Determination of Median Lethal Concentration (LC50) and Nitrite Accumulation in the Blood and Tissue of Blood Cockle (*Tegillarca granosa*, Linnaeus 1758)

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Abstract: This study aimed to determine the nitrite toxicity of blood cockle *Tegillarca granosa*, with the objectives being to identify the median lethal concentration (LC50) and the accumulation level of nitrite in *Tegillarca granosa*, and to determine the relationship of nitrite accumulation with mortality percentage. The levels of LC50 and accumulation of nitrite were determined after 72 h of exposure to different nitrite concentrations (0, 0.5, 1.0, 1.5, and 2.0 mg/L). Nitrite accumulation was analysed using Method 8153 and a DR2800 spectrophotometer (HACH, Loveland, CO, USA). LC50 was identified at 1.53 mg/L, and nitrite accumulated in the ranges of 0.012 to 0.106 mg/L wet weight and 0.002 to 0.089 mg/L wet weight in the blood and soft tissue samples, respectively. Accumulation concentration in both tissue and blood cells increased proportionally with the exposure concentration, and had a strong positive relationship with the percentage of mortality. Our findings suggest that prolonged exposure of nitrite led to accumulation in the blood and tissues and caused cockle mortality.

Keywords: blood cockle; bivalves; bio-accumulator; nitrite; toxicity; pollution

1. Introduction

The presence of high nutrient residue in aquatic environments has been known to harm receiving water bodies. In addition, the presence of nutrient byproducts in water may cause aquatic life mortality. For example, the presence of nitrite (NO$_2^-$) due to the reduction of nitrates under conditions of an oxygen deficit increases the risk of accumulation of toxic nitrite, and may result in mass aquatic life mortality [1,2]. Examples of nitrite toxicity to aquatic lives are listed in Table 1. In a study of nitrite toxicity on post larvae of *Peneaus setiferus* (white shrimp), nitrite exposure significantly decreased the larvae’s temperature tolerance [3], while a study of amphibians showed nitrite-induced behavioural and morphological changes, reduced feeding activity, and also caused disequilibrium and paralysis, abnormalities, and death [4,5]. In fish, nitrite has resulted in the inhibition of chloride ion uptake at the bronchi, impairment of the acid–base balance and the electrolyte balance, reduction of the oxygen-carrying capacity of blood by the oxidation of haemoglobin (Hb) to methaemoglobin.
(MetHb), changes in gill histopathology and gross growth efficiency, and the survival of extracellular hyperkalaemia [6–8]. Moreover, the passage of nitrite into the bloodstream results in the irreversible conversion of Hb to MetHb, thus compromising its oxygen-binding capacity, causing respiratory deficiencies in aquatic animals and human beings [9,10].

Table 1. Examples of the nitrite toxicity to aquatic fauna.

| Species                          | Test         | Nitrite (mg/L) | Reference |
|---------------------------------|--------------|----------------|-----------|
| Gilthead seabream (12-day-old larvae) | LC50: 24 h   | 607            | [11]      |
| Australian crayfish             | LC50: 24 h   | 42.9           | [12]      |
| European eel                    | LC50: 96 h   | 144            | [13]      |
| Juvenile grass carp             | LC50: 96 h   | 10.6           | [6]       |
| Short-nose sturgeon             | LC50: 96 h   | 10             | [14]      |
| Nile tilapia                    | LC50: 96 h   | 8-81           | [15]      |
| Amphibian                       | LC50: 15 d   | <2             | [16]      |
| Tiger prawn                     | LC50: 96 h   | 14             | [17]      |
| White shrimp (Penaeus setiferus) | LC50: 24 h   | 1.49           | [18]      |
| Geoduck clam (Panopea japonica) | LC50: 72 h   | 1.12           |           |
|                                 | LC50: 96 h   | 112.76         | [19]      |

Nitrite toxicity has been known to depend greatly on the salinity of the water, with less toxic effects evident in marine organisms compared to freshwater organisms. However, because estuaries and coastlines receive terrestrial effluent, and both water bodies are productive areas of benthic life, the presence of nitrite is expected to have an impact on benthic life. Due to poor understanding of the effects of nitrite in estuaries, therefore, there is a need to continuously monitor and study the effects of nitrates in marine and estuarine organisms. In addition, very limited information exists addressing the effects of nitrite on bivalves, particularly on blood cockles Tegillarca granosa (Linnaeus, 1758). The production of Tegillarca granosa has reportedly experienced a tremendous decline in Southeast Asian countries, particularly Malaysia, over the past decade [20]. Although the most common reason for the decline of cockle production is excessive nutrients from agricultural activities [21], limited studies have been conducted on the toxicity effect of nitrite on the species. Hence, this study was conducted to provide baseline information on the effect of nitrite on Tegillarca granosa by determining the median lethal concentration of nitrite and the level of nitrite accumulation in the blood and soft tissues of Tegillarca granosa, as well as to study the relationship between nitrite accumulations and cockle mortality.

