THE PHOTOTACTIC BEHAVIOR OF *DAPHNIA MAGNA* AS AN INDICATOR OF CHRONIC TOXICITY

Linda J. Whitman* and Rudolph J. Miller

Department of Zoology, Oklahoma State University, Stillwater, Oklahoma 74078

The objective of this study was to develop a chronic bioassay technique to rapidly screen chemicals for potential toxic effects. The phototactic behavior of *Daphnia magna* was used for that purpose. Negative phototaxis was elicited by switching the direction of light imposed on a population of 20 adult *Daphnia.* The percent population that responded by swimming a marked vertical distance in 30 seconds was recorded. At least 5 replicates were used for each experiment or treatment, and the results presented as the mean response (%) of the 5 replicates. A number of variables influencing phototactic behavior were explored, including light intensity, circadian rhythms, annual rhythms, food deprivation, culture techniques, and temperature. Finally, the effects of naphthalene on phototactic behavior was determined. The present report deals with light intensity and temperature effects, and the effects of exposure to three concentrations of naphthalene.

At a light intensity of at least 250 foot-candles, the mean phototactic response of healthy *Daphnia* populations was at least 75% in most of the experiments. Of the variables explored, the effects of experimental water temperature had a dominating influence over phototactic behavior. Naphthalene completely inhibited phototactic responses at a concentration of 2.0 mg/*l* and phototaxis was significantly depressed at 1.5 mg/*l* and 1.0 mg /*l*.

INTRODUCTION

A multitude of bioassay techniques have been developed in the past few decades in response to a growing concern that too little is known about the effects of chemicals and wastewaters before they are released into natural aquatic systems. The task of assessing the effects of toxic substances involves the monitoring of complex biological systems, at both the individual and community level. The realization that no single method can fully evaluate how a substance affects the survival and normal functions of the aquatic biota has resulted in the development of a series of protocols designed to institute a systematic approach to the problem.

At the level of the organism, toxicity testing has developed along two major lines: (a) the lethal, or acute bioassay (1); and (b) the sublethal, or chronic bioassay (2). While the methodology for the former is now highly standardized (3, 4) and widely applied, the chronic bioassay is yet in the developmental stages. The reasons are readily apparent. The acute bioassay deals with but one variable, survival. It is a short-term test, usually one to four days in duration, during which the organisms are not fed. Experimental variations caused by changes in water quality or competitive interactions of organisms are thus kept to a minimum. The chronic bioassay, on the other hand, can involve any of a number of variables dealing with the ability of the organism to carry on its normal functions; e.g. reproduction (5, 6, 7), growth (5, 8), respiration (9, 10), activity (11, 12), and behavior (13). One inherent difficulty of the chronic bioassay is that of defining and quantifying "normality" and relating departure from the norm to the ecological impact on the organisms (2). In addition, many of the chronic bioassays require containment and monitoring of the organisms over extended periods of time, and therefore necessitate feeding of the organisms and careful monitoring and control of water chemistry, which places a greater demand on available time. Such complications have impeded development of chronic bioassays into routine biological monitoring systems, and the need for continued improvement and standardization of the techniques remains prominent (2, 14-16). "Safe" concentrations of wastes must be determined to insure not only survival, but also growth, reproduction, and the general well-being of a species under conditions of continuous exposure (17).

The current demand in biological moni-

*Present address: 5017 Alcova Rt., Box 30, Casper, Wyoming, 82601
Proc. Okla. Acad. Sci. 62:22-33 (1982)

toring is for quantifiable, short-term bioassays that can effectively predict long-term effects of sublethal toxication (18). To meet these requirements, a behavioral bioassay was devised for the following research based on a number of qualifying factors. Sprague (1) suggested that behavioral responses may be even more sensitive than survival, growth, and reproduction, and thus of over-riding importance to species continuity. Warner (19) recognized the relevance of behavior to toxicological studies because (a) the behavior of animals is likely to have distinct survival value and thus any impairment in the behavioral repertoire is likely to be deleterious, (b) behavior involves an integration of many biochemical and physiological processes and thus may provide a more comprehensive measure of effects than a single biochemical or physiological parameter, and (c) behavioral patterns appear to be very sensitive to changes in environmental quality.

Sherer (13) recommended the use of locomotor behavioral patterns as the most suitable for assaying toxicological effects. Activities such as swimming performance, spatial selection (such as preference-avoidance reactions), and circadian and seasonal rhythmicity can be objectively described and quantified. The circadian patterns of zooplankton migrations are particularly well suited for laboratory investigations. Representative members of the migratory zooplankton, such as *Daphnia,* are easily cultured in the laboratory, have proven to be highly sensitive to toxicants, and are commonly employed for the biological assay of many substances (20-29), providing a frame of reference for sublethal exposures. Because the vertical migrations of zooplankton are cued primarily by light (30-34) it is not difficult to simulate the pattern in the laboratory (30). The phototactic response itself is relatively easy to stimulate and measure in terms of distance traveled over a specified period of time (33).

Finally, the relevance of vertical migrations to the survival of zooplankton populations is easily accounted for. That it is a worldwide phenomenon with a diverse array of participating species is well-documented (34), and suggests that the behavior evolved as a result of strong selective pressures. Defining these pressures has been the topic of some debate. The avoidance of physiologically adverse radiation (35, 36), predator avoidance and niche diversification (37), and increased feeding, growth, and reproductive efficiencies (7, 38-40) have all been suggested as possible evolutionary mechanisms. Hutchinson (34) was more inclined to believe that vertical migrations were initially an expression of photic sensitivity which acquired additional significance because of advantages derived from other mechanisms such as those listed above.

