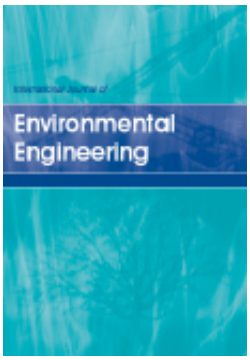

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Acute and chronic toxicity of difenoconazole fungicide on freshwater shrimp (*Macrobrachium lanchesteri*)

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Acute and chronic toxicity of difenoconazole fungicide on freshwater shrimp (*Macrobrachium lanchesteri*)

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Abstract: The toxicity of difenoconazole, a fungicide belonging to the triazole group of compounds, upon the freshwater shrimp (*Macrobrachium lanchesteri* sp.) was assessed within a laboratory setting. Shrimps were exposed to five different concentrations (0.33, 0.65, 1.30, 3.25, and 6.50 mg/L) of difenoconazole. The lethal concentration for 50% mortality in the population (LC50) determined the acute toxicity level of the compound, whilst chronic toxicity was derived from its no-observed effect concentration (NOEC). Subsequently, the LC50 and NOEC values observed for *M. lanchesteri* were determined to be 2.91 mg/L and 0.79 mg/L, respectively. This study demonstrated that aside from the intended anti-fungal effects of difenoconazole fungicides, they may also be toxic to off-target organisms, particularly *M. lanchesteri*.

Keywords: difenoconazole; acute toxicity test; chronic toxicity; *Macrobrachium lanchesteri*; LC50.

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1 Introduction

Pesticides came into widespread application post-World War II, where they were utilised to kill or control unwanted organisms including insects, weeds, fungi and nematodes. Due to the notoriously high and hazardous levels of toxicity borne by early-generation pesticides, their use has gained negative connotations over the years, evidently as they could be detrimental to both the environment and human health (Chambers et al., 2010). Besides that, pesticides might also be toxic to aquatic life and their usage is known to adversely affect water quality (Helfrich et al., 2009). Acute and chronic toxic effects of excessive pesticide exposure include neurological manifestations, respiratory diseases, genetic damage, and reproductive disorders in various organisms (Bhardwaj and Saraf, 2014; Bhardwaj et al., 2018).

Pesticides are grouped based on their formulations, where some could contain as many as over eight hundred active ingredients (Hernández et al., 2013). According to Abdullah et al. (1997), over tens of thousands of formulations are found in the pesticide manufacturing industry. The chemicals are often classified further by their targeted organism and therefore may be referred to as fungicides, insecticides, herbicides, molluscicides, nematocides, rodenticides, and so forth (Aktar et al., 2009). Over the years, the agricultural sector of Malaysia has reported an increasing trend in the implementation of pesticides as a routine part within crop management. In paddy fields, fungicide is one of the crucial groups of pesticides used by farmers to avoid damage from diseases caused by fungi (Abd Rahman et al., 2019) and to preserve the quality and yield of profitable agricultural products (Hamsan et al., 2017). Many fungicides on the market should be applied in between the reproductive and harvesting stages of paddy planting. Difenoconazole is an effective and widely used variant commonly found in many countries including Malaysia. The IUPAC name of difenoconazole is 1-[2-[2-chloro-4-(4-chloro-phenoxy)-phenyl]-4-methyl-1,3-dioxolan-2-ylmethyl]-1H-1,2,4-triazole. It interferes with mycelia growth by inducing systemic sterol demethylation (DMI), which successively inhibit the germination of pathogenic fungal spores and prevent further fungal growth (Reuveni and Sheglov, 2002).

