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Total hemocyte and hemoglobin changes in nypa palm worm (*Namalycastis rhodochorde*) to *Aeromonas* sp. NrBF9 infection

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Abstract. *Yanti AH, Arinendo HI, Setyawati TR, Kurniatuhadi R. 2025. Total hemocyte and hemoglobin changes in nypa palm worm* (Namalycastis rhodochorde) *to* Aeromonas *sp. NrBF9 infection. Biodiversitas 26: 4274-4283.* Nypa palm worms (*Namalycastis rhodochorde*) are polychaetes essential to mangrove ecosystems as detritivores and decomposers. They also hold economic value for coastal communities through cultivation. However, pathogenic infections threaten their survival and aquaculture. *Aeromonas* sp. NrBF9, isolated from the worms' fecal pellets, is an indigenous pathogen with hemolytic and DNase activities that likely cause hematological damage. Previous studies observed blanching symptoms in cultured worms exposed to *Aeromonas* from their feces, hypothesized to result from blood and hemocyte damage, though this remains unconfirmed. This study examined the hematological profile of nypa palm worms after dorsal injection with *Aeromonas* sp. NrBF9 at 10^3, 10^5, and 10^7 cells/mL. Parameters assessed included total hemocyte counts, hemoglobin levels, and hemocyte diameters. Hemolytic activity was tested using a smear method on worm blood agar. Results showed infected worms developed anemia, with significant reductions in total hemocyte counts (32.9%, 39.2%, and 39.9% decreases at 10^3, 10^5, and 10^7 cells/mL, respectively; $p = 0.007$, $p<0.05$), and hemocyte diameters (8.8%, 12.5%, and 15.0% decreases at 10^3, 10^5, and 10^7 cells/mL, respectively; $p = 0.00$, $p<0.05$). Infected worms also exhibited paler hemocytes and hemocyte aggregation, while hemoglobin levels remained unchanged ($p = 0.679$, $p>0.05$). *Aeromonas* sp. NrBF9 displayed β-hemolytic activity on worm blood agar. These findings show changes in hemocyte counts and colonization caused by *Aeromonas* infection, emphasizing the need for early detection and management to minimize losses and boost nypa palm worm aquaculture productivity.

Keywords: *Aeromonas* pathogenesis, dorsal blood vessel injection, hemocyte, nipah worm, polychaeta worm

INTRODUCTION

Nypa palm worms (Nereididae: Polychaetes) play a crucial ecological role as detritivores in mangrove ecosystems, aiding organic matter decomposition and nutrient cycling (Junardi 2021; Miri et al. 2023). They also serve as bioindicators of water pollution and contribute to sediment stabilization, improving estuarine environments (Putro et al. 2025). In biomedicine, these worms show promise due to bioactive compounds with antimicrobial and anti-inflammatory effects, supporting novel wound regeneration therapies (Ghazaly et al. 2024). Furthermore, their adaptability and high nutritional value make them a sustainable aquaculture feed alternative, reducing reliance on costly commercial feeds (Setyawati et al. 2020; Junardi 2021; Kurniatuhadi et al. 2025).

The increasing use of polychaete-based products in aquaculture has driven demand for cultivable species. Polychaetes provide essential nutrients, including nutrients —proteins, lipids, amino acids, and vitamins—offering a sustainable alternative to traditional fish feed (Dorgham et al. 2015; Pajand et al. 2017; Phoonsamran et al. 2017; Monteiro et al. 2024). They are also preferred feed for shrimp broodstock (Jayaseelan et al. 2021), highlighting the need for standardized cultivation methods (Serebiah 2015).

Nypa palm worms (*Namalycastis rhodochorde* (Glasby, Miura, Nishi & Junardi, 2007)), native to West Kalimantan, are economically valuable to coastal communities as fishing bait and aquaculture feed due to their high protein content, exceeding 58% (Junardi et al. 2020; Setyawati et al. 2020; Yanti et al. 2020). Despite laboratory-scale cultivation using in vitro fertilization, challenges such as slow growth and susceptibility to protozoan and bacterial infections remain (Junardi et al. 2020). These worms are especially vulnerable to pathogens throughout their life cycle (Yanti et al. 2022). Junardi et al. (2020) identified bacterial infections as a major cause of high larval mortality during cultivation. Their deposit-feeding behavior increases exposure to sediment-borne pathogens. Desrina et al. (2018) noted that introducing polychaetes into aquaculture ponds raises infection risks, as infected polychaetes consumed by brood shrimp can transmit pathogens. Thus, ensuring aquaculture products from these worms are free of pathogens is essential.

N. rhodochorde play key ecological and aquacultural roles by contributing to sediment bioturbation and nutrient cycling in estuaries. Their susceptibility to bacterial infections, such as *Aeromonas*, makes them valuable for studying invertebrate immunity and health management in aquaculture (Setyawati et al. 2020; Yanti et al. 2022). Invertebrates, including aquatic worms, rely on innate immune defenses like hemocytes for phagocytosis and encapsulation, and humoral factors such as antimicrobial peptides and the prophenoloxidase (proPO) melanization cascade to combat pathogens (Melillo et al. 2018; Qadeer

and Cheng 2024). Polychaete hemocytes, diverse in form and function, include granular amoebocytes for pathogen defense, agranular hyalinocytes for recognition and cell development, eleocytes for nutrient storage and repair, and pigment-containing cells for oxygen transport. They support immunity, healing, nutrient distribution, detoxification, and respiration in some species (Cuvillier-Hot et al. 2014; Canesi et al. 2022). Hemocyte responses can also exhibit trained immunity-like memory, enhancing defense upon secondary exposures. As a sediment-dwelling annelid with a complex hemocyte system, *N. rhodochorde* offers a valuable in vivo model for investigating defense mechanisms, hematological pathology from pathogen stress, and early-warning biomarkers for aquaculture health.