2. Materials and Methods

2.1. Sampling

Samples of Tegillarca granosa were collected from Bagan Pasir Laut, which is located 10 km from Bagan Datoh, Perak, Malaysia (Figure 1). The coordinates for the sampling site are 3°51′20.556″ N, 100°49′26.144″ E. Sampling was undertaken on 4 October 2018 to avoid the cockle’s spawning season. Samples of Tegillarca granosa were collected with a hand dredge net of 1.5 cm mesh. Approximately 100 cockles with sizes in the range 2–3 cm (Figure 1) were collected and transported alive to the laboratory.
2.2. Preparation of Artificial Seawater

Artificial seawater was used instead of natural seawater to minimize interference from other nutrients present in natural seawater that would affect the study findings. Artificial seawater with salinity between 25 to 30 ppt was prepared by dissolving 30 g of fine sea salt in 1 L of distilled water that was heated to 80 °C to accelerate the dissolving time, and then cooled to 28–35 °C. Other water parameters were monitored and controlled according to their natural habitat, as shown in Figure 2 and as in the Organization for Economic Co-operation and Development [22]. The oxygen content was monitored at a level of least 90% concentration (8.26–7.43 mg O₂/L at 25 °C) after aeration and stabilization for 24 h [23].

![Figure 1. Tegillarca granosa samples used for nitrite exposure.](image1)

2.3. Preparation of Nitrite Stock Solution

Stock solution of nitrite with concentration 100 mg/L was prepared by dissolving 0.15 g dried sodium nitrite (NaNO₂) in artificial seawater and bringing to a volume of 1000 mL. Before preparing the stock solution, NaNO₂ powder was dried at 105 °C and kept in a desiccator prior to utilization, in order to remove any excess moisture that would affect the result. The stock solution was prepared daily according to the Standard Method for the Examination of Water and Wastewater, Nitrogen (Nitrite) Section 4500-NO₂ Colorimetric Method.

![Figure 2. Mortality (%) of Tegillarca granosa after 72 h of exposure to a series of nitrite (NO₂-N) concentrations.](image2)
2.4. Cockle Acclimatization

The cockle samples were acclimatized for 24 h in 8 L of aerating artificial seawater, and the water was changed twice per day to simulate a suitable environment for cockles. The water quality parameters were monitored. Cockles were not fed, in order to excrete the contents of the digestive tract, including contaminants that may be associated with digested food and fecal matter, as well as to optimize the exposure rate [24]. This process is also known as depuration [24].

2.5. Cockle Exposure

A group of 45 blood cockles was selected randomly and used for the nitrite exposure test. Three individual cockles were placed in each treatment tank by 1 L beakers and exposed to 300 mL of the test solution. Each tank corresponded to different concentrations of nitrite (0, 0.5, 1.0, 1.5, and 2.0 mg/L), including a control group (0 mg/L) containing only artificial seawater. The exposure was undertaken for 72 h, and observations were recorded daily. The series of nitrite concentrations selected in the study were based on a 24 h range-finding test method. The test solutions for cockle exposure were replaced twice per day to maintain the test concentration and simulate the cockles’ natural environment. A static renewal water delivery system was applied throughout the exposure, and the exposure tanks were not aerated to minimise loss of nitrite content in the test solution, due to nitrite oxidation [25,26]. Cockles were not fed during the exposure in order to reduce nitrogen excretion, maintain water quality, and prevent other interference [27,28]. The death of a cockle was confirmed by its inability to close its valve upon mechanical stimulus, such as by touching with a glass rod, and it was then immediately removed from the treatment tank [29]. The essential water parameters were recorded and maintained: salinity 27 ± 2 ppt, dissolved oxygen above 7 mg/L, pH 7 ± 0.5, and temperature 30.5 ± 0.3 °C. The nitrite toxicity test was conducted in three replicates.

2.6. Nitrite Analysis

The blood and whole-body soft tissue samples were collected from cockles that survived the 72 h nitrite exposure. The blood samples were extracted from the forced opening of the cockles’ shells, and were obtained using a 1 cc insulin syringe fitted with a 29 G needle. Blood samples were pooled together according to the testing concentration in the ethylene diamine tetra-acetic acid (EDTA) bottles, centrifuged to obtain the plasma, and then 100 µL of the plasma used for ELISA reading. The tissue samples were pooled together according to the test concentration; homogenized in phosphate buffer saline (PBS), using a homogenizer; and centrifuged to obtain the supernatant. Then 100 µL of the supernatant was used for ELISA reading. If not processed immediately, both plasma and tissue supernatant samples were frozen at −80 °C until analysis to prevent nitrite oxidation. It is of note that all analyses (blood and soft tissue samples) were performed in three experimental replicates using an ELISA reader. This procedure was based on the Cayman Chemical Nitrate/Nitrite Colorimetric Assay Kit procedure manual.

