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Lethal concentration 50 (LC_{50}) and effects of Diuron on morphology of brine shrimp *Artemia salina* (Branchiopoda: Anostraca) Nauplii

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Abstract

Diuron is an alternative biocide to replace tributyltin in antifouling paints. However, its effect on non-targeted organisms are not fully studied, hence this study was conducted to investigate toxicological effects of Diuron in the early life stage (24hr post-hatch nauplii) of brine shrimp *Artemia salina*. The lethal concentration 50 (LC_{50}) of Diuron on *A. salina* nauplii was determined after 24 hours, 48 hours and 72 hours exposure after conducting a range definitive tests. Additional investigations on morphological abnormalities and total length were also conducted. Results showed that LC_{50} of Diuron were 23.27 $mg.L^{-1}$, 12.19 $mg.L^{-1}$, 6.00 $mg.L^{-1}$ in 24, 48 and 72 hours, respectively. Some external abnormalities were also observed. The total length was found to decrease with the increase in Diuron concentration. These results indicate that Diuron is an environmentally toxic substances. Furthermore, in-depth investigation should be conducted to establish *A. salina* as a bioindicator for Diuron contamination.

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1. Introduction

Organotin, especially tributyltin (TBT) was documented to affect nontarget organisms, causing, for example, organ

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deformation, endocrine disruption, and the formation of unnecessary organs [1,2]. Due to its severe effects to the environment, tin-free antifoulings have been proposed as replacement [2]. Diuron (1-(3,4dichlorophenyl)- 3,3 dimethyl urea) is a chemical substance which has been used worldwide as an alternative active ingredient in antifouling materialsand widely used in agriculture as herbicide [3,4]. It has been found in marine environments at different concentrations. The most likely sources of Diuron are from agricultural runoff and leaching from antifouling coatings. High concentrations of Diuron in estuaries in several EU countries and Japan have been measured [5,6] and a maximum permissible concentration of 430 $ng.L^{-1}$ has been derived by the appropriate Dutch authorities. However, it is no longer approved for use in the UK as an active ingredient in antifouling paints, on any size of vessel. Previously, up to 10 tons of Diuron were used on leisure craft in the UK [7]. Later reports revealed that Diuron concentrations were in a range between 6.74 $\mu g.L^{-1}$ in coastal waters of the UK [8] and up to 1.3 $mg.L^{-1}$ in the US surface waters [9]. Concerns rose because Diuron, as low as 0.3 $\mu g.\ L^{-1}$, can inhibits photosynthesis in aquatic environments [10].

Unfortunately, little is known regarding the effects of Diuron on the organisms inhabiting contaminated aquatic environments. A few studies exist that report LC_{50} (48 hour) values of Diuron ranging from 4.3 mg. L^{-1} to 42 mg L^{-1} in fish, and from 1 mg. L^{-1} to 2.5 mg. L^{-1} for aquatic invertebrates [11]. In addition, Diuron has also been reported to disrupt mitosis in the early development of the Pacific oyster, *Crassostrea gigas* [12]. Based on this evidence, classification of Diuron has been deemed moderately toxic to fish and aquatic invertebrate.

Artemia is one of the most used species in toxicity assessment because of its ease of culture, low cost, and its commercial availability in dry cysts [13]. The acute toxicity of organotin, cadmium and chromium on *Artemia franciscana* has been reported by [14]. The present study is aimed to determine lethal concentration 50 (LC_{50}) and investigate morphological effects of Diuron in the early life stage (24hr post-hatch nauplii) of *Artemia salina* after 24 hours, 48 hours and 72 hours exposure.

1. Materials and method

1.1. Dilution water

Standard artificial seawater of 35 ± 1‰ was used for the hatching as well as for the test. Whenever possible, the sea artificial salt mixture of Instant Ocean dissolved in distilled water was utilized. After aeration and stabilization for 24hours, the dilution water should have a pH of 8.0 ± 0.5 and the oxygen content should be at least 90% saturation. If necessary the pH should be adjusted with concentrated hydrochloric acid or sodium hydroxide. Before the artificial sea water is used, it should preferably be filtered through a l-µm filter under vacuum [15].

