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ACUTE TOXICITY TEST USING CYANIDE ON *DAPHNIA MAGNA* BY FLOW-THROUGH SYSTEM

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This study describes the acute toxicity of sodium cyanide on the crustacean Daphnia magna (Cladocera). A new flow-through system was innovated in which the microorganism continuously exposure to the toxicity of sodium cyanide during the test. We used twelve different concentration of sodium cyanide from low values (0 mg CN/L) to high values (1 mg CN /L) on bioassay test. D. magna were exposed to the concentrations for 24, 48, 72 and 96 h at 20 – 25°C. We controlled the three important parameters such as temperature, pH and DO to meet the standard requirements. The LC_{50} values for 24, 48, 72 and 96 h (95% confidence limits in parentheses) were estimated statistically by the probit methods and were 0.171 (0.163 – 0.179), 0.12 (0.112 – 0.128), 0.07 (0.062 – 0.078) and 0.019 (0.011 – 0.027) mg/L respectively. Finally, we proposed two new values for SAR (safe application rate) and SAFE Coefficients.

Key words: acute toxicity test, bioassay, *Daphnia magna,* sodium cyanide.

1. Introduction

Environmental pollution with a variety of toxic compounds has become a threat to the aquatic flora and fauna and is one of the issues of concern. These pollutions are mainly imported into the water bodies from industrial effluent and agricultural area and many of these compounds are highly resistant. This level of emissions over time is endangering aquatic life (Susan et al., 2010). The environmental analysis helps to preserve the natural environment and human health from contaminants such as pesticides, metals and other dangerous toxins and pollutants in the air, water, soil and nutrients (Mansour and Gad, 2010). The need to protect plant and animal life in the land and water ecosystems from pollutants released into the environment, which during the past five decades have increased, have been led to the development of methods to assess adverse effects of chemicals. Today usage of the variety of standard methods for determining the acute and chronic toxicity of pollutants has increased (USEPA, October, 2002a).

The most comprehensive chemical and physical specifications of effluent, does not show adverse effects on the water bodies ecosystems. In general, the standard method for evaluating the toxicity of pollutants in wastewaters, effluents and water bodies is biological toxicity test or bioassay (USEPA 1984., USEPA, October, 2002a, 2005). The recent progress of biomarkers based on the study of the response of organisms to pollutants has provided essential tools for vigilant monitoring of pollution (Susan et al., 2010).

There are many methods for doing bioassay test. Two common types, mainly used, include evaluated of acute and chronic toxicity. Selection of the type test depends on the existing rules and regulations, test objectives, available resources, wastewater characteristics, including fluctuations in the level of its toxicity, and etc (USEPA, October, 2002b).

Maintain stable concentrations of toxic compounds are essential in order to obtain reliable results of toxicity tests. Different methods and equipments are needed to reach this aim. Although roughly, the static or static-renewable toxicity test can be performed easily and with minimum cost; but these methods for many compounds, including absorbent compounds, compounds with the potential for rapid biodegradation, volatile compounds or insoluble materials are not suitable. Hence, the test with flow-through system overcomes to these limitations (Lauth et al., 1996; Tisler and Zagorc-Koncan, 1998). Other advantages of the flow-through toxicity test to static test are constant water quality in terms of dissolved oxygen, NH_3, salinity and concentration of toxic substances. In addition, a flow-through method reduces the impaction stress from seining organisms during static renewal toxicity testing (Dietrich et al., 2002).

There are many types of organisms, including fish (Capkin et al., 2010; Carriger et al., 2011; Lammer et al., 2009), green alga (Grade et al., 2000), bacteria (Venturaa et al., 2012; Lopez-Roldana et al., 2012) and etc. that can be used as an bioindicator to determine the toxicity of chemicals and control of effluent toxicity and polluted water but one of the international of them is toxicity tests with crustaceans called *Daphnia*, especially *D. magna* (USEPA, October, 2002a; Yegane et al., 2008; Persoone et al., 2009). The selection of *D. magna* as a standard test species has several advantages including its small size, easy to culture and hold in the laboratory and its wide distribution in the habitat. Parthenogenesis reproduction of *D. magna* under non-stressed conditions increases the reproducibility and repeatability of the test results. In addition, the organism has a higher sensitivity to chemicals compared to other freshwater invertebrates and its relatively short life cycle and reproduction is ideal for chronic

experiments. Therefore, *D. magna* is the most dominant species of freshwater in acute and chronic toxicity tests (USEPA, October, 2002a, 2005; Meinertz et al., 2008; Yegane et al., 2008). The sex of *D. magna* is listed as an indicator organism in toxicity tests by the American Public Health Association (APHA), the Central Institute Board of India Guidelines (CIBIG), the American Standard Testing Methods (ASTM), the Food and Agriculture Organization (FAO), U.S. Environmental Protection Agency (USEPA) and the Organization of European Committee for Determining (OECD) (Jamil, 1999).