Difenoconazole is relatively persistent, particularly in water bodies. It is estimated that just 15% of the compound would have been degraded after two months in aqueous solutions (Rodríguez-Cabo et al., 2013). As such, it is most typically found as a

component in seed treatment and foliar sprays. This miscible nature of difenoconazole means that it may become accidentally transported via spray drift or runoff into nearby sources of water (Schulz, 2004). Water flow from paddy fields could carry along pesticide residues (Inao et al., 2003). A higher amount of rainfall following the application of pesticides would also increase the runoff rate of difenoconazole into water systems (Sultana et al., 2005). Furthermore, the compound is presumed to be toxic to aquatic organisms. In time, the heavy usage of difenoconazole could therefore negate its fungal growth control benefits, as it might evolve to become harmful to river species including *M. lanchesteri*.

Shrimp production output has maintained a declining trajectory for years (Reyes et al., 1999). Although one could not attribute decreased shrimp hauls solely unto pesticides, it is obvious that the progressive shrinking of their population has coincided with the increased dependency on pesticides in agricultural practices. In the case of difenoconazole, its persistence could also pose long-term effects as the compound is transferred horizontally through the food chain (Fuad et al., 2012). Contamination of rivers or irrigation channels with pesticide residues can therefore not only threaten the shrimp population, but may eventually lead to public health crises.

Consequently, an evaluation on the potential toxicology effects of difenoconazole fungicides on freshwater shrimp is most critical. Many test methods can be used to determine the toxicity of pesticides, chemicals and other effluents to aquatic organisms [Organization for Economic Cooperation and Development (OECD), 1984; International Organization for Standardization (ISO), 1996; Lewis et al., 1994]. Contrarily, a standard method of assessment specifically for freshwater shrimp is yet to be described. In Malaysia, acute toxicity data on the species are limited. The current standard practice in determining toxicity relies on the cross-referencing of obtained data against the publicly available data for the crustacean *Daphnia magna*. However, it is undesirable to directly compare *M. lanchesteri* to *D. magna*, primarily as the latter is not a tropical species. It is likely that the types of pesticides used locally are different from the ones common to temperate climates and therefore making such parallel observations may not accurately represent the immediate ecotoxicological risk a pesticide poses on freshwater shrimps. Additionally, key differences affecting species response against pollutants exist between temperate and tropical environments. Amount of light exposure, light intensity and weather volatility (Segers and Martens, 2005) could all factor into a pesticide's toxicity. Uddin et al. (2018) have discussed that the negative effects of increased water temperatures beyond optimal levels upon the enzyme-catalysed pathways within aquatic organisms could severely reduce resistance to pollutants, thus increasing the mortality rate.

As an important source of food to various fish species in the tropics (Samuel et al., 1988; De Grave et al., 2007), it is vital that a thorough characterisation of pesticide toxicity towards *M. lanchesteri* is conducted. The shrimps are known to be specifically sensitive to GABA-gated chloride channel antagonist and sodium channel modulator insecticides. Additionally, they exhibit moderate and low sensitivity for acetylcholinesterase inhibiting insecticides and fungicides, respectively (Daam and Rico, 2016). However, no studies have thus far evaluated the toxicity of difenoconazole on freshwater shrimps. Methodologically, the acute toxicity test previously performed with *C. laevis* may be suitably applied to *M. lanchesteri* as well. In Sucahyo et al. (2008), *C. laevis* was shown to be moderately sensitive to diazinon and lambda-cyhalothrin

whilst displaying a much greater sensitivity towards endosulfan and paraquat. Conversely, *C. laevis* appeared completely invulnerable to carbofuran.

Deriving similar acute toxicity data for *M. lanchesteri* would be critical, despite how acute data is on its own considered inadequate for developing protective water quality guidelines (Kumar et al., 2010). Acute data is nonetheless required in estimating a chronic toxicity value, which in turn is applied towards predicting the long-term effects of pesticides on organisms. Chronic toxicity assessments will not only minimise livestock loss but will also help save cost and time. In current toxicity evaluation practices, the acute-to-chronic (ACR) extrapolation is a method utilised to predict chronic toxicity levels from acute data (May et al., 2016).