1.2. Reference toxicant

Preparation of Diuron stock solution was made by dissolving in 0.3% acetone following Koutsaftis and Aoyama [16]. The working solutions were made immediately before use by dilution in artificial seawater of 35 ± 1‰ (ASW) to appropriate concentrations.

1.3. Hatching and preparation of the Nauplii A. salina

The hatching procedure followed the one described in ARC-test, standardized short-term toxicity test with *Artemia* nauplii [17]. The hatching medium, artificial seawater of normal seawater salinity (35‰), was prepared on site. For test approximately 0.5 g cysts of brine shrimp *A. salina* was incubated in 500 ml seawater in a cylindroconical tube at a temperature of 25 ± 1 C° and with lateral illumination by a light tube (1000 Lux) during the test period. All cysts were kept in continuous suspension with aeration provided by a small air tube extending to the bottom of the hatching device. After 18 to 24 hours, aeration was stopped and the hatched larvae (instar I) were transferred to new petri dish, each petri dish had ten individuals of nauplii and incubated at 25 C° for 24 hours, 48 hours and 72 hours. After 24, 48 and 72 hours from the start of the test, all larvae would have moulted to instar 2 or instar 3 stages.

1.4. Toxicity test

Each test concentration had 5 replicates and ten animals per replicate, and was conducted with 5 nominal concentrations of biocides. A range finding test for Diuron toxicants was conducted. The concentrations of Diuron dilutions used were 5 mg. L^{-1}, 10 mg. L^{-1}, 15 mg. L^{-1}, 20 mg. L^{-1}, 25 mg. L^{-1}, 30 mg. L^{-1}, 40 mg. L^{-1}, 50 mg. L^{-1}, and 100 mg. L^{-1}. Control experiments were carried out with 0.3% of acetone, which was identical to the content at the highest concentration for Diuron. The mortality results showed that the toxicity range from 0% to 100% mortality was between 10 mg. L^{-1} to 100 mg. L^{-1} for 24 hours, 5 mg. L^{-1} to 40 mg. L^{-1} for 48 hours and 5 mg. L^{-1} to 20 mg. L^{-1} for 72 hours. Based on these data the definitive test was designed and the concentration used for Diuron.

Ten nauplii were transferred with a Pasteur pipette into a petri dish. The volume of seawater carried over with the nauplii was minimal. After that, the toxicant solutions were prepared. About 10 ml of respective toxicant solutions was transferred to petri dish and incubated at a temperature of 25 ±1 C° for 24, 48 and 72 hours. After treatment ended, the petri dish was placed on the stage of a dissection microscope.

Mortality was recorded by determining the number of dead individuals if no movement of the appendages was observed within 10 Seconds. The percentage mortality was calculated from the total number of dead larvae for each concentration. The survivors were used to study the morphological effect of toxicant on the total length.

1.5. Morphological analysis

Morphological disorders on nauplii after a 24, 48 and 72 hours toxicity period were investigated under an optical microscope (Olympus BX51TF, 10× objective lens) connected with a microscope camera (Dino-Eye camera). The total body length for *A. salina* nauplii was also measured using the camera software (DC2.0-AM7023) of the optical microscope.

2. Results

Results on *A. salina* nauplii mortality after exposure to different Diuron concentrations for 24, 48 and 72 hours are shown in Fig. 1. Mortality increases with the increase of Diuron concentration. LC_{50} was determined by the Probitanaysis. The results of this analysis showed that yhe toxicity range of minimum and upper limits from 0% to 100% mortality of Diuron were between 10 mg.L^{-1} to 100 mg.L^{-1} for 24 hours, 5 mg.L^{-1} to 40 mg.L^{-1} for 48 hours and 5 mg.L^{-1} to 20 mg.L^{-1} for 72 hours, respectively. Probit analysis also showed that the LC_{50} values for Diuron determined in this study are 23.27 mg.L^{-1}, 12.19 mg.L^{-1} and 6.00 mg.L^{-1} for 24, 48 and 72 hours of exposures, respectively (Fig. 2). The effect of Diuron on nauplii of brine shrimp *A. salina* was further investigated by observing their morphological changes during developmental growth as well as total length. The further formation of one pair of mandibles and two pairs of antennae occurred, where the second pair consists of exopod, endopod, endite and swimming setae etc, during the ages of 24, 48, and 72 hours of non-significant change. The variation was due to the increase in dose of Diuron, thereby, increasing the effect on the *A. salina*. The regration analysis show with the increase dose of diuron, the total length of *A. salina* will signaficatly decrease (Fig. 3). The minimum and maximum toxicity concentrations show a variation in the total length (TL) at 24, 48 and 72 hours in range of up to 870.50 µm. This demonstrated that Diuron is responsible for the change in total length of body *A. salina* after exposure in test period. Generally, TL decreases when Diuron concentration is increased (Fig. 3). Significant differences in morphology were observed in all survived *A. salina* nauplii exposed to all concentration of Diuron. Abnormalities observed include improper development of mandibles and under developed endopod, endite, and the swimming setae of the second pair antenna, which resulted in an imbalanced and irregular swimming pattern.