Aeromonas poses a major pathogenic threat in aquaculture, causing severe diseases in fish, annelids, and crustaceans that lead to significant economic losses (Setyawati et al. 2020; Semwal et al. 2023). It is known to cause opportunistic and primary infections such as septicemia, ulceration, and hemorrhage, which reduce survival rates and product quality in invertebrates (Gallani et al. 2020; Kari et al. 2022). Infection with *Aeromonas* causes pale coloration in nypa palm worms, linked to the pathogen's hemolytic activity on sheep blood agar (Setyawati et al. 2020). This pallor diminishes the appeal of *N. rhodochorde*, whose color depends largely on their blood or hemocytes. Additionally, mature worms are especially susceptible to infection. It induces pale coloration in nypa palm worms due to hemolytic activity, lowering their market appeal (Setyawati et al. 2020). Mature worms are particularly vulnerable to infection.

Aeromonas sp. NrBF9, isolated from nypa palm worm fecal pellets, exhibits hemolytic and DNase activity (Setyawati et al. 2020), potentially causing hematological damage and threatening palm worm cultivation. Previous studies have reported color changes in infected worms (Yanti et al. 2022) and immune responses involving various coelomocytes (Yanti et al. 2022; Ramadani et al. 2023). However, detailed investigations into the hematological effects on hemocytes are lacking, as prior research has focused mainly on coelomocytes and general symptoms. Further study of hemocyte hematology post-*Aeromonas* infection is needed to better understand the nypa palm worm immune system in aquaculture.

Hemocyte parameters are key indicators of invertebrates' physiological and pathological states (Canesi et al. 2022; Lynch et al. 2022). Infectious diseases significantly impact aquaculture, yet the effects of bacterial pathogens on hemocyte responses in nypa palm worms, specifically *N. rhodochorde* from West Kalimantan, Indonesia, remain understudied. This study investigates hemocyte responses following injection with *Aeromonas* sp. NrBF9, a potential pathogen isolated from their digestive tract. The findings may aid in health assessment and monitoring of nypa palm worms and support management strategies to reduce losses and improve productivity in their aquaculture.

MATERIALS AND METHODS

Materials

The materials utilized in this study comprised sub-mature nypa palm worms, measuring between 90 to 100 cm in length and weighing between 20 to 25 g. Additionally, *Aeromonas* sp. NrBF9, Nutrient Agar (NA), Luria-Bertani Agar (LBA), blood worm-derived agar, 0.9% physiological saline (NaCl), McFarland 0.5 standard solution, Hayem's solution, and Wright's stain (HCl) were employed. *Aeromonas* sp. NrBF9 was isolated from the fecal pellets of *Namalycastis rhodochorde*, as reported by Setyawati et al. (2020).

Research design

The research was conducted from April to July 2024 at the Hydrobiology Laboratory, Department of Biology, Universitas Tanjungpura, Pontianak, West Kalimantan, Indonesia. A completely randomized design was used to study the effects of different concentrations of *Aeromonas* sp. NrBF9, with three distinct treatment levels based on the 50% Lethal Dose (LD_{50}) for nypa palm worms, ranging from 10^3 to 10^7 cells/mL (Setyawati et al. 2020). Bacterial injections included three concentrations: 10^3 cells/mL (P1), 10^5 cells/mL (P2), and 10^7 cells/mL (P3). Controls consisted of a positive control group injected with sterile distilled water and a negative control group with no bacterial injection. Negative controls assessed symptom development and hemocyte data in uninfected worms, while positive controls evaluated injection effects on hematological profiles and excluded bacterial infection. Each treatment was replicated five times with five worms each, totaling 25 worms. Measured parameters included total hemocyte count, hemoglobin levels, hemocyte diameter, and qualitative blood smear observations. Clinical symptoms on the worms' body surfaces during exposure to *Aeromonas* sp. NrBF9 were also recorded. All parameters and symptom development were observed daily for up to 72 hours, with symptoms characterized qualitatively after injection.

Procedures

Nypa palm worm (Namalycastis rhodochorde) *preparation*

The experiment used 25 sub-mature nypa palm worms, 90-100 cm long and weighing 20-25 g, showing no clinical signs of *Aeromonas* infection. Sub-maturity was confirmed by detecting early-stage gamete cells in 0.3 mL of coelomic fluid, examined microscopically at 1000× magnification. Immature sperm and ova among eleocytes indicated gamete presence. Worms were acclimated for seven days in a controlled tank with mud substrate and nypa palm leaf litter as food, maintained at 28°C and salinity of 10-20 ppt. Sterile estuarine water was sprayed to keep the soil moist (Yanti et al. 2022).

Aeromonas *sp. NrBF9 preparation*

The *Aeromonas* isolate from previous research on *N. rhodochorde* feces has not been molecularly identified; its strain was determined using cell morphology, physiology, and biochemical tests based on Bergey's Manual of Determinative Bacteriology. *Aeromonas* sp. NrBF9 was first rejuvenated on Nutrient Agar (NA) medium by streaking a

pure culture onto sterile NA (2 g NA powder in 100 mL distilled water) and incubating at 37°C for 24 hours, following Yanti et al. (2022). After incubation, the bacteria were suspended in 0.9% NaCl and vortexed to match the turbidity of McFarland 0.5 standard (~1.5 × 10^8 cells/mL). Turbidity was confirmed by UV-Vis spectrophotometry at 600 nm with an absorbance of 0.12. The suspension was then serially diluted to 10^7, 10^5, and 10^3 cells/mL using sterile saline. Cell density was determined by direct counting with a hemocytometer (Yanti et al. 2022).