2.7. Statistical Analysis

The 72 h LC50 was obtained by plotting the mortality percentage against the nitrite concentration using Probit analysis. The variation in nitrite concentrations of tested samples was performed using the t-test and the relationship of nitrite accumulation against exposure concentration, and the mortality percentage was conducted using linear regression analysis.

3. Results

Overall Results

The mortality percentage of Tegillarca granosa exposed to different concentrations of nitrite is shown in Figure 2. Findings indicate the mortality percentage proportionally increases with the
The concentration of nitrite exposure. The median lethal concentration (LC50) of nitrite in blood cockles was calculated to be 1.53 mg/L based on Probit analysis, and the 95% confident interval was between −1.76 and 4.56 mg/L, as shown in Table 2. The concentrations of nitrite in the blood and soft tissue samples ranged from 0.012 to 0.106 mg/L wet weight and 0.002 to 0.089 mg/L wet weight, respectively, as shown in Figure 3. No significant differences of nitrite concentration were found between the blood and soft tissue samples at each nitrite concentration, with p > 0.05. The relationship between nitrite accumulations in the cockles (blood and soft tissue) against the exposure concentration was performed using linear regression analysis, as shown in Table 3. A strong positive relationship was observed between the nitrite accumulation in the soft tissue and the exposure concentration, with R² = 0.9876 (Table 3). The true mean nitrite accumulation in the soft tissue samples was 0.046 mg/L wet weight, and the 95% confident interval was between 0.033 and 0.06 mg/L wet weight (Table 3). The relationship between nitrite accumulation and cockle mortality was performed using linear regression analysis. A strong positive relationship was found between nitrite accumulation in the blood and the soft tissue, as well as the percentage of mortality, with R² = 0.8482 and R² = 0.9979, respectively (Figure 4).

Table 2. Estimated concentration–response curves of Tegillarca granosa mortality after 72 h of exposure to nitrite a.

| Sample    | Mean (mg/L) Wet Weight | Intercept | Standard Error | Slope | LC50 | 95% Confidence Interval |
|-----------|------------------------|-----------|----------------|-------|------|-------------------------|
| Blood     | 0.0458                 | −0.0002   | 0.015          | 0.046 | 0.8839 | (0.004, 0.088)          |
| Soft Tissue| 0.046                 | 0.0002   | 0.005          | 0.0458 | 0.9876 | (0.033, 0.06)          |

a the median lethal concentration (LC50) and the corresponding 95% confidence limits are reported.

Table 3. Estimated nitrite accumulation curves of Tegillarca granosa after 72 h of exposure to nitrite b.

| Sample    | Mean (mg/L) Wet Weight | Intercept | Standard Error | Slope | LC50 | R² | 95% Confidence Interval |
|-----------|------------------------|-----------|----------------|-------|------|----|-------------------------|
| Blood     | 0.0458                 | −0.0002   | 0.015          | 0.046 | 0.8839 | (0.004, 0.088)          |
| Soft Tissue| 0.046                 | 0.0002   | 0.005          | 0.0458 | 0.9876 | (0.033, 0.06)          |

b the corresponding 95% confidence limits are reported.

