Study on acute toxicity of amoxicillin wastewater to Zebrafish

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Abstract. The main research in this paper is to obtain the effect of pharmaceutical wastewater on the acute toxicity of Zebrafish. The experimental method of exposure is used in this research. Experiments were carried out with different groups of pharmaceutical wastewater. Zebrafish was cultivated in a five liter fish tank. In the experiment, according to mortality, initially a 96h preliminary test was carried out at exposure concentrations to determine if the amoxicillin wastewater was toxic and to define the concentration range (24h LC100, 96h LC0) to be employed in the definitive tests. Based on the half lethal concentration of Zebrafish, the acute toxicity of amoxicillin wastewater to Zebrafish was calculated and the toxicity grade of wastewater was determined. In the experiment, the Zebrafish was exposed with amoxicillin wastewater during 96h. The 24h, 48h, 72h and 96h LC50 of amoxicillin wastewater on the Zebrafish were 63.10%, 53.70%, 41.69% and 40.74%, respectively. At 96h, the test time is the longest, and the value of LC50 is the smallest. In the observation period of 96 hours, the LC50 of amoxicillin wastewater were in the range of 40% ~ 60% and the value of Tau is 1 ~ 2. It indicates amoxicillin wastewater is low toxic wastewater when the experimental time is shorter than 48h, amoxicillin wastewater is moderate toxicity wastewater when the experimental time is higher than 48h. According to the experimental data, with the exposure time and the volume percentage of amoxicillin wastewater increases, the mortality rate of Zebrafish is gradually increased and the toxicity of amoxicillin wastewater increases. It indicates that the toxicity of amoxicillin wastewater is the biggest and the effect of wastewater on Zebrafish is greatest. In some ways, the toxicity of amoxicillin wastewater can be affected by the test time.

1. Introduction

Antibiotics are a class of so-called emerging contaminants that have raised concern in recent decade. They are widely used in human and veterinary medicine to prevent or to treat microbial infections. The large production of antibiotics in the world, where the annual production is 100,000 to 200,000t, is one of the main reasons for the gradual increase of pharmaceutical contaminants in waters[1-2]. Amoxicillin is one of the most widely used drug, due to its chemical composition, level of consumption, solubility, pharmacological characteristics and environmental toxicity, it is known as one of the main persistent pollutantvs in the aquatic environment. Compared with other pharmaceuticals, amoxicillin has a very low metabolic rate in anima, so 80~90% of amoxicillin is released to the water unchanged[3-5].

In the frame of the present research, Zebrafish is rapidly becoming a promising model organism for both toxicological and neuropharmacological in-vivo studies. Not only antibiotics can be found in the environment but also their transformation products about which little information is generally
available. The development of bacterial resistance to antibiotics is particularly worrying as it can lead to sanitary and health problems. Due its cost-efficiency, easy housing, rapid reproduction, and high genetic and physiological homology to humans, fish have become a popular tool in toxicological screening disorders. The present study aimed to examine amoxicillin wastewater aquatic for toxicity by assessing acute behavioral in adult Zebrafish.

Aquatic organisms are exposed to a wide variety of environmental contaminants to assess the possible biological impacts. Fish have been extensively used as toxicity test organisms. Zebrafish is a small tropical fish, cyprinididae, Danio. Zebrafish have become model organism which recommended by the international organization for standardization, because they are sensitive to the change of environment. Besides, it has been widely used in the areas such as gene embryos[6-8]. Amoxicillin wastewater that used in this experiment comes from a pharmaceutical enterprise in Shijiazhuang. This experiment takes Zebrafish as the research object. The toxicity of amoxicillin wastewater was estimated by the mortality of Zebrafish. The toxicity of amoxicillin wastewater was determined by calculating the median lethal concentration to Zebrafish.

2. Materials and methods

2.1. Test materials
Test organism Zebrafish were obtained from aquafarm in Shijiazhuang. In this experiment, mean length of fish used was 23±3mm. Mean weight of fish used in this experiment was 0.20±0.05g. Before entering the laboratory, disinfection treatment employ 5% salt water, and then domesticate a week in fully aeration of dechlorinated tap water. No feeding before the experiment 24 hours andduring the experiment.

2.2. Experimental equipment
Aquarium air pump (SB-648A); thermometer; measuring cylinder; pH meter (FE20); dissolved oxygen meter (JPBJ-608); water hardometer (YD300); 5L glass fish tank.

2.3. Experimental wastewater
Amoxicillin wastewater was obtained from a pharmaceutical factory in Shijiazhuang. Wastewater is odorless and bright yellow (show in Figure 1). Wastewater was stored at 4℃ in freezer.

2.4. Water quality index of amoxicillin wastewater
Before the experiment, the COD, TOC, ammonia nitrogen and pH of wastewater were assayed. The results of the measurements and the instruments used in experiment are shown in table 1.

| Project                  | Value (ppm) | standard value | Result  | Analytical method                          |
|--------------------------|-------------|----------------|---------|--------------------------------------------|
| COD                      | 1802.24     | 120            | Excessive | Potassium dichromate method                |
| Ammonia nitrogen         | 22.54       | 20             | Excessive | Nessler's reagents spectrophotometer        |
| TOC                      | 6759.09     | 35             | Excessive | Shimadzu TOC-VCPn analyzer                |
| pH                       | 4.46        | 6-9            | Excessive | pH meter (FE20)                            |

Note: The unit of measurement is mg·L⁻¹, except pH.

2.5. Test methods
The results showed that COD, ammonia nitrogen and TOC were seriously beyond the standard. The pH of the wastewater should be adjusted to neutral before the experiment, principally because of wastewater is acidic.

