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

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PREFACE

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CHAPTER I

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, both at the individual and community level. The realization that no single methodology 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 (Cairns and Dickson, 1978).

At the level of the organism, toxicity testing has developed along two major lines: (1) the lethal, or acute bioassay (Sprague, 1969); and (2) the sublethal, or chronic bioassay (Sprague, 1971). While the methodology for the former is now highly standardized (APHA, 1975; Peltier, 1978) 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 (Mount, 1968; Brungs, 1969; Buikema, 1980), growth (Mount, 1968; Gilderhaus, 1966), respiration (Cairns and Scheier, 1964; Morgan and Kuhn, 1974), activity (Cripe, 1975; Shirer et al, 1965), and behavior (Sherer, 1977). 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 (Sprague, 1971). 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 implementation of chronic bioassays into routine biological monitoring systems, and the need for continued development and standardization of the techniques remains prominent (Sprague, 1971; Warren, 1971; Cairns and van der Schalie, 1980; Anderson and D'Apollonia, 1978). "Safe" concentrations of wastes must be determined not only to insure survival, but also growth, reproduction, and the general well-being of a species under conditions of continuous exposure (Tarzwell, 1962).

The current demand in biological monitoring is for quantifiable, short-term bioassays that can effectively predict long-term effects of sublethal toxication (Geiger et al, 1978). To meet these requirements, a behavioral bioassay was devised for the following research based on a number of qualifying factors. Sprague (1979) suggested that behavioral responses may be even more sensitive than survival, growth, and reproduction, and thus of over-riding importance to species continuity. Warner (1966) recognized the relevance of behavior to toxicological

studies because (1) the behavior of animals is likely to have distinct survival value and thus any impairment in the behavioral repertoire is likely to be deleterious, (2) 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 (3) behavioral patterns appear to be very sensitive to changes in environmental quality.

Sherer (1977) 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 (Ellis, 1937; Anderson, 1944 and 1950; Frear and Boyd, 1967; Morgan, 1972; Baudouin and Scoppa, 1974; Canton et al, 1975 and 1978; Rawash et al, 1975; Durkin, 1978; LeBlanc, 1980), providing a frame of reference for sublethal exposures. Because the vertical migrations of zooplankton are cued primarily by light (Harris and Wolfe, 1955; Cushing, 1955; Clarke, 1930 and 1932; Ringelberg, 1964; Lincoln, 1970; Hutchinson, 1967) it is not difficult to simulate the pattern in the laboratory (Harris and Wolfe, 1955). The phototactic response itself is relatively easy to stimulate and measure in terms of distance traveled over a specified period of time (Ringelberg, 1964).

Finally, the relevance of vertical migrations to the survival of

zooplankton populations is easily accounted for. That it is a world-wide phenomenon with a diverse array of participating species is well-documented (Hutchinson, 1967), and suggests that the behavior evolved as a result of strong selective pressures. Defining exactly what those pressures are have been the topic of some debate. The avoidance of physiologically adverse radiation (Russell, 1927; Hairston, 1976), predator avoidance and niche diversification (Wetzel, 1975), and increased feeding, growth, and reproductive efficiencies (McLaren, 1963; Buikema, 1973 and 1974; Enright, 1977) have all been suggested as possible evolutionary mechanisms. Hutchinson (1967) 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 in consideration that 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 (1) to define and quantify the normal, or average, phototactic response of Daphnia magna to a light stimulus, and (2) to determine the effects of naphthalene, one of the more toxic substituents of crude oils and coal tar, on the response defined.

CHAPTER II

LITERATURE REVIEW

Daphnia magna

Daphnia are freshwater crustaceans belonging to the order Cladocera; the water fleas. Members of the genus are distributed world-wide, inhabiting a variety of habitats including lakes, ponds, and rivers. *Daphnia magna* is more commonly found in ponds and small lakes of the northern and western regions of North America (Ward and Whipple, 1959).

General Morphology

Cladocerans range in size from about 0.2 mm to 5.0 mm and are characterized by a distinct head with a beak-like projection ventrally, and a body enfolded in the transparent cuticle, or carapace; a bivalve-like structure. The carapace lacks any true valve or joint, and terminates posteriorly with a structure characteristic of the species, such as a single spine in the case of *Daphnia*. Two pairs of antennae project from the head; the first pair are short and bear sensory hairs, the olfactory setae, and the second pair are much larger with long plumose setae. The latter are attached proximally by powerful muscles and serve as locomotor organs (Ward and Whipple, 1959).

Five or six pairs of appendages project from the thoracic region of the trunk of the Cladoceran. These are flattened structures bearing fine filtering setae. Movement of the appendages creates a current of

water around them where food particles are trapped by the set oxygen is provided to the respiratory surfaces. The epipodit trunk appendages may be major areas of gas exchange, though th appendage and general integumentary surface are also sites of respiratory activity (Barnes, 1974). Food particles are collected in the ventral food groove at the bases of the appendages, and are passed forward to the mouth where they are ground up by the mandibles and passed into the esophagus. The esophagus widens into the stomach which is continuous with the intestine posteriorly. Two digestive caeca attached to the stomach store food and assist in digestion. The rectum opens ventrally near the postabdomen. Daphniidae have a row of spines on either side of the postabdomen and two terminal claws which comb the carapace and appendages, keeping them free of parasites and foreign matter (Ward and Whipple, 1959).

Cladocerans have a globular heart with two ostia which lies at the anterior trunk on the dorsal side. Blood is expelled anteriorly from the heart and flows through regular pathways in the hemocoel, though there are no closed blood vessels. Hemoglobin is often present in the blood, especially in populations inhabiting stagnant waters, but is usually absent in well-aerated water (Barnes, 1974).

Daphnia have two light-sensitive organs; a single compound eye composed of 22 ommatidia near the apex of the head, and the ocellus, or nauplius eye, located ventral to the brain and composed of a few retinal cells (Baylor and Smith, 1958). The optic ganglion provides numerous nerves to the eyes. The compound eye is attached by six muscles capable of rotating the eye over 160° in the saggital plane (Downing, 1974). Frost (1975) observed several types of movement of the compound

eye; (1) a fast tremor possibly functioning to prevent loss of information through adaptation, and to increase acuity, (2) rhythmic scanning movements of 5° - 6° in amplitude, the function of which is unclear, and (3) large fast movements of up to 150° in amplitude which occur in response to stimulus displacement. Denervation of the compound eye results in a loss of sensitivity to various wavelengths of light, whereas destruction of the nauplius eye disrupts the behavioral patterns associated with vertical migrations (Baylor and Smith, 1957). Resolution of pattern in the visual field is probably poor (Frost, 1975).

Physiological Parameters

Temperature and pH have a profound effect on various physiological systems of Daphnids. McArthur and Baillie (1929) found that the optimum temperature range is 17° C to 23° C, and the optimum pH is between 8.1 and 8.5, though the animals survive over a larger range of 5.4 to 9.5. Ringelberg (1973) determined that 20° C is ideal for maximum reproduction. In general, Daphnia raised at higher temperatures have an increased rate of development, molting, and brood production, and a decreased longevity (Wetzel, 1975).

Buikema (1973) found that light intensity and wavelength affected cultures of Daphnia pulex in various ways. Light intensity significantly affected adult body length, the average number of young produced per adult, and abortion rate. Fourteen foot-candles was optimal lighting with respect to size, longevity, and reproduction. Increased mortality was observed when animals were raised under only a portion of the visible spectrum, particularly green wavelengths. Under red wavelengths growth was retarded and release of the first brood was delayed. Green

wavelengths stimulated molting and retarded growth between molts. Blue wavelengths increased the number of young per brood and per adult.

An adequate food supply has been shown to be important for rapid growth and maximum reproduction (Ingle et al, 1937). Canton and Adema (1978) found that 48 h is the maximum time that Daphnids can be deprived of food without showing increased mortality. Mean and maximum longevity were greater for Daphnia reared on an algal diet compared to a trout-granule diet, though brood sizes were two times greater on the trout-granule diet (Winner et al, 1977). Dewey and Parker (1964) achieved greater reproductive success with Daphnia magna fed on yeast (Fleischman's fresh active) and algae (Scenedesmus), either used alone or combined, than on any of 11 other diets they investigated, including bacterial and manure-soil infusion diets.

