A SHORT-TERM BEHAVIORAL BIOASSAY FOR ACUTE TOXICITY OF WATER-BORNE POLLUTANTS

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Submitted to the Faculty of the Graduate College
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in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1984

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PREFACE

The purpose of this study has been to develop a rapid and sensitive method for detecting toxic conditions in fresh water systems. The completed test, which can be performed within as little as two hours, allows more sensitive detection of toxic conditions than is now obtained with standard 24- or 48-hour lethal bioassays. Financial support has been provided by the National Wildlife Federation, American Petroleum Institute, and an O. S. U. Presidential Challenge Grant.

I wish to express my gratitude to thesis advisor, Dr. Rudolph Miller for his guidance, encouragement, and exemplification of scholarly thought throughout the course of the study. I also thank Dr. Sterling Burks, Dr. Anthony Echelle, and Dr. William Warde for their guidance as committee members, and especially Dr. Margaret Essenberg for her encouragement and guidance. I thank Dr. E. C. Nelson for participating in the final examination in Dr. Essenberg's absence.

I extend my gratitude to my fellow students and office mates for their camaraderie and stimulating exchange of ideas.

I express my deep appreciation to my parents, my mother for exemplifying integrity of character, and my father for his demonstration of true scholarship.

Especially I thank the many past teachers and friends who have communicated in their various ways the primacy of the pursuit of truth, whether it be scientific, historical or poetic.

Finally I wish to express my greatest debt to my wife Mida, my most steadfast friend. Amo, lloro, canto, sueno, siempre contigo.

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CHAPTER I

INTRODUCTION

The proliferation of chemical substances, while imparting many benefits to our society, has also created many problems associated with their production, use and disposal. In certain instances the dangers of a chemical may become obvious during development or early use and may be of such a magnitude that the decision to discontinue production is the only reasonable option. In other cases the use of the substance will become widespread before the deleterious effects are realized, leading to such disasters as the thalidomide tragedy and the ubiquity of such compounds as DDT and PCBs in the environment. An important difference exists between protochemicals (naturally occurring chemicals) and allochemicals (those produced only by man). Natural selection produced mechanisms which minimize the potentially harmful effects of protochemicals.

Butler (1978