ACKNOWLEDGEMENTS

This investigation was supported by funds provided by Grant R200-W-5540 from the Department of Health, Education and Welfare, U.S. Public Health Service.

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EFFECTS OF SUB-LETHAL CONCENTRATIONS OF PHENOL ON EVENTS IN THE PRE-REPRODUCTIVE PERIOD OF THE CLADOCERAN, *DAPHNIA MAGNA*[1]

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ABSTRACT

The purpose of this investigation was to determine the effect of continuous exposure of pre-adult *Daphnia magna* to low, presumably sub-lethal doses of phenol.

The experimental data were obtained through use of a standard 24-hour toxicity bioassay and a modified long-term toxicity bioassay. Control and test animals were cultured in a synthetic pond water and fed with dried yeast. Six concentrations of phenol were tested.

Data were applied to least squares linear regression analysis, multiple linear regression analysis, crossed covariance analysis, and several related tests in order to quantitatively interpret the total effects of the chronic poisoning.

It was found that phenol exhibited strong interaction with temperature of the culture medium and with the age of the individual to retard ecdysis. This results in a prolonged generation time. Mortality was also increased and reproduction was greatly inhibited. Combinations of these effects act to greatly reduce population growth rates.

It is concluded that even minute amounts of phenol introduced into a natural environment may have deleterious effects on the ecological balance of an ecosystem.

[1]The funds for this research were received from the Federal Water Pollution Control Administration, Grant WP-00927-03.

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INTRODUCTION

With a wealth of biological data present pertaining to daphnids, it is reasonable that they would be used as indicators in biological tests. Their small size, rapid reproduction, and the ease with which they are cultured make them ideal for testing toxicities of various water-borne substances. In ascertaining the normal reactions of daphnids the most comprehensive data available concerning events in *D. magna* life history are those found in Anderson and Jenkins (1942). Their data was based on hourly observations of clones.

Numerous workers have tested daphnids under both normal and stressed conditions. Belleuvre (1938) worked on physiological reactions to pyrethrins. Blaska tested *D. magna* and *D. pulex* reactions to metal salts. Sanders and Cope (1966) tested *D. pulex* reactions to several pesticides at different temperatures. They reported a general decrease in toxicity with a temperature increase. Anderson (1945) reported an IC_{50} (median immobilization concentration) of 1.0 ppb (parts per billion) for DDT. In another paper (1960), he found EPN to be more toxic than DDT, parathion, malathion, and chlorobenzilate.

Several workers have utilized daphnids in toxicity tests of herbicides. Crosby and Tucker (1966) reported on the acute toxicity of several herbicides to *D. magna*. Like Anderson (1945), they derived an IC_{50} for the toxicants. Utilizing 26-hour studies they found that such herbicides as Dichlone, Molinate, Propanil, sodium arsenite, and Dichlobenil are usually applied at concentrations higher than those lethal to *Daphnia*. However, other herbicides such as 2,4D were completely innocuous. Their findings also suggested that tests on mammals and those on *Daphnia* were not comparable and neither held potential as a predictor of harm for the other variety of animal. Findley (dissertation, 1969) subjected *D. magna* to sub-lethal concentrations of amino triazole weed-killer. She observed that older animals were more resistant than immatures and that doses of 2 and 3.5 ppm (parts per million) effect their ability to lay eggs.

Phenol, a common component of oil refinery effluents, gashouse wastes, and some pulp mill wastes, has not been widely studied with regards to its effects on daphnids. Three papers mention it directly. Adams (1927), while testing bactericides in Nile River water, found that phenol and cresols were lethal to *Daphnia* sp. and *Cyclops* sp. at 10 ppm. Ellis (1937) has a good review of the available literature on toxicants to fish and *Daphnia*. In his own findings he reported that 8 ppm phenol killed *D. magna* in soft water. Anderson (1944) attempted to find toxicity thresholds of various substances using *D. magna* as an indicator. Using immobility as the end point and centrifuged Lake Erie water as the diluent, he reported the toxicity threshold of phenol to be 0.001 mole or 94 ppm.