Oxygen consumption varies with age. At 20° C, 25 one to two-day old Daphnids consume about 100 ug of oxygen per day, compared to 25 adult egg-bearing Daphnids which consume about 850 ug per day (Adema, 1978).

Anderson et al (1937) found that the rate of reproduction is directly correlated with the growth increment. Reproduction rate, determined by the number of young released or the number of eggs or embryos in the brood chamber, is thus considered a satisfactory indicator of the general metabolic condition of a Daphnid (Anderson and Jenkins, 1942).

Reproduction and Development

Under favorable conditions, Daphnia reproduce by diploid parthenogenesis. Eggs are deposited in the brood chamber, a cavity on the dorsal side of the body, where they hatch in a form similar to that of the parent (Wetzel, 1975). These females go through four to six instars

(Anderson and Jenkins, 1942), marked by shedding of the carapace, before they produce their first eggs at about one week of age and every two or three days thereafter (Dewey and Parker, 1964). The number of eggs per clutch varies from two up to forty in the larger Daphnidae raised under optimal conditions (Wetzel, 1975).

Males begin to appear in populations as the production of parthenogenic eggs dwindles due to any of a number of factors. Depletion of the food supply, evaporation of habitat, overpopulation of the culture, accumulation of metabolic wastes in the medium, a decrease in temperature, and a decrease in daylength have all been implicated as stimuli for the production of males and sexual eggs (Banta, 1939; Stross and Hill, 1965). The sexual eggs are haploid, requiring fertilization. They are enclosed in a thickened and darkly pigmented shell, the ephippium, which is shed with the carapace at the next molt. These "resting" eggs remain dormant, often withstanding freezing and drying. and develop into parthenogenic females when conditions become favorable again (Ward and Whipple, 1959).

Vertical Migrations and Phototaxis

Cladocerans distribute themselves vertically in the water column by means of diurnal, or twilight migrations (Hutchinson, 1967). The migrations vary considerably in amplitude depending primarily on the transparency of the water. Burkhardt (1910) and Worthington (1931) recorded migrations of up to 50 m in depth in clear Swiss lakes, whereas Juday (1904) rarely found migrations exceeding 5 m in the more turbid waters of Wisconsin. There is also evidence of seasonal variations in migratory behavior. Cunningham (1972) found that under the ice *Daphnia galeata* showed only a slight tendency toward nocturnal migration, and

other members of the migratory zooplankton, Diaptomus minutus and Cyclops scutifer, showed a pronounced reverse nocturnal migration. In polar seas during the arctic summer, vertical migrations do not occur, but take place in the autumn when there is a typical diurnal rhythm of light intensity (Harris, 1963). Variations in pattern of migrations also occur under heavy cloud cover (Wetzel, 1975).

The primary stimulus for vertical migrations is clearly light (Ringelberg, 1964). Harris (1963) suggested that an intrinsic rhythm can initiate a rise before "true dawn", at which time depth is then governed by light intensity. Harris and Wolfe (1955), however, found no evidence of intrinsic rhythms in laboratory animals subjected to various light cycles. They found the pattern of vertical migrations could be repeated at any time of day, and with any periodicity between 2 h and 13 h.

A number of theories have been proposed to explain the mechanism of the light response which produces a vertical migration. Loeb and Groom (1890) believed that phototaxis was continuous in the presence of a light stimulus until a secondary stimulus, elicited by some property inherent in the water, caused a change in the phototactic sign (direction of movement in relation to the light source). Subsequently, Loeb (1904) showed experimentally that CO_2, acids, salts, and temperature changes all effected a change in phototactic sign, and postulated that the by-products of phytoplankton activities combined with temperature changes encountered with depth produced the migrations.

Loeb's theory was not widely accepted. Cushing (1951) stated that the artificial conditions under which Loeb was able to stimulate migratory behavior in the lab were unlikely to occur in nature with any degree

of regularity.

Ewald (1910) proposed the idea of a preferendum hypothesis of migration; a migration produced as a result of the animals maintaining themselves within a preferred range of light intensity. This hypothesis required the existence of absolute upper and lower limits of light intensity to which the animals respond. As yet there is no evidence for this hypothesis (Ringelberg, 1964).

The concept of a relative optimum intensity of light was developed by Clarke (1930) as a result of his laboratory investigations with Daphnia magna. A relative optimum implies that light adaptation occurs and a phototactic response is elicited by a change in light intensity. This mechanism of response was supported by Harris and Wolfe's (1955) investigations of vertical migrations in a cylindrical vessel in which a steep light gradient was introduced with India ink. Daphnia maintained themselves within a narrow range of light intensity (relative to the range applied in the cycle) by varying depth. When subjected to sudden increases or decreases of light intensity, the animals reacted initially by rapidly swimming to a zone of intensity similar to that which they formerly occupied. Within minutes, however, they drifted back to their original depth. The latter reaction suggested that a process of adaptation to light was occurring to accomodate depth regulation.

Ringelberg (1964) designed his phototactic experiments with Daphnia magna to explore the relative optimum intensity theory of migration. He found that a continuous decrease in light intensity at a slow rate elicits a step-like upward swimming. Instantaneous decreases in intensity elicited a similar response. He concluded that changes in light intensity are the direct cause of a swimming response and suggests that upward

migrations in nature occur in response to a decrease in light intensity. Ringelberg (1964) also demonstrated that as long as light intensity decreases at a sufficient rate, the stimulus is continuously renewed. If the rate of increase is slow, upward swimming is interrupted by periods of no swimming.

Cushing (1955) suggested that the dominating mechanism of migration is photokinesis rather than phototaxis. Harris and Masons's (1956) observation that Daphnia still respond to a light stimulus even after the compound eye had been extirpated supported this view. Numerous experiments however have shown that Daphnia have the capacity to orient to the direction of light (Jander, 1973; Ringelberg, 1964; Baylor and Smith, 1957). Vertical movement (Harris and Wolfe, 1955) as well as horizontal movement (Clarke, 1930 and 1931) can be stimulated by the propagation of light in the appropriate direction. In the absence of a compound eye, the nauplius eye probably serves in some orienting capacity (Baylor and Smith, 1958).

A number of factors have been found to modify the phototactic response. Clarke (1931) found that rapid changes in temperature caused a reverse in the primary sign of phototaxis; negatively phototactic Daphnia became positively phototactic following a rapid decrease in temperature. He also observed that a latent period of reaction to a light stimulus increased in duration with a decrease in temperature. Baylor and Smith (1957) reported that Daphnids raised at 15° C and exposed to a horizontal light beam showed a vigorous negative phototaxis at 30° C. Small, but continuous decrements of temperature increased the velocity of upswimming. The latter temperature response was believed to accommodate vertical movements in spite of rapidly changing light

intensities (Baylor and Smith, 1957). McNaught and Hasler (1964) were able to establish a linear relationship between the rate of vertical migrations and the rate of decrease in the log of absolute light intensity, the slope of which increased with higher temperatures. This suggested that populations would migrate at greater speeds in warmer water for a given light decrease.

Lincoln (1970 and 1971) explored the effects of pressure changes on the migratory behavior of Daphnia magna. He determined that in the presence of light only a brief response is elicited by a large increase in pressure, and it appeared to be largely a kinetic response that did not involve a specific orientation of the swimming activity. Lincoln (1970) concluded that it is unlikely that pressure changes play any real part in the natural behavior of Daphnia.

Baylor and Smith (1957) found that Daphnids reared at pH 8.0 were photopositive at pH 8.5 and negative at pH 7.0. They also reported that redox potentials affect phototaxis; the oxidizing substance, catechol, produced strong upswimming, and the reducing substance, cysteine, produced strong downswimming. It was suggested that the food type which the Daphnia are reared on (e.g. green algae vs. bacterial culture) affects the redox potential and thus the phototactic behavior of the culture (Baylor and Smith, 1957).

Different wavelengths of light have also been shown to have an effect on phototaxis (Smith and Baylor, 1953). Under red light (6000 Å) Daphnia are calm, orientation is upright, and the vertical vector of motion is greater than the horizontal. Under blue light (5000 Å) populations are more agitated, the animals lean forward more, and the major vector of motion is horizontal. Smith and Baylor

(1953) suggested that the color response may provide a mechanism for concentrating populations in areas of dense phytoplankton by keeping them in place under clumps of phytoplankton, which filter out more of the short wavelengths, and stimulating more wandering in clear water. Stearns (1975) verified the color responses, but challenged the assumption that the color of ambient light is a reliable clue to the presence of phytoplankton, and that the clumping of phytoplankton is such that it is energetically feasible for Daphnia to wander between patches.