The present study was undertaken with the aim of determining the acute toxicity of difenoconazole and its LC50 on the freshwater shrimp, *M. lanchesteri*. Subsequently, the acute toxicity data achieved was applied in ACR extrapolation to predict the chronic toxicity of the fungicide towards *M. lanchesteri*. A simple dilution method was used to identify five different concentrations of difenoconazole to be tested in this toxicology study. Finally, statistical analyses with one-way ANOVA were conducted to validate the data obtained in this study and to justify its applicability for ecological risk assessment purposes.

2 Methodology

2.1 Shrimp collection and culture maintenance

M. lanchesteri were purchased from the local fish shop and transferred to the laboratory. Live individuals were transferred to a big aquarium tank containing dechlorinated tap water. Water temperature was strictly maintained between 26 and 27°C (the optimum breeding and growth temperature for large shrimps).

2.2 Test chemical

Difenoconazole was purchased from an agricultural store in Permatang Pauh, Penang, Malaysia. The formulaic composition of active ingredients in the product are 23% w/w (250 g/L) of EC with the patented trade name ARIMO 23EC, produced and trademarked to Advansia Sdn Bhd. All working stock solutions were made shortly prior to use.

2.3 Toxicity test

Five different concentrations of difenoconazole and a negative control were used in the toxicity test. For each concentration, 20 *M. lanchesteri* shrimps (~30 mm long and weighing ~0.1 g each) were released into a one litre beaker containing the test solution. Conductivity, pH, and dissolved oxygen levels were measured daily throughout the 96-hour experimental period. The beaker was covered with plastic film to reduce volatilisation and kept aerated by motorised oxygen supply. No additional feed was supplied to the shrimps during the experiment. The number of dead shrimps was counted and cleared every 12 hours, from which the mortality value was calculated at the end of every 24-hour cycle. The criteria for death were the absence of movement when the shrimp was gently probed with a glass rod and an observed change in body colour to a

deeper red. The assay was repeated twice for all concentrations, and final values were averaged from the triplicated experiment.

2.4 Chronic toxicity prediction

A probit analysis was performed in SPSS Statistical Software to calculate the LC50 value. The log-log method was used based on a regression of log LC50 against the log of exposure time (day) as shown in equation (1):

$$ACR_T = \exp \beta[(\ln 4 - \ln T)] + \varepsilon \quad (1)$$

where β is the regression slope, T is the estimated chronic time measured in days (21 and 90) and ε is the random error (Kumar et al., 2010). The formula was used to calculate the ACR value. This model was generated from the time-toxicity relationship proposed by Heming et al. (1989).

2.5 Pesticide analysis

At the end of 96 hours of exposure, 500 mL of water was collected from each beaker using the solid phase extraction (SPE) method. Briefly, samples extracted using HyperSep™ C18 cartridges (Thermo Scientific™) were evaluated by gas chromatography-mass spectrometry (GC-MS) for difenoconazole concentration. Upon collection, water samples were first filtered with filter paper. The DSC-C18 cartridges were prepared through a series of washing, first with 10 mL of acetone, followed by 10 mL of acetonitrile and 3 mL of distilled water in succession. A final post-extraction wash step with 6 mL of acetone eluted any difenoconazole residue trapped in the cartridge. The resulting solution was analysed on the Shimadzu GCMS-QP2010 platform to confirm the retention of difenoconazole in water samples even after the 96-hour test period. Specifications of the capillary column used are 0.25 mm ID by 0.25 mm film thickness. The parameters applied during GC-MS were an injector temperature of 250°C, a detector temperature of 280°C, and a splitless time of 0.75 min. Temperature increments occurred at a rate of 10°C/min from 100°C to the set injector and detector temperatures, then cooled at a rate of 30°C/min. Table 1 shows the nominal and measured concentrations of difenoconazole in extracted water samples. Measured concentrations of difenoconazole were found to be close to the nominal concentrations (>90%). The average recoverability of spiked difenoconazole was at least 87% (n = 3). Based on the standard deviation of the response and its slope, the limit of detection (LOD) for difenoconazole was estimated to be 10 µg/L with a retention time of 4.7 minutes.

