Research Article

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Insights in aquaculture and biotechnology

2017
Vol.1 No.2:8

Acute Toxicity Test of Four Disinfectants to Juvenile *Pinctada maxima*

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Citation: Song J, Ningning S, Mingge Z, Falin Z, Jianhua H, et al. (2017) Acute Toxicity Test of Four Disinfectants to Juvenile *Pinctada maxima*. Insights Aquac Cult Biotechnol Vol.1 No.2:8

Abstract

The acute toxicity of glutaraldehyde, dibromohydantoin, methionine iodine and bleaching powder to juvenile *Pinctada maxima* was studied. The results showed that the order of toxicity was bleaching powder>dibromohydantoin>m ethionine iodine>glutaraldehyde and the median lethal concentrations (LC50) of above disinfectants were 13.05 mg/L, 27.49 mg/L, 53.20 mg/L and 67.36 mg/L, respectively in 24 h and the LC50 were 10.15 mg/L, 9.35 mg/L, 29.27 mg/L and 35.58 mg/L respectively in 48 h; and their SC (safe concentration) were 1.86 mg/L, 0.32 mg/L, 2.66 mg/L and 7.52 mg/L respectively. The research indicated that we can use glutaraldehyde to disinfect *P. maxima* under safe concentration, but should not use bleaching powder, and can use dibromohydantoin and methionine iodine carefully.

Keywords: *Pinctada maxima*; Juvenile shell; Acute toxicity; Disinfectant

Received: August 12, 2017; **Accepted:** November 23, 2017, **Published:** November 30, 2017

Introduction

Pinctada maxima is widespread on the coast of South-East Asian countries, Australia and western Pacific, which is the biggest and has highly economic value among the cultured pearl [1-3]. With the enlarging of the area of aquaculture and the increasing of the total amount of aquaculture, the pollution of water quality in offshore area is aggravating and the breeding and production of kinds of economic shellfish are becoming more and more unstable and large-scale bacterial disease always breaks out. Therefore, various kinds of disinfection and sterilization drugs which inhibited the breeding of germs and ensure the success of shellfish breeding and cultivation are used gradually [4-6]. Zhao et al. [7] studied the acute toxicity of benzalkonium bromide, dibromohydantoin, methionine iodian and glutaraldehyde on the acute toxicity of *Babylonia areolata*, the LC and SC of those four kinds of sanitizers act on *B. areolata* were determined in this experiment. Liu and Wang [8] studied the SC and the effect on vitality, ingestion rate and daily growth rate of D-larvae and the umbo-larvae of Pinctada fucata when faced to several antibacterial agents. The acute toxicity of juvenile *P. maxima* with Hg^{2+} and Cd^{2+} was conducted to determine its tolerance scope to Hg^{2+} and Cd^{2+} by Fan [6]. However, there are few studies on toxicity effect of common disinfectant to *P. maxima* larvae at present, and it often couldn't achieve good sterilization effect or cause death of shellfish due to improper use of drugs during production. Therefore, the acute toxicity experiment of glutaraldehyde, dibromohydantoin, methionine iodian and bleaching powder to juvenile *P. maxima* was carried out in order to understand the effect of the disinfect on *P. maxima* larvae to determine the safety concentration. It provides scientific basis for the rational use of disinfectants in the cultivation of *P. maxima* larvae.

Materials and Methods

Experimental materials

P. maxima with shell height (SH) of 1.36 ± 0.17 mm were obtained from one family of the same batch breeding larvae from Lingshui, Hainan and four kinds of disinfectants were glutaraldehyde (20%

effective concentration, Beijing zhongnong huazheng veterinary drug limited liability company), dibromohydantoin (20% effective concentration, Beijing biological and fisheries technology limited liability company), methionine iodine (50% effective concentration, Shanxi Shenlong Tianyi science and technology limited company) and bleaching powder(50% effective chlorine, Guangxi Nanning chemical industry group corporation). Four kinds of disinfectants were prepared into mother liquor before the test. The experimental container was a polyethylene bucket with a volume of 10L, and the sea water was filtered by sand firstly and then inflated by protein skimmer, besides, the dissolved oxygen content in the water was more than 5 mg/L and the temperature of water during the experiment was 27 ± 1˜, pH 8.3 ± 0.1, salinity 31~ 33 and the experiment was carried out indoor.

