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Effect of 3, 4-dichloroaniline on growth of juvenile zebrafish (*Danio rerio*)

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Abstract
Present study was designed to evaluate the effect of 3, 4-Dichloroaniline (DCA) on the growth rate of juvenile zebrafish (*Danio rerio*) according to guidelines of OECD 215 and EC 14. Healthy juvenile zebrafish (1.5 month old) having individual body weight 50 -100 mg was exposed to 0.016, 0.031, 0.063, 0.125, and 0.250 mg DCA/L along with control group for 28 days. Study was performed under semi-static condition where media was renewed at every 24 hour intervals. Surviving juvenile fishes were weighted (blotted dry) replicate vise on days 14 and 28. Based on body weight average specific growth rate was calculated replicate wise. Test medium was analysed for active ingredient concentration and stability for the lowest (0.016 mg/L) and the highest (0.5 mg/L) tested concentration along with control when freshly prepared and immediately prior to renewal on days 0, 7, 14, and 21. The active ingredient concentration and stability of test item in test media was 80% of nominal concentration. All juvenile fishes were normal and no mortality was observed at the concentration levels of 0.016, 0.031, 0.063, 0.125, and 0.250 mg DCA/L and in control group. The No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) up to 28 day exposure period of DCA were found 0.063 and 0.125 mg/L, respectively. The EC_{50} values of DCA for average specific growth rate between days 0 and 14, 14 and 28, and 0 and 28 were 0.144, 0.178, and 0.257 mg/L, respectively. Based on the results, it can be concluded that DCA has toxic effect on growth of juvenile zebrafish. DCA affected body weight of juvenile zebrafish at the test concentration levels of 0.031, 0.063, 0.125, 0.250, and 0.500 mg DCA/L.

Keywords: 3, 4-dichloroaniline, NOEC, LOEC, growth rate, juvenile fish, zebrafish

1. Introduction
The aromatic amine, 3,4-Dichloroaniline (DCA) a chlorinated agent has been used for the synthesis of several herbicides (e.g. Diuron, Linuron and Propanil), and it has been found as one of the major residue in the effluent from dye-manufacturing plants such as azo dyes for polyester fabrics, textile and pharmaceuticals [1, 2, 3]. DCA endangers growth, development and propagation of fish, other aquatic organisms and human [4, 5].
The safety of the fish community is an important concern because fish play a very important role in trophic cascades and as food resource for human beings. The zebrafish is a vertebrate animal model which is increasingly used for *in vivo* drug toxicity, efficacy screening, and for assessing chemical toxicity and safety [6, 10]. Substantial information gathered from developmental and genetic research has placed zebrafish in an attractive position for use as a toxicological model.
DCA cannot degrade easily and remains in the environment for longer time. The aromatic amine, DCA was detected in various surface waters in Western Europe [11]. It has toxicity impact to fish at low concentrations in extended exposures [12]. DCA showed different modes of toxic action and well documented effects on *Daphnia* reproduction. Regulatory authority has also selected DCA as a reference substance for fish embryo toxicity test. The results obtained in our in-house validation study on fish embryo toxicity test and fish short term embryo and sac fry studies using zebrafish embryos showed that DCA have toxic effects on the development of zebrafish embryos and affected the normal growth of developing embryos. Hence, the present study was designed to assess the effects of DCA on the growth rate of juvenile zebrafish (*Danio rerio*) according to OECD (Organisation of Economic Co-operation and Development) 215.

2. Materials and Methods

2.1. Chemical

3, 4-dichloroaniline (97.6%) was procured from Sigma-Aldrich, Switzerland.

2.2. Animal

Juvenile zebrafish (wild type, 1.5 month old) was used as test system for the experiment. Total juvenile fishes were divided into seven groups (3 replicates/group) with each group comprising thirty juvenile fish. Juvenile fishes were acclimatise for 15 days before use in the study. Juvenile fishes were fed at a rate of 8% of their body weight per day. Juvenile fishes with body weight between 50.1 and 75.9 mg were distributed among the test vessels. An individual juvenile fish body weight was kept within ± 25% of the arithmetic mean weight at the start of the test.

2.3. Fish, Juvenile Growth Test

The present study was conducted according to OECD guideline no. 215 (OECD 2000) [13] and EC No. 440/2008 (EC 2008) [14].

