeys Honest Significant Difference test ($P<0.05$).[26]

Results

Synthesis of AgNps from *C. gigantea* leaf extracts

The synthesis of AgNps was detectable following a color change in the reaction solution (*C. gigantea* leaf extract+0.75 mM silver ions) from Day 2 of incubation (UV-Vis spectra), and a distinct peak was observed at 410 nm. The UV-Vis spectra show a slow shift in peak values and wavelength for the AgNps through the first ten days of the synthesis (Figure 1A). The peak values continued to increase during the incubation period (Figure 1B). After ten days, the change in the peak value and peak wavelength was reduced, and saturation was observed from Day 15. The depth profile using atomic force microscopy (AFM) shows the spherical arrangement of the synthesized AgNps particles, with a diameter range of 6.3-12.67 nm (Figure 2).

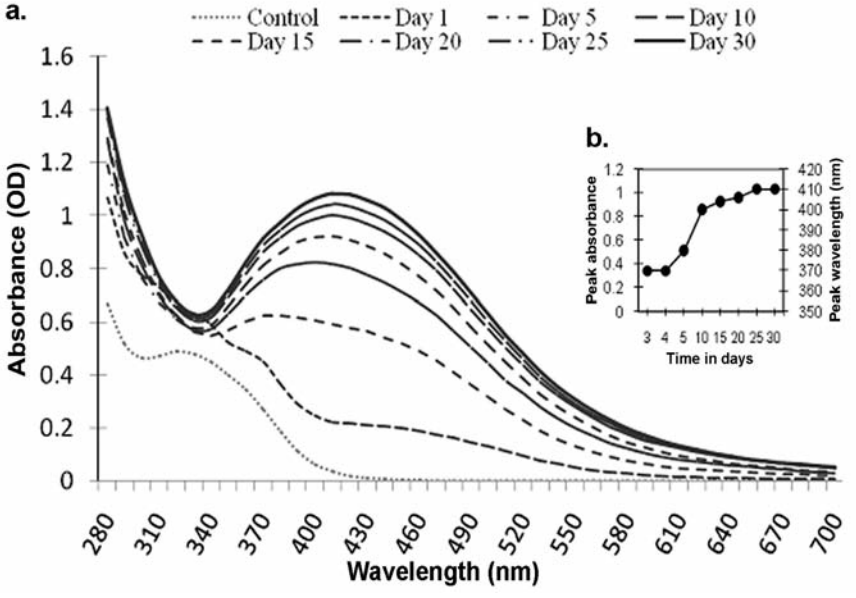

Figure 1. a. UV-visible reflective spectra of AgNps synthesis at different time intervals. b. Change in absorption peak and wavelength of the peak absorbance in time (Days).

In vitro inhibitory activity of AgNps against *V. alginolyticus*

AgNps synthesized with *C. gigantea* leaf extract showed a strong inhibitory activity against *V. alginolyticus* (well diffusion method). The inhibition zone for AgNps was 16 mm (±0.25) in diameter, while that for the silver nitrate solution without synthesized AgNps was 2 mm in diameter. No zone of inhibition was observed for *C. gigantea* leaf extracts. Figure 3 shows the colony counts of *V. alginolyticus* (10^4 CFU) grown on LB agar plates, supplemented with varying concentrations of AgNps (5, 10, 15, and 20 μg/mL). More than 300 colonies were observed on the LB agar plates containing 5 μg/mL concentration of green synthesized AgNps (Figure 3). Higher concentrations of AgNps (10 and 15 μg/mL) restricted the number of colonies on the LB agar plates to 116 and 17, respectively. At a concentration of 20 μg/mL, AgNps totally inhibited the growth of *V. alginolyticus*.

In vivo antibacterial activity of AgNps in an *Artemia* hatching setup

The efficacy of AgNps *in vivo* against *V. alginolyticus* was tested in *Artemia* nauplii at hatching. The hatch rate for *A. franciscana* cysts treated with 5 μg/mL AgNps was significantly higher (84%) than that of untreated cysts (64%) infected with *V. alginolyticus* (Figure 4).