Experimental Method

Pre-experiment

5 L of seawater was added in the polyethylene bucket, the number of the *P. maxima* larvae in each barrel was 30 ind and different volume of disinfectant mother liquor was added to each bucket. During the experiment, the water was kept inflated. The *P. maxima* larvae was not fed and disinfectant solution was changed every 12 h, and the death of the *P. maxima* larvae was recorded to determine the highest survival LC0 after 48 h of treatment and the LC100 when all larvae dead at 24 h.

Formal experiment

Based on the experimental results of pre-test, five treatment groups of different mass concentrations and one control group were set according to the numerical arithmetic interval method. Each treatment involved 3 replicates and each group *P. maxima* of 30 capsules were placed. The survival of *P. maxima* larvae was observed in 48 h and the dead ones were removed in time. The standard of death was as below: shell opened, continuously touching adductor muscle of *P. maxima* without any active reaction and the muscle was rigid [9].

Statistical analysis

The mortality rates of 24 h and 48 h in each group were calculated according to the following formula. The mortality rate (%)=number of death/total number of experimental *P. maxima* × 100, The regression equation of the probability unit of the mortality rate and the usual logarithm of drug mass concentration, the death concentration of the drugs (LC50) were obtained by using the linear interpolation method, The X-axis of the regression equation was the usual logarithm of drug mass concentration and the Y-axis of the regression equation was the probability unit of the mortality rate. Then the safety quality concentration (SC) is obtained according to the following formula [10]:

$SC=48\ h\ LC50 \times 0.3/(24\ h\ LC50/48\ h\ LC50)^2$

Results

Acute toxicity test of glutaraldehyde to juvenile *P. maxima*

According to the pre-test results, the LC0 and LC100 treated by glutaraldehyde were 16.0 mg/L and 158.0 mg/L respectively. Five mass concentration of glutaraldehyde were determined according to the two mass concentrations above by equal spacing method, the results of the test of the toxicity of glutaraldehyde to the larvae of *P. maxima* were shown in **Table 1**. During the whole test period, there was no death in the control group. Regression analysis showed that regression curve equation between the unit of mortality of 24 h and the drugs concentration can be expressed as Y=5.07 × -4.27, R2=0.99 **(Figure 1a)**, It calculated that the 24 h LC50 was 67.36 mg/L with the method of linear interpolation and the confidence limit of 95% was 61.66 ~ 74.00 mg/L. The regression equation of the medicine bath for 48 h was Y=4.79 × -2.43, R2=0.99 **(Figure 1b)** and the 48 h LC50 was 35.58 mg/L with the method of linear interpolation. The 95% confidence limit was 39.80 ~ 59.22 mg/L and the SC was 7.52 mg/L **(Table 1)**. Test results of acute toxicity of glutaraldehyde to the *P. maxima* larvae **(Figure 1)**. Effect of glutaraldehyde treatment for 24 h (a) and 48 h (b) on the mortality of *P. maxima* larvae.

Acute toxicity test of dibromohydantoinm to juvenile *Pinctada maxima*

Based on the results of the pre-experiment, the LC0 and LC100 treated by dibromohydantoinm were 3.0 mg/L and 94.0 mg/L, respectively. Five mass concentration of dibromohydantoinm were determined according to the two mass concentrations above by equal spacing method, the results of the test of the toxicity of dibromohydantoinm to the larvae of *P. maxima* were shown in **Table 2**. During the whole test, there was no death in control groups. Regression analysis showed that the regression curve equation of the rate of death rate of 24 h and drug concentration was Y=3.12 × +0.51, R2=0.98 **(Figure 2a)**. It calculated the 24 h LC50 was 27.49 mg/L with the method of linear interpolation and the 95% confidence limit was 23.94 ~ 31.78 mg/L. The regression

Table 1 Test results of acute toxicity of glutaraldehyde to the *P. maxima* larvae.