The probable reason for discrepancies between the various phenol concentrations found to be critical to daphnids lies in the composition of the diluent. In an effort to make toxicity tests more standard Freeman (1953) developed a synthetic test medium he called "Standard Reference Water". Frear and Boyd (1967) simplified Freeman's formula so that only three chemical compounds were needed to synthesize it.

Even though such advances as a standard diluent have been developed to improve bioassay work, experiments concerning toxic materials have sorely ignored the long-term effects of small doses. Most criteria have been based on acute reactions. Recent papers on chronic reactions in invertebrates are very scarce.

Set forth in the following paper is a series of experiments designed to test certain aspects of long-term exposure to phenol. Effects of low, sub-lethal concentrations of phenol on *Daphnia magna* Straus were ascertained using a modified long-term bioassay. Results were subjected to several statistical tests in order to evaluate more fully the effects of the phenol under several conditions.

MATERIALS AND METHODS

Daphnia magna were mass cultured in gallon jars containing a synthetic pond water (Frear and Boyd, 1967). The culture jars were placed at room temperature (approximately 24 C), and small increments of Fleishmann's dry active yeast were added daily to provide food. Waste was periodically removed with a pipette.

Once every two or three weeks the daphnids were removed from the jar and rinsed three to five times in clean water in a device similar to that used by Frear and Boyd (1967). Cultures were generally maintained with good success as long as such a routine was followed.

The toxicant used was non-substituted phenol. A stock solution was made from analytical grade, crystalline phenol dissolved in distilled, demineralized water. Approximately 100 grams of the water-laden phenol were dissolved in a liter of distilled, demineralized water and the solution was standardized according to the method given in Standard Methods (1960). This resulted in a stock with a concentration of 12297.82 milligrams of phenol per liter. The solution was then stored in a brown glass bottle. Subsequent standardization of this solution showed no change in concentration. The phenol stock was then added in appropriate amounts to the synthesized medium to give desired concentrations.

In order to establish a range of sub-lethal doses suitable for the planned tests, it was decided to first use a preliminary short-term test. A pilot bioassay was used to set the upper concentration limit and give indications of the possible range of concentrations. A 24-hour study was chosen to establish the median tolerance limit (TL_m) and two such tests were run. These tests were conducted at a temperature of 20 C in a constantly lighted incubator. The animals received no food during the tests. Results of the two 24-hour tests were combined in the end analysis. Immatures were selected as test animals because the planned long-term tests were to be concerned primarily with the immature stages. A large number of animals were placed at 20 C overnight for temperature conditioning. Then ten *Daphnia* were randomly selected and put into jars containing 30ml of test medium. Seven concentrations were used in the tests, and controls containing no phenol were run simultaneously. The final sequence was as follows: 12.7 ppm, 25.4 ppm, 63.5 ppm, 76 ppm, 88 ppm, 101 ppm, and 127 ppm. Two replicates of each concentration were used in each of the tests. The total number of animals held at each concentration was 40, combining all four replicates.

The results of the TL_m test were extrapolated from the low end of the curve until mortality theoretically equalled 2% for a 24-hour period. This point was then used as the mid-point of a series of six concentrations ranging from 0.05 ppm to 1.54 ppm.

In the long-term tests each bottle contained 30 milliliters of test solution, and one newborn female. The same medium and phenol stock were used to make the dilutions as were used in the preliminary tests. The concentrations were arranged as follows: 0.05 ppm, 0.10 ppm, 0.19 ppm, 0.38 ppm, 0.77 ppm, 1.54 ppm, and a control.

Several replications were started at each concentration. One series of tests was run at 20 C and another at 25 C. Because of limited space available within the incubators and the length of time required to make observations on each individual, the number of replicates that could be run simultaneously was limited.

In total, 119 individuals were observed for varying lengths of time. Each individual was, at most, two hours old when first isolated and had been hatched at the temperature in which it was used. Fifty-nine individuals were kept at 20 C and 60 individuals were kept at 25 C. The test individuals were watched during their pre-adult instars through the first adult instar.