In summary, the phototactic behavior of *Daphnia magna* was selected for a bioassay because it is amenable to laboratory investigations, it provides a rapid and quantifiable response, and it is the primary mechanism of the vertical migrations essential to the ecology of the species. The objectives of the research were (a) to define and quantify the normal, or average, phototactic response of *Daphnia magna* to a light stimulus, and (b) to determine the effects of naphthalene, one of the more toxic constituents of petroleum refinery effluents and coal tar, on the response defined.

MATERIALS AND METHODS

Culture Methods

Daphnia magna used in this project were from cultures maintained in the laboratory for approximately one year prior to the beginning of this research project. The cultures were kept in large wooden and glass tanks which contained about 160 *l* of tap water aerated with one air stone in each tank. The water level was maintained by replenishing it weekly with aerated tap water from a reserve tank. Brewer's Yeast, (Plus Products of Irvine, CA, Formula 250) was sprinkled on the water surface whenever the culture medium appeared dilute (determined by clarity of the water) and was the only source of food provided. Bacterial and algal growth in the culture tanks served as additional sources of food. Densities of *Daphnia* fluctuated widely in the tanks, but in general this method provided substantial populations.

Development of Technique

Preliminary observations of *D. magna* in a variety of glass vessels indicated that movements toward or away from a given light source were erratic and difficult to define

unless all extraneous sources of light were eliminated. Therefore, preliminary experiments were conducted in a completely dark room, with only a single source of light, or in a black box in which the lighting could be controlled. Only adult animals were used in the following investigations for several reasons: (a) neonates are known to exhibit phototactic responses different from those of adults (32): (b) personal observations of neonates indicated that there is more individual variation in their responses than for adults; and (c) adults are easier to observe by virtue of their larger size.

Several procedures for observing phototaxis were tried before a final method which met the requirements for a bioassay was developed. With the final procedure, described below, *Daphnia* could be made to swim repeatedly up and down a clear column of water in response to changing the direction of the light, though some fatigueing was evident if the stimulus was repeated in rapid succession. The negative phototactic response elicited in this manner became the basis for the bioassay because it induced a response from a large majority of a population of *Daphnia*, and could be easily quantified in terms of time, distance traveled, and the number of animals responding. The final methodology permitted a single observer to run a complete bioassay within a single day with inexpensive and readily available equipment.

Description of Apparatus

A large box, 1.2 × 0.3 × 0.3 m (Fig. 1), made of 1.3-cm plywood and painted flat black on the inside, was used as an observation chamber. Two shelves with 25-mm-diameter holes drilled in the centers were placed in the center of the box, spaced approximately 190 mm apart so that a 200-mm test tube suspended from the upper shelf extended just down to the hole in the lower shelf. These shelves served to direct light into the tube while blocking any rays outside the diameter of the tube. Two more shelves supported lamps (30-W indoor flood lamps) positioned 25 cm above and 25 cm below the center shelves. The lamps were connected by a switch box which simultaneously turned one off and the other on when a single switch was flipped. Midway between the lamps and center shelves, 0.7-cm plate glass was placed to reduce heat convection to the tube.

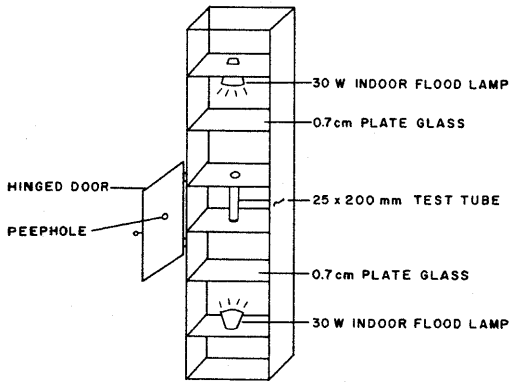

FIGURE 1. Black box for observing phototactic response of *Daphnia* in a test tube.

Pyrex test tubes, suspended by the lips of the tubes in the center of the box, served as test vessels for the *Daphnia*. A hinged door on the front of the box allowed easy placement and removal of the test tubes. Observations were made through a 2.6-cm-diameter peephole in the door.

Experimental Procedures

Part I: Non-exposure Experiments

The following general procedures were used for all experiments. Modifications for specific purposes are noted in the appropriate following sections.

Daphnia from large culture tanks were siphoned into a 1-*l* beaker, care being taken to keep the siphon under water so as not to expose the animals to air.

Twenty mature *Daphnia* (based on size) were pipetted from the beaker into 25-mm × 200-mm test tubes containing approximately 70 m*l* of aged tap water (from a reserve tank also used to replenish culture water), at approximately the same temperature.

A pre-test acclimation period of 2 h was used to overcome any physiological disturbances caused in transferring the animals, and to allow time for adjustment to the test tube environment (also to retain consistency with naphthalene experiments, which required a 2-h exposure period for maximum uptake by the *Daphnia* (41). During the acclimation period, the test tubes were left uncovered at room temperature and under normal laboratory lighting (fluorescent lights, approximately 125 foot candles at the level of the test tubes).

At the end of the acclimation period each test tube population was individually observed in the black box. A 2-min pre-stimulus period was used for each tube in which the animals were exposed to approximately 350 foot candles of light at the surface of the test tube from the overhead light. Response time was defined as 30 s following the switch in direction of the light stimulus; i.e. when the overhead light was turned off and the light underneath turned on simultaneously with the start of the clock.