			24 h		48 h	
Drug concentration (mg/L)	Logarithm of drug quality concentration	Number of organisms (grains)	Mortality rate (%)	Probability unit	Mortality rate (%)	Probability unit
0.00		30	0.00		0.00	
16.00	1.20	30	0.00	1.91	3.33	3.160
28.41	1.45	30	3.33	3.160	40.00	4.75
50.44	1.70	30	18.89	4.120	80.00	5.84
89.55	1.95	30	72.22	5.59	96.67	6.84
159.00	2.20	30	97.78	7.01	100.00	8.09

curve equation of the medicine bath for 48 h was Y=3.07 × +2.02, R2=0.99 **(Figure 2b)**, it calculated that the 48 h LC50 was 9.35 mg/L with the method of linear interpolation and the 95% confidence limit was 8.06 ~ 10.80 mg/L, the SC was 0.32 mg/L **(Table 2)**. Test results of acute toxicity of dibromohydantoinm to *P. maxima* larvae **(Figure 2)**. Effect of dibromohydantoinm treatment for 24 h (a) and 48 h (b) on the mortality of *P. maxima* larvae.

Acute toxicity test of methionine iodine to juvenile *P. maxima*

Based on the results of the pre-experiments, the LC0 and LC100 treated by methionine iodine were 16.0 mg/L and 100.8 mg/L, respectively. Five mass concentration of methionine iodine were determined according to the two mass concentrations above by equal spacing method, the results of the test of the toxicity of methionine iodine to the larvae of P. maxima were shown in **Table 3**. During the whole test period, there was no death in control groups. Regression analysis showed that the regression curve equation of the rate of death rate of 24 h and drug concentration was Y=6.13 × -5.58, R2=0.98 **(Figure 3a)**. The 24 h LC50 was 53.20 mg/L with the method of linear interpolation and the 95% confidence limit was 49.35 ~ 57.74 mg/L. The regression curve equation of the medicine bath for 48 h was Y=6.11 × -3.96, R2=0.96 **(Figure 3b)**. The 48 h LC50 was 29.27 mg/L with the method of linear interpolation and the 95% confidence limit was 26.98 ~ 31.89 mg/L and the SC was 2.66 mg/L.

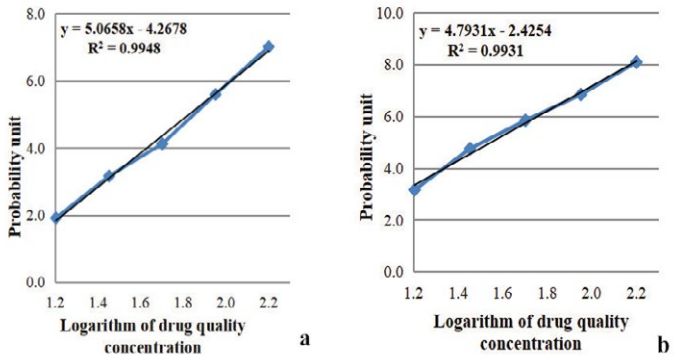

Figure 1 Effect of glutaraldehyde treatment for 24 h (a) and 48 h (b) on the mortality of *P. maxima* larvae.

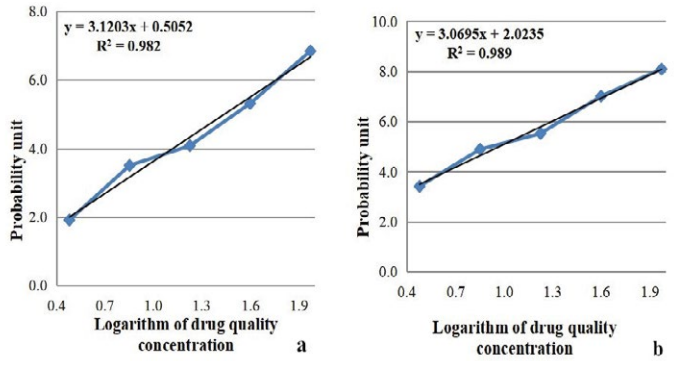

Figure 2 Effect of dibromohydantoin treatment for 24 h (a) and 48 h (b) on the mortality of *P. maxima* larvae.