Naphthalene

Physiocochemical Properties

Naphthalene ($C_{10}H_8$) is a bicyclic aromatic hydrocarbon with a molecular weight of 128.18, melting point 80.5° C, boiling point 218° C (Kingsbury et al, 1979), and vapor pressure of 0.492 mm Hg at 19.8° C (Gil'denblat et al, 1960). Solubility ranges between 30 mg/l and 40 mg/l, and increases substantially in organic solvents such as ethanol (Bohon and Claussen, 1951). At room temperature nephthalene is a white crystalline solid.

Sources and Uses

Naphthalene is a by-product of the coke industry, and is a major constituent of coal tar (Kingsbury et al, 1979). It is also produced in petroleum refining and is believed to be one of the primary contributors to the toxicity of crude and refined oils (Rice et al, 1977). It is used in chemical and dye manufacturing, in preserving wood, and as a moth repellant, antihelminthic, vermicide, and intestinal

antiseptic (U.S. EPA, 1980a).

Industrial effluents and oil spills are the major sources of naphthalene in the aquatic environment. Up to 32,000 ug/l have been found in industrial effluents, and 22 ug/l in the final effluents of sewage treatment plants receiving those discharges. Up to 1.4 ug/l have been found in drinking water (U.S. EPA, 1980a).

Effects on Organisms

Bioconcentration of naphthalene has been shown to occur in many species of fish (Anderson, 1975; DiMichele, 1978; Lee et al, 1972; Neff et al, 1976; Roubal and Stranahan, 1978), various zooplanktons and crustaceans (Corner et al, 1973; Lee, 1975; Southworth, 1978), and algae (Vandermeulen and Ahern, 1976). The bioconcentration factors ranged from 32 to 77 for three different species of marine fish exposed for one to three hours (Lee et al, 1972), 50 to 60 for copepods in one-day exposures (Harris et al, 1977), up to 100 in Daphnia in two hours (Southworth et al, 1978), and 5,000 for a copepod exposed for nine days (Harris et al, 1977). Fish selectively accumulate naphthalene in the gills, liver, gut, and gall bladder (Lee et al, 1972).

Ninety-six-hour LC50 values range from 2.3 mg/l to 8.9 mg/l in freshwater fish, with the exception of the mosquitofish which has an LC50 of 150 mb/l (U.S. EPA, 1980a). LeBlanc (1980) determined 24-hour and 48-hour LC50's for Daphnia magna to be 17 mg/l and 8.6 mg/l respectively.

The major effects of sublethal doses in fish include generalized blood stasis, degeneration of neurosensory organs, and gill hyperplasia (DiMichele, 1978). Concentrations as low as 0.67 mg/l naphthalene

reduced growth in coho salmon fry during a 40-day exposure period (Moles et al, 1981). In algae, cell growth and photosynthesis were inhibited (Vandermeulen and Ahern, 1976). The principle consequence of naphthalene poisoning in man is hemolytic anemia. Other symptoms include jaundice, hemoglobinuria, cerebral anoxia caused by capillary blockage, and cataracts (DiMichele and Taylor, 1978; Ghetti and Mariani, 1956).

Many fish and invertebrates studied are capable of purging themselves of naphthalene when placed in uncontaminated water, though the metabolic products have been found to be retained for prolonged periods of time in some crustaceans (Malins, 1977). The ability to metabolize naphthalene depends on the presence of mixed-function oxygenase enzymes. These enzymes are believed responsible for conversion of naphthalene in rat tissues as well as in some invertebrates such as houseflies, crabs, and lobsters (Malins, 1977).

CHAPTER III

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 maintained in large wood and glass tanks which contained about 42 gallons 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. Two to three grams of Brewer's Yeast, manufactured by Plus Products of Irvine, California (Formula 250), were 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 to the culture. Bacterial and algal growth in the culture tanks probably served as additional sources of food. Densities fluctuated widely in the tanks, but in general this method provided substantial populations and consumed little time for maintenance.

Development of Technique

Preliminary observations of *Daphnia 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 impinging on the vessel were eliminated. Therefore, preliminary

experiments were conducted in a completely dark room, using 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: (1) neonates are known to exhibit phototactic responses different from that of adults (Clarke, 1932); (2) personal observations of neonates indicated that there is more individual variation in their responses compared to adults; and (3) adults are easier to observe by virtue of their size.

Several procedures for observing phototaxis were tried before a final method which adequately met the requirements for a bioassay was developed. Daphnia placed in a tall cylinder (60 cm height), and exposed to a single overhead beam of light, showed obvious reactions to variations in light intensity, but the population observed did not react in any identifiable pattern as a whole. A chamber devised with a perforated partition in the center, which diffused the light, but allowed the Daphnia to pass through, demonstrated that the animals unanimously preferred the area of lower light intensity. Switching direction of the light, and hence the areas of direct and diffuse light, produced a marked response in which a large majority of the population moved rapidly away from the light source. Since the partition seemed more to inhibit than enhance movement, it was removed. The next series of observations of phototaxis were elicited simply by changing direction from which the light was propagated. With this procedure, 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 fatiguing was evident if the stimulus was given 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 was designed to allow a single observer to run a complete bioassay within a single day with inexpensive and readily available equipment.

Description of Apparatus

A large box, 4' X 1' X 1' (Figure 1), made of ½" 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-watt 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 by flipping a single switch. Midway between the lamps and center shelves, ¼" plate glass was placed to reduce heat convection to the 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 1" diameter peephole in the door.

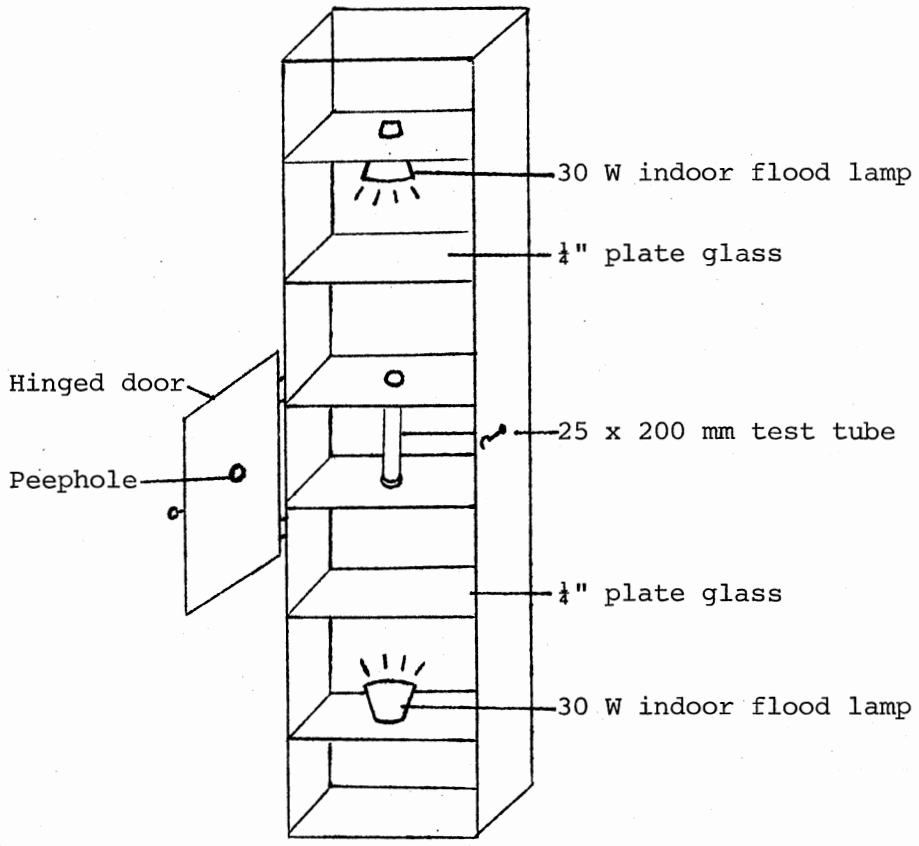

Figure 1. Black box for observing phototactic responses of Daphnia in a test tube.

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 sections following:

Daphnia from large culture tanks were siphoned into a one-liter beaker, taking care to keep the siphon underwater so as not to expose the animals to air.