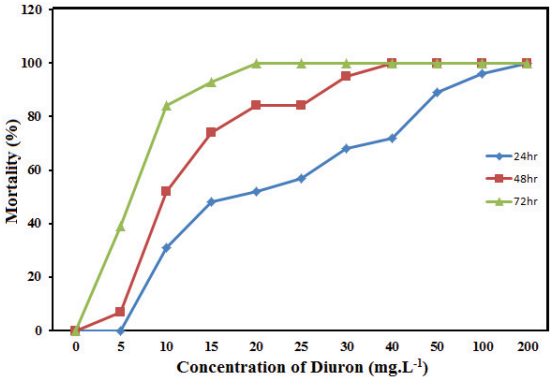

Fig. 1. Relationship between mortality rates and increasing concentrations of Diuron

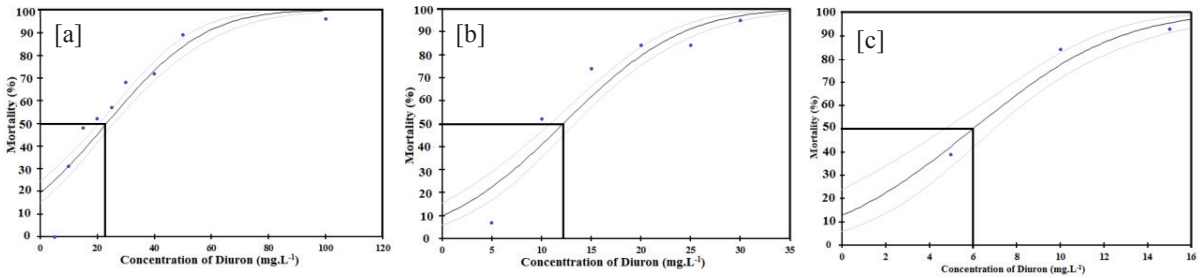

Fig. 2. The relationship between mortality percentage and different Diuron concentration after [a] 24hr (LC_{50} = 23.27 mg. L^{-1}); [b] 48hr (LC_{50} = 12.19 mg. L^{-1}); [c] 72hr (LC_{50} = 6.00 mg. L^{-1})

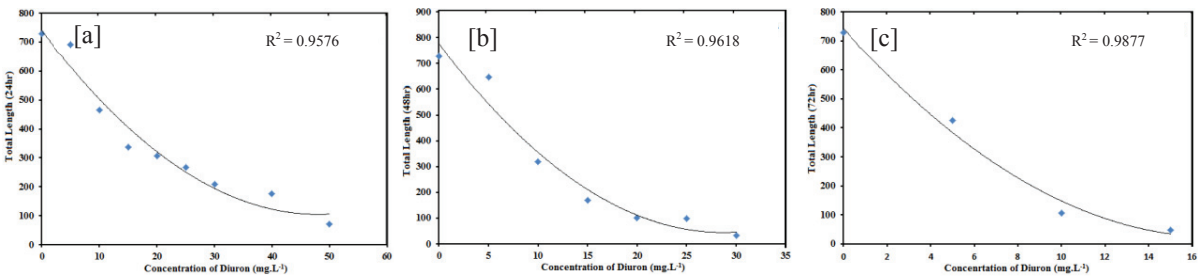

Fig. 3. Relationship between total length of *A. salina* nauplii and different Diuron concentrations after [a] 24hr; [b] 48hr; [c] 72hr

