Bull. Environ. Contam. Toxicol. (1998) 61:355-362
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Bulletin of Environmental Contamination and Toxicology

Development of a 7-Day *Daphnia magna* Growth Test Using Image Analysis

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Received: 9 March 1998/Accepted: 6 July 1998

In the United Kingdom there is increasing recognition by regulators of the value of using direct toxicity assessment (DTA) procedures as part of an "integrated approach' to control the discharge of toxic substances into the aquatic environment (Wharfe and Tinsley 1995). This need to apply ecotoxicologial methods stems from the realisation that it is not possible to control the myriad of substances entering the aquatic environment in increasingly complex mixtures by the use of single substance-specific standards alone. Furthermore, in the United States toxicity criteria have been included in regulatory permits to discharge for more than a decade (US EPA 1991).

Procedures promulgated by international organisations such as the Organisation for Economic Cooperation and Development and the International Standards Organisation are available for assessing the short-term lethal effects of effluents and leachates on crustaceans (such as the water flea *Daphnia magna* and the marine copepods such as *Acartia tonsa* and *Tisbe battagliai*) and fish (Calow 1993; Rand 1995). To assess sub-lethal toxicity tests measuring algal growth inhibition over 72 hr, *Daphnia magna* juvenile production over 21 days and fish growth over 28 days have been developed. However, the *Daphnia magna* reproduction and fish growth tests are not ideal for assessing the toxicity of environmental samples such as effluents and receiving waters due to their cost and the duration of exposure for these potentially unstable test solutions (Johnson 1996). Therefore, attention has focused on developing shorter-term tests of comparable sensitivity which can be used in this role. In the United States short-term (7 day) methods have been developed to monitor the effects of effluents on the reproduction of *Ceriodaphnia dubia* and the growth of fathead minnows (*Pimephales promelas*) as part of the National Pollutant Discharge Elimination System (US EPA 1994).

This study was conducted to ascertain whether growth can provide a sensitive short-term (7 day) measure of sub-lethal effects *in Daphnia magna* relative to existing immobilisation and juvenile production responses. It also investigated whether image analysis could provide a more cost-effective way of assessing growth than methods which require test operators to assess the length of organisms microscopically using an eyepiece graticule or estimating growth using dry weight.

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Microscopic examination is time-consuming and laborious compared to the use of image analysis. Measurement of growth using dry weight terminates the test and means only one set of data can be obtained from experimental animals during the exposure period unlike the non-invasive image analysis technique which means repeat measurments can be made throughout the test. Although image analysis has only been applied occasionally in this way, Tollrain (1994) used image analysis to assess the effects of chemicals released by fish predators on the morphology of *Daphnia lumholtzi*. In this paper results are presented for the reference toxicants zinc and 3,4-dichloroaniline and those for effects on growth measured using image analysis are compared with data from 48-hr immobilisation and 21-day juvenile production tests with these substances.

MATERIALS AND METHODS

Tests were performed using juvenile (<24-hr old) *Daphnia magna* which were cultured in hard (250 mg $CaCO_3 l^{-1}$) groundwater at the Medmenham laboratory. The *Daphnia* had been typed as IRCHA clone 5 and had been cultured at the laboratory since 1985.

The zinc and 3,4-dichloroaniline (3,4-DCA) test solutions were prepared on the day of the test using freshly made stock solutions. A 10 mg l^{-1} zinc stock solution was prepared by dissolving 44 mg of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) in 1 litre of groundwater in a volumetric flask. The nominal test concentrations of zinc used were 0 (Control), 100, 200, 400, and 800 µg Zn l^{-1}. A 50 mg l^{-1} 3,4-DCA stock solution was prepared by dissolving 50 mg of 3,4-DCA in 1 litre of groundwater in a volumetric flask. The solution was stirred on an autostirrer for 24 hours prior to use. The nominal test concentrations of 3,4-DCA used were 0 (Control), 10, 20, 50, and 100 µg l^{-1}.