Twenty mature Daphnia (based on size) were pipetted from the beaker into 25 mm x 200 mm test tubes containing approximately 70 ml 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 (Southworth et al, 1978). During the acclimation period, the test tubes were left uncovered at room temperature and under normal laboratory lighting (flourescent 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- minutes 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 seconds 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 seconds. The total response for an experiment was the mean percent response of 5 test tube populations (a total of 100 Daphnia). A sample data sheet with calculations of response rates are provided in the Appendix.

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 problem of floaters is usually attributed to air being 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 experiemnts will be considered 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. In addition, Experiments A and B 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 included (C) the role of intrinsic annual rhythms, (D) effects of food deprivation, (E) effects of culture technique and use of specific age classes, and (F) effects of temperature on phototactic responses. All experiments were performed within the basic design of the procedures described and with the additional conditions presented below.

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 (Steel and Torrie, 1960).

Experiment B: Circadian rhythms and phototaxis. Phototaxis experiments were performed between 800 h and 2200 h of one day, at 2 h to 4 h intervals to determine whether responses were more or less sensitive at any particular time of day. Duncan's multiple-range test was used to test for significant differences in responses using an OSL of 0.05.

Experiment C: Role of intrinsic annual rhythms. The results of

all non-exposure (control) experiments, performed within the framework of the basic procedures described, were averaged for each month of an entire year, and analyzed graphically for the possibility of seasonal or annual trends.

Experiment D: Effects of food deprivation. Adult Daphnia were siphoned from a large culture tank and distributed into each of four 1-liter beakers containing 800 ml of aged tap water. Approximately 110 Daphnia were placed in each beaker to provide the required 100 animals per treatment and allow for a 10% mortality. No aeration was provided to the beakers. A 10% yeast solution (one gram of brewer's yeast per 100 ml of water) (Dewey and Parker, 1964) was fed to each beaker culture according to the schedule in Table I.

Following five days in the beaker cultures, the Daphnia were pipetted from each beaker and randomly placed into a set of 5 test tubes. The most actively swimming animals were selected first, and any dead or immobile ones were excluded from the experiment. Experiments were performed as described above. The entire procedure was repeated four times, using Daphnia from two different cultures.

TABLE I

FEEDING SCHEDULE FOR TESTING EFFECTS OF FOOD DEPRIVATION

Beaker	Day 1	Day 2	Day 3	Day 4	Day 5	No. days without food
A	2 ml	1 ml	1 ml	1 ml	1 ml	0
B	2 ml	1 ml	1 ml	--	--	2
C	2 ml	1 ml	--	--	--	3
D	2 ml	--	--	--	--	4

Experiment E: Effects of culture technique and use of specific age classes. Several authors have emphasized the need to rigidly control culture conditions, and to use Daphnia of known age for toxicity testing (Anderson, 1944; Geiger et al, 1978; Frear and Boyd, 1967). To determine whether these variables affected the phototactic responses of populations, Daphnia were cultured using a modification of Frear and Boyd's (1967) technique. Two adult Daphnia were placed in each of 80 four ounce bottles containing 100 ml of aged tap water. Two drops of a 10% yeast solution were added to each bottle daily. Noenates ($\leq$ 48 h) were separated from the adults every two days and transferred to four-liter culture jars containing aged tap water. Adults were replaced in the four ounce bottles with fresh medium at that time. Neonates were fed approximately 2 ml of yeast solution per bottle daily and cultured 7 to 14 days before testing their phototatic responses. Various densities were tried in the rearing jars in attempts to maximize growth efficiencies while minimizing laboratory space and effort.

Experiment F: 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 h. 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 h 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 h pre-test period, the time required for maximum uptake of naphthalene (Southworth et al, 1978). 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 ml 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 along with each naphthalene bioassay. Ethanol controls, containing the maximum quantity of ethanol that would be present in any of the naphthalene dilutions 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 X 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 final concentration of naphthalene remaining in the test tubes (or cylinders) after a bioassay was completed was determined by flourescence analysis (Aminco-Bowman Spectrophotoflourometer with ellipsoidal condensing system) for all of the 1981 experiments. Data analysis, however, was based on initial concentrations since they were accurately known, and a discussion of naphthalene losses during the course of the experiments is included in the following chapter.

Statistical differences between controls and the various treatments were determined for each bioassay using Dunnett's procedure (Steel and Torrie, 1960) with an OSL $\leq$ 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.

CHAPTER IV

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 II) 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; this discrepancy is discussed under Experiment B: Circadian rhythms and phototaxis).

A mean response ($\bar{x}$) of 75% or greater was achieved in 80% of the experiments. A majority of those experiments had a standard deviation (S.D.) of less than 10.00. These figures, $\bar{x} \geq 75\%$ and S.D. ≤ 10.00, were considered to represent the expected normal response of non-exposed animals.

Experiments to determine the optimal light intensities and effects of circadian rhythms were included in this phase of the research. The following results were obtained.

TABLE II

THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.)
OF NON-EXPOSURE PHOTOTAXIS EXPERIMENTS
PERFORMED 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

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 III and IV). 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 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: Circadian Rhythms and Phototaxis

Of seven phototaxis experiments performed between 800 h and 2200 h of one day (see Appendix for raw data) there was no significant difference among the mean response rates (Duncan's multiple-range, OSL = 0.05). A diurnal pattern of response, however, is apparant in the graphical presentation of the data (Figure 2). Phototactic responses were higher in the morning (800 h and 1000 h) and evening (2030 h and 2200 h) than at mid-day (1200 h to 1630 h). The mid-day experiments also had significantly greater variations in response rates among replicates. The exceedingly high standard deviation at 1200 h can be attributed to a single replicate of the five observed at that time deviating substantially in percent response from the other four. Whether this deviant population represents a real variation present among animals during mid-day or is a result of some extraneous source of experimental error

TABLE III

THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.) OF 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 IV

THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.) OF 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

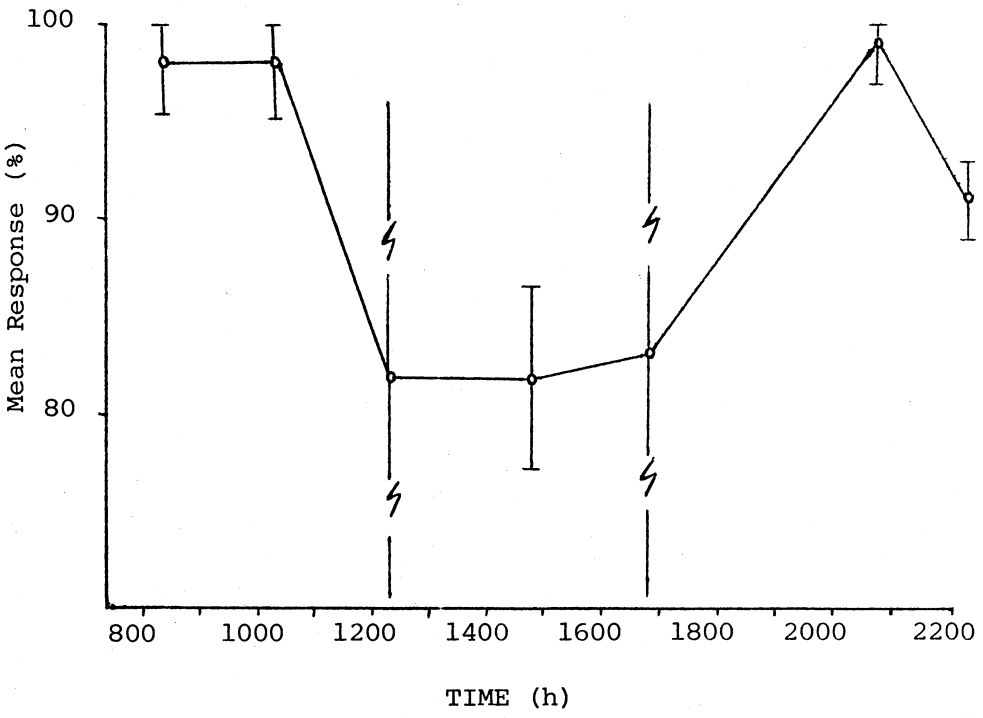

Figure 2. The mean response of Daphnia populations to a light stimulus between 800h and 2200h of one day

(e.g. contaminated test tubes) is difficult to determine. The latter case seems more likely. If the phenomenon of increased variability in behavioral patterns among animals during mid-day hours is real, one would expect a certain proportion of each test tube population to react uncharacteristically, rather than a whole subpopulation (one test tube population) reacting differently from four other subpopulations. Thus a decrease in the mean response of the five replicates would be expected rather than an increase in standard deviation. The point at 1430 h represents such a case. The point at 1630 h represents both a lower mean and a higher standard deviation (not attributable to a single replicate). Thus variations in reactivity of populations may be a realistic occurrence.