Acute toxicity test of bleaching powder to juvenile *P. maxima* larvae

According to the pre-test results, the LC0 and LC100 treated by bleaching powder were 4.8 mg/L and 17.5 mg/L respectively. Five mass concentration of bleaching powder were determined according to the two mass concentrations above by equal spacing method, the results of the test of the toxicity of bleaching powder to the larvae of *P. maxima* were shown in **Table 4**. During the whole test period, there was no death in control group. Regression analysis showed that the regression equation of the rate of death rate of 24 h and the concentration of drug concentration was Y=6.22 × -1.94, R2=0.95 **(Figure 4a)**, the 24 h LC50 was 13.05 mg/L with the method of linear interpolation and the 95% confidence limit was 12.17 ~ 14.04 mg/L. The regression curve equation of the medicine bath for 48 h was Y=4.63 × +0.34, R2=0.93 **(Figure 4b)**. It calculated that the 48 h LC50 was 10.15 mg/L with the method of linear interpolation and the 95% confidence limit was 9.52 ~ 10.91 mg/L, the SC was 1.86 mg/L. Tab.4 the results of acute toxicity test of bleaching powder to juvenile *P. maxima* larvae **(Figure 4)**. Effect of bleaching powder treatment for 24 h (a) and 48h (b) on the mortality of *P. maxima* larvae.

Discussion

When choosing the microbicides during the aquaculture of the *P. maxima*, it is necessary to consider the safety of the pearl oyster larvae, the convenience and cost of disinfectants, the degree of pollution to water and effectiveness of the pathogen inhibition. In the culture of *P. maxima*, especially in the stage of larvae breeding, it is prone to break out the bacterial disease and

Table 2 Test results of acute toxicity of dibromohydantoinm to *P. maxima* larvae.

			24 h		48 h	
Drug concentration (mg/L)	Logarithm of drug quality concentration	Number of organisms (grains)	Mortality rate (%)	Probability unit	Mortality rate (%)	Probability unit
0.00		30	0.00		0.00	
3.00	0.48	30	0.00	1.91	5.56	3.41
7.10	0.85	30	6.67	3.5	45.56	4.89
16.79	1.23	30	17.78	4.08	70.00	5.52
39.73	1.60	30	62.22	5.31	97.78	7.01
94.00	1.97	30	96.67	6.84	100.00	8.09

Table 3 The results of acute toxicity test of methionine iodine to juvenile *P. maxima* larvae.

			24 h		48 h	
Drug concentration (mg/L)	Logarithm of drug quality concentration	Number of organisms (grains)	Mortality rate (%)	Probability unit	Mortality rate (%)	Probability unit
0.00		30	0.00		0.00	
16.00	1.20	30	0.00	1.91	2.22	2.99
25.35	1.40	30	3.33	3.16	43.33	4.83
40.16	1.60	30	14.44	3.94	92.22	6.42
63.62	1.80	30	57.78	5.2	96.67	6.83
100.80	2.00	30	97.78	7.01	100.00	8.09

Table 4 The results of acute toxicity test of bleaching powder to juvenile *P. maxima* larvae.

			24 h		48 h	
Drug concentration (mg/L)	Logarithm of drug quality concentration	Number of organisms (grains)	Mortality rate (%)	Probability unit	Mortality rate (%)	Probability unit
0.00		30	0.00		0.00	
4.80	0.68	30	0.00	1.91	3.33	3.16
6.63	0.82	30	6.67	3.5	26.67	4.38
9.17	0.96	30	23.33	4.27	53.33	5.08
12.66	1.10	30	50.00	5	66.67	5.43
17.50	1.24	30	70.00	5.52	81.11	5.88

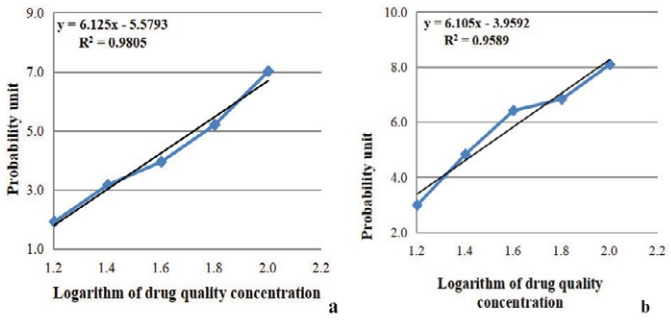

Figure 3 Effect of methionine iodine treatment for 24 h (a) and 48h (b) on the mortality of *P. maxima* larvae.