Aeromonas *infection into nypa palm worm*

Aeromonas sp. NrBF9 infection was conducted using a modified injection method based on Setyawati et al. (2020) and Yanti et al. (2022). Instead of injecting the bacterial suspension into the worm's ventral region to reach the coelomic cavity, this study targeted the dorsal vessel blood near the setae using a 1 cc syringe. Nypa palm worms were anesthetized with clove oil (1 mL per 20 L sterile seawater) before injection. A 0.1 mL bacterial suspension at concentrations of 10^3, 10^5, and 10^7 cells/mL, plus a positive control, was injected intramuscularly into the dorsal vessel blood at the anterior, median, and posterior segments. Each treatment was replicated five times, totaling 25 worms. After injection, worms were returned to the maintenance tank and observed for 72 hours, with morphological assessments every 24 hours to detect bacterial damage (Yanti et al. 2022).

Worm's blood sampling and hemocyte extraction

Blood sampling was conducted by making incisions in the anterior, median, and posterior regions of the worm's body with a scalpel. Worms were anesthetized in ice water at 4°C for five minutes beforehand (Setyawati et al. 2020). Hemocytes were extracted by inserting a sterile needle or micropipette into a dorsal longitudinal incision targeting the ventral blood vessels (Abdurrahman et al. 2022). Blood was collected into 3 mL microtubes containing anticoagulant (e.g., EDTA) to prevent clotting. Samples were then centrifuged gently at 500 × g for 5-10 minutes at 4°C to separate hemocytes from plasma. The hemocyte pellet was used for total hemocyte count analysis.

Total hemocyte count procedure

The total hemocyte count of the nypa palm worm was measured using a hemocytometer (Abdurrahman et al. 2022; Yanti et al. 2022). A blood sample was drawn to the 0.5 mark, then mixed with Hayem's solution up to the 101 mark on the erythrocyte pipette. The mixture was thoroughly agitated and transferred to the hemocytometer counting chamber. Under 400× magnification, hemocytes were counted in 80 small squares (16 small squares within five medium squares). Counting followed a zigzag pattern from top left to right, then down to the next row. The total hemocyte count per mm^3 was calculated using: N = (number of erythrocytes/80) × 4000 × 200, where N represents the number of hemocytes per mm^3 of blood.

Hemocyte smear preparation

Hemocyte smears were prepared using the smear technique and Wright staining. Blood was applied to one slide's edge, and a second slide was angled at 45° to spread the blood across the first slide. After air-drying, Wright stain was applied for 1-2 minutes to fix the cells, followed by an equal volume of phosphate buffer and incubation for 5-7 minutes for optimal staining. The smear was rinsed with water, air-dried, and examined microscopically from low to high magnification (up to 1000× with oil immersion) for detailed cell observation (Juanda et al. 2023). Hemocyte diameter was measured by sampling ten representative sample of ten representative cells.

Determination of hemoglobin (Hb) concentration

Hemoglobin levels were measured using the Sahli method (Pathak et al. 2024). A Sahli tube was filled with 0.1 N hydrochloric acid (HCl) solution to the second mark. Blood (20 µL) was drawn from the dorsal vessel with a Sahli pipette and added to the HCl. Residual blood was cleared by aspirating and expelling HCl two to three times. After 3-5 minutes of incubation, distilled water was added and gently mixed to match the standard color. Hemoglobin concentration was read from the Sahli tube scale and reported in g/dL.

Hemolytic activity in Aeromonas *sp. NrBF9*

The hemolytic activity test involved culturing *Aeromonas* sp. NrBF9 on modified blood agar, where sheep blood was replaced with nypa palm worm (*N. rhodochorde*) blood, following Setyawati et al. (2020). This modification aimed to assess *Aeromonas* sp. NrBF9's activity against nypa palm worm hemocytes in vitro. Based on Li et al. (2023), 1 mL of fresh nypa palm worm blood was mixed with 20 mL of Luria-Bertani Agar. The bacterial smear was applied to the agar surface and incubated at 37°C for 48 hours. Hemolytic activity was determined by observing clear zones around bacterial growth.

Data analysis

The total counts of hemocytes, hemoglobin (Hb) levels, and hemocyte diameters were assessed for normality at a significance level of $\alpha = 0.05$. When the data conformed to a normal distribution, subsequent analyses were conducted using Analysis of Variance (ANOVA) with a significance threshold of 0.05. Post-hoc comparisons were performed using Duncan's test, maintaining the same significance level, with all analyses carried out in SPSS Version 28.

RESULTS AND DISCUSSION

Hematocyte response of the nypa palm worm following injection of *Aeromonas* sp. NrBF9

The hematological profile examined in this study encompassed total hemocyte counts, hemoglobin concentrations, and hemocyte diameters, which collectively indicated the presence of anemia in the nypa palm worms subjected to injection with *Aeromonas* sp. NrBF9 bacteria. The average total hemocyte count for the nypa palm worms

in this investigation ranged from 0.86 × 10^6 cells/µL to 1.44 × 10^6 cells/µL (Table 1). It was noted that total hemocyte counts diminished with increasing doses of the *Aeromonas* sp. NrBF9 bacterial suspension. In the control group, total hemocyte counts varied from 1 × 10^6 cells/µL to 2.12 × 10^6 cells/µL, with the highest average recorded in the sterile aquabidest injection treatment at 1.44 × 10^6 cells/µL (n = 5). In contrast, the injection treatment exhibited total hemocyte counts ranging from 0.65 × 10^6 cells/µL to 1.2 × 10^6 cells/µL, with the lowest average observed in the injection treatment at a concentration of 10^7 cells/mL, which was 0.87 × 10^6 cells/µL (n = 5). All data demonstrated a normal distribution (p-value>0.05). Statistical analysis using ANOVA confirmed a significant effect (p = 0.007; p<0.05) of *Aeromonas* sp. NrBF9 injection on the total hemocyte counts of the nypa palm worms.