The response criterion was defined as the percent of a test tube population migrating 9 cm vertically up the tube in 30 s. The total response for an experiment was the mean percent response of 5 test tube populations (a total of 100 *Daphnia*).

Individual *Daphnia* were used only once and discarded. If an animal was already above the 9-cm mark on the tube before the initiation of the stimulus, it was not counted as a responder. Frequently *Daphnia* were found floating on the surface of the water during the acclimation period as if trapped in the surface layer. The presence of floaters is usually attributed to air trapped underneath the carapace as a result of exposure to air. However, the problem was seemingly unavoidable, even with the most careful handling techniques, and varied daily from 0% to 80% using the same handling techniques performed by the same person. Prior to each test, floaters were driven down from the surface by gently directing a stream of water through an eye dropper onto the surface of the water. Generally this was sufficient to release the animals from the surface layer, and usually they swam directly downward as the tube was placed in the experimental box with a bright overhead light.

Non-exposure experiments were conducted in two phases. Phase I experiments were performed during the developmental stages of the research to establish a normal range of responses for non-exposed *Daphnia*. Experiments were performed to determine the appropriate light intensity required for maximum response, and to determine whether a circadian rhythm of phototactic responses exists or affects the results. Phase II experiments were performed during the latter months of the research in an attempt to identify the factors that caused an interruption of the phototactic patterns established during the development of the technique. These experiments investigated the role of intrinsic annual rhythms, effects of food deprivation, effects of culture technique and use of specific age classes, and effects of temperature on phototactic responses. This paper deals with the effects of light intensity and temperature; other parameters will be discussed elsewhere and are available in Whitman (42).

Experiment A: Effects of light intensity on phototaxis: To determine the light intensity required to produce the maximum response level, a series of experiments were performed using intensities of 15, 50, 150, 250, 500 and 600 foot-candles. Five replicates (test tubes with 20 *Daphnia* each) were used at each intensity. The experiment was repeated a second time using intensities of 50, 100, 250 and 500 foot-candles. Results were analyzed using Duncan's multiple-range test for significance with an observed significance level (OSL) of 0.05 (43).

Experiment B: Effects of temperature. Temperature effects were determined in four stages. First, a regression analysis of experimental temperatures vs. response rates was performed using data from all control experiments for which temperatures were recorded. The determination of a significant correlation between phototactic responses and temperature led to three subsequent sets of experiments in which temperatures were manipulated to maximize responses.

The first approach was to test the effects of manipulating experimental temperatures only (as opposed to culture water temperatures or both). *Daphnia* were placed in test tubes at the same temperature as the culture water from which they were obtained. One set of five test tubes was incubated in a cold water bath at approximately 15 C, another set in an incubator at 25 C, and two other sets (controls) at room temperature, which ranged between 20 C and 22 C. The incubation period was 2 hr. The experiment was repeated on three different days.

The second approach was to test the effects of manipulating culture water temperatures. An aquarium heater was placed in one of the large culture tanks (Culture A) to raise the water temperature to about 25 C. The animals were allowed to acclimate

to the increase for one week. Culture B was maintained as usual at room temperature (culture water temperature was 20.5 C). The phototactic responses of the two cultures were then compared using the normal procedures with 10 replicates (200 *Daphnia*) per treatment. Experimental temperature was 22.5 C.

Finally, both experimental and culture water temperatures were controlled for culture A animals, while culture B animals were maintained at room temperature. Culture A *Daphnia* came from a heated aquarium and were placed in test tubes containing experimental water at, or slightly above the culture water temperature. These test tubes were then incubated in a water bath to maintain that temperature throughout the 2-hr pre-test period. Culture temperatures for the experimental group (Culture A) ranged from 25.1 C to 25.8 C, and experimental temperatures were between 25.1 C and 26 C. In the control group (Culture B) culture temperature was 21 C and experimental temperature was 22.2 C. Experiments were performed on three different days using 10 replicates per treatment.

Part II: Naphthalene Bioassays

Naphthalene experiments were conducted using the basic format outlined in Part I. *Daphnia* were exposed to various concentrations of naphthalene in the test tubes during the 2-hr pre-test period, the time required for maximum uptake of naphthalene (37). A fresh stock solution was made each day that a naphthalene bioassay was conducted. Two hundred milligrams of scintillation grade, 99+% naphthalene (Aldrich Chemical Company) were dissolved in 100 m*l* of 95% ethanol, and an appropriate aliquot was diluted in aged tap water to make nominal concentrations of 0.5, 1.0, 1.5, and 2.0 mg/*l* naphthalene. Each naphthalene solution was then poured into 5 test tubes, and 20 *Daphnia* were placed in each test tube immediately following. Five test tubes containing aged tap water only (controls) were run simultaneously with each naphthalene bioassay. Ethanol controls, containing the maximum quantity of ethanol that would be present in any of the naphthalene solutions, were also run several times to eliminate the possibility that phototactic responses were adversely affected by ethanol.

The final two bioassays (June 30 and July 1, 1981) were conducted in 38-mm × 200-mm glass cylinders to facilitate the analysis of dissolved oxygen (D.O.). A YSI B.O.D. oxygen probe equipped with a stirrer could be placed directly in these cylinders for rapid and accurate D.O. readings immediately following the bioassay. Because the cylinders had a greater volume than the test tubes, only four were used for each treatment, with 25 *Daphnia* in each one, bringing the total number of observations *(Daphnia)* per treatment to 100, the same as that used in the test tube experiments. The slight increase in populations per vessel did not seem to impair the observer's accuracy in counting responses.