Test concentrations of zinc and 3,4-DCA were prepared in 1 litre volumetric flasks by adding the appropriate volumes of the relevant stock solution and then diluting these with groundwater.

Ten animals were used at each test concentration and these were exposed individually to 100 ml of test solution in 150 ml Pyrex beakers. The *Daphnia* were randomly added to each vessel from the isolation vessel. The transfer of daphnids was carried out rapidly and carefully whilst minimising the stress to the organisms. Animals were transferred using a glass tube and were released under the surface of the test solution. Any organisms which were floating or injured following transfer were replaced.

On day 0 the body length of each daphnid was determined by the following procedure. Initially, the image analysis equipment was calibrated to 1 mm for the required microscope magnification using a suitable graticule. Each daphnid was then placed in a drop of water on a glass slide so that it was immobilised and lying laterally. The slide was then placed on a Leitz Ortholux 2 binocular microscope to which the Seescan image analysis equipment had been attached. The system

comprised a 0.33 inch monochrome CCD camera and standalone image processing apparatus. An image was captured and the daphnid carefully returned to the test solution as quickly as possible by immersing the slide in the test solution. The magnification at which each measurement was made was recorded and the captured image was used to measure the body length of the *Daphnia* from the point immediately above the eyespot to the base of the dorsal spine. If the magnification used to measure the daphnid was changed from the original setting, this change was noted and taken into account during subsequent data analysis.

After starting a test, the temperature, pH, and dissolved oxygen of the solutions in each preparation flask were measured. Duplicate samples from each test concentration were taken for chemical analysis after the test vessels had been filled.

The test vessels were observed each day and any anomalies in the behaviour of the *Daphnia* or mortalities were noted. The body length of each daphnid was measured on days 2, 4, and 7. Test dilutions were renewed every Monday, Wednesday, and Friday from the start of the test until day 7 (test end day). Care was taken in transferring the daphnids from old to new solutions. Temperature, pH, and dissolved oxygen of the old and new test dilutions were measured. The *Daphnia* were fed *Chorella vulgaris var. viridis* at 0.2 mg C l^{-1} daily. Immediately after measuring the length of the *Daphnia* at the end of the test, the temperature, pH, and DO in the controls and test concentrations were measured.

The resulting regression equations of length against day were compared by analysis of covariance and treatment groups different from the control in each experiment were identified by Dunnett's test (Zar 1984).

RESULTS AND DISCUSSION

The initial test on *Daphnia* neonate growth in groundwater controls established that the change in body length was linear over a 7 day period with the regression equation showing a correlation of 0.959. Table 1 shows the regression equations for length against day for this initial test and the control groups in the zinc and 3,4-DCA tests. Calculated organism length on days 0, 2, 4, and 7 for each control group are also given.

In the subsequent control tests, growth was also linear over the 7 day period and the absence of mortality in the tests indicated that organisms were not stressed by making the repeat measurements of length over time. Comparison of the regression equations for these different groups of control organisms (ANCOVA) showed that the slopes ($F=5.46$, $p<0.001$) and intercepts ($F=17.13$, $p<0.001$) were significantly different indicating that the initial length of neonates and the rate of growth of control organisms can vary between test occasions.

Table 1. Regression equations of *Daphnia magna* growth under control conditions

Control test	Calculated organism length ± SD) on given day (mm)				Regression equation (3 value)
	0	2	4	7	
1	1.04	1.60	2.16	3.02	Length = 1.042 + 0.28 Day (r^2 = 0.959)
2	1.10	1.77	2.44	3.45	Length = 1.10 + 0.335 Day (r^2 = 0.994)
3	1.05	1.61	2.17	3.01	Length = 1.05 + 0.284 Day (r^2=0.889)