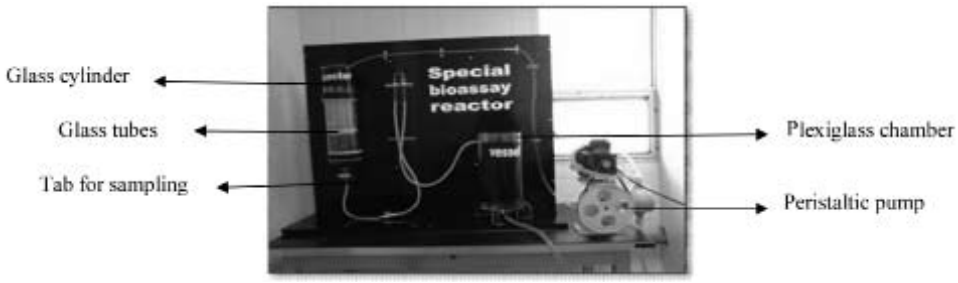
Cyanide is one of the most toxic chemicals and it is very toxic for most of the aquatic and human life even at very low concentrations. So cyanide registered by the USEPA as priority pollutant (Dash et al., 2009). Cyanides are used widely in factories of electroplating, photo-processing, plastics, and metal mining operations, as chemical-fertilizer, printed circuit board manufacturing, intermediates in agricultural chemical production and for some therapeutic applications and can be exist in different concentrations in the wastewater industry (Muniswamy et al., 2010; White et al., 2000; Sirianuntapiboon et al., 2008). Residual cyanide in wastewater is a very dangerous substance due to strong effects on humans and the environment (White et al., 2000). Thus the wastewater must be treated before discharge to the environment to an acceptable level of cyanide (<1 mg/L) (Muniswamy et al., 2010). Free cyanide is not bio-accumulate in the food chain and its toxicity is acute and immediate (Muniswamy et al., 2010). Free cyanide concentrations from about 50 to 200 μg/L were fatal to juveniles of most of the more sensitive fish species, with concentrations much above 200 μg/L being rapidly fatal to most juvenile fish. Thus, there is a relatively narrow range of species sensitivity for fishes (USEPA, 1984). Hence, the use of biological toxicity testing for cyanide is very important and several studies have been conducted in this area. A study has been done by EPA's Animal Biology Laboratory in 1977 on the effects of sodium cyanide on freshwater fish, rainbow trout. The results showed that sodium cyanide was categorized as highly toxic to rainbow trout (LC_{50} was 0.118 ppm) (USEPA, 1994). In the last decade, many researchers have been worked on flow-through toxicity test (Susan et al., 2010; Yegane et al., 2008; Conradson et al., 1989; Welsh et al., 2008); however, very little information is available relating to the flow-through bioassay with *D. magna*. On the other hand, because of effluent controlling in Iran is still based on chemical and physical parameters. Therefore, the preparation of an appropriate strategy for monitoring of effluent is necessary. Consequently, it was decided to study in more detail on the acute toxicity of sodium cyanide to *D. magna*.

2. Materials and Methods

2.1. Test solutions. A stock solution of cyanide was prepared by dissolving of weighted sodium cyanide in dionized/distilled water and either used immediately or preserved at 4 °C to keep them in good condition for the duration of the study. The stock solution was diluted to the desired concentration before beginning the tests. Nomination concentration of sodium cyanide was 0 (control), 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/L and were prepared fresh for each renewal period.

2.2. Test organisms. The used organisms for test were *Cladocerans, D. magna*. This stock daphnids were purchased at microbiology laboratory, Health College, Tehran University of Medical Science. These organisms were acclimated for 20 – 30 days in 4 l container containing deionized water and aerated/dechlorinated tap water (in ratio 2:1). The photoperiod is adjusted to 16 h light and 8 h dark. *Daphnia* food was prepared with mixture of green alga and a suspension of trout chow and yeast according to the standard methods (2005). The *D. magna* selected for each test were third generation and ≤ 24 h old. Water quality measurements such as dissolved oxygen, pH, temperature, total alkalinity, total hardness, calcium, magnesium, lithium, potassium, sodium and nitrite were taken in the water which was used for daphnia culturing.