The concentration of naphthalene remaining in the test tubes (or cylinders) after a bioassay was completed was determined by fluorescence analysis (Aminco-Bowman spectrophotofluorimeter with ellipsoidal condensing system) for all of the 1981 experiments. Data analysis, however, was based on initial concentrations because they were accurately known, and a discussion of naphthalene losses during the course of the experiments is included in a following section.

Statistical differences between controls and the various treatments were determined for each bioassay using Dunnett's procedure (43) with an OSL ≤ 0.05. In the case where only one treatment was compared to the control (July 10, 1980), Student's *t*-test was performed. A regression analysis of treatment effects was also done for both 1980 and 1981 experiments.

RESULTS AND DISCUSSION

Part I: Non-exposure Experiments

During the first phase of the non-exposure research, June through September, 1980, thirty-eight control experiments were performed (Table 1) within the basic format of the procedures described. Mean responses varied between 61% and 99% with standard deviations between 1.98 and 16.05 (with the exception of one experiment on August 15 with a S.D. of 29.31).

A mean response (0) of 75% or greater was achieved in 80% of the experiments. Most experiments had a standard deviation (S.D.) of less than 10.00 (Table I). These figures, $0 \geq 75\%$ and S.D. ≤ 10.00, were

TABLE 1. *Mean response ($\bar{x}$) and standard deviation (S.D.) in non-exposure phototaxis experiments in phase I research, June — September, 1980.*

Date	$\bar{x}$	S.D.
June 25	84%	4.18
	69	13.18
26	72	2.74
	76	11.94
27	78	5.70
	78	9.08
30	86	6.52
	82	10.37
July 1	83	4.83
2	90	9.35
	82	12.70
10	75	11.70
	64	4.13
11	68	16.00
12	80	14.46
14	71	9.62
15	77	7.78
16	66	7.42
30	79	13.42
	85	11.73
Aug. 12	93	8.37
	88	9.75
13	90	7.91
15	98	2.82
	98	2.94
	82	29.31
	82	4.49
	83	16.05
	99	2.13
	96	1.98
27	82	10.37
Sept. 15	82	4.53
	83	16.05
18	95	5.13
	97	4.47
	93	5.70
29	61	7.60
	75	14.08

TABLE 2. *Mean response ($\bar{x}$) and standard deviation (S.D.) in phototaxis experiments using various light intensities.*

	Light intensity (foot-candles)					
	15	50	150	250	500	600
$\bar{x}$	54.6	57.4	72.6	94.9	97.0	93.0
S.D.	11.76	9.94	12.50	5.13	4.47	5.70

TABLE 3. *Mean response ($\bar{x}$) and standard deviation (S.D.) in phototaxis experiments using various light intensities.*

	Light intensity (foot-candles)			
	50	100	250	500
$\bar{x}$	29.2	21.0	61.4	74.6
S.D.	9.91	11.40	7.60	14.08

considered to represent the expected normal response of non-exposed animals.

Experiment A: Effects of Light Intensity on Phototaxis

The results of experiments in which different light intensities were used on different test tube populations (five replicates at each intensity) showed that the maximum response levels were obtained at 250 foot-candles or greater (Tables 2 and 3), though absolute response levels varied considerably in the two experiments. Below 250 foot-candles responses to the light stimulus were significantly depressed. For the bioassays, therefore, it is desirable to use a light source that generates greater than 250 foot-candles. This intensity maximizes the responses of non-exposed animals, and provides for a greater range of differentiation between the responses of control animals and those that are potentially impaired as a result of exposure to toxicants.

Experiment B: Effects of Temperature

Phototactic responses unexpectedly declined and reversed as the year progressed, particularly from the fall to winter months. A regression analysis of the data collected over the year demonstrated that mean response rates were strongly correlated to changes in culture water temperature (Fig. 2, R = 0.88). A similar relationship was found for experimental temperatures (room temperature) and mean responses (R = 0.84), which would be expected because culture temperatures were consistently 1 to 2 C lower than room temperature owing to the cooling effects of aeration. Culture temperatures throughout the year varied between 18 C and 25 C. Experimental temperatures varied between 20 C and 26 C.

Response rates declined as laboratory temperatures decreased during the cooler months of the year. By regression, 25 C was determined to be the optimal temperature for achieving a perfect response (100%).

Subsequently, various temperature manipulations were performed to better evaluate the role of temperature and the extent to which this factor can be used to the experimenter's advantage.

Of the three series of experimental temperature manipulations—(a) warming or cooling experimental water temperatures during the 2-hr pre-test period, (b) raising the temperature of the culture water only, and (c) raising the culture water temperature and maintaining that temperature throughout the experiment—only the latter proved successful in significantly affecting response rates when compared to populations raised and tested at room temperature.

Variable results were obtained when experimental temperatures were increased or decreased over the 2-hr pre-test period. In general, average response rates were very low, ranging between 21% and 57%, and variation within treatments and between days was very high (Table 4). There was no clear evidence that short-term changes in experimental temperatures influenced phototactic behavior consistently one way or the other.