2.4. Preparation of Test Concentrations

DCA Stock solution (0.1 mg/mL, A) was prepared by dissolving 10 mg of DCA in 100 mL of water, via stirring overnight on magnetic stirrer. Aliquots of stock solution A were added in test media to achieve the concentrations of 0.016, 0.031, 0.063, 0.125, 0.250 and 0.500 mg DCA/L.

2.5. Growth Analysis

Tank average specific growth rates were calculated for each test vessels. The average specific growth rate was calculated according to the following formula:

Where,

w1, w2 weights of particular juvenile fish at times t1 and t2, respectively

(Juvenile fish body weight w1 at the initiation was calculated from individual fish body weight)

Log w1 average of the logarithms of the values w1 for the fish in the tank at the start of the study period

Log w2 average of the logarithms of the values w2 for the fish in the tank at the end of the study period

t1, t2 time (days) at start and end of study period

2.6. Active Ingredient Concentration and Stability Analysis

The test solutions were subjected for active ingredient concentration and stability analyses using the validated analytical method. Sampling was performed for active ingredient concentration on days 0, 7, 14, 21 and for stability on days 1, 8, 15, 22 from the highest and the lowest test concentrations (i.e., sample was analysed from the same solution - when freshly prepared and at renewal) by using HPLC [Shimadzu, LC-2010 AHT with LC- Solution software].

2.7. Instrumental Parameters

Instrument: HPLC [Shimadzu, LC-2010 AHT with LC-Solution software]

Column: Waters X-select CSH, Flouro Phenyl [250×4.6 mm (i.d.); 5.0 µm particle size]

Wavelength: 246 nm

Flow Rate: 1.0 mL/minute

Injection Volume: 80 µL

Mobile Phase: Acetonitrile (40): 0.1% Formic acid in Milli-Q water (60), v/v

2.8. Statistical Evaluation

EC_{50} with 95% confidence limits was calculated following the Probit analysis method (Finney, 1971) [15], using in-house developed, validated software at the end of study. To estimate LOEC based on an analysis of variance (ANOVA) of the tank-average specific growth rate, heterogeneous variance (Bartlett's test) was performed. As the required assumptions for parametric methods were not met with heterogeneous variance (Bartlett's test), data were transformed to homogenize variances prior to performing the ANOVA. Data were transformed by adding 5 followed by square root of individual values.

3. Results

3.1. Average Specific Growth Rate

Individual juvenile fish body weight was recorded immediately prior to exposure (on day 0). All surviving juvenile fishes in test chambers were weighed (blotted dry) individually on days 14 and 28 (at the end of study). The average specific growth rate was calculated for each test chamber between days 0-14, 14-28, and 0-28. Statistically significant decrease in average specific growth rate was observed in fish exposed to the test concentrations of 0.031, 0.125, 0.250, and 0.500 mg DCA/L between days 0-14 and 0-28 when compared with the control group (Table 1).

3.2. Active Ingredient Concentration and Stability Analysis

The active ingredient concentration and stability of test medium was analyzed, and recovery is presented in Table 2 to 5.

3.3. Mortality and Behaviour

The juvenile fishes were examined daily during the test period for mortality, external abnormalities and abnormal behavior. All juvenile fishes were normal, and no mortality was observed at the test concentrations of 0.016, 0.031, 0.063, 0.125, 0.250 mg DCA/L, and in control group. Fish exposed to 0.500 mg DCA/L settled at bottom as an abnormal behavior from day 19 to 28. 6.67% mortality of juvenile fish was observed at the test concentration of 0.500 mg DCA/L. 3.33% mortality of juvenile fish (mortality of 1 fish out of 30 fish) was observed while transferring the fish for weighing of day 14.

3.4. Environmental Parameters

The pH values of test media were ranged between 7.16 and 8.01. Dissolved oxygen and temperature values of test media were ranged between 61.4 and 100.0% and 21.8 to 24.5 °C, respectively. Total hardness and alkalinity of test media were ranged between 201.6 and 212.8 mg/L as $CaCO_3$ and 2 to 3 mg/L as phenolphthalein, respectively [16].

3.5. Statistical Analysis

The EC_{50} values of 3, 4-dichloroaniline for average specific growth rate between days 0 and 14, 14 and 28; and 0 and 28 were calculated (Table 6) using the probit of analysis method14.

The EC_{50} of DCA for average specific growth rate between days 14 and 28 was determined as 0.144 mg/L with the 95% fiducial limit ranged between 0.074 and 0.281 mg/L. The

regression equation established [probit of growth rate reduction (y) vs log concentration (mg/L) of DCA (x)] was y = 1.692 + 1.533x (Table 6).