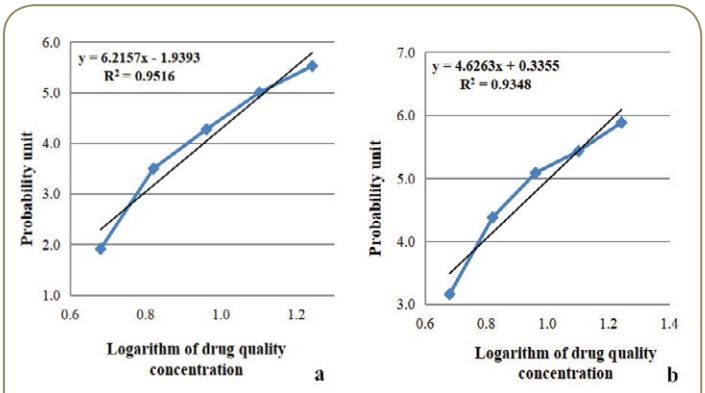

Figure 4 Effect of bleaching powder treatment for 24 h (a) and 48h (b) on the mortality of *P. maxima* larvae.

cause large-scale death of shellfish. Therefore, the phenomenon that disinfectant and antibiotic abused in production occurs, the arising of the resistance problem further aggravated the epidemic of disease. So the selection of suitable disinfectants as well as rational use of sterilization drugs is particularly crucial.

The **Tables 1-4** showed that 24 h LC50 of the four kinds of disinfectant was all higher than that of 48 h LC50. When prolonging the contact time, the toxic effect of *P. maxima* larvae enhanced, which caused the pearl oyster larvae mortality increased. From the perspective of setting range of the disinfectant concentration, the sensitivity of the *P. maxima* larvae to four kinds of disinfectants was different from the range of the concentration of each disinfectant. *P. maxima* larvae was most sensitive to bleaching powder which concentration was 4.8-17.5 mg/L, dibromohydantoin, methionine iodine and glutaraldehyde followed, the concentration was 3.0-94.0 mg/L, 16.0-100.8 mg/L, 16.0-159.0 mg/L, respectively. The result was similar to what Zhao did to juvenile *B. areolata* [7]. The results of four kinds of disinfectants to larvae showed that the 24 h LC50 value of bleaching powder to *P. maxima* larvae was 13.05 mg/L, 48 h LC50 was 10.15 mg/L, the difference between the two data was the least, which illustrated the toxicity of bleaching powder was more stronger than others to larvae. The toxicity of three kinds of disinfectants in descending order was dibromohydantoin, methionine iodine and glutaraldehyde. By calculating the safe concentration (SC), the sensitiveness of *P. maxima* larvae to four kinds of disinfectants in descending order was bleaching powder, dibromohydantoin, methionine iodine and glutaraldehyde.

Bleaching powder is a kind of white flour with strong chlorine odor, the effective ingredient is hypochlorous and hydrochloric acid decomposed by calcium hypochlorite, which is further decomposed into hydrochloric acid and oxygen atom, the oxygen atom has a strong oxidation capacity, which can effectively kill microorganism that come into contact [11-13]. At the same time, part of hypochlorous contants with hydrochloric acid and liberates chlorine, and kill the bacteria such as vibrio, *Aeromonas hydrophila* and so on. Chlorine and water combined to produce atomic oxygen to sterilize it. At present, the sterilization efficacy of bleaching powder to the bacteria in aquaculture water has been

studied by many scholars, it was found that the MBC of bleaching powder to marine vibrio was 8-19 mg/L; the MIC of vibrio was 4.6 mg/L [14,15]. According to the results of this experiment, the SC of bleaching powder to *P. maxima* larvae was inferior to the MIC above, which was only 1.86 mg/L. It stated that sterilizing effect will not be obvious, if the use of bleaching powder under SC. Therefore the use of bleaching powder is not recommending for disinfection of the water of the *P. maxima* larvae.