Table 1 Measured concentrations of difenoconazole following the 96-hour toxicity test period

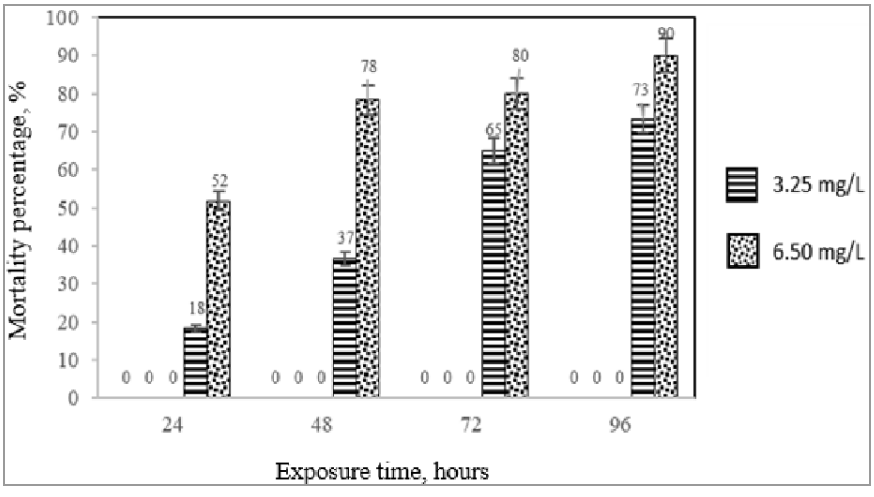
Difenoconazole concentration	*Measured concentration (mg/L)*
1	0.33 ± 0.23
2	0.65 ± 0.53
3	1.30 ± 0.26
4	3.25 ± 0.31
5	6.50 ± 0.11

3 Results and discussion

3.1 Acute toxicity data

Figure 1 shows the mortality percentage of freshwater shrimp *M. lanchesteri* within 96 hours of exposure to five different concentrations of a difenoconazole fungicide. Mortality was calculated at the 24th-hour, 48th-hour, 72nd-hour and 96th-hour points after exposure. Three replicates were performed per treatment. At lower concentrations ranging from 0.33 mg/L to 1.30 mg/L, difenoconazole was not potent enough to kill any *M. lanchesteri* within the 96-hour period. However, upon 24 hours of exposure to greater concentrations (3.25 mg/L and 6.5 mg/L) of the compound, 18% and 52% mortalities were recorded, respectively. At 48 hours post-exposure, these mortality rates have increased to 35% and 78%. Eventually, exposure to 3.25 mg/L respectively caused 65% and 73% mortality rates at the 72nd-hour and 96th-hour checkpoints, whereas the *M. lanchesteri* population in the 6.5 mg/L difenoconazole solution displayed 80% and 90% mortality at the same time intervals. These difenoconazole toxicity tests on *M. lanchesteri* demonstrated both reproducibility in outcomes and the controlled responsiveness of the organism towards a potentially toxic substance. This suggests the potential of utilising freshwater shrimp as a model organism in standard toxicity tests.

Figure 1 Average mortality percentage against duration of exposure, in hours, of *M. lanchesteri* to five different concentrations of difenoconazole fungicide



The mortality rate was interpreted via the one-way ANOVA test and probit analysis. Probit analysis was also used to determine the LC for certain percentages of the surviving population. The LC values of difenoconazole on *M. lanchesteri* over the 96-hour experimental period is summarised in Table 2.