The average hemoglobin levels of nypa palm worms in this study ranged from 5.68 g/dL to 6.72 g/dL (Table 1). It was noted that hemoglobin levels exhibited a decreasing trend with increasing doses of *Aeromonas* sp. NrBF9 bacterial suspension. The control treatment resulted in hemoglobin levels between 6.28 g/dL and 6.72 g/dL, with the highest average recorded in the aquabidest injection treatment at 6.72 g/dL (n = 5). In contrast, the injection treatment demonstrated hemoglobin levels ranging from 5.68 g/dL to 6.12 g/dL, with the lowest average observed in the 10^7 cell/mL injection treatment at 5.68 g/dL (n = 5). Statistical analysis using ANOVA confirmed that the *Aeromonas* sp. NrBF9 injection did not have a statistically significant effect (p = 0.679; p>0.05) on the hemoglobin levels of nypa palm worms. Additionally, a positive correlation (R^2 = 0.758706) was observed between hemocyte counts and hemoglobin levels (Figure 1), suggesting a linked physiological response, although the reductions in hemoglobin levels remained within normal limits.

Table 1. Hematology profile of nypa palm worm (*Namalycastis rhodochorde*) following injection with *Aeromonas* sp. NrBF9

Treatments (cells/mL)	Parameters		
	Average hemoglobin level (g/dL)	Total hemocyte count average (× 10^6 sel/µL)	Average of hemocyte diameter (µm)
Without injection	6.28±1.22^{ns}	1.43±0.42^{a}	5.21±0.33^{a}
Aquabidest injection	6.72±0.93^{ns}	1.44±0.38^{a}	5.21±0.10^{a}
Aeromonas 10^3	6.12±1.13^{ns}	0.96±0.17^{b}	4.75±0.10^{b}
Aeromonas 10^5	6.00±1.47^{ns}	0.87±0.15^{b}	4.56±0.21^{bc}
Aeromonas 10^7	5.68±0.67^{ns}	0.86±0.19^{b}	4.43±0.09^{c}

Note: The occurrence of identical numerical values accompanied by the same letter denotes the absence of a statistically significant difference between treatments at the 5% level, as assessed by Duncan's test. Conversely, this designation indicates that there is no substantial difference; thus, further analysis utilizing Duncan's test is unnecessary

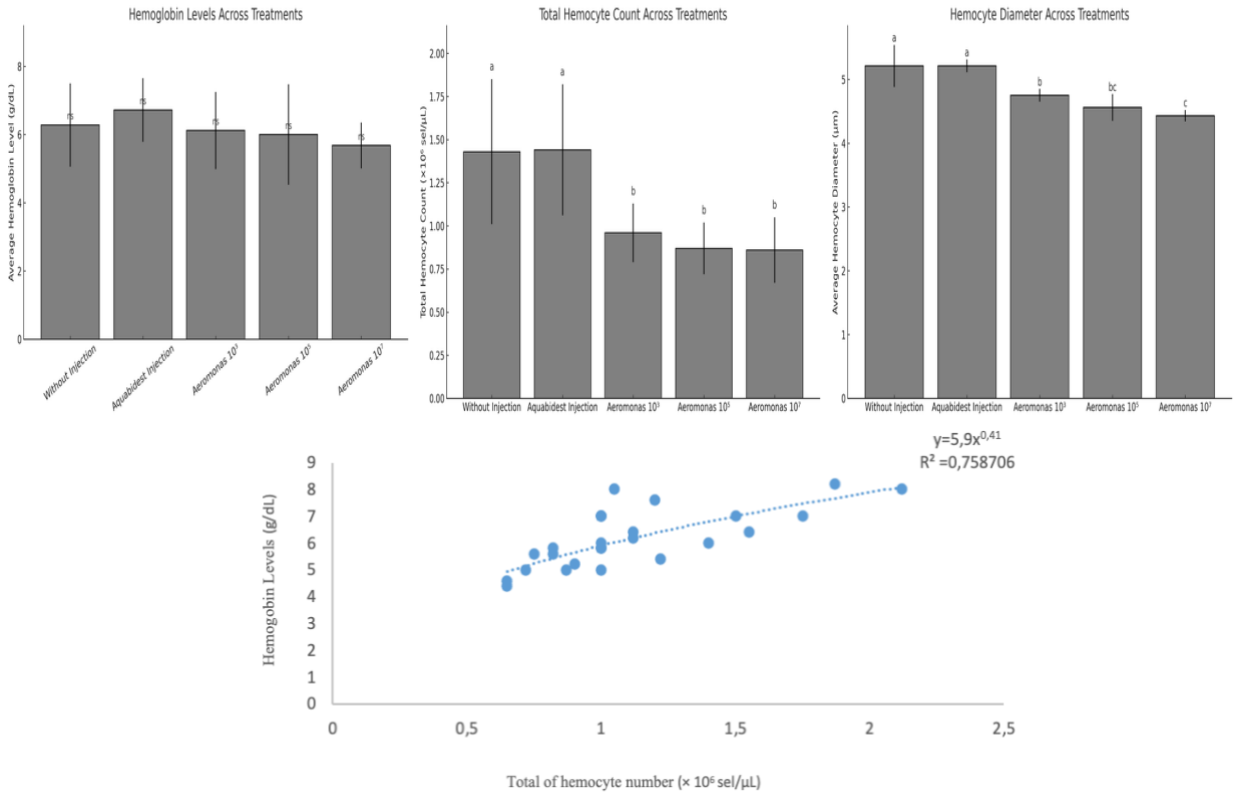

Figure 1. Quantitative charts with error bars and statistical significance (above) and correlation between total hemocyte count and hemoglobin levels in nypa palm worms (*Namalycastis rhodochorde*) (below)