Dibromohydantoin is an excellent brominating agent with stable broad-spectrum bactericidal action. Dibromohydantoin is mainly formed by bromate in water and bromine is released in the form of the hypobromous acid. The reaction of dibromohydantoin release of bromine is very fast, the continuously released Br^- plays the bactericidal role [6]. The reports about the research on bactericidal effect of dibromohydantoin in aquaculture can be easily found in shrimps and crabs, fish, *Stichopus japonicas, B. areolata* and the SC to the animals above was ranged from 0.6 mg/L to90 mg/L, however, there were few reports on the bactericidal effect of dibromohydantoin to *P. maxima* [7,16-19]. Compared with the aquatic animals reported above, the *P. maxima* larvae were more sensitive to the toxicity of dibromohydantoin and the SC was only 0.32 mg/L. Therefore, in the production, it should not use dibromohydantoin disinfect aquatic water, avoiding the damage to *P. maxima* larvae.

Methionine iodine is the dibasic complex of methionine and iodine. Iodine works mainly in molecular form, the releasing free iodine molecule in water plays a sterile role. The research has found that the five species of fish, such as *Megalobrama amblycephala, Carassius auratus, Ctnopharyngodon idellus, Hypophthalmichthys molitrix, Spinibarbus sinensis* were sensitive to methionine iodine and the SC of methionine iodine was 2.03-2.38 mg/L in these fish mentioned above [20].The highest MIC and MBC of methionine iodine to *Pseudomonas fluorescens, Aeromonas sobria, Aeromonas hydrophila* was 1.25 mg/L, which was lower than the SC, so methionine iodine can be used for sterilization and disease prevention and treatment in these five kinds of fish. This study found that the SC of methionine iodine was 2.66 mg/L to *P. maxima* larvae, which was slightly lower than the manufacture's recommended SC 2.0 mg/L. But in production, farmers are accustomed to use several times higher than the recommended dosage of disinfection drugs. Therefore, the dosage of disinfectant should be strictly controlled when using methionine iodine to the aquatic water of *P. maxima* larvae.

Glutaraldehyde disinfection solution is a new type, high efficiency, low toxicity neutral strengthening disinfection solution, mainly through its two active aldehyde groups to kill the microorganism. Its activity was influenced by the factors such as pH and temperature. Under the condition of alkaline, the glutaraldehyde-aldol unsaturated poly (pathogen) saturated can be polymerized to form a higher form of polymerization, which can kill pathogenic microorganisms such as bacterial reproduction, bacterial spore, hepatitis virus and so on. Glutaraldehyde has ability of broad spectrum to kill microorganisms [21,22]. Some studies showed that MBC was 1.6-4.2 mg/L; MIC was 0.9-3.2 mg/L of glutaraldehyde to the vibrio and aeromonas in medical pathogens and the bacteria in aquaculture water [23-27]. The results of our study showed that the SC of the glutaraldehyde to *P. maxima* larvae was 7.52 mg/L, which was much higher than the minimum bactericidal concentration of some pathogens. Therefore, it is safe and effective to prevent and treat bacterial diseases related to bacterial diseases in the culture water of the *P. maxima* larvae with glutaraldehyde.

The experiment was a semi-hydrostatic test. The *P. maxima* larvae did not feed the bait during the experiment, so the drug toxicity was similar to the real value. However, the toxicity of the drug is also closely related to the water temperature, water flow, organic matter, dissolved oxygen and the size of *P. maxima* larvae. Therefore it is necessary to use the drug according to the actual situation and observe the active situation of *P. maxima* to ensure the safe and effective use of the drug.

Conclusion

In this study, we examined the acute toxicity of glutaraldehyde, dibromohydantoin, methionine iodine and bleaching powder to juvenile *P. maxima*. Results from this study indicated that we can use glutaraldehyde to disinfect *P. maxima* under safe concentration, but should not use bleaching powder, and can use dibromohydantoin and methionine iodine carefully.

Acknowledgement

This research was supported by the Special Scientific Research Funds for Central Non-profit Institutes, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (2015TS10); Special Scientific Research Pilot Project of Sanya (2014KS03).

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