Natural populations of Daphnids generally avoid intense radiation during mid-day hours by migrating downward in the water column. Attempting to elicit negative phototactic responses at mid-day, then, would not conflict with this natural tendency.

In the early stages of this research, experiments were generally performed between 1300 h and 1600 h with satisfactory results. Because several hours are required for experimental preparations and exposure periods, it is convenient to run experiments during early afternoon hours. The analysis of circadian patterns of response in the laboratory needs to be further investigated. It may prove to be advantageous to alter the laboratory photoperiod to obtain animals in their most reactive state during convenient laboratory hours.

The second phase of non-exposure research, experiments C - F, determined the effects of a number of variables that influence the phototactic behavior of Daphnia magna.

Experiment C: Role of Intrinsic Annual Rhythms

The average monthly responses (the mean of all experiments performed each month) over a period of one year, indicate that a distinct change in behavior occurred (Figure 3). In the summer and early fall, around 80% of any population of Daphnia responded negatively to light. In late fall, and through the winter months, fewer and fewer animals responded negatively to the light. During that period observations of reverse responses (positive phototaxis) or no response (Daphnia alive, but remaining at the lower end of the tube regardless of the direction of light) became more and more frequent. Data points are missing for some months because many experiments in which responses were far below the normal range established previously were terminated or not recorded, and attempts were being made to increase responses by manipulation of various experimental parameters. Data from experiments in which the technique deviated from the basic format outlined in "Procedures" was not included in Figure 3.

In early spring and summer of the second year of experimentation population responses appeared to be in an upward trend. Response levels similar to those of the previous summer were never achieved, though a few unrecorded observations in early July gave strong indications that a complete cycle may have been attained, with response levels around 80% again. Time did not allow for continuation of this aspect of phototactic behavior.

Assuming that there exists an annual cycle of phototactic behavior in Daphnia, it is worthwhile to speculate as to whether it is an intrinsic characteristic or environmentally cued. The feasibility of using phototaxis as a bioassay technique may depend on whether conditions

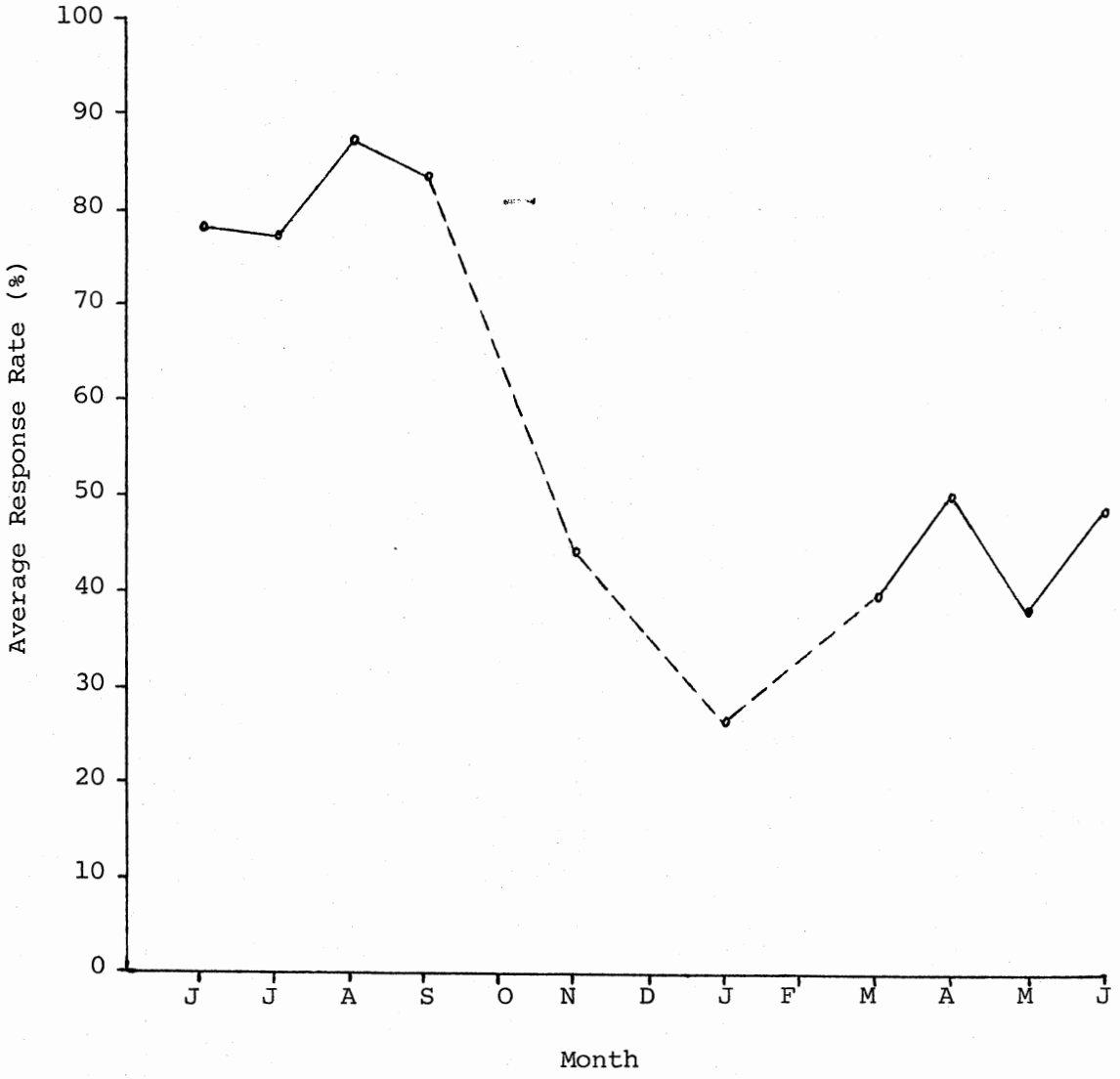

Figure 3. Monthly averages of phototaxis experiments over a period of one year

can be manipulated to produce consistent responses year-round.

The photoperiod in the lab was maintained on a 16 h light:8 h dark cycle throughout the year. Laboratory windows covered only by blinds, however, may have allowed enough light through to be perceived by the Daphnia. Thus, the effects of a change in photoperiod cannot be entirely eliminated as a factor influencing the behavior of the animals.

Air temperatures in the laboratory varied between 20° C in the winter, and 24° C (and occasionally warmer) in the summer. Daphnia have been shown to respond to light stimuli differently at different temperatures. Clarke (1931) and Baylor and Smith (1957) showed that rapid temperature changes caused a reversal of phototactic responses. McNaught and Hasler's (1964) data suggest that migrations are more rapid at higher temperatures, and Cunningham (1972) found that migrations under ice were minimal or reversed. The small temperature changes that occurred over long periods of time during the course of this research were not at first considered to be great enough to significantly influence phototactic behavior. A closer look at the data did, however, reveal a significant relationship (see Experiment F: Effects of Temperature).

Intrinsic rhythms have still not been entirely ruled out. Though Daphnia have a short life span, several months at best, the concept of annual rhythms in this animal cannot be disregarded since parthenogenic reproduction provides a vehicle for passing on the required genetic information intact to an indefinite number of succeeding generations. It is conceivable, though, that intrinsic rhythms are closely linked to environmental cues which take precedence once they are perceived.

Harris (1963) for example demonstrated that the diurnal migrations of Daphnia may be initiated by endogenous rhythms, but are governed by light intensity as soon as that cue is available to them. Though it would be desirable to continue the analysis of annual rhythms for another year or two, it appears likely that for the purposes of the bioassay, environmental conditions can be manipulated to elicit responses that might normally be suppressed by intrinsic mechanisms. The remainder of the non-exposure investigations explored a number of environmental conditions that may affect phototactic behavior.