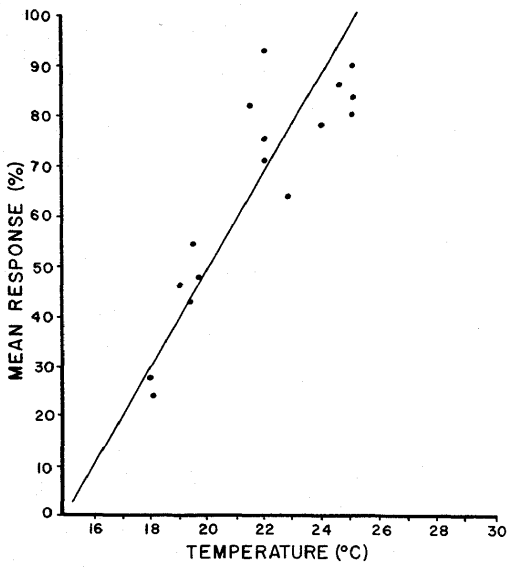

FIGURE 2. Effects of culture water temperature on the mean response of phototaxis experiments.

Increasing the water temperature of culture A over a period of one week did not significantly improve the response of culture A compared to culture B. Both were tested at room temperature which was a few degrees cooler than culture A water, and a few degrees warmer than culture B water. The mean response for culture A was 50.6% (S.D. = 14.18) and for culture B was 49.5% (S.D. = 10.42).

A significant effect was ultimately demonstrated by maintaining the water tem-

TABLE 4. *Effects of varying experimental water temperature on phototaxis.*

Date		Cool 15C	Room temperature 20C - 22C		Warm 25C
May 5, 81	$\bar{x}$	56.8%	42.5%	51.4%	29.2%
	S.D.	15.94	14.99	12.90	10.84
May 11, 81	$\bar{x}$	40.1%	26.2%	28.7%	27.8%
	S.D.	14.83	12.28	10.63	13.24
May 12, 81	$\bar{x}$	35.6%	21.1%	24.8%	30.0%
	S.D.	4.04	4.61	5.26	7.91

TABLE 5. *Effects of increasing culture water and experimental water temperatures on mean phototactic responses.*

	Culture A 25 - 26C		Culture B 21 - 23C	
Date	Mean	S.D.	Mean	S.D.
June 22, 81	80.6%	7.87	32.4%	9.60
June 23, 81	77.6%	7.61	51.3%	10.92
June 24, 81	83.5%	9.20	36.4%	13.64

perature in the test tubes at about the same temperature as the heated culture water during the 2-hr pre-test period (Table 5). The experimental group, cultured and tested between 25 C and 26 C, had significantly higher mean response rates and less variation than the control group on each of the three days the experiment was performed.

It appears that the relationship between culture temperatures and experimental temperatures is as critical as the absolute temperature of the experiments. Those animals taken from heated culture tanks and allowed to cool several degrees to room temperature did not perform satisfactorily. The *Daphnia* needed to be maintained at, or a few degrees above, their culture temperature throughout the experimental period in order to elicit the appropriate responses.

The temperature factor was not suspected of being so critical initially because the range of temperatures that occurred in the laboratory over an entire year, approximately 20 C to 26 C, was very small in comparison to daily and seasonal fluctuations that wild populations would likely encounter. One might speculate that laboratory populations lacking the environmental cues available to wild populations, such as a changing photoperiod and large temperature fluctuations, become very "finely tuned" to environmental stimuli. These temperature experiments demonstrated that even within a narrow range of temperature fluctuations there is a significant relationship between temperature and the phototactic behavior of laboratory animals. It appears then that experimental variation can be minimized by maintaining cultures and experimental containers at an optimal temperature, approximately 25 C.

Part II: Naphthalene Bioassays

Naphthalene bioassays conducted in July of 1980 showed a strong inverse relationship between mean response and the concentration of the toxicant (Fig. 3, R = -0.93). Among the controls the mean responses ranged between 68% and 80% with an overall average of 74.4% for all five experiments. Standard deviations ranged from 7.78 to 16.00. *Daphnia* exposed to 2.0 mg/*l* naphthalene were unable to respond to the stimulus but were obviously alive and moving at the bottom of the test tubes. When exposed to 1.5 mg/*l* a few animals were able to respond, but the mean response was always significantly lower than that of the controls. In four out of five experiments there was also a significant difference between controls and a 1.0 mg/*l* level of exposure. At the 0.5 mg/*l* level there was a significant difference in three out of five experiments (Table 6).

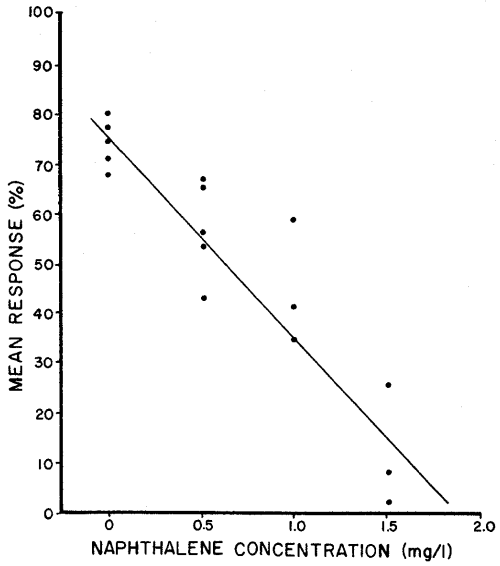

FIGURE 3. Effects of naphthalene on phototactic responses of *Daphnia* in July, 1980 experiments.

Three additional naphthalene bioassays were conducted in January and March of 1981. These proved unsuccessful in that mean responses among the controls (ranging from 7% to 31%) were far below expected levels, making it difficult to ascertain whether or not naphthalene was causing a decrease in response among the treatments. In most cases, mean responses among treatments were lower than the controls, but apparently some other factor had an over-riding effect on the ability of all the animals, treatments and controls, to respond to the given stimulus.