2.6. Experimental design
The static test method was used in the experiment. Nitially a 96-h preliminary test was carried out at exposure concentrations, fish in treatment group were exposed to five concentrations (0, 0.2%, 0.3%,
0.4%, 0.5% and 0.6%) for 96h, respectively. All assays were done in replicated three times for each concentration. Dissolved oxygen (DO), pH, and temperature (T) should be measured daily. PH is maintained between 7.4 and 7.6. DO is about 5 mg/L. T is controlled at (22±1)℃. During the experiment, continuous aeration was required. The experiment was carried out for 96h. The number of Zebrafish deaths was observed at 24h, 48h, 72h and 96h. LC50 is determined by mortality.

2.7. Data statistics method

(1) Median lethal concentration (LC50): According to the method of probability unit weighted regression (Bliss), statistics were performed using Probit Probability Unit Analysis Method for SPSS19.0 Software [9-10].

(2) Acute Toxic Unit (Tua):

\[ Tua = \frac{1}{LC_{50}} \]

3. Results and discussion

3.1. The toxicity effect of amoxicillin wastewater on zebrafish

While the volume percentage of amoxicillin wastewater increases, the mortality of Zebrafish mortality increases significantly and the acute toxicity effect of amoxicillin on Zebrafish also increases (Table 2). There was a significant dose-response relationship between the Zebrafish mortality and the logarithm of the experimental concentration at different volume percentage of wastewater (Figure 1).

![Figure 1. Regression curves of the logarithm of the volume percentage of amoxicillin and the death probability of Zebrafish](image)

**Table 2. Zebrafish mortality in different group of amoxicillin wastewater**

| Volume percentage (%) | The cumulative mortality rate of fish at different test times (%) |
|-----------------------|--------------------------------------------------------------|
|                        | 24h   | 48h   | 72h   | 96h   |
| 0.2                   | 16.7  | 16.7  | 16.7  | 16.7  |
| 0.3                   | 16.7  | 16.7  | 16.7  | 16.7  |
| 0.4                   | 16.7  | 33.3  | 33.3  | 50    |
| 0.5                   | 33.3  | 50    | 66.7  | 66.7  |
| 0.6                   | 50    | 83.3  | 83.3  | 83.3  |
| 0.7                   | 50    | 83.3  | 100   | 100   |
| 0.8                   | 50    | 83.3  | 100   | 100   |

According to the experimental data, the 24h LC50 and 96h LC100 volume percentage for the acute toxicity of the amoxicillin wastewater were 0.6% and 0.8%, respectively. As the exposure time and the
concentration of amoxicillin wastewater increases, the mortality rate of Zebrafish is gradually increased. It indicates that amoxicillin wastewater have greater toxicity and have a certain impact on the growth of Zebrafish.

3.2. Acute Toxicity Effects of Amoxicillin Wastewater on Zebrafish

According to the mortality rate of Zebrafish at different time periods, LC50 and Tua were calculated by regression equation (Table 3).

| Experimental time (h) | Probability unit (y) percent concentration (x) equation | Correlation coefficient (R2) | LC50 (%) | Tua | 95% Confidence limit |
|-----------------------|----------------------------------------------------------|-----------------------------|----------|-----|---------------------|
| 24                    | y=4.8335x-3.6855                                          | 0.798                       | 63.10    | 1.6 | 56.22–73.93         |
| 48                    | y=5.3901x-4.3244                                          | 0.8387                      | 53.70    | 1.9 | 47.40–61.86         |
| 72                    | y=7.2062x-6.636                                          | 0.8973                      | 41.69    | 2.4 | 35.85–48.29         |
| 96                    | y=7.2184x-6.6016                                          | 0.9004                      | 40.74    | 2.5 | 35.03–47.19         |

Note: The values of probability unit in the table are derived from the percent of probability unit conversion table.

In this experiment, with the exposure time and the concentration of amoxicillin wastewater increases, the mortality rate of Zebrafish is gradually increased. The study is similar to the mortality of Zebrafish increases with the concentration of pharmaceutical wastewater[11].

3.3. Identification of wastewater toxicity

Wastewater is classified according to the Standard for Classification of Industrial Wastewater Toxicity (Table 4) [12-14]. In the observation period of 96 hours, the LC50 of amoxicillin wastewater were in the range of 40% ~ 60% and the value of Tua is 1 ~ 2. It indicates amoxicillin wastewater is low toxic wastewater when the experimental time is shorter than 48h; amoxicillin wastewater is moderate toxicity wastewater when the experimental time is higher than 48h.

| Toxicity grade         | LC50 (%) | Tua |
|------------------------|----------|-----|
| Advanced Toxicity      | <1       | >100|
| Highly toxic           | 1–10     | 10–100|
| Moderately toxic       | 10–50    | 2–10|
| Low toxicity           | 50–100   | 1–2 |
| Micro-toxic or non-toxic| >100 | <1  |

4. Conclusion

(1) The LC50 value of amoxicillin wastewater to in 96 hours was 40% ~ 60%, Tua was between 1 and 2. This shows that amoxicillin wastewater in the 48h experimental time is low toxic wastewater; higher than 48h test time is poisoning wastewater.

(2) In the experiment, the Zebrafish was exposed with amoxicillin wastewater during 96h. The 24h, 48h, 72h and 96h LC50 of amoxicillin wastewater on the Zebrafish were 63.10%, 53.70%, 41.69% and 40.74%, respectively. At 96h, the test time is the longest, and the value of LC50 is the smallest. It indicates that the toxicity of amoxicillin wastewater is the biggest and the effect of wastewater on Zebrafish is greatest. To a certain extent, the toxicity of amoxicillin wastewater is affected by the test time. The toxicity of amoxicillin wastewater was estimated by the mortality of Zebrafish. As the exposure time and the volume percentage of amoxicillin wastewater increases, the mortality rate of Zebrafish gradually increased.

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