Experiment D: Effects of Food Deprivation

Using the normal culture methods described, the Daphnia cultures probably did not suffer any effects from food deprivation. However, since food requirements for the cultures were subjectively determined, based on the clarity of the culture medium, this experiment was designed to objectively determine whether any effects on phototactic responses can occur as a result of food deprivation of up to four days.

Daphnia cultured in 1-liter beakers for the five days of controlled feeding, generally had a low mortality rate ($\leq$ 10%), and the animals in all treatments were active and reproducing. The results of the phototactic experiments using these animals were highly variable (Table V). In trials 1 and 4 where responses were below 25% among controls (zero days without food), there was no significant difference among treatments. In trials 2 and 3 animals fed every day did significantly better than those deprived of food for three or four days (Duncan's multiple-range, OSL = 0.05). The results of trials 2 and 3 suggest that if the responses of control animals are sufficiently high (in this case

TABLE V

THE EFFECTS OF FOOD DEPRIVATION ON THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.) OF PHOTOTAXIS EXPERIMENTS

Trial	Daphnia Culture		No. Days Without Food 0	2	3	4
1	A	$\bar{X}$	23.2%	23.0%	28.0%	31.0%
		S.D.	4.31	10.78	7.59	13.48
2	B	$\bar{X}$	61.0%	61.0%	42.6%	42.0%
		S.D.	15.17	8.94	11.62	6.71
3	A	$\bar{X}$	46.0%	42.4%	30.2%	25.7%
		S.D.	8.22	8.29	16.87	13.70
4	B	$\bar{X}$	22.4%	25.4%	24.2%	27.0%
		S.D.	14.77	8.70	11.23	4.47

at least 46%), then a significant effect due to food deprivation will be detected.

These experiments were performed during the second phase of research when phototactic responses in general were highly variable compared to those recorded during the first phase. Based on the results, it did not appear that food deprivation was the primary cause of this overall variability. However, trials 2 and 3 provide some evidence that more than three days without food may significantly affect the ability of the animals to respond to the stimulus. To eliminate this potential source of variability, Daphnia cultures were provided with additional food approximately 24 h prior to all subsequent experiments.

Experiment E: Effects of Culture Technique and Use of Specific Age Classes

The method used for culturing the Daphnia provided large numbers of animals in various stages of development. Adults required for the phototactic experiments were easily separated from neonates either by careful pipetting techniques or by using a 1/16" mesh net held under the water surface to allow neonates to pass through while adults remained trapped within. While these procedures provided reasonably uniform experimental populations, some variation in size (which generally reflects age) could not be avoided, and the age of the animals was not known.

Using Frear and Boyd's (1967) rearing techniques to reduce this source of variation proved to be less than successful. To obtain adult animals of observable size, at least eight to ten days of rearing in gallon containers were required. No aeration was provided because

it caused too much turbulence for the animals to maintain their orientation. Thus water quality was not consistent day to day and probably deteriorated considerably by the end of the rearing period. This factor was compounded with greater densities. Rearing densities, however, had variable effects on phototactic responses and no clear relationship was discernable (Table VI).

Qualitative observations of Daphnia raised under controlled conditions indicated that variations in cultures were not readily eliminated. Size differences were apparent within and between cultures of the same age. Ephippial eggs were produced abundantly in some culture jars and not in others. These variables did not appear to be related to population densities.

Phototactic responses using Daphnia of known age were variable (Table VI). In general, response rates comparable to earlier experiments with Daphnia from the mass rearing cultures were not attained. In three different experiments comparing phototactic responses of Daphnia from controlled cultures and mass cultures (Table VII), only one showed a significant difference.

Clearly the rearing procedures required much more time and exact monitoring to prove useful. Considering the extra time, effort, and space required and the variable results obtained with Daphnia from the jar cultures, the original mass culturing method was decidedly more favorable. Provided food and space are not limiting, the culture techniques and the absolute age of the animals probably have a lesser affect on phototactic respones than other environmental variables (such as temperature).

TABLE VI

THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.)
OF PHOTOTAXIS EXPERIMENTS WITH
DAPHNIA OF KNOWN AGE

Date of Experiment	Daphnia Age (days)	$\bar{X}$	S.D.	Density of culture (neonates per gal.)
Nov 12, 80	7	59%	21.33	unknown
	9	54%	5.48	
Nov 20, 80	9	92%	10.37	unknown
Jan 12, 81	7-9	0	-	100
Mar 2, 81	9	21%	16.55	unknown
Mar 9, 81	10-11	71%	13.42	175
	11-12	96%	5.48	
Mar 11, 81	8-10	30%	7.07	unknown
Mar 17, 81	10	58%	12.55	200
Mar 20, 81	12	65%	5.93	200
	12	52%	35.11	200
Mar 23, 81	12	63%	9.08	120
	14	47%	15.25	120
Mar 25, 81	10	49%	15.17	unknown

TABLE VII

THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.) OF PHOTOTAXIS EXPERIMENTS USING DAPHNIA FROM CONTROLLED CULTURES VS MASS CULTURES

Date		Controlled Culture	Mass Cultures A	B	C
Nov 20, 80	$\bar{X}$	92%	44%	-	-
	S.D.	10.37	21.62		
Mar 2, 81	$\bar{X}$	21%	34%	-	-
	S.D.	16.55	11.94		
Mar 3, 81	$\bar{X}$	58%	63%	54%	22%
	S.D.	12.55	33.07	9.62	7.58

Experiment F: 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 (Figure 4, R = .88). A similar relationship was found for experimental temperatures (room temperature) and mean responses (R = .84), which would be expected since culture temperatures were consistently 1° to 2° C lower than room temperature due 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 - - (1) warming or cooling experimental water temperatures during the 2 h pre-test period, (2) raising the temperature of the culture water only, and (3) 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 h pre-test period. In general,

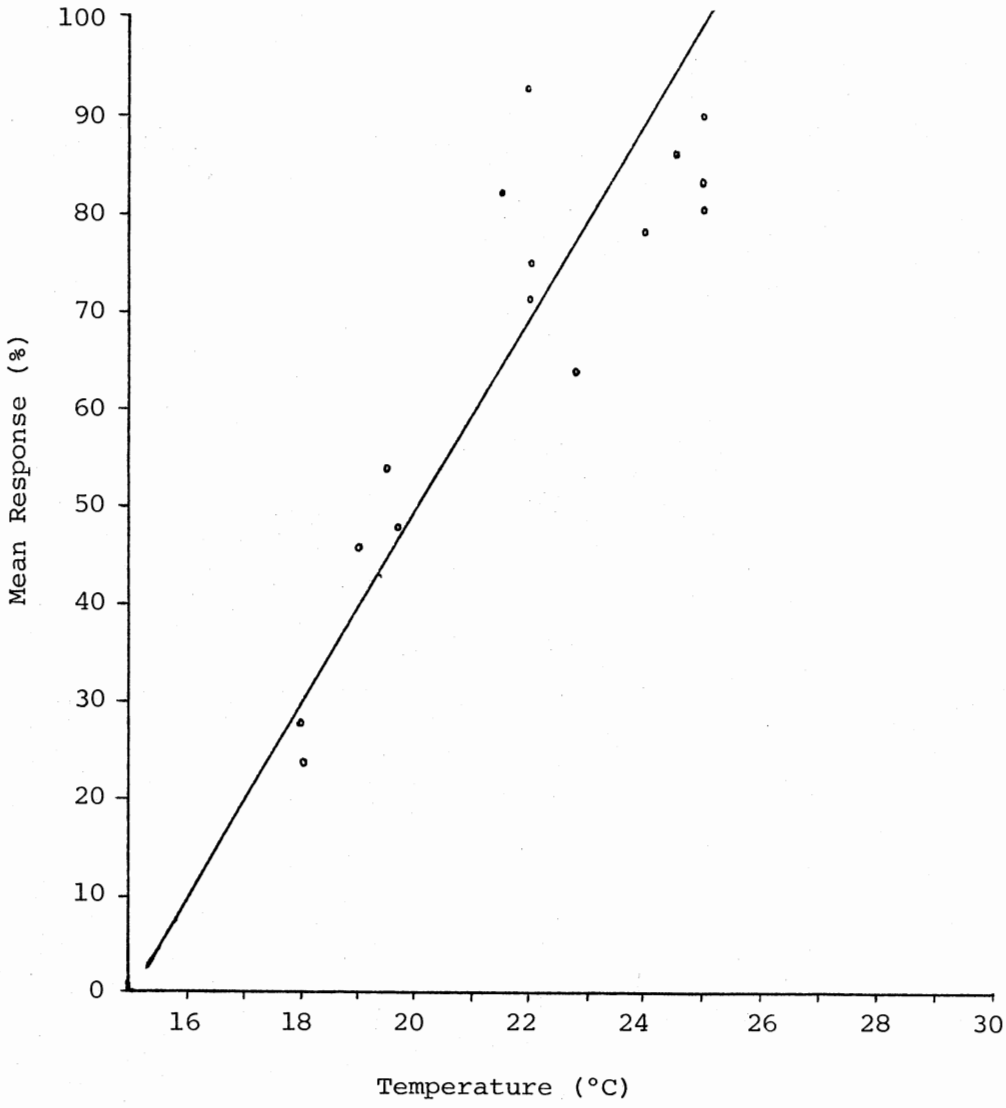

Figure 4. Effects of culture water temperature on the mean response of phototaxis experiments

average response rates were very low, ranging between 21% and 57%, and variation within treatments and between days was very high (Table VIII). There was no clear evidence that short-term changes in experimental temperatures influenced phototactic behavior consistently one way or the other.