The EC_{50} of DCA for average specific growth rate between days 14 and 28 was determined as 0.178 mg/L with the 95% fiducial limit ranged between 0.080 and 0.398 mg/L. The regression equation established [probit of growth rate reduction (y) vs log concentration (mg/L) of DCA (x)] was y = 2.002 + 1.332x (Table 6).

The EC_{50} of DCA for average specific growth rate between days 0 and 28 was determined as 0.257 mg/L with the 95% fiducial limit ranged between 0.103 and 0.645 mg/L. The regression equation established [probit of growth rate reduction (y) vs log concentration (mg/L) of DCA (x)] was y = 2.466 + 1.051x (Table 6).

3.6. Validity criteria

a) No mortality was observed in the control group at the end of the test.
b) The mean weight of fish in the control was increased 2.3 times from day 0 to day 28.
c) The dissolved oxygen concentration was maintained within 61.4 - 100% of the air saturation value (ASV) throughout the test.
d) The water temperature was not differ by more than ± 1 °C between test chambers during the test and was maintained within 21.8 – 23.8 °C.

Table 1: Average specific growth rate

Different Concentration of 3,4-dichloroaniline (mg/L)	Growth Rate Between Days (Mean ± SD)	
	0 -14	0-28
0.0	3.36 ± 0.31	3.04 ± 0.09
0.016	3.07 ± 0.26	2.71 ± 0.09
0.031	2.67 ± 0.39↓	2.35 ± 0.17↓↓
0.063	2.64 ± 0.38↓	2.49 ± 0.14↓
0.125	2.43 ± 0.22↓↓	2.20 ± 0.08↓↓
0.250	0.69 ± 0.23↓↓	1.17 ± 0.40↓↓
0.500	-3.48 ± 0.43↓↓	-1.61 ± 0.82↓↓

Key: Data are expressed as Mean ± SD; where SD = Standard deviation, ↓ = significantly lower than control at 5% ($p \leq 0.05$) level, ↓↓= significantly lower than control at 1% ($p \leq 0.01$) level

Table 2: Active ingredient concentration and stability analysis of 3, 4-Dichloroaniline (at 0 day/0h and; 0 day/24 h)

Concentration (mg/L)	Theoretical Concentration in Test Media Based on Purity (mg/L)	(0 day/0 h)		(0 day/24 h)	
		Analyzed Concentration (mg/L)	% Recovery	Analyzed Concentration (mg/L)	% Recovery
Control	0.0000	-	-	-	-
0.016	0.0156	0.0158	101.28	0.0151	96.79
0.500	0.4880	0.4797	98.30	0.4704	96.39

Key: ND= Not detected, Purity of Test Item= 97.60%, - = Not applicable

Table 3: Active ingredient concentration and stability analysis of 3, 4-Dichloroaniline (7 day/0h and 7 day/24 h)

Concentration (mg/L)	Theoretical concentration in test media based on purity (mg/L)	(7 day/0 h)		(7 day/24 h)	
		Analysed Concentration (mg/L)	% Recovery	Analysed Concentration (mg/L)	% Recovery
Control)	0.0000	-	-	-	-
0.016	0.0156	0.0148	94.87	0.0165	105.77
0.500	0.4880	0.5151	105.55	0.5266	107.91

Key: ND= Not detected, Purity of Test Item= 97.60%

Table 4: Active ingredient concentration and stability analysis of 3, 4-Dichloroaniline (14 day/0h and 14 day/24 h)

Concentration (mg/L)	Theoretical Concentration in Test Media Based on Purity (mg/L)	(14 day/0 h)		(14 day/24 h)	
		Analysed Concentration (mg/L)	% Recovery	Analysed Concentration (mg/L)	% Recovery
Control)	0.0000	-	-	-	-
0.016	0.0156	0.0167	107.05	0.0166	106.41
0.500	0.4880	0.4499	92.19	0.4454	91.27

Table 5: Active ingredient concentration and stability analysis of 3, 4-Dichloroaniline (21 day/0h and 21 day/24 h)

Concentration (mg/L)	Theoretical Concentration in Test Media Based on Purity (mg/L)	(21 day/0 h)		(21 day/24 h)	
		Analysed Concentration (mg/L)	% Recovery	Analysed Concentration (mg/L)	% Recovery
Control)	0.0000	-	-	-	-
0.016	0.0156	0.0156	100.0	0.0160	102.56
0.500	0.4880	0.4834	99.06	0.4799	98.34