3. Discussion

Diuron has long been used in agricultural and other activities. A large amount of Diuron has been discharged into river water and transported into the sea with little or no degradation [18]. In this study LC_{50} values for 24, 48 and 72 hours mortality at 25C^{o} and 35‰, range finding tests were performed at the beginning for the Diuron. Control experiments were carried out with 0.3% of acetone, which was identical to the content at the highest concentration of Diuron. The concentration response curves for Diuron are presented in (Fig. 2). The LC_{50} values for Diuron were 23.27 mg.L^{-1},12.19 mg.L^{-1}, 6.00 mg.L^{-1}, respectively. Study by Alyürük and Çavaş [19] found that the EC_{50} value of Diuron was found as 12.01 mg.L^{-1}. Another study by Koutsaftis and Aoyama [16] found that the LC_{50} values of Diuron on *A. franciscana* was 12.5 mg.L^{-1}. In addition the results were found by Kwok and Leung [20] revealed the toxicity of Diuron is a much higher degree compared to the toxicity of TBT for equitoxic concentrations against the copepod *Tigriopus japonicas*.

The toxicity of Diuron was tested on *A. salina* nauplii and the morphological changes are the nauplii of brine shrimp, which undergo certain developments in prominent growth, as well as the total length during the ages 24, 48 and 72 hours. This is proven by regration analysis which indicated that the morphological development decrease when increasing the doses of Diuron (Fig. 3). However, Alyürük and Çavaş [19] reported that the morphological observation of the test group treated with Diuron did not alter the morphology of cysts or hatchlings, but only affected the hatching ability of cysts during 24-hour age. Some nauplii exposed to the 25 mg/L Diuron concentration were unable to break the cyst wall completely. Retardations and arrests were observed in developing *Artemia* embryos exposed to Diuron at the prenauplius E-1 and E-2 stages. Alyürük and Çavaş [19] also reported that the effects on total lengths of newly hatched nauplii were not affected by Diuron. The mean total length of newly hatched nauplii for the control group was 0.54 ± 0.01 mm, which was similar in the mean total length for 25 $mg.L^{-1}$ Diuron-treated groups (0.56 ± 0.02 mm). These results indicated that Diuron had no effect on the total body length of *A. salina* of newly hatched nauplii. This study that was conducted using 24-hour post-hatched nauplii showed a different pattern. This could be due to the fact that after 24 hours, all larvae will be molted to the instar 2 and 3 stages. These instars have been shown to be the most sensitive stage [17,21,22] and were accordingly used for our toxicity tests. Thus, results in this study suggested that Diuron is harmful on the early life stage of marine invertebrates, as indicated by *A. salina* nauplii. This study showed that the brine shrimp assay could be a simple and accurate to assess the marine aquatic toxicity profile of any toxicant, including heavy metals [23]. It also demonstrated that Diuron alters the morphology of brine shrimp, *A. salina* during test period. Between the toxicants, Diuron exhibited prominent adverse effects on the test organism. Further experiments are warranted to study the effects of extensively used Diuron against different marine aquatic organisms. Based on these findings, there is a strong concern in the use of Diuron, therefore, an alternative provided should be less hazardous than Diuron. Also the alternatives must be thoroughly evaluated to ascertain it is less hazardous. The results of the present study may add some information towards this direction, clarifying the higher the magnitude of Diuron toxicity.

4. Conclusion

The increase in Diuron concentration increases mortality as well as influence the morphological development and total length of *A. salina* nauplii. The LC_{50} of Diuron was identified as 23.27 $mg.L^{-1}$, 12.19 $mg.L^{-1}$, 6.00 $mg.L^{-1}$after 24, 48 and 72 hours exposure, respectively. Nauplii of *A. salina* at instar 2 and 3 are sensitive to Diuron contamination to reflect early life stage effects of toxicants. Further long-term toxicity studies and synergistic effects investigations would permit the complete evaluation of Diuron as hazardous chemicals to aquatic organisms.

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