When it was determined that culture and experimental water temperatures played a significant role in phototactic responses, three final naphthalene bioassays were conducted in June and July of 1981 under controlled temperature conditions. Again an inverse relationship was found between mean responses and the concentration of naphthalene used (Fig. 4, R = -0.70).

though it was not as strong a correlation as in the previous year. Mean responses among controls averaged 60%. Using Dunnett's procedure (OSL ≤0.05) there was no significant difference between controls and animals exposed to 0.5 mg/*l* naphthalene. In one experiment there was no significant difference among any of the treatments. The other two experiments demonstrated a significantly lower mean response at the 1.0 and 1.5 mg/*l* level of exposure (Table 6).

Of four experiments comparing responses of animals in control water with those in a 0.075% ethanol solution (the maximum ethanol concentration present in naphthalene treatments), half of them showed no significant difference and half showed an enhanced response in the ethanol treatments (Table 7). The quantity of ethanol used in the naphthalene dilutions probably did not have any detrimental effects on phototactic responses of the *Daphnia.*

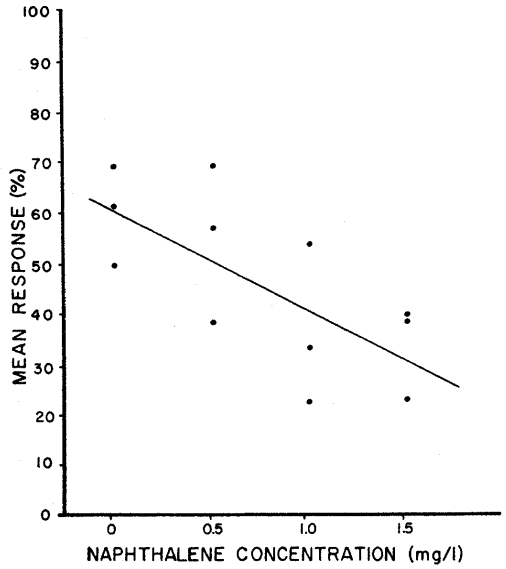

FIGURE 4. Effects of naphthalene on phototactic responses of *Daphnia* in June-July, 1981 experiments.

Dissolved oxygen measurements taken in the final two bioassays showed that an adequate supply of oxygen was present in the test solutions for all treatments. On June 30 and July 1, 1981, the cylinders contained 7.4 ± 0.1 ppm and 7.3 ± 0.1 ppm oxygen respectively. There was no difference in dissolved oxygen among the treatments.

Fluorescence analysis of naphthalene present in solution at the termination of each bioassay showed that concentrations were

TABLE 6. *The mean response* ($\bar{x}$) *and standard deviation (S.D.) of Daphnia populations exposed to naphthalene,* 0.5-2.0 mg/*l.*

Date		Naphthalene concentration (mg/*l*)				
		Controls	0.5	1.0	1.5	2.0
Jul 10, 80	$\bar{x}$	75.0%	57.0%			0
	S.D.	11.7	11.0			
Jul 11, 80	$\bar{x}$	68.0%	43.0%	42.0%	0	
	S.D.	16.0	10.4	7.6	—	
Jul 12, 80	$\bar{x}$	80.5%	66.0%	34.4%	8.4%	
	S.D.	14.46	19.39	21.45	9.42	
Jul 14, 80	$\bar{x}$	71.0%	66.7%	60.9%	27.1%	
	S.D.	9.62	9.65	9.17	5.54	
Jul 15, 80	$\bar{x}$	77.3%	53.8%	34.5%	2.4%	
	S.D.	7.78	15.11	7.91	3.32	
Jan 14, 81	$\bar{x}$	31.0%	16.0%	0	0	
	S.D.	8.22	8.94	—	—	
Jan 20, 81	$\bar{x}$	7.0%	16.0%	4.0%		
	S.D.	6.71	19.17	4.18		
Mar 11, 81	$\bar{x}$	30.0%	27.0%	18.0%		
	S.D.	7.07	14.40	10.37		
Jun 29, 81	$\bar{x}$	69.0%	69.3%	33.7%	39.3%	
	S.D.	15.57	13.48	11.93	16.28	
Jun 30, 81	$\bar{x}$	61.0%	57.0%	54.0%	40.0%	
	S.D.	10.52	15.45	13.27	13.47	
Jun 31, 81	$\bar{x}$	50.0%	39.0%	23.0%	24.0%	
	S.D.	18.04	10.52	13.22	12.65	

reduced slightly during the course of the experiments (Table 7). In general, the proportion of initial naphthalene lost was very similar among treatments. The higher losses on June 29 are probably attributable to increased volatilization due to the higher temperature (29.5 C) at which the test tubes were incubated during the pre-test period compared to incubation temperatures of 27.5 C and 27.0 C in the last two experiments. Losses were minimal on June 30 when the cylinders were loosely capped with clear plastic petri dishes.