Table 2 shows the regression equations (and correlation coefficients) of growth against exposure time *in Daphnia magna* exposed to zinc. Growth was not significantly affected by zinc concentrations of 100-400 µg Zn l^{-1}. However, there was significant inhibition of growth in organisms exposed to 800 µg Zn l^{-1}. No mortalities were recorded at any of the exposure concentrations. ANCOVA showed that both the slope and elevation of the regression equation for the *D. magna* exposed to 800 µg Zn l^{-1} were significantly different from those of the control indicating that the effect of zinc increased with exposure duration. This is evident from Table 3 which shows the lengths of zinc-exposed *D. magna* after 4 and 7 days estimated from the regression equations in Table 2. At the lower exposure concentrations there was evidence of slight stimulation of growth. The results for zinc are consistent with those of Berglind (1986) who showed that zinc exerted no inhibitory effects on the growth of *Daphnia magna* at concentrations up to 200 µg l^{-1} after 8 days exposure. In that study, measurements of organism length (from the top of the head to the base of the dorsal spine) were made from photographs taken through a dissection microscope.

Table 2. Regression equations of *Daphnia magna* growth during zinc exposure

Zinc level (µg l^{-1})	Regression equation	r^2 value	Signficant difference from control	
			Slope	Elevation
0	Length = 1.10 + 0.335 Day	0.959	-	-
100	Length = 1.00 + 0.362 Day	0.964	NS	NS
200	Length = 1.13 + 0.358 Day	0.961	NS	NS
400	Length = 1.16 + 0.339 Day	0.979	NS	NS
800	Length = 1.16 + 0.285 Day	0.959	p<0.01	p<0.01

Table 3. Estimated lengths of zinc-exposed *Daphnia magna* after 4 and 7 days and the % change in length from controls

Zinc level ($\mu g l^{-1}$)	Estimated length (mm) after given exposure time (% change from controls)	
	4 days	7 days
0	2.44	3.45
100	2.45 (+0.4)	3.53 (+2.3)
200	2.57 (+5.3)	3.64 (+5.5)
400	2.52 (+3.3)	3.53 (+2.3)
800	2.30 (-5.7)	3.16 (-8.4)

Table 4 shows the regression equations (and correlation coefficients) of growth against exposure time in *Daphnia magna* exposed to 3,4-DCA. In the 3,4-DCA-exposed *Daphnia* there were no significant effects on growth at toxicant concentrations of 10-100 µg l^{-1}. Mortality was ≥ 30% at 3,4-DCA concentrations of 20-100 µg l^{-1} and the 7-day LC_{50} was 100 µg l^{-1}.

Table 4. Regression equations of *Daphnia magna* growth during 3,4 dichloroaniline exposure

3,4-DCA level (µg l^{-1})	Regression equation	r^2 value	Signficant difference from control	
			Slope	Elevation
0	Length = 1.05 + 0.284 Day	0.886	-	-
10	Length = 1.05 + 0.288 Day	0.906	NS	NS
20	Length = 1.04 + 0.290 Day	0.934	NS	NS
50	Length = 1.09 + 0.235 Day	0.752	NS	NS
100	Length = 1.08 + 0.249 Day	0.888	NS	NS

Figure 1 compares the threshold concentration for effects on growth with those for reproduction (juvenile production after 21 days for zinc and 3,4-DCA in studies carried out at the WRc laboratory (WRc unpublished observations). It also provides information on the effects of these substances on reproduction (juvenile production after 7 days) in *C. dubia* and growth (7 days) in fathead minnows. Threshold effect concentrations for juvenile production and growth are the geometric means of NOEC and LOEC values.

For both zinc and 3,4-DCA, the threshold for growth effects after 7 days occurred at a higher concentration to that for juvenile production after 21 days, but a lower concentration than that causing effects on immobilisation after 48hr. The 48-hr EC_{50} values for *D. magna* immobilisation measured at WRc were 1570 µg l^{-1} for zinc and 800 µg l^{-1} for 3,4-DCA.