Figure 3. Mean concentration of nitrite in the blood and tissue of exposed blood cockles, with p > 0.05.
when exposed to air, compared to the normal blood, which has a red color [38]. Dark-colored 2020 Water This contradiction between the current findings and the literature is to be expected, because the pollutants [36] therefore, nitrite accumulation in the blood and soft tissue is directly proportional to the exposure concentration (Table 3). The possible risk due to the high accumulation upon entry of nitrite ions directly increases the mortality percentage of cockles. (a) The strong positive relationship observed between the concentrations of nitrite in the cockle 2020 Water (Figure 4). Blood cockle mortality percentages vs. the concentration of nitrite in the blood (a) and soft tissue (b) samples of nitrite-treated cockles. 4. Discussion 4.1. Median Lethal Concentration (LC50) Due to limited information regarding the LC50 of nitrite in bivalves, the findings could not be compared with other results from the same species or the same phylum. In a study of the tremendous reduction of Tegillarca granosa production, the most common reason was given as excessive nutrients from agricultural activities [20]. Therefore, the LC50 of nitrite obtained in the present study proves the potential risk of nutrient contamination affecting bivalve communities within coastal areas. 4.2. Nitrite Accumulation in the Blood and the Soft Tissue The lack of significant differences between nitrite concentration in the blood samples and the soft tissue samples (p > 0.05) at each exposure concentration is expected, due to the presence of blood cells in the wet soft tissue samples used in the analysis. Thus, the readings were almost equal between the two sets of the data (Figure 3). The soft tissue samples were not distinguished according to the organs, as this study represented the common human feeding habit in which the whole soft tissue of the cockle is consumed [30,31]. Findings were inconsistent with previous studies (of the same species), where a higher nitrite level was reported in the blood compared to the gills, liver, brain, and muscles [32,33]. This contradiction between the current findings and the literature is to be expected, because the concentrations in the cockle’s entire soft tissue would be higher than those in specific organs. The strong positive relationship observed between the concentrations of nitrite in the cockle samples with the exposure concentration, as shown in Table 3, is expected, due to the possibility of nitrite ions diffusing into respiration organs and transferring to the whole body through the bloodstream [34,35]. Gills are known to act as an active site for the sorption of nutrients and pollutants [36] therefore, nitrite accumulation in the blood and soft tissue is directly proportional to the exposure concentration (Table 3). The possible risk due to the high accumulation upon entry is oxidization by nitrite of the iron in hemoglobin (Hb), resulting in methaemoglobinemia in the cockles [28,37]. Methemoglobin (MetHb) under naked-eye observation is a dark brownish color when exposed to air, compared to the normal blood, which has a red color [38]. Dark-colored blood has undergone hemolysis—that is, the rupturing of red blood cells (RBCs) and release of their contents, causing RBCs to lose their properties and affecting their roles in the body system [28–40]. Methemoglobinemia prevents oxygen binding and transportation; hence, sample individuals with a high level of MetHb suffer functional anemia, owing to the reduction of the total oxygen-carrying capacity in the blood [28], thereby causing tissue hypoxia [41], in which a certain region of the tissue is deprived of adequate oxygen supply [42].
In this study, the nitrite-treated samples were found to have ruptured RBCs, as the blood sample turned a dark brownish color compared to the RBC of the control sample, which had an evenly distributed red color, suggesting that nitrite exposure groups experienced methemoglobinemia, as shown in Figure 5. Thus, this finding indicates that nitrite has the potential to cause methemoglobinemia, thereby reducing the oxygen-carrying capacity in blood and eventually leading to respiratory deficiency in blood cockles. In other words, the accumulation of nitrite in the tissue of blood cockle *Tegillarca granosa* leads to further consequences for the tissue, such as tissue damage [28].

![Figure 5. Differences of the extracted blood samples' color between the nitrite exposed sample and the control group.](image)

A strong positive relationship between nitrite accumulation in the blood and tissue against the mortality percentage (Figure 4) suggests that the accumulation of nitrite in blood and tissue led to mortality of the cockles. The potential reason for the fatality is as discussed above. This is expected due to consistent exposure intensity and increased duration exposure; cockles eventually died due to methemoglobinemia and tissue hypoxia, thus leading to oxygen deficiency in the blood. It is important to know the LC50 value of nitrate in blood cockles, as this compound is expected to cause damage to red blood cells (RBCs) or hemolysis. Even at a low concentration with a prolonged exposure, this could become toxic and represent a hazard to cockles.

Based on the Interim National Water Quality Standard for Malaysia (INWQS), the allowable nitrite concentration in estuarine habitats (Class E) is 0.055 mg/L, and concentrations higher than this value will be toxic to aquatic lives [43]. Although the tested concentration used in this study was greater than the allowable concentration stated in the INWQS, the findings suggest that continuous monitoring of nitrite contamination is of great concern, due to the rapid development of anthropogenic activities leading to excessive nutrient pollution, particularly from the agriculture and aquaculture sectors in coastal areas.

5. Conclusions

This study identified that the LC50 of nitrite after 72 h of exposure in adult *Tegillarca granosa* was 1.53 mg/L. Furthermore, no significant differences were found for nitrite concentration in blood and wet soft tissue samples at each concentration exposure. It is revealed that there was a strong relationship between nitrate accumulation in blood and soft tissue and the mortality percentage, thus indicating that an accumulation of nitrite in the blood and soft tissue leads to the mortality of cockles. Hence, it can be said that these current findings serve as important baseline information for future studies.
Areas with poor understanding and that require further investigation are the metabolism of nitrite in *Tegillarca granosa* and the effects of chronic exposure on the life stages of *Tegillarca granosa*.

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