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 temperature in the test tubes at about the same temperature as the heated culture water during the 2 h pre-test period (Table IX). 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

TABLE VIII

EFFECTS OF VARYING EXPERIMENTAL WATER TEMPERATURE IN PHOTOTAXIS EXPERIMENTS

Date		Cool 15°C	Room Temperature 20°C - 22°C		Warm 25°C
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 IX

EFFECTS OF INCREASING CULTURE WATER AND EXPERIMENTAL WATER TEMPERATURES ON MEAN RESPONSES OF PHOTOTAXIS EXPERIMENTS

	Culture A 25° - 26°C		Culture B 21° - 23°C	
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

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 (Figure 5, R = -.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 X).

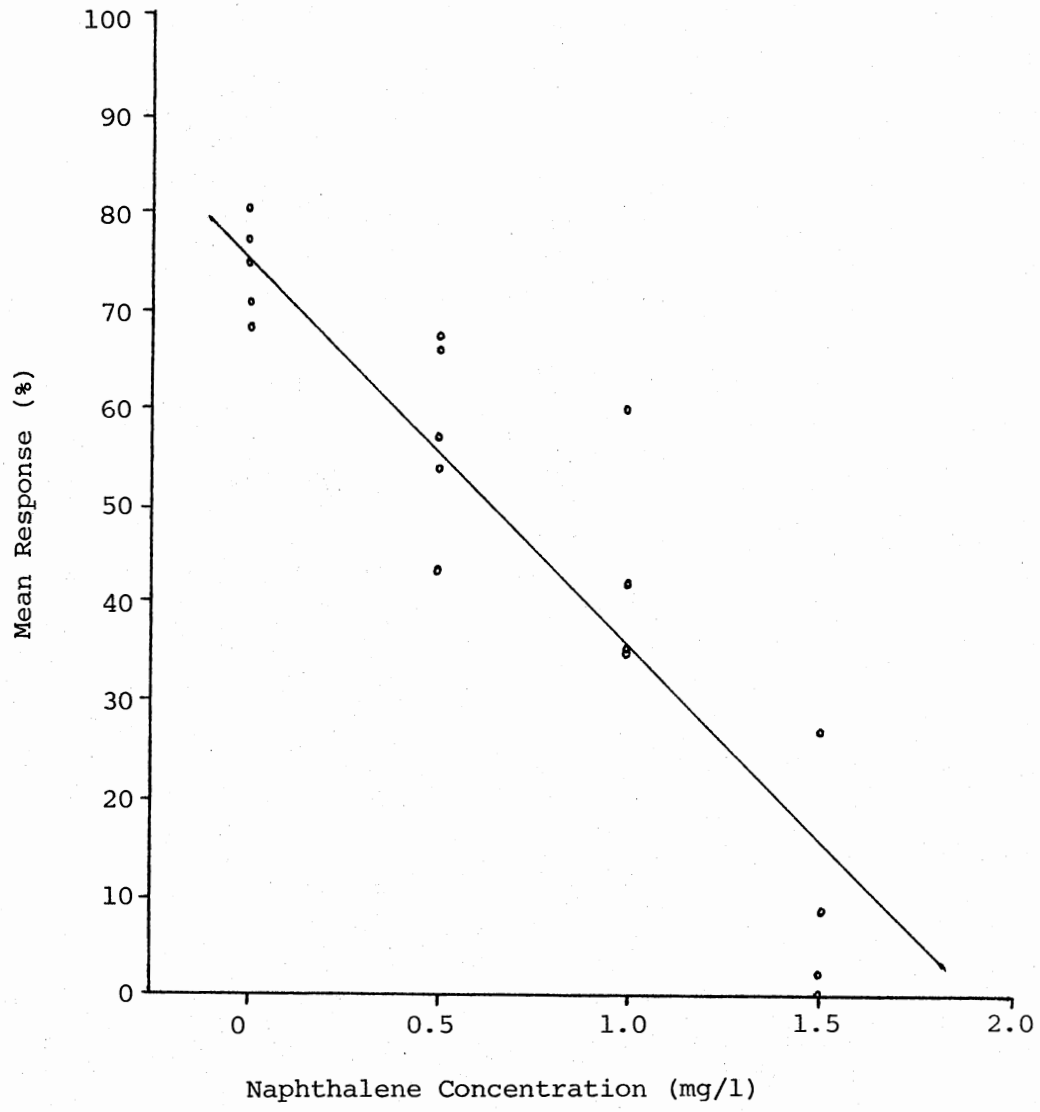

Figure 5. Effects of naphthalene on phototactic responses of Daphnia in July, 1980 experiments

TABLE X

THE MEAN RESPONSE ($\bar{X}$) AND STANDARD DEVIATION (S.D.) OF DAPHNIA POPULATIONS EXPOSED TO 0.5 mg/l TO 2.0 mg/l NAPHTHALENE

		Naphthalene Concentration (mg/l)				
Date		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	

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 (Figure 6, $R = -.70$), though it was not as strong a correlation as in the previous year. Mean responses among controls averaged 60%. Using Dunnett's procedure ($OSL \leq 0.05$) 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 X).

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 XI). The quantity of ethanol used in the naphthalene dilutions probably did not have any detrimental effects on