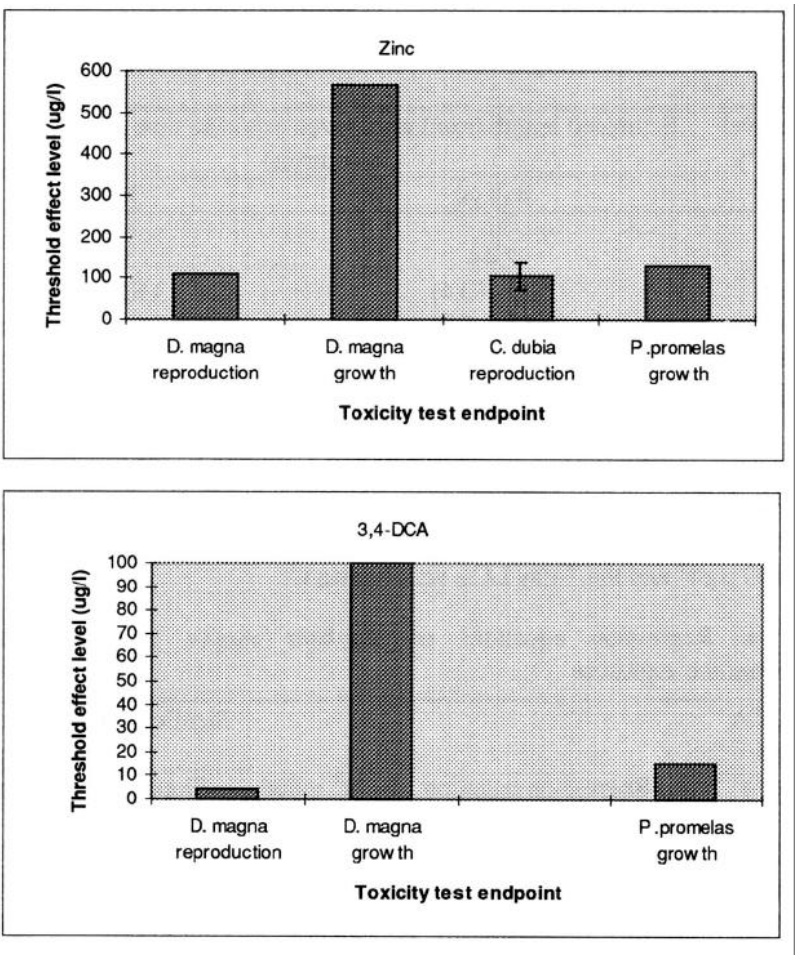

Figure 1. Summary information on threshold effect concentrations for zinc and 3,4-DCA on *D. magna* growth (7-day) and reproduction (21-day), *C. dubia* reproduction (7-day) and fathead minnow growth (7-day)

These results indicate that growth may be a useful short-term indicator of chronic toxicity. Indeed Winner (1981) showed that the body *size* of primiparous *Daphnia* magna was predictive of lifetime exposure effects of copper and zinc. The results are also slightly higher than threshold levels causing effects on reproduction in *Ceriodaphnia dubia* and growth in *Pimephales promelas.* Masters *et al.* (1991) showed that for zinc the threshold concentration for effects on C. *dubia* reproduction was 70-140 µg l^{-1}. For growth in fathead minnows, reported threshold effect concentrations are 130 µg l^{-1} for zinc (BEAK, personnel communication) and 5-26 µg l^{-1} for 3,4-DCA (Call *et al.* 1987).

Further investigations are currently being conducted at the WRc laboratory to assess responses of the technique to a wider range of substances with different modes of toxic action. Available data from the literature indicates that growth in

Daphnia sp. is impaired following exposure to sub-lethal concentrations of atrazine (Schober and Lampert 1977), carbaryl (Hanazato 1991), and dithiocarbamates and related compounds (van Leeuwen *et al.* 1985). In addition, since image analysis is a non-invasive technique the possibility of using the method in combination with other endpoints such as juvenile production is being investigated.

The test takes about 25-30 h to carry out for a control and four treatments each consisting of 10 beakers. This timeframe comprises the period from obtaining test organisms, to preparing and conducting the test and analysing the data. The test duration is comparable with the periods of 25-26 h reported by Kszos and Stewart (1991) for conducting these phases of the 7-day *C. dubia* reproduction and 7-day fathead minnow growth tests.

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