As shown in Table 2, the geometric mean of LC50 between 0.33, 0.65, 1.30, 3.25, and 6.50 mg/L of difenoconazole on the mortality of *M. lanchesteri* was estimated to be 2.881 mg/L. Difenoconazole becomes lethal to shrimps at a concentration of 1.313 mg/L, where 5% of the population will be affected. Complete elimination of a whole population would occur at 8.757 mg/L concentrations of the compound. Compared to other triazole fungicides, difenoconazole purportedly poses relatively high acute toxicity to a wide

range of aquatic organisms (Dong et al., 2013). To the best of our knowledge, no data has been reported on the acute toxicity of difenoconazole towards *M. lanchesteri*. However, difenoconazole is widely known to be toxic to *D. magna* (EC, 2006). The established geometric mean of LC50 between 430, 770, 830, 940, and 1,100 µg/L difenoconazole concentrations on the mortality and immobilisation of *D. magna* was 778 µg/L (0.78 mg/L) (Mensink, 2008).

Table 2 Calculated lethal concentrations (LC) of difenoconazole to *M. lanchesteri* over 96 hours

Endpoint	*Difenoconazole concentration (mg/L)*	*95% confidence limit*	
		Lower	*Upper*
LC5	1.313	0.835	1.702
LC10	1.562	1.065	1.960
LC50	2.881	2.381	3.400
LC80	4.308	3.639	5.417
LC95	6.323	5.093	9.049
LC99	8.757	6.640	14.269

A separate study reaffirmed this value by reporting a nearly identical LC50 for *D. magna* after a 96-hour period of difenoconazole exposure (Arysta, 2010). The same authors also showed that the LC50 for the mysid shrimp – a smaller prawn species – was significantly lower at 0.15 mg/L. Apart from crustacea, difenoconazole has been shown to assert a negative effect on zebrafish during its adult and early life stages (Mu et al., 2013). In a nutshell, it can be concluded that difenoconazole is comparatively less toxic on *M. lanchesteri* than it is on other freshwater invertebrates. However, *M. lanchesteri* remains relevant to be used as the acute toxicity test organism, especially for assessments involving species from tropical climate countries.

3.2 Chronic toxicity prediction

Based on Figure 2, 21- and 90-day chronic toxicities of *M. lanchesteri* were estimated to be 0.79 mg/L and 0.25 mg/L, respectively. The predicted chronic toxicity in this study displayed good reproducibility with a standard error of 0.14. Kumar et al. (2010) has nonetheless advised against the usage of single species in acute toxicity tests as it rendered the determination of standard error during chronic toxicity estimation difficult. However, this study chose to focus on a singular species. Not only did this strategy inevitably come with cost and time benefits, but an opposing opinion from Slaughter et al. (2005) has claimed that chronic toxicity tests from one species-based evaluations would generate more environmentally accurate tolerance information than would acute toxicity tests on multiple species.

The log-log model proposed by Heming et al. (1989) was observed to give a near linear regression with an R-square value of 0.9936. Regression methods for predicting chronic toxicity (lethality) from acute lethality data with shrimp were developed and compared to pre-existing methods. It was found that the log-log model gave the most reliable probability of death predictions as a function of extended exposure times (Kumar et al., 2010). As discussed by May et al. (2016), ACR extrapolation is an approach to

predict acceptable no-effect levels from less reliable acute data, and could be invaluable in the risk assessment of chemical substances.

Figure 2 Estimation of chronic lethality of difenoconazole for 21- and 90-day exposure with a log-log method (see online version for colours)