Previous experimentation with *Daphnia pulex* was used as a guideline in establishing a schedule for observing the test animals. These prior experiments had shown greater variability than is reported in the literature. Apparently, the reported lack of variability has been due to the time span between observations that previous workers have employed. An extensive time between observations tends to mask variability. During the experiments with *D. pulex* and some preliminary experiments with *D. magna* the animals were observed regularly every two hours for lengthy periods of time. Lags between hatching of a brood of eggs and subsequent molting of the parent often occurred. Also, laying of a brood of eggs after the parent molted was noticeably delayed at times. These events are easily overlooked if a typical schedule of observing the animals every 24 hours is employed.

Because observations on a two-hour schedule were difficult to maintain, observations every four hours were scheduled and, with few exceptions, were successfully carried out for a period of 548 hours. This schedule allowed for detection of the variability discussed above.

Observations made on each individual every four hours were: age in hours from hatch; molting during previous interval; eggs laid, eggs lost, eggs hatched during previous interval; color changes; physical abnormalities; and death. Exuviae were removed with a pipette. Newly hatched young were removed and counted.

RESULTS

Preliminary Bioassay

The results of the preliminary bioassay are given in Table 1. The original data were corrected to compensate for death in the controls. The median tolerance limit was determined graphically by picking the point on the corrected line which represented response by 50% of the animals. This median occurred at a concentration of 83.5 ppm. Extrapolation of the high-survival section of the tolerance curve was used to establish an arbitary range of concentrations which would be more or less below lethal doses.

Table 1. Results of Preliminary Bioassay

Concentration	Percent Survival	
	Uncorrected	Corrected to 100% Survival in Controls
0.00	92.50	100.00
12.70	82.50	90.00
25.40	80.00	87.50
63.50	60.00	67.50
76.20	47.50	55.00
88.90	35.00	42.50
101.60	32.50	40.00
127.00	17.50	25.00

Long-term Bioassay

In the long-term, low-mortality tests, three independent variables were recognized. These variables were as follows: concentration of toxicant, temperature, and age, by instar. The dependent variable was the length of time spent in an instar, measured in hours.

Linear Regression Analysis

A line was fitted by least squares regression for each temperature-concentration combination with age as the independent variable. Twelve combinations were fitted. Results of the twelve analyses are given in Table 2. The regression coefficients were tested for significant deviation from zero by one-way analysis of variance. Results of these tests are given in Table 3.

Multiple Regression Analysis

The variables were analyzed simultaneously to establish the relationship of the three independent variables to the dependent variable. The test was run by a computer using a stepwise multiple regression program.

Using the results of this test, the independent variables were ranked in decreasing order according to their partial regression coefficients. Signs indicate whether a direct or inverse relationship exists and have nothing to do with the absolute magnitude of the number used for ranking the variables. The multiple regression equation was:

$E(Y) = 39.27 - 6.65 (X_1 - 22.65) + 4.08 (X_2 - 2.95) + 1.40 (X_3 - 0.192)$ where X_1 is temperature, X_2 is age, and X_3 is concentration level.

An analysis of variance test was run on the partial regression coefficients and all were significantly different from zero at the 1% significance level.

Table 2. Results of Analyzing Data by Least Squares Method of Regression.

Temperature-Concentration	Regression Equation
(20 C, 0.00 ppm)	$Y = 38.87 + 2.02 (X_i - 2.84)$
(20 C, 0.05 ppm)	$Y = 38.31 + 2.68 (X_i - 2.19)$
(20 C, 0.10 ppm)	$Y = 41.50 + 3.49 (X_i - 2.90)$
(20 C, 0.19 ppm)	$Y = 49.02 + 4.18 (X_i - 3.02)$
(20 C, 0.38 ppm)	$Y = 44.13 + 7.11 (X_i - 3.26)$
(20 C, 0.77 ppm)	$Y = 45.96 + 11.27 (X_i - 2.61)$
(25 C, 0.00 ppm)	$Y = 30.52 + 0.10 (X_i - 2.70)$
(25 C, 0.05 ppm)	$Y = 41.34 + 6.37 (X_i - 3.78)$
(25 C, 0.10 ppm)	$Y = 39.54 + 5.34 (X_i - 3.32)$
(25 C, 0.19 ppm)	$Y = 39.46 + 6.04 (X_i - 2.92)$
(25 C, 0.38 ppm)	$Y = 40.33 + 4.36 (X_i - 3.52)$
(25 C, 0.77 ppm)	$Y = 35.00 + 1.19 (X_i - 2.31)$