TABLE 7. *Mean percent response (and standard deviation) of Daphnia in control water and .075% ethanol using student's t-test of significance.*

Date	Controls	.075% Ethanol	*t*	OSL
Jul 14, 80	71.0 (9.62)	91.0 (8.94)	6.81	.001
Jul 15, 80	77.3 (7.78)	74.7 (11.14)	0.86	.50
Jan 14, 81	31.0 (8.22)	32.0 (10.37)	1.72	.10 OSL .20
Mar 11, 81	30.0 (7.07)	37.0 (10.95)	2.40	.02 OSL .05

In general the phototaxis bioassays proved to be an effective means of demonstrating toxic effects of naphthalene to *Daphnia magna*. As is often the case with chronic bioassays, the concentrations at which toxic effects were detected were well below LC_{50} values for the same animal. LeBlanc (29) determined 24 hr and 48 hr LC_{50} values for *Daphnia magna* to be 17 mg/*l* and 8.6 mg/*l* respectively, and estimated a "no discernible effect concentration" of 0.60 mg/*l* naphthalene. The phototactic response was completely inhibited at only 2.0 mg/*l* naphthalene, and concentrations as low as 1.0 mg/*l* significantly reduced responses in the majority of the bioassays performed. The LC_{50} data were determined by LeBlanc (29) with neonates less than 24 hr old, which are generally more sensitive to toxicants than adults (44). The phototaxis bioassay which measures toxicity to adult animals appears to be a particularly sensitive technique.

The phototaxis bioassay offers several advantages over the more conventional life-cycle and acutely lethal bioassays. Complications due to food requirements, particularly troublesome in life-cycle bioassays and acute bioassays greater than 24 hr in duration, were virtually eliminated in the phototaxis bioassays. The *Daphnia* were obtained from well-fed cultures and the entire experimental procedure lasted only a few hours. Tap water aerated for several days in a large holding tank was the source of the experimental water (control and dilution water) and provided ample dissolved oxygen for the duration of the experiments. Complex diluter systems required to maintain chemical concentrations in longer-term experiments were avoided because behavioral effects occurred after only a 2-hr period of exposure to naphthalene.

The exposure period was determined specifically for naphthalene, however, based on a study of bioconcentration potentials over time (41). A similar study would have to be performed or obtained from the literature to determine exposure periods required (minimum time for maximum uptake) for other chemicals to be used for bioassays. Bioconcentration of polycyclic aromatic hydrocarbons tends to take longer for compounds of higher molecular weight (41). However, the larger molecules are generally less volatile, so longer exposure periods would not necessarily require a flow-through system to maintain concentrations for this class of chemicals.

TABLE 8. *Final naphthalene concentrations determined by fluorescence spectroscopy after a bioassay.*

	Initial concentrations of naphthalene (mg/*l*)		
Date	0.5	1.0	1.5
14 Jan 81	.47	.95	1.18
20 Jan 81	.54	.89	—
11 Mar 81	.48	.77	—
29 Jun 81	.28	.59	.90
30 Jun 81	.40	.81	1.30
1 Jul 81	.33	.68	1.07

Though it is generally undesirable to complicate experiments with additional chemicals, the carrier solution used to increase the solubility of naphthalene (ethanol) did not inhibit phototactic responses (at the concentrations used). Because phototaxis experiments can be easily and rapidly replicated, the evaluation of complicating factors such as carrier solutions presents little difficulty.

The major drawbacks to the phototaxis bioassays are the possibility of intrinsic rhythms having a significant influence on behavior, and the comparability of results among experiments performed on different days and at different times of the year. The problem of intrinsic rhythms may require another year of experimentation under more controlled environmental conditions (with particular attention to photoperiods and temperatures). Comparability of results might be improved by using more controlled culture techniques, using uniform age classes, and controlling experimental temperatures. It appears that among experiments in which the response of controls was similar, treatment effects were also similar. Treatment effects were most clearly demonstrated when the mean response of the controls was at least 70%. Perhaps 70% to 75% (the normal expected response established in Part I) should be considered as a minimum acceptable level of response among controls in toxicity experiments. Bioassays in which that requirement is not met may not provide an accurate measurement of toxic effects.

The concentration of naphthalene that caused behavioral effects in the phototaxis experiments probably would not have serious detrimental effects on populations of Daphnids if it was a short-term exposure as a result of a spill, for example. Southworth *et al*, (41) found that *Daphnia* placed in uncontaminated water following exposure to sublethal concentrations of naphthalene were quickly able to excrete the chemical and regain vigor. Prolonged exposure, however, would probably result in more serious consequences. Concentrations as low as 2.0 mg/*l* naphthalene reduce mobility to the extent that the animals would not be able to maintain themselves in the water column where food is available. Other suggested advantages derived from the migratory patterns, such as predator avoidance, niche diversification, and growth efficiency, would also be lost as a result of chronic naphthalene exposure.

On the basis of toxicity tests for a number of different organisms, including Cladocerans, the United States Environmental Protection Agency (45) determined the chronic toxicity of naphthalene to aquatic life to be 0.62 mg/*l*, and the acute toxicity to be 2.30 mg/*l*. Behavioral dysfunction was detected at these same levels of exposure in the phototaxis experiments. Phototactic responses were significantly depressed btween 0.5 mg/*l* and 1.0 mg/*l*, and were completely inhibited at 2.0 mg/*l*. Thus a behavioral bioassay can provide results that are highly comparable to those obtained using other methods presently employed.

Phototaxis bioassays provide evidence that sublethal concentrations can critically effect the behavioral repertoire of Daphnids. No single biological technique can adequately determine "safe limits" for water quality control, and thus it is desirable to use all available methods of assessing pollutant effects on organisms. The time and expense required to perform numerous tests, however, are usually limiting. Phototaxis bioassays may provide the speed and simplicity required to screen numerous potential pollutants for harmful effects.