Table 6: Relationship between Per cent Growth Rate and Concentration of 3, 4 Dichloroaniline

Duration (Days)	EC_{50} Value (mg/L)	95% Fiducial Limits (mg/L)		Regression Equation (y = a + bx)
		Lower Limit	Upper Limit	
0-14	0.144	0.074	0.281	y = 1.692 + 1.533x
14-28	0.178	0.080	0.398	y = 2.002 + 1.332x
0-28	0.257	0.103	0.645	y = 2.466 + 1.051x

Key: y = Probit of mortality, x = Log concentration of 3, 4-Dichloroaniline, a = Intercept, b = Slope

3. Discussion

Various guidelines have been developed for the Eco-toxicological biomonitoring based to the importance of fish in aquatic pollution. Regulatory authorities like OECD, US-EPA and EC have suggested *Danio rerio* as an experimental model for assessing toxicity levels at different developmental stages [17, 18, 19]. Toxicity analysis is an important phenomenon in assessing the impact of various pesticides on environment as they indicate the toxic properties of chemicals on organisms by changing their behavior, morphology and survival rate [20]. It gives initial data on the effect of pesticides on aquatic organisms, especially fishes. These data are important in providing awareness about harmful effects of pesticide in the environment [21].

In the present work Zebrafish exposed to 0.500 mg DCA/L settled at bottom as an abnormal behavior from day 19 to 28. Various scientists in their studies observed the abnormal behavior. Behavioral abnormalities in juveniles of african catfish exposed to endosulfan were reported by various workers [22, 23, 24]. Various swimming behaviors were observed after exposure to endosulfan in spotted snakehead *Channa punctatus*, Channel catfish *Ictalurus punctatus* and *Barbus stigma*, respectively [25, 26, 27].

During the present study a statistically significant decrease in average specific growth rate was observed in fish exposed to the test concentrations of 0.031, 0.125, 0.250, and 0.500 mg DCA/L between days 0-14 and 0-28 when compared with the control group. The growth of red drum *Sciaenops ocellatus* was significantly affected by exposure to the concentrations of 40 and 80 lg/L of atrazine or 1–10 lg/L of malathion [28]. Similar to the present study the weight reduction was also found in australian catfish *Tandanus tandanus* [29], in Nile tilapia *Oreochromis niloticus* [30], in *Barbus stigma* [27] and in spotted snakehead contaminated with diazinon [31]. The adverse effects of lindane, pentachlorophenol and propoxur on the growth of Nile tilapia, African catfish and Bagrid catfish *Chrysitchtys nigrodigitatus* were studied [32].

Zebrafish is also widely used as an experimental model to study toxicity of chemicals, to understand developmental process and to study different human diseases. Concentrations for fish juvenile growth test were selected based on the results obtained from our in-housed validation studies on zebrafish embryos and fish embryo and sac fry stages. All juvenile fishes were found normal and no mortality was observed at any of the tested concentrations except 0.500 mg DCA/L during experiment. Active ingredient concentration and stability analysis of test medium showed that the concentration of DCA was greater than 80% of nominal concentration.

Based on data collected for body weight on days 0, 14 and 28; the growth rate was calculated for each concentration and per cent decrement in growth rate was calculated. There was dose dependent decrement in average specific growth rate observed in fish exposed to various concentration of DCA/L between days 0-14 and 0-28. Previous reports of terbutryn nitrite and diclofenac exposure to juvenile zebrafish showed that growth suppression effects on juvenile fish [32]. These reports also indicated that oxidative stress one of the major contributors for growth inhibitory effects. Different modes of action of DCA have been reported for toxic effects on aquatic vertebrate and invertebrate animals. DCA has also known for inducing oxidative stress via free radical generation. In the present study, depletion of antioxidants due to oxidative stress might have affected the growth of one of juvenile fish. The exact underlying mechanism for growth inhibitory effect is yet to be identified.

4. Conclusions

The No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) up to 28 day exposure period of DCA was found to be 0.063 and 0.125 mg/L, respectively.

The EC_{50} values of DCA for average specific growth rate between days 0 and 14, 14 and 28; and 0 and 28 were 0.144, 0.178 and 0.257 mg/L, respectively. Based on the results, it can be concluded that 3, 4-dichloroaniline has toxic effect on growth of juvenile zebrafish up to day 28 at the test concentrations of 0.031, 0.063, 0.125, 0.250 and 0.500 mg DCA/L.

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