A. franciscana nauplii survival rate

A. franciscana nauplii treated with AgNps (5 μg/mL) (VN) showed significantly higher survival rate when compared to *V. alginolyticus* infected *Artemia* nauplii (V). There was no survival recorded for Control *A. franciscana*, following a 12 h exposure to *V. alginolyticus*. Experimental infection studies also showed that *V. alginolyticus* infected *Artemia* nauplii, treated with AgNps (10 μg/mL seawater), showed greater survival (>40%) than that of non-AgNps treated *Artemia* nauplii (Figure 5).

Discussion

The application of metallic nanoparticles to control microbial mediated diseases remains largely unexplored. Several methods have been employed to synthesize AgNps, including green chemistry. Although a number of physical and chemical methods for the synthesis of AgNps are available, there is a need to develop an eco-friendly method for the synthesis process.[27] The green synthesis of AgNps has been reported in bacteria and angiosperms.[28-29] In the present study, aqueous extracts of *C. gigantea* were used to synthesize AgNps and tested against *V. alginolyticus*. Plant extracts are widely applied to synthesize AgNps by reducing Ag^+ ions into Ag^0 ions, which increases the optical density of the solution.[30] This is due to the excitation of SPR (surface plasmon resonance) and the reduction of silver nitrate during the incubation period.[31] The shift in the spectral peak value observed in the present study is due to a variation in the size and shape of the synthesized AgNps (Figure 1).[32] An increase in absorption spectra (350-600 nm) did not affect the spectral region of ~410 nm, indicating that the particles present in the broth were not aggregating.[12] AFM revealed that the size of the AgNps synthesized during this study was 6-12 nm. Several studies have reported that efficient antimicrobial properties of AgNps were dictated by the small size and surface planar accessibility of the AgNps.[18, 33,13]

Lower concentrations of AgNps (5, 10, 15 and 20 μg mL^{-1}) on LB agar plates effectively reduced the number of *V. alginolyticus* colonies. A gradual decrease in CFUs on LB agar plates was observed at 5μg/mL AgNps, while a total inhibition of *V. alginolyticus* was achieved at 20μg/mL concentration of AgNps. According to Nanda and Saravanan,[16] 2 μg of biosynthesized AgNps failed to show a zone of inhibition against *V. cholera*. When different concentrations of AgNps were incorporated on the LB agar plates, the bacterial growth was completely inhibited at or above a desired concentration. The concentration of the nanoparticles and the decrease in bacterial CFU influences the complete inhibition. The mechanism of the bactericidal action of AgNps is only partially understood. Nanosilver particles can enter into the bacterial cells,[26] possibly by dis-

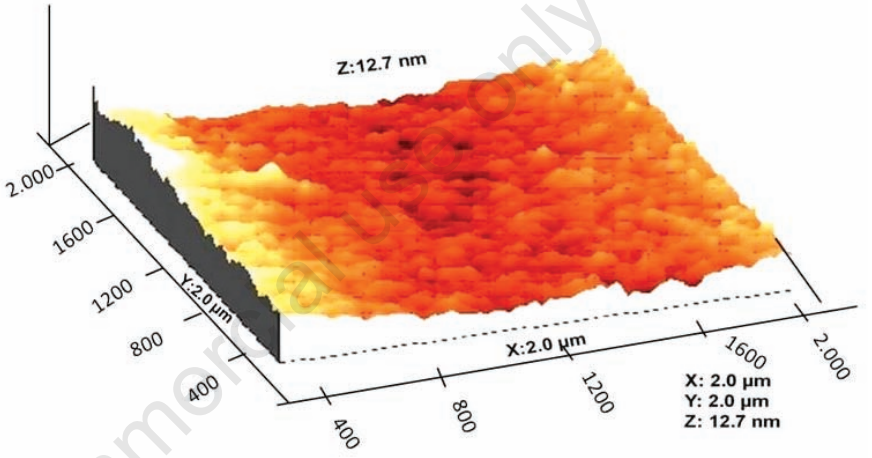

Figure 2. Atomic force microscopy depth resolved image of AgNps synthesized by *C. gigantea* leaf extract.