Crossed Covariance Analysis

After establishing the degree of effect of each variable, a crossed covariance analysis was applied to the data. This test allowed simultaneous analysis of the effects of temperature and concentration on instar length, taking age into account as a concomitant variable. The analysis of covariance results are shown in Table 4.

Additional tests were run on the covariance data. The first analysis established that all of the concentration lines were not representative of a common line. The same analysis applied to the temperature lines showed they also did not represent a common line. Both test results were highly significant. The second analysis established that not all of the concentration lines were parallel. The same analysis showed that the temperature lines were parallel.

Table 3. One-way Analysis of Variance of Regression Coefficients for Individual Temperature-Concentration Combinations.

Temperature-Concentration	Sums of Squares Due to Regression	df	Error Sums of Squares	df	Calculated F Value
(20 C, 0.00 ppm)	273.08	1	3442.27	35	2.78
(20 C, 0.05 ppm)	118.08	1	1433.36	14	1.15
(20 C, 0.10 ppm)	1165.66	1	6680.34	38	6.63*
(20 C, 0.19 ppm)	1763.88	1	12183.10	39	5.65*
(20 C, 0.38 ppm)	5656.46	1	10231.45	44	23.22*
(20 C, 0.77 ppm)	4270.21	1	19856.75	26	5.59*
(25 C, 0.00 ppm)	1.98	1	23269.48	104	0.01
(25 C, 0.05 ppm)	5009.43	1	7837.79	30	19.14*
(25 C, 0.10 ppm)	2082.35	1	7208.61	26	7.51*
(25 C, 0.19 ppm)	1672.60	1	9545.36	22	3.86
(25 C, 0.38 ppm)	1832.79	1	4421.51	25	10.36*
(25 C, 0.77 ppm)	32.02	1	739.98	11	0.56

*Significant $P<0.05$

Table 4. Crossed Analysis of Covariance Results.

Source	Sums of Squares	df	Mean Squares	Calculated F Value
Concentration	13328.52	10	1332.85	26.27*
Temperature	9082.94	2	4541.47	89.52*
Interaction	84404.21	10	8440.42	166.38*
Error	21001.83	414	50.73	
Total	127817.50	436		

*Significant $P<0.01$

Analysis of Egg Duration

The length of time an individual spends in the egg stage can have a drastic effect on the dynamics of a population. Edmondson (1960, 1968) gave relationships between egg duration and birth and growth rates. In the long-term tests, animals which carried eggs and hatched them during the experiment were carefully watched. A comparison was made between egg duration for controls and egg duration for various concentrations at 20 C.

Apparently, phenol does not permeate the shell as no disturbance of the eggs seemed to take place. However, egg mortality was high and a large number of eggs failed to hatch or were lost before hatching. This was probably indicative of physiological imbalance in the female laying the egg rather than a direct effect on the egg itself.

DISCUSSION

In assuring ourselves that we were working with a relatively normal population of *D. magna*, comparisons were made between our 25 C control populations and the populations followed by Anderson and Jenkins (1942). A comparison can be found in Table 5. As can be seen, only slight disparity existed between the populations and this lay more in the standard deviations. Most likely this a result of comparing mixed populations with clonal populations. In addition our populations were smaller than the Anderson and Jenkins populations and this added to the disparity between the standard errors.

It was concluded that the number and length of pre-adult instars for the control populations under discussion were similar to other laboratory-reared populations and that the population could be considered normal. Therefore, reactions of the population to the toxicant could be attributed to effects of the phenol and not necessarily to abnormalities of the population.