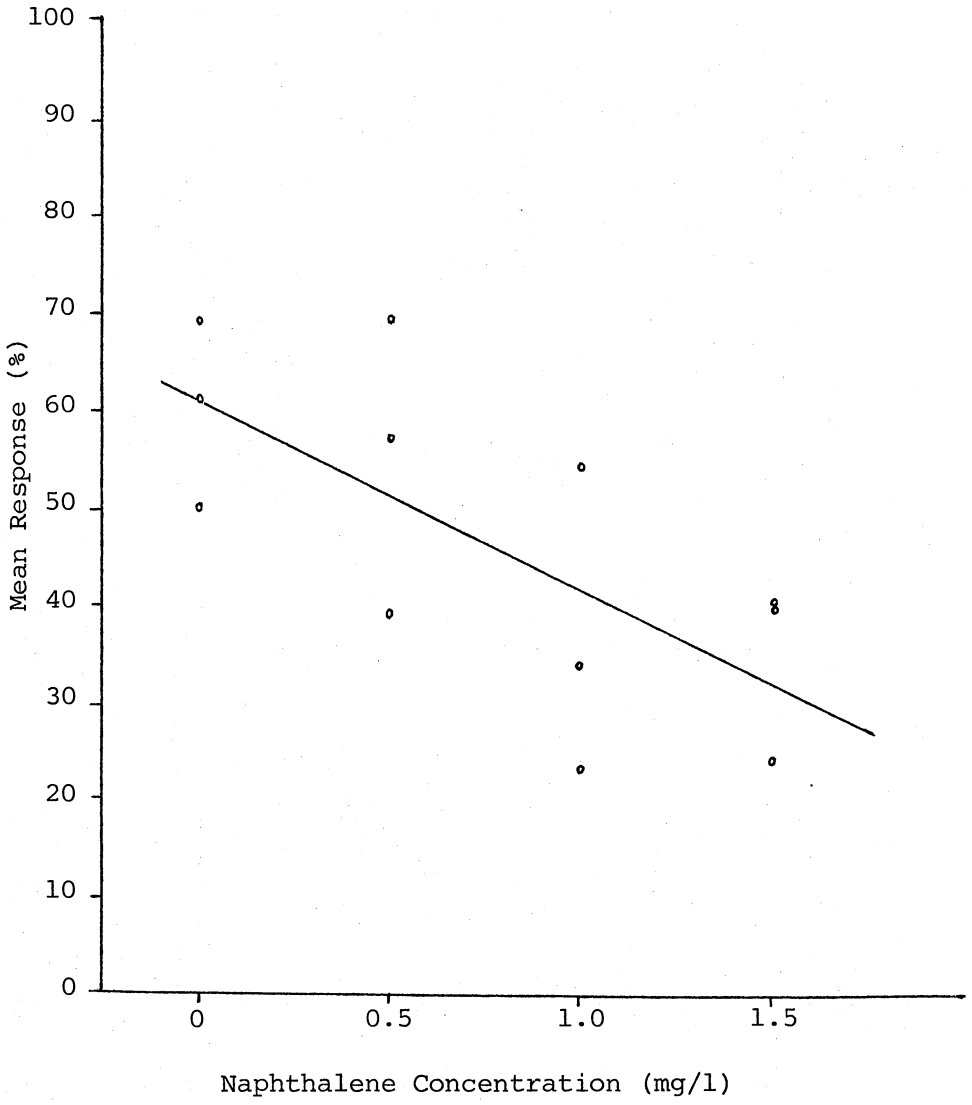

Figure 6. Effects of naphthalene on phototactic responses of Daphnia in June-July, 1981 experiments

TABLE XI

THE 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

phototactic responses of the Daphnia.

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.

Flourescence analysis of naphthalene present in solution at the termination of each bioassay showed that concentrations were reduced slightly during the course of the experiments (Table XII). In general, the proportion of initial naphthalene concentrations 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.

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 LC50 values for the same animal. LeBlanc (1980) determined 24 h and 48 h LC50's 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. 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 LC50 data was determined by LeBlanc (1980) with neonates less than 24 h old which are generally more sensitive to

TABLE XII

FINAL NAPHTHALENE CONCENTRATIONS DETERMINED BY FLOURESCENCE SPECTROSCOPY FOLLOWING A BIOASSAY

	Initial concentrations of Naphthalene (mg/l)		
Date	0.5	1.0	1.5
14 Jan 81	.4764	.9557	1.1811
20 Jan 81	.5466	.8916	-
11 Mar 81	.4897	.7771	-
29 Jun 81	.2883	.5917	.9083
30 Jun 81	.4076	.8155	1.3043
1 Jul 81	.3360	.6856	1.0755

toxicants than adults (Anderson, 1942). The phototaxis bioassay, which measures toxicity to adult animals appears then 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 h 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 h period of exposure to naphthalene.

The exposure period was determined specifically for naphthalene, however, based on a study of bioconcentration potentials over time (South et al, 1978). 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 (Southworth et al, 1978). 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.

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). Since 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 (1978)

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.

Based on toxicity tests for a number of different organisms, including Cladocerans, the United States Environmental Protection Agency (1980b) 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 between 0.5 mg/l and 1.0 mg/l, and were completely inhibited at 2.0 mg/l. This demonstrates that 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. Photo-

taxis bioassays may provide the speed and simplicity required to screen numerous potential pollutants for harmful effects.

CHAPTER V

SUMMARY

A method of eliciting and measuring phototactic responses of Daphnia magna was developed in order to determine the effects of naphthalene on the animal's behavior. Twenty adult Daphnia were observed in a large test tube inside a black box with a bright light placed equidistant above and below the tube. With the overhead light on alone the Daphnia were driven to the bottom of the tube. When the light underneath the tube was turned on and the one overhead turned off simultaneously, the Daphnia moved rapidly up the water column. The number of animals that swam at least halfway up the tube within 30 seconds was recorded. Each experiment, or treatment, had five replicates (test tubes with 20 Daphnia per tube). The results were converted to percentages and presented as the mean percent response of the five replicates.

In 38 control experiments a mean response of 75% or greater was observed in 80% of the experiments. This was determined to be the expected normal response of healthy animals.

A light intensity of at least 250 foot-candles maximized responses. Significantly fewer animals responded when the light stimulus was less than 250 foot-candles.

There was no significant difference among mean responses of experiments performed at different times of the day, but there was more

variation among replicates during mid-day than during either morning or evening hours.

Mean phototactic responses were higher and more consistent during summer and fall months than during winter and early spring months. It was postulated that annual rhythms in phototactic behavior may occur in response to changing photoperiods and temperatures. Intrinsic rhythms cannot as yet be ruled out as a potential source of variation in phototactic behavior.

Phototactic responses of Daphnia that have not been fed for three or more days may be significantly depressed. Experimental animals should be adequately supplied with food prior to the experiments.

Modification of culture techniques to reduce variation in culture conditions and produce animals of equal age for experiments did not reduce variation in phototaxis experiments. Though adults from mass cultures varied in age and size, their phototactic behavior was similar. Thus the mass culture method used was considered adequate for these experiments.

A significant relationship was found between water temperatures and phototactic responses. By regression, 25° C was determined to be the optimum temperature for maximum responses. Both the culture water and experimental water should be maintained at approximately 25° C.

In naphthalene bioassays, Daphnia were exposed to naphthalene for 2 h in the test tubes prior to the phototaxis experiments. A strong inverse relationship was found between mean phototactic responses and the concentration of naphthalene in the experimental water. Phototaxis was completely inhibited following exposure to 2.0 mg/l naphthalene. Responses were significantly reduced in 1.5 mg/l for all

experiments, and in 1.0 mg/l for most experiments. Exposure to 0.5 mg/l produced variable results. Treatment effects were most clearly differentiated when the mean response of controls was at least 70%. The carrier solution, ethanol, used to increase solubility of naphthalene did not inhibit phototaxis at the concentrations required for the naphthalene dilutions.

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APPENDIX

Date: September 15, 1980

Experiment: Effects of Circadian Rhythms on Phototaxis

Test Tube	Time of Day (h) 800	1000	1200	1430	1630	2030	2200
1	20/20	19/20	19/20	17/20	20/20	20/20	19/20
2	19/20	21/21	20/21	16/19	17/20	20/20	19/20
3	18/19	20/21	6/20	18/21	19/20	20/21	20/21
4	18/18	19/19	20/20	15/20	12/20	20/20	20/20
5	20/20	20/20	18/20	16/20	15/20	20/20	19/20
$\bar{X}$	98%	98%	82%	82%	83%	99%	96%
S.D.	2.82	2.94	29.31	4.49	16.05	2.13	1.98

Date: July 15, 1980

Experiment: Naphthalene Bioassay

Test Tube	Naphthalene Conc. (mg/l) 1.5	1.0	0.5	control	Ethanol 0.075%
1	0/20	4/20	15/20	15/20	13/20
2	1/20	7/20	7/20	12/20	19/20
3	0/20	5/20	11/20	15/20	13/20
4	0/20	6/20	12/20	17/20	14/20
5	1/20	9/20	8/20	17/20	15/20
$\bar{X}$	2.4%	34.5%	53.8%	77.3%	74.7%
S.D.	3.32	7.91	15.11	7.78	11.14

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VITA

Linda Jean Whitman

Candidate for the Degree of

Master of Science

Thesis: THE PHOTOTACTIC BEHAVIOR OF DAPHNIA MAGNA AS AN INDICATOR OF CHRONIC TOXICITY

Major Field: Zoology

Biographical:

Personal Data: Born in Neenah, Wisconsin, January 29, 1954 to Thomas and Dorothy Gardner. Married to Rick Whitman on December 23, 1978.

Education: Received Bachelor of Science degree in Zoology from the University of Wisconsin, Madison, Wisconsin in 1977; attended Oklahoma State University in spring of 1979, and enrolled in masters program in summer of 1979; completed requirements for the Master of Science degree at Oklahoma State University in December, 1981.

Professional Experience: Laboratory technician in the Laboratory of Limnology at the University of Wisconsin, 1976-1977. Field research assistant for Salmon Research Project in Two Rivers, Wisconsin in the fall of 1977. Technician for the Oklahoma Cooperative Fishery Research Unit, 1979. Research Assistant at Oklahoma State University, 1979-1981.