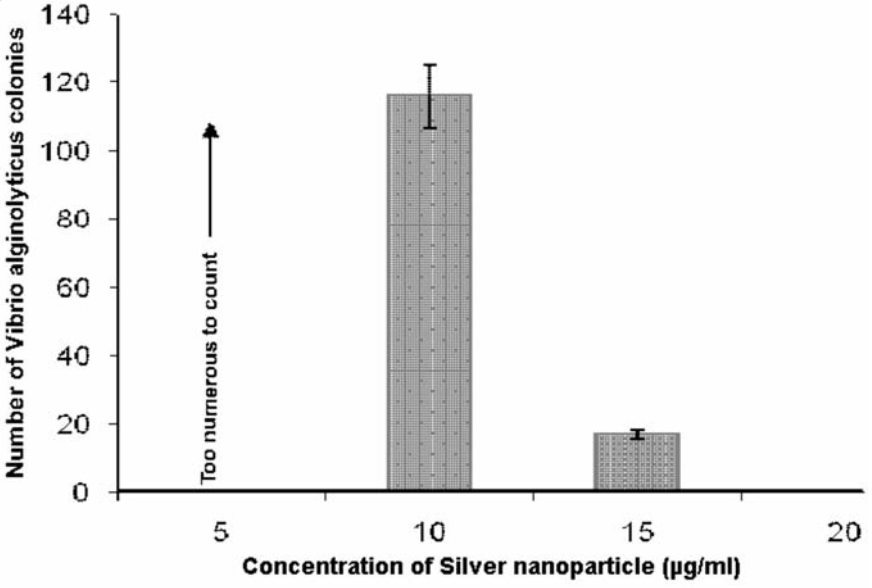

Figure 3. Inhibitory activity of AgNps synthesized by *C. gigantea* leaf extract at different concentrations (μg/mL) against *V. alginolyticus*.

turbing the cell permeability and respiratory functions.[25] Ag^+ ions suppress the bacteria by affecting the sulphur group of the biomolecules.[32] It has been suggested that the nanoparticles cause cell wall protein and other protein denaturation and prevent the replication process taking place.[14,17] We observed that 5 μg (per 100 mL of hatching media) of AgNps present in hatching medium improved the hatching percentage. The nauplii challenge tests provide convincing evidence that AgNps can control and eradicate *V. alginolyticus* from the hatching medium. *Artemia* cysts and nauplii are suitable for analyzing the activity of antibacterial substances in seawater systems.[15] Application of antibiotics to control the pathogens may involve the development of a risk factor of resistance as well as transfer of virulent genes. Therefore, immediate attention should be given to trying alternative strategies to control microbes. Alternatively, the safe use of plant-based AgNps in higher organisms requires relatively a low concentration for effective control activities. Silver has a long history as a germicide and has little or no toxic impact at low concentrations.[22] Nano material research and risk assessments will ultimately need to address multiple potential health related aspects of aquatic animal diseases. Even though there is no report yet available on the application of AgNps in controlling *Vibrio* species, it appears that aquatic animals can be employed to evaluate nanoparticle toxicity.[34] In conclusion, the green synthesis of metallic nanoparticles on overcoming multidrug resistant bacterial pathogens is still an unexplored area of research, and the present study has made an attempt to show the capability of AgNps to control *Vibrio* as an experimental model. The study successfully demonstrated that AgNps synthesized from *C. gigantea* leaf extract can control *V. alginolyticus* in an *Artemia* culture system. Further studies are needed to assess the long-term toxic effects of AgNPs in the system, and to extend this knowledge to other experimental models.