REFERENCES

1. J. B. SPRAGUE, Water Res. 3: 793-821 (1969).
2. J. B. SPRAGUE, Water Res. 5: 245-266 (1971).
3. AMERICAN PUBLIC HEALTH ASSOCIATION, *Standard Methods for the Examination of Water and Wastewater,* 14th ed., APHA, Washington, D.C., 1975.
4. W. PELTIER, *Methods for measuring the acute toxicity of effluents to aquatic organisms, U.S.* Environmental Protection Agency, Environmental Monitoring and Support Laboratory, EPA-600/4-78-012, 1978.
5. D. I. MOUNT, Water Res. 2: 215-233 (1968).
6. W. A. BRUNGS, Trans. Am. Fish. Soc. 98: 272-279 (1969).
7. A. L. BUIKEMA, JR., Hydrobiologia 41: 391-418 (1973).
8. P. A. GILDERHAUS, Trans. Am. Fish. Soc. 95: 289-296 (1966).
9. J. CAIRNS and A. SCHEIER, Notul. Nat. 370 (1964).
10. W. S. G. MORGAN and P. C. KUHN, Water Res. 8: 129-137 (1972).
11. C. R. CRIPE, J. Fish. Res. Board Can. 32: 1884-1885 (1975).
12. H. W. SHIRER, J. CAIRNS, W. T. WALLTER, Water Resources Bull. 4: 27-43 (1968).
13. O. E. SHERER, *Behavioral Assays—Principles, Results, and Problems.* Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Nov. 2-3, 1976. Environmental Protection Service Technical Report No. EPS-5AR-77-1, Halifax, Canada, 1977, pp. 33-40.
14. C. E. WARREN, *Biology and Water Pollution Control.* W. B. Saunders Co., Philadelphia, PA., 1971, pp. 192-213.
15. J. CAIRNS and W. H. VAN DER SCHALIE, Water Res. 14: 1179-1196 (1980).
16. P. D. ANDERSON and S. D. 'APOLLONIA, Aquatic animals. In G. C. Butler (ed.), *Principles of ecotoxicology*. Scope 12.

John Wiley & Sons, New York, 1978, pp. 187-221.
17. C. M. TARZWELL, Can. Fish. Cult. 31: 35-41 (1962).
18. J. G. GEIGER, A. L. BUIKEMA, and J. CAIRNS, JR., Proceedings of the Third Annual Symposium on Aquatic Toxicology, ASTM Special Technical Publication 707, 1978, pp. 13-26.
19. U. S. ENVIRONMENTAL PROTECTION AGENCY. *Part 5: Water Quality Criteria Document.* Federal Register 45 (231), 1980.
20. M. M. ELLIS, Bull. Bureau Fish. 48: 365-437 (1937).
21. B. G. ANDERSON, Sewage Works J. 16: 1156-1165 (1944).
22. B. G. ANDERSON, Trans. Am. Fish. Soc. 78: 96-113 (1950).
23. J. R. MORGAN, Bull. Environ. Contam. Toxicol. 8: 129-137 (1972).
24. M. F. BAUDOUIN and P. SCOPPA, Bull. Environ. Contam. Toxicol. 12: 745-751 (1974).
25. J. H. CANTON and D. M. M. ADEMA, Hydrobiologia 59: 135-140 (1978).
26. J. H. CANTON, P. A. GREEVE, S. SLOOF, and G. J. VAN ESCH, Water Res. 9: 1163-1169 (1975).
27. I. A. RAWASH, E. A. GAABOUB, F. M. EL GAYER, and A. Y. EL SHAZILI, Toxicology 4: 133-144 (1975).
28. P. R. DURKIN, TAPPI Environ. Conf. (Proc.). 165-169 (1978).
29. G. A. LEBLANC, Bull. Environ. Contam. Toxicol. 24: 684-691 (1980).
30. J. E. HARRIS and U. K. WOLFE, Proc. Roy. Soc. B144: 329-354 (1955).
31. G. L. CLARKE, J. Exp. Biol. 7: 109-131.
32. G. L. CLARKE, J. Exp. Biol. 9: 180-211 (1932).
33. J. RINGELBERG, Netherl. Sea Res. 2: 319-406 (1964).
34. G. E. HUTCHINSON. *A Treatise on Limnology. Vol. II. Introduction to lake biology and the limnoplankton*. New York, John Wiley & Sons, Inc., 1967.
35. E. S. RUSSELL, Biol. Rev. 2: 213-263 (1927).
36. N. G. HAIRSTON, Proc. Natl. Acad. Sci. USA. 73: 971-974 (1976).
37. R. G. WETZEL, *Limnology*, W. B. Saunders Co., Philadelphia, PA., (1975).
38. L. A. McLAREN, J. Fish. Res. Board Can. 20: 685-727 (1963).
39. A. L. BUIKEMA, Hydrobiologia 47: 43-58 (1974).
40. J. R. ENRIGHT, Limnol. Oceanogr. 22: 856-872 (1977).
41. G. R. SOUTHWORTH, J. J. BEAUCHAMP, and P. K. SCHMIEDER, Water Res. 12: 973-977 (1978).
42. L. WHITMAN, *The phototactic behavior of* Daphnia magna *as an indicator of chronic toxicity*, M.S. Thesis, Okla. State Univ., Stillwater, 1981.
43. R. G. D. STEEL, Principles and Procedures of Statistics. McGraw-Hill Book Co., New York, 1960.
44. B. G. ANDERSON, and J. C. JENKINS, Biol. Bull. 83: 260-272 (1942).
45. U.S. ENVIRONMENTAL PROTECTION AGENCY. Part 5: *Water Quality Criteria Document*. Federal Register 45 (231), 1980.