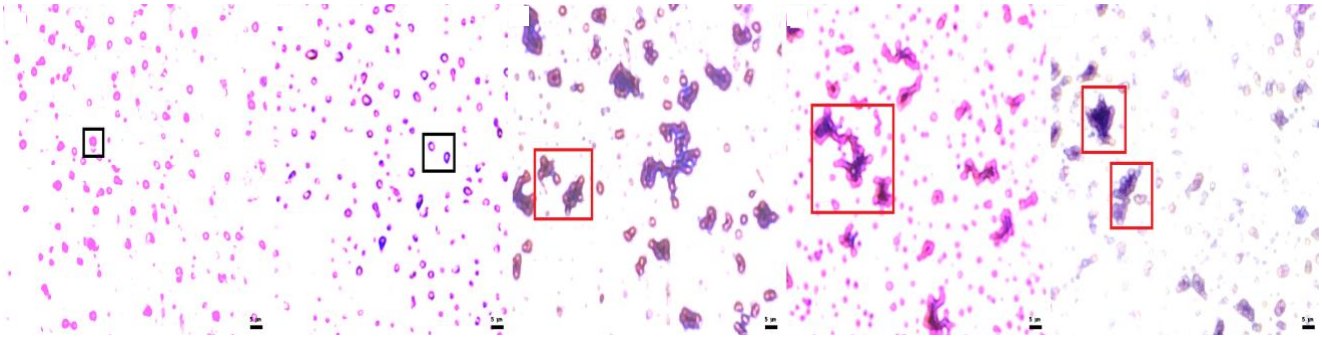

Figure 2. The visualization of hemocytes from the Nypa palm worm across various treatment conditions is presented. Control Treatment: A. Negative control (no injection), B. Positive control (injection with sterile aquadest). Bacterial Injection Treatment: C. *Aeromonas* suspension at a concentration of 10^3 cells/mL, D. *Aeromonas* suspension at a concentration of 10^5 cells/mL, E. *Aeromonas* suspension at a concentration of 10^7 cells/mL. The aggregation of cells, as indicated by the red box, represents the clustering of hemocytes observed in the blood smear results following the bacterial injection treatment. The cells enclosed within the black box represent individual hemocytes (Scale bar corresponds to 10 µm)

Figure 3. The visualization of the hemolytic activity of *Aeromonas* sp. NrBF9 on blood agar media supplemented with the blood of the nypa palm worm is presented as follows: A. Observations made after 24 hours of incubation did not indicate any hemolytic activity, B. Observations conducted after 48 hours of incubation revealed a distinct clear zone and a significant fading of the agar color, which suggests the occurrence of hemolysis (the clear zone is delineated by a white circular line)

The diameters of hemocytes ranged from 4.43 µm to 5.21 µm (Table 1), demonstrating a decrease in size with increasing bacterial concentration. The control group exhibited hemocyte diameters between 4.66 µm and 5.51 µm, with the highest average diameter of 5.21 µm recorded in the non-injection treatment (n = 5). In contrast, the bacterial injection treatment resulted in hemocyte diameters ranging from 4.26 µm to 4.84 µm, with the lowest average diameter of 4.43 µm observed in the 10^7 cells/mL injection treatment (n = 5). Analysis of Variance (ANOVA) revealed that the injection of *Aeromonas* sp. NrBF9 bacteria had a statistically significant effect ($p = 0.00$; $p<0.05$) on the diameter of hemocytes in nypa palm worms. Hemocytes from nypa palm worms injected with *Aeromonas* sp. NrBF9 exhibited a reduced diameter and tended to display a paler coloration compared to the control group (Table 1).

Microscopic evaluation demonstrated significant differences in hemocyte characteristics between the control treatment and the bacterial injection treatment. In the infected worms, hemocytes exhibited aggregation, forming rouleaux structures in which cells adhered to one another (Figures 2.C-2.E). The blood smears of nypa palm worms that were not subjected to bacterial injection did not show any aggregation of multiple hemocytes. Furthermore, hemocytes from the bacterial injection group displayed a paler coloration compared to the control samples, which retained a deeper purple staining due to enhanced dye absorption (Figure 2).

Hemolytic activity of *Aeromonas* sp. NrBF9

The hemolytic activity assay demonstrated that *Aeromonas* sp. NrBF9 possesses the ability to lyse hemocytes when cultured on blood agar derived from nypa palm worms. This β-hemolytic activity, indicated by a clear zone surrounding the bacterial colony, became apparent after 48 hours of incubation (Figure 3.B). The observed hemolysis is likely associated with the lysis of the hemocytes from the worm, resulting in the degradation of hemoglobin (Figures 2.C-2.E).

Symptoms manifesting in the body of *N. rhodochorde* following injection of *Aeromonas* sp. NrBF9

Following the injection of bacteria, nypa palm worms exhibited various pathological changes, including wounds, erythema of the setae, and thickened tissue (Figures 4.A-4.C). Morphological abnormalities were observed 72 hours post-injection, particularly in worms treated with *Aeromonas* sp. NrBF9. Notable symptoms included the presence of white lesions along the anterior-posterior axis (Figure 4.A), the formation of hardened tissue resulting in segmental protrusions (Figure 4.B), and the appearance of red spots near the injection site (Figure 4.C). Despite exposure to different concentrations of bacteria, no mortality was recorded among the worms. Symptoms began to show signs of improvement on the fourth day, as evidenced by the disappearance of the wound at the infected site and the resolution of white lesions on the affected segments of the nypa palm worm.