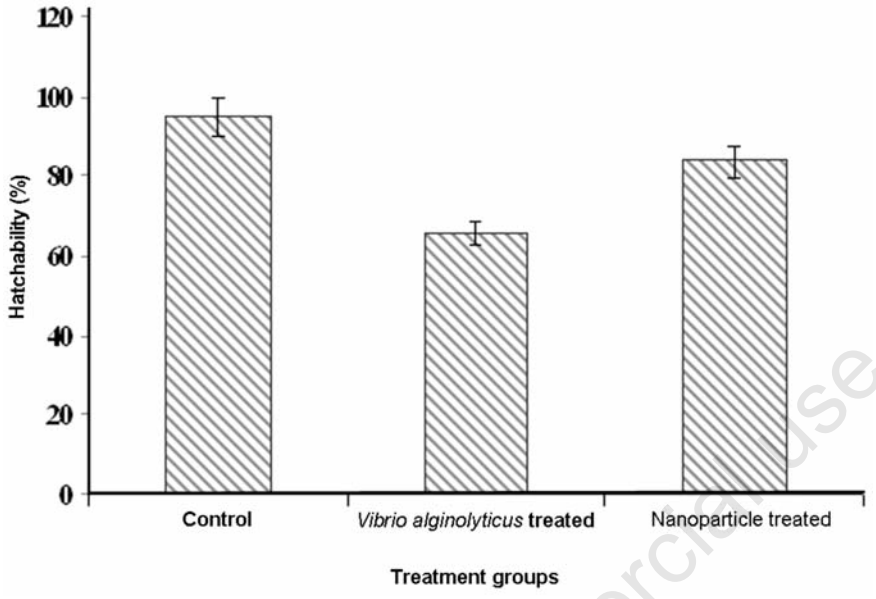

Figure 4. Hatch rate for of *Artemia franciscana* cysts after *V. alginolyticus* challenge (4×10^5 CFU)

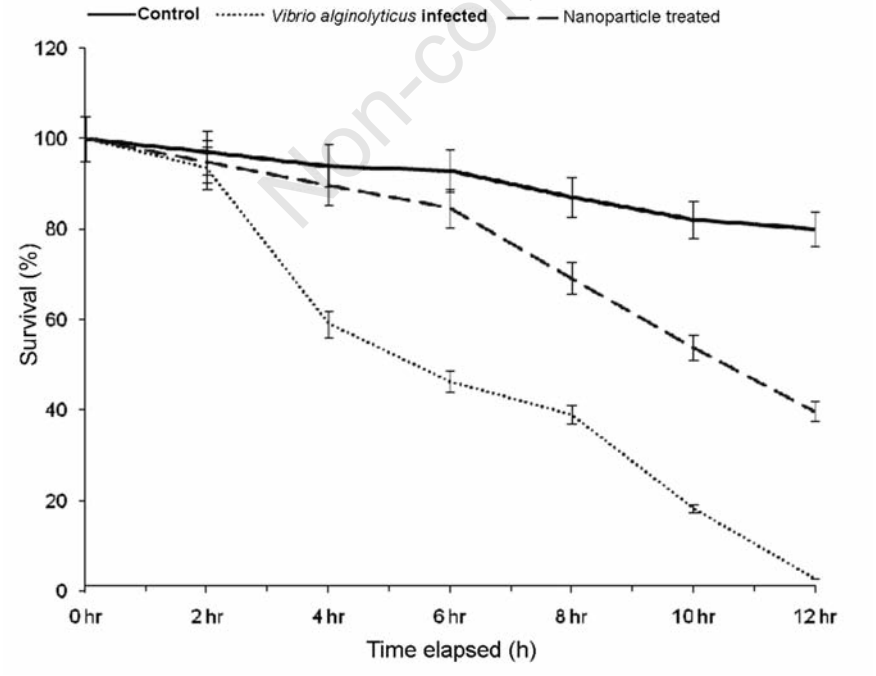

Figure 5. Effect of AgNps on the survival rate (%) of *Artemia* nauplii during *V. alginolyticus* challenge (4×10^5).

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