Table 5. Comparison of Duration of Instars for Anderson and Jenkins Population* and Cox and Bunting Control Population.

	Mean length and standard deviation of instars in hours for animals primiparous in			
	Fifth Instar		Sixth Instar	
Instar	Anderson and Jenkins	Cox and Bunting	Anderson and Jenkins	Cox and Bunting
1	20.3 ± 0.2	25.1 ± 1.4	20.4 ± 0.2	25.8 ± 2.0
2	21.3 ± 0.1	27.3 ± 8.2	21.3 ± 0.2	25.0 ± 2.7
3	24.8 ± 0.1	29.2 ± 7.6	24.1 ± 0.2	23.8 ± 1.7
4	32.5 ± 0.2	39.1 ± 9.0	25.5 ± 0.1	19.8 ± 6.2
5	50.1 ± 0.3	50.9 ± 16.2	32.5 ± 0.4	20.5 ± 5.4
6	–	–	50.8 ± 0.4	63.5 ± 19.8

*Data taken from Anderson and Jenkins, 1942.

In general, *D. magna* showed greatly increased mortality, inhibited reproductive ability, and a lengthened pre-reproductive period when subjected to long-term exposure of phenol.

Based on the preliminary bioassay, mortality in the long term studies should not have exceeded 2% of the population. However, mortality was such that no individuals at 1.54 ppm survived past the third instar. The major mortality began to be expressed after the completion of the third instar at all concentrations of toxicant. The sudden increase in mortality beginning with the fourth instar could have two possible explanations.

First, the young used in the tests were taken from mothers raised under normal conditions. Fixation of certain genetic characters prior to eggs being laid has been demonstrated in *D. magna*. This factor may have been at work in the test animals. Three instars could possibly have been required before the effects of the phenol overrode genetically influenced molting times.

A second explanation, which we prefer, involves the probable mode of phenolic action which affects *D. magna*. Visual observation of test animals showed most mortality due to an inability to shed the previous exuvium. This observation suggests that phenol interferes with separation of old and new cuticle layers. Therefore, the surface area of the animal could be critical concerning the ease with which the carapace is shed. The first three instars may have a small enough surface area to overcome the disturbing effects of phenol, but by the beginning of the fourth instar a critical area had been reached, causing difficulty in molting. Early exuviae were usually complete, where later exuviae were ragged or in pieces. Thus age became an important factor when phenol was present. Mortality continued to climb with aging as well as increased concentration.

Phenol in low concentrations clearly inhibited reproduction and retarded the onset of reproduction in some of those that succeeded in bearing broods. It was noted that all but one of the controls at 20 C had successfully borne at least one brood of young by the sixth instar while only 9 of 52 animals exposed to phenol had successfully borne a brood by the sixth instar. Two other test animals succeeded in hatching a brood in the eighth instar. The same effect held true at 25 C. However, only 13 of 26 controls and 1 of 33 test animals hatched broods by the sixth instar. Three other test animals hatched broods during the seventh instar.

The lengthening of the pre-reproductive period occurred as a result of the increase of time spent in each succeeding instar when influenced by phenol. Controls did not show such an increase in instars with age. This instar lengthening

most likely stems from the difficulty in molting already discussed in relation to mortality. The fact that in most cases the lengthening of instars did not occur until the onset of the fourth instar lends support to this theory.

Under the influence of phenol it can be seen that age is an important factor, but temperature appears to have even more effect. Increased temperature caused phenol to have a more drastic effect on mortality. Higher temperatures also caused higher variability. The length of each succeeding instar increased at both temperatures, when taking all concentrations into account. The rate of increase was the same for both temperatures. The average length of each instar was longer at 20 C than at 25 C.

The three primary effects of phenol on *D. magna*, when interacting, serve to drastically reduce the birth rate and also the population growth rate. This serves to produce a population that is smaller than normal at any given time. Since daphnids may be a very important link in food chains, the presence of small quantities of phenol in the environment may have far-reaching effects. If one followed the dictates of a short-term bioassay, the entire population could conceivably be lost over a period of days.

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