2.3. Flow-through system. The acute toxicity test carried out using EPA standards under continuous flow-through conditions that based on immobilization of the *D. magna* due to the action of cyanide. The flow-through system comprise a 10-L test chamber for holding test solutions, 5 glass tubes joined together for house the test organisms, 8-L glass cylinder and prestalting pump. 5 glass tubes fit firmly onto the cylinder and can be removed minimal effort for washing and transporting the organisms. The external diameter, height and wall thickness of the cylinder are 15 cm, 50 cm and 5 mm, respectively. End of the cylinder has a tap for sampling. The size of glass tubes is 3 cm in diameter and 23 cm in height. Up and end of the five glass tube covered by lace with mesh 10 micron. The cause of selecting glass equipment for this system is enabling a clear view of the internal of the tube containing organisms and test solution. Test chamber and cylinder were covered with plastic wrap to minimize losses of HCN due to evaporation and to minimize the entry of dust or other particulates in to the solutions. Solutions are recycled from Plexiglas container into the cylinder using pump and then back in to container. Pumping rate was 0.278 L/min. At this rate the tank volume is replaced approximately each 20 min. The whole equipments attached to a Plexiglas sheet as show in Figure.



Schematic of flow-through bioassay reactor

Neonates younger than 24 h were exposed to the concentrations for 24, 48, 72 and 96 h at 20 – 25°C. Twenty neonates younger than 24 h were used for each concentration examined in a 5 glass tube so that each glass tube containing 4 neonates. The toxicity of the sodium cyanide was evaluated by estimation of the LC_{50} value (the effect concentration of the tested substance causing 50 % immobilization on *D. magna*). The number of the alive daphnia and chemical parameters such as pH, DO and temperature in each replicate were recorded for 24, 48, 72 and 96 h observations. Counting of the immobilized daphnia was carried out by eyes. All glassware was washed with distillated water before and after beginning the test.

2.4. Data interpretation. Mortality data was used for calculating lethal concentration LC_{50}, 95% confidence interval using probit analysis in SAS software v.9.1. SAR (safe application rate) and SAFE Coefficients were also calculated on the basis of 96 h acute toxicity test. The formula was used as indicated below (Basak and Konar, 1977):

$$\text{SAFE} = \frac{LC_0 \text{ at } 96\text{ h}}{LC_{100} \text{ at } 96\text{ h}}; \qquad (1)$$

$$\text{SAR} = 96\text{ h } LC_{50} \cdot \text{SAFE}. \qquad (2)$$

The test was considered to be valid and acceptable if mean control survival was greater than 90 percent and water quality parameters were maintained within acceptable limits. The experiment was conducted with 3 replicate at each concentration and control.

3. Results and discussions

The Average physicochemical characterizations of the tab water for daphnia culturing are tabulated in Table 1. It is obvious from Table 1; these parameters meet the standard requirements. Therefore, we have no limitation to use tab water for daphnia culturing.

Table 1. Physicochemical characteristic of the tab water for daphnia culturing

Parameter	Unit	Concentration
Temperature	°C	21 – 25
pH	Dimensionless	7.2 – 7.6
DO	mg/L	6 – 7.5
Total alkality	mg/L as $CaCO_3$	130 – 140
Total hardness	mg/L as $CaCO_3$	300 – 315
Permanent hardness	mg/L as $CaCO_3$	130 – 135
Magnesium hardness	mg/ L as $CaCO_3$	135 – 140
Magnesium ion	mg Mg^{2+}/L	33 – 35
Calcium ion	mg Ca^{2+}/L	65 – 70
Lithium	mg/L	2 – 2.2
Sodium	mg/L	110 – 120
Potassium	mg/L	2.5 – 3
Nitrites	mg N/L	0.1 – 0.15
Phosphorous	mg P/L	0.045 – 0.05

We wanted to make a situation in which the only reason for *D. magna* mortality was cyanide concentrations. Therefore, the other important factors such as temperature, pH and DO were recorded continuously during the flow-through test to be in the values (temperature: 22.25+0.14, pH: 7.22+0.14, DO: 6.99+0.14).

The Probit Method was used for estimating the LC_{50} and the associated 95% confidence interval. In this method, the test for heterogeneity was not significant because the calculated Chi-square (496.87) was less than the tabular value (1781.65). Thus, the Probit Method appears to be appropriate for this data. The goodness of chi-square test for the two independent variables used in toxicity tests (cyanide concentration and exposure time) were calculated 33.6491 and 13.1338, respectively. According to p-value that were significant for both variables

(< 0.0001) it can be concluded that the cyanide concentration and exposure time have an effect on *Daphnia* mortality. In this model, effect factor of cyanide concentration, effect factor of exposure time and intercept were estimated 8.6066, 0.0181 and -1.9025, respectively. Therefore, the probable equation of daphnia mortality is obtained using the available data:

$$Y=\beta_0+(\beta_1 X_1) + (\beta_2 X_2). \quad (3)$$

Where Y – probable response; β_0 – intercept; β_1 – independent variable number 1; X_1 – effect factor of independent variable number 1; β_2 – independent variable number 2; X_2 – effect factor of independent variable number 2;