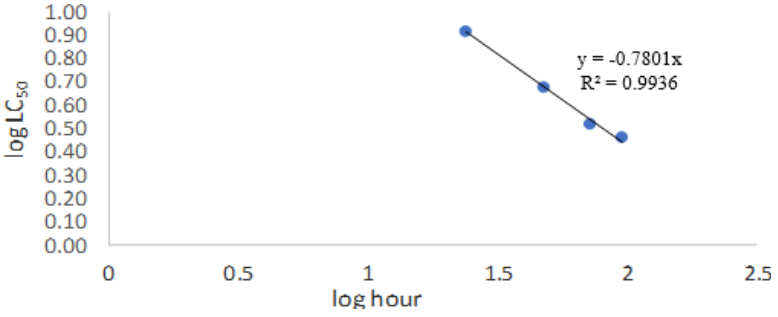
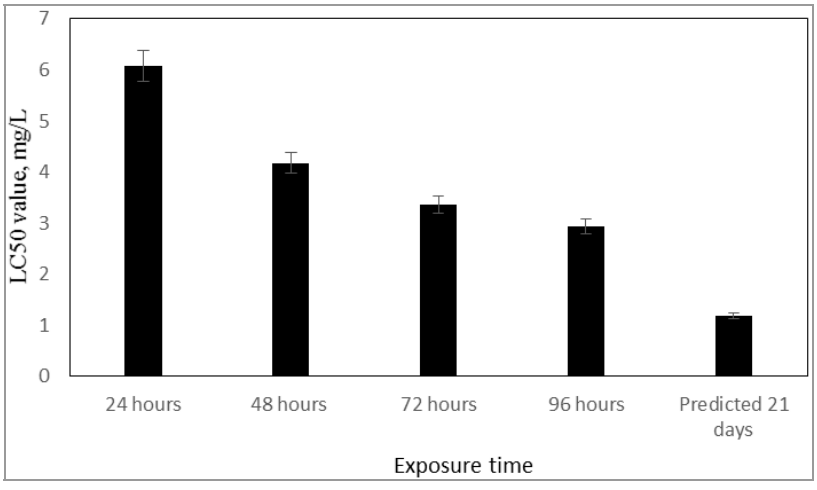


Figure 3 shows the average LC50 values on *M. lanchesteri* produced by outcomes from acute toxicity tests as well as the predicted chronic 21-day toxicity of difenoconazole. Clearly, increasing the time of exposure to the fungicide will increase its toxicity levels. According to Kumar et al. (2010), the value of LC10 from acute toxicity data was closer to the estimated chronic LC50. Based on this statement and the findings of the present study, the acute LC50 value was therefore used for extrapolation over the no-observed effect concentrations (NOEC) of LC10 and LC0.1 (Slaughter et al., 2007; Kumar et al., 2010). The usage of NOEC in ACR calculations has long been discouraged in the toxicology field. NOEC is both a poor toxicity indicator and can greatly vary between tests, and therefore its application may lead to inaccuracies in the relative measurements of toxicity (Jager, 2012). By contrast, not only does LC50 have the highest statistical confidence, but it is also less model dependent (Slaughter et al., 2007).

Figure 3 The LC50 from three replicates of *M. lanchesteri* acute toxicity tests and predicted chronic 21-day toxicity of difenoconazole



4 Conclusions

The freshwater shrimp *M. lanchesteri* was proposed to be a suitable test organism in toxicity tests as it could reliably represent other freshwater aquatic organisms inhabiting the paddy field area. As a control, *M. lanchesteri* displayed resilience against low-level toxicity, demonstrated by the 0% mortality rate recorded even when they were not fed throughout the 96-hour testing period. Results in this study furthermore derived a relatively high difenoconazole toxicity LC50 value of 2.91 mg/L for *M. lanchesteri*. Meanwhile, the chronic toxicity concentrations of the compound were estimated to be 0.79 mg/L and 0.26 mg/L for a 21-day and a 90-day exposure period, respectively. These predictions of chronic toxicity can therefore be used as additional information prior to ecological risk assessments with reasonable confidence, especially where no chronic toxicity estimation is available. Finally, this study provided more evidence that difenoconazole fungicides could be lethal to non-targeted organisms and particularly *M. lanchesteri*, supporting the argument that the agricultural usage of any pesticide should be preceded with careful risk assessment and planning.

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