Discussion

Hemocytes are motile cells found within the hemolymph of invertebrates, playing crucial roles in both immune and transport systems. Annelids, particularly polychaetes, are distinguished by the presence of two compartments containing circulating cells: (i) the blood system, which includes hemocytes; the role of this compartment in immunity has been previously investigated, and (ii) the coelom, which houses various populations of coelomocytes that contribute to immune defense (Cuvillier-Hot et al. 2014; Kurenkova et al. 2023). In certain species, coelomocytes exhibit similarities to vertebrate macrophages, performing essential functions in cellular immunity through mechanisms such as pathogen phagocytosis, encapsulation of large structures (e.g., eggs from parasitoids), and humoral responses mediated by the production of Antimicrobial Peptides (AMPs) (Yang et al. 2021). Differentiated hemocytes also play a significant role in maintaining homeostasis by facilitating tissue repair, synthesizing extracellular matrix components, and clearing apoptotic cells (Hartenstein 2020; Soderhall et al. 2022). Additionally, they are involved in the storage of lipids and nutrients, as well as the transport of iron (Cattenoz et al. 2020; Cattenoz et al. 2021).

The results of the study revealed a significant decrease in the total number of hemocytes in *N. rhodochorde* injected with *Aeromonas* sp. NrBF9 when compared to the control treatment. The average total hemocyte counts for the negative and positive control treatments were recorded at 1.43×10^6 cells/µm and 1.44×10^6 cells/µm, respectively. In contrast, the hemocyte counts following bacterial injection (ranging from 10^3 to 10^7 cells/mL) were measured at 0.96×10^6 cells/µm, 0.88 x 10^6 cells/µm, and 0.87×10^6 cells/µm, respectively. Previous research indicated that the total hemocyte counts in *N. rhodocohorde* under normal conditions ranged from 1.5×10^6 cells/µm to 2×10^6 cells/µm (Ramadani et al. 2023), the observed values exhibit similarities with the control treatment worms in this study, thereby indicating a clear tendency for a decrease in hemocytes as a result of *Aeromonas* infection. A study conducted by Dolar et al. (2020) on the crustacean *Porcellio scaber* (Latreille, 1804) demonstrated that the reduction in total hemocyte counts was associated with the migration of hemocytes to the site of infection caused by pathogenic bacteria (*Rhabdochlamydia porcellionis*) and Iridovirus, as well as processes such as aggregation, lysis, and the dissemination of infection to the hemopoietic tissue. A comparable phenomenon was documented in lobsters infected with the PaV1 virus, as indicated by Pascual et al. (2022), who demonstrated a reduction in hemocyte counts in juvenile *Panulirus argus* (Latreille, 1804) infected with the virus.

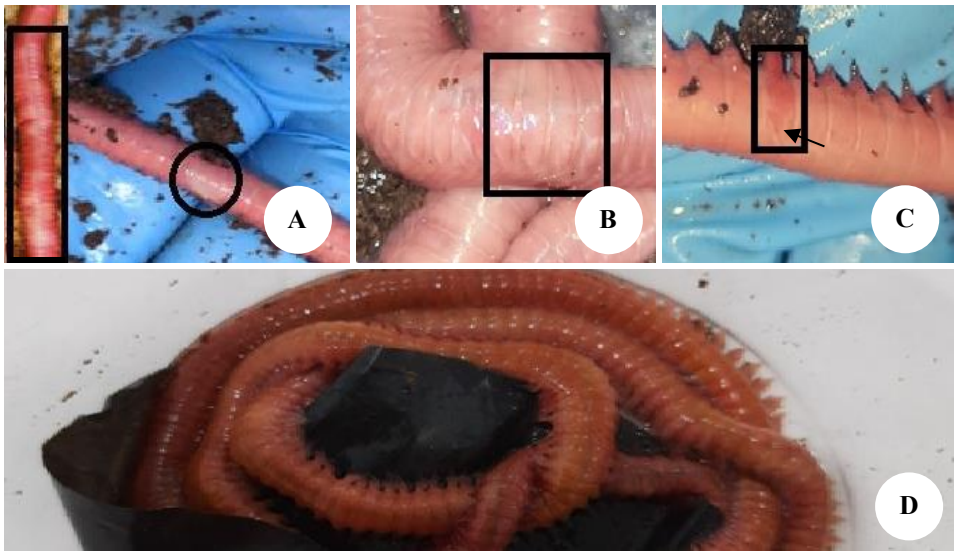

Figure 4. Symptoms observed in nypa palm worms following *Aeromonas* sp. NrBF9 into the body surface included: A. The development of white lesions or wounds (indicated by round and square boxes), which were the initial symptoms to manifest within 24 hours of incubation, B. The thickening and wrinkling of skin tissue (indicated by a square box), which represented the second set of symptoms appearing within 48 hours of incubation, C. The emergence of red spots on the setae (indicated by a square box), which constituted the third set of symptoms observed within 72 hours of incubation. In contrast, D. The control group of normal worms did not exhibit any symptoms, and their body coloration remained reddish

Hemocyte quantity positively correlates with hemoglobin levels in nypa palm worms (Table 1). Worms injected with *Aeromonas* sp. NrBF9 showed a decrease in hemoglobin compared to controls, but this reduction was not statistically significant and remained within the normal range. Average hemoglobin levels were 6.28 g/dL (negative control), 6.78 g/dL (positive control), and 6.12, 6.00, and 5.68 g/dL for bacterial doses of 10^3, 10^5, and 10^7 cells/ml, respectively. Normal hemoglobin levels in uninfected nypa palm worms range from 6 to 8 g/dL (Ramadani et al. 2023). Although *Aeromonas* infection reduced hemoglobin levels, they stayed within physiological norms. Qosimah et al. (2023) also reported decreased hemoglobin in *Aeromonas*-infected zebrafish, but data on its effects in Polychaetes, especially *Namalycastis*, are lacking. Unlike vertebrates, in which hemoglobin is contained within red blood cells, many annelids possess hemoglobin that circulates freely outside of cells. Only a limited number of annelid species have red blood cells that internally carry hemoglobin. We assumed that the extracellular globin structure in these annelids differs significantly from vertebrate hemoglobin. Instead of a heterotetramer composed of globin subunits, many annelids exhibit a large hexagonal bilayer hemoglobin molecule (HBL-Hb, formerly known as "erythrocruorin"), consisting of at least 144 globin peptides assembled with linker proteins. Erythrocruorin is an extracellular protein freely dissolved in the hemolymph plasma of annelids, unlike vertebrate hemoglobin, which is contained within blood cells (Song et al. 2020). This suggests that the reduction in hemocytes from *Aeromonas* infection does not correspond to the decrease in hemoglobin, known as erythrocruorin. Consequently, it is necessary to address the limitations and termination criteria associated with hemoglobin level testing in nypa palm worms to enhance the specificity and appropriateness of the testing methodology.

Further analysis indicated a reduction in the diameter of hemocytes and a paler coloration in worms infected with *Aeromonas* sp. NrBF9 when compared to the control group (Figure 2). Although there is a lack of data from previous studies regarding the normal size range of hemocytes in nypa palm worms, existing evidence suggests a trend of decreasing hemocyte diameter correlating with an increase in bacterial cell numbers (Table 1). This alteration is likely associated with reduced iron levels in nypa palm worms as a consequence of *Aeromonas* sp. NrBF9 infection. Iron is crucial for hemoglobin synthesis in polychaetes (Vogt et al. 2021) and also plays a significant role in bacterial growth, reproduction, and virulence (Teng et al. 2018). *Aeromonas* species are known to utilize iron through the production of siderophores and heme-binding receptors (Maltz et al. 2015), which diminishes the availability of extracellular iron and may lead to hemocyte dysfunction. This depletion of iron may account for the observed alterations in hemocyte morphology and aggregation in blood smears.

The aggregation of hemocytes, as indicated by the red box in Figure 2, reflects the clustering of hemocytes observed in blood smear analyses following *Aeromonas* injection treatment. Tishkowski and Zubair (2023) noted that erythrocyte aggregation or sedimentation tends to increase under various conditions associated with the inflammatory response. Furthermore, a study by Chen and Keddie (2021) demonstrated that sepsis induced in *Galleria mellonella* by *Escherichia coli* results in heightened microaggregation of hemocytes, which is attributed to elevated levels of plasma fibrinogen and other acute-phase reactants. Furthermore, hemocyte aggregation may occur due to the adhesive properties of bacteria binding to the surfaces of two or more hemocytes. This interaction facilitates significant cell aggregation through a bridging mechanism, leading to the phenomenon of hemagglutination, as reported by Dara et al. (2025) in the hemocytes of *Sabella spallanzani* following infection with *E. coli*. This process is influenced by the hemocytes' ability to bind and engulf various bacterial species, as well as the differential susceptibility of these bacteria to intracellular killing. Additionally, the role of soluble factors, such as agglutinins and opsonins, along with surface-bound factors, in the phagocytosis of bacteria by hemocytes in the predominant marine bivalve species *Bathymodiolus japonicus* (Hashimoto & Okutani, 1994), *B. platifrons* (Hashimoto & Okutani, 1994), and *B. septemdierum* (Hashimoto & Okutani, 1994) has been emphasized (Tame et al. 2015).

Microbial infections involve complex host-pathogen interactions, with microbes employing various strategies to evade immune defenses (Thakur et al. 2019). *Aeromonas* species' pathogenicity stems from their ability to invade host cells, spread systemically, and produce virulence factors (Fernández-Bravo and Figueras 2020). Virulence varies by strain and host model (Li et al. 2023), but hemolysin activity significantly damages the bloodstream. This study confirmed hemolytic activity of *Aeromonas* sp. NrBF9 on nypa palm worm blood agar (Figure 3), showing β-hemolysis that lysed red blood cells, degraded hemoglobin, and formed clear zones around colonies. *Aeromonas* secretes extracellular products such as ochratoxin A, proteases, lipases, enterotoxins, and hemolysins (Awan et al. 2018), which aid bacterial adherence and attack on host cells, weakening immune responses and promoting disease (Ghatak et al. 2016). Hemolysin is the primary virulence factor, causing hemolytic and enteric toxicity by binding and damaging host cells (Takahashi et al. 2014). Hemolysin-related host responses and signaling pathways are key to pathogenicity. In *Aeromonas hydrophila*, hemolytic activity depends on hemolysin and aerolysin genes; their inactivation abolishes hemolysis (Petersen et al. 2017; Erickson et al. 2023). The cytolytic enterotoxin (Act) of *A. hydrophila* exhibits hemolytic, cytolytic, and enterotoxic effects (Pessoa et al. 2019).

Hemocytes are key to invertebrate defense, involved in recognizing, processing, and amplifying immune responses (Yang et al. 2021). Hemocytes are crucial for phagocytosis, encapsulation, cytotoxicity, and releasing reactive oxygen species and antimicrobial peptides. Humoral factors like lysozymes, lectins, agglutinins, and phenoloxidase-like cascades provide additional antimicrobial defense and pathogen recognition. These immune responses regulate sediment microbial communities, enhance nutrient recycling, and support population resilience under environmental stress. In aquaculture, the immune competence of polychaetes ensures their survival as live feed and bioremediators,

suppresses opportunistic pathogens such as *Vibrio* spp., and offers immune molecules with potential as natural antimicrobial agents to improve farm biosecurity and sustainability (Eleftherianos et al. 2021).

However, Boidin-Wichlacz et al. (2024) found that most annelid hemocytes primarily transport or store oxygen via intracellular hemoglobin. Studies of nypa palm worms infected with *Aeromonas* sp. NrBF9 showed a non-significant decrease in total hemocyte concentration with higher bacterial doses. Hemocyte response inversely correlated with coelomocyte activity in infected worms. Yanti et al. (2022) reported increased coelomocyte numbers in *N. rhodochorde* after *Aeromonas* injection, indicating an immune role. While hemocytes in the ventral blood vessels likely contribute to immunity, their response mechanisms differ, with coelomocytes functioning similarly to vertebrate leukocytes (Cuvillier-Hot et al. 2014; Yanti et al. 2022). In nypa palm worms infected with *Aeromonas* sp., hemocyte levels slightly decreased, while coelomocyte numbers rose, indicating their immune role (Yanti et al. 2022). Coelomocytes function similarly to vertebrate leukocytes, suggesting distinct immune mechanisms from hemocytes (Cuvillier-Hot et al. 2014; Yanti et al. 2022).

Although some studies suggest that hemocytes are involved in the immune response of invertebrates, the findings of the present study indicate a decrease in total hemocyte count following infection with *Aeromonas* sp. NRBF9 in the nypa palm worm (*N. rhodochorde*). This observation contrasts with the results reported by Yanti et al. (2022), who documented an increase in total coelomocytes upon infection with the same pathogen. These findings imply that, within Annelida, including Polychaeta, coelomocytes may play a more prominent role in immune function compared to hemocytes. The ability of hemocytes in nypa palm worms to form extracellular traps and encapsulate pathogenic microbes remains unclear, unlike that of coelomocyte cells (Topa et al. 2025). Coelomocytes are specialized motile cells residing in the coelomic fluid of annelids and contribute to immune defense mechanisms (Stanonova et al. 2023). While it remains uncertain whether hemocytes possess similar or distinct characteristics, this study provides a detailed description of hemocyte profile and characteristics during *Aeromonas* infection in the nypa palm worm, which were previously attributed primarily to coelomocytes.

This study shows that changes in hemocyte parameters in the examined species align with those in other annelids like *Arenicola marina* (Linnaeus, 1758) and *Capitella* sp. Hemocytes are central to immune functions, and their morphological changes indicate responses to environmental stressors. In *A. marina*, pathogen and pollutant exposure alters hemocyte and coelomocyte gene expression linked to defense (Stanovova et al. 2023). *Capitella* sp., a eutrophic bioindicator, has a single coelomic or hemocyte cell type performing metabolic and immune roles, so cell size changes likely reflect activation rather than proliferation (Boidin-Wichlacz et al. 2024). The stable Total Hemocyte Count (THC) alongside diameter changes suggests activation without recruitment, consistent with other polychaete studies. Hemoglobin level variations relate to its roles in oxygen transport, metal binding, and antioxidant defense (Coates and Decker 2017). Overall, hemocyte metrics effectively indicate organic pollution and pathogen stress in coastal ecosystems (Ayhan et al. 2021; Impellitteri et al. 2022).

Morphological changes in nypa palm worms injected with *Aeromonas* sp. NrBF9 include white lesions, red spots near the injection site, and tissue hardening (Figure 3). Although the LD_{50} was not reached at any bacterial concentration, all worms recovered within 3 to 4 days after symptom onset. Similar symptoms without mortality were reported by Setyawati et al. (2020) and Yanti et al. (2022) following bacterial injection into the coelomic cavity. These effects are likely caused by hemolysin secreted by *Aeromonas* sp. NrBF9. Grim et al. (2014) showed that hemolysin from *A. hydrophila* facilitates bacterial infection and spread, causing cellular damage and death. Li et al. (2023) found that hemolysin induces vesicle formation, erythema, and ulceration in fish abdominal cavities, indicating damage beyond the host's immune capacity. However, a rapid increase in diverse coelomocytes can reduce symptoms caused by *Aeromonas* injection (Yanti et al. 2022). Hemocytes play a key role in mitigating infection symptoms through agglutination, a process documented by Cuvillier-Hot et al. (2014), who noted that both hemocytes and coelomocytes contribute to the immune response in Polychaeta worms.

This study highlights experimental infection's role in understanding immune variability in nypa palm worms, focusing on hemocyte responses to *Aeromonas* infection. *Aeromonas* injection in *N. rhodochorde* caused dose-dependent changes in total hemocyte counts, reduced hemoglobin levels, and hemocyte anomalies, including adherent cells. These findings highlight the need for further research on hematological responses during natural and artificial infections. Limitations include small worm sample size, injection methods, and unclear disease mechanisms from tissue pathology, which restrict insights into pathogen effects on immunity and resistance. Treating each worm as an independent replicate, despite rearing over 25 individuals per treatment in a single container, inflates statistical power. Therefore, results are preliminary regarding nypa palm worm mortality from bacterial infection. Future studies should rear worms individually, increase sample size based on power analysis, and quantify mortality using dose-response curves to determine LD_{50} or LT_{50} with standardized bacterial doses. Additionally, research should examine hemocyte function and cellular traits beyond counts and hemocyte-to-coelomocyte ratios. Nonetheless, this study demonstrates that experimental infection is a valuable model for studying host-pathogen interactions and developing immunological defenses. Controlled infections also reveal how environmental factors —such as temperature, heavy metals, and pathogens— affect polychaete immunity, enhancing understanding of resistance mechanisms in natural and cultured ecosystems.

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