Then, we have:

$$Y=-1.9025 + (8.6066\ \text{Con.}) + (0.081\ \text{h}). \quad (4)$$

The concentrations that killed 10% (LC_{10}), 50% (LC_{50}) and 90% (LC_{90}) of *D. magna* are shown in Table 2. LC_{50} values for 24, 48, 72 and 96 h (95% confidence limits in parentheses) were estimated statistically by the probit methods and were 0.171 (0.163 – 0.179), 0.12 (0.112 – 0.128), 0.07 (0.062 –0.078) and 0.019 (0.011 – 0.027) mg/L respectively. In all tests, the rate of *D. magna* mortality is increased corresponding to the elevated toxic concentrations and duration of exposure and no control *D. magna* died during toxicity tests.

Table 2. Lethal concentrations ($LC_{10\text{-}90}$) of sodium cyanide for D. magna

Concentration (mg/L) (95% confidence intervals)				
Point	24 h	48 h	72 h	96 h
LC_{10}	0.013	0.08	0.21	0.029
	(0.0122 – 0.0138)	(0.07 – 0.088)	(0.013 – 0.029)	(0.021 – 0.037)
LC_{50}	0.171	0.12	0.07	0.019
	(0.163 – 0.179)	(0.112 – 0.128)	(0.062 – 0.078)	(0.011 – 0.027)
LC_{90}	0.320	0.269	0.219	0.168
	(0.312 – 0.328)	(0.261 – 0.277)	(0.211 – 0.227)	(0.160 – 0.177)

Anderson et al. in 1944 found that 48 h – LC_{50} of NaCN for immobilization of *D. magn*a by static renewal test was < 3.4 mg/L (Anderson, 1946) while in this paper; 0.12 mg/L were obtained by flow through test on *D. magna*. The new proposed method leads us to high accurate results. In this approach, because of

the continuous flow during the test respect to the traditional static methods, it is easily overcome to the short comings and coverage us rapidly to the real results.

The sensitivity of the organism has a great effect on the results. Pablo et al. in 1977 estimated the 48 h – LC_{50} value of NaCN by static renewal test for Larvae of the Doughboy Scallop (*Chlamys asperrimus)*, 28.6 ppb (Pablo et al., 1997). Comparison of the results between Pablo et al. and present study shows that the larvae are more sensitive to sodium cyanide than *D. magna*. The renewal test was used by Muniswamy et al. in 2010 to obtain the acute toxicity (LC_{50}) of sodium cyanide over 96 h period for *Catla catla, Labeo rohita, Cirrhinus mrigala, Cyprinus carpio* and *Oreochromis mossambicus* and they achieved 0.11 mg/L, 0.19 mg/L, 0.33 mg/L, 1 mg/L and 0.420 mg/L, respectively. In this study we considered the same parameter (96 h – LC_{50}) on the *D. magna* and its value obtained as: 0.019 mg/L. The LC_{50} for sodium cyanide in this study were approximately 6 – 53 times higher than the LC_{50} value reported by Muniswamy et al. study (Muniswamy et al., 2010). Therefore, from the mentioned studies it can be inferred that cyanide is very toxic to the *D. magna*. Generally, the use of a bioassay to direct measurement of toxicity of toxic materials is an accurate and reliable method to assess its effect.

When any organism sensitivity were examined to any toxic substance, reporting a reliability or safety factor is necessary. Equations 1 and 2 are used to calculate the safety factor of SAFE and SAR Coefficients which were 0.006 and 0.0001, respectively. By considering the obtained value of SAR (0.0001 mg/L), it can be seen that effluents with this SAR can be inter to the body water without any concerning. In this situation, all the organisms in the body waters have adequate safety under this cyanide concentration.

4. Conclusion

In this paper, the toxicity of sodium cyanide on the crustacean *D. magna* (*Cladocera*) by flow-through method has been studied. The results provide baseline information in formulating strategy for controlled release of treated industrial effluents into the receiving water bodies. The estimated LC_{50} values for cyanide (using nominal concentrations) for the *D. magna* show that cyanide is very toxic to these organism. Further tests using more test organisms (e.g. fish species, invertebrates, plant species) by applying flow-through method need to be conducted to validate results. For application of toxicity data in regulation of wastewater discharges and prediction of environmental affects both acute and chronic toxic levels have to be determined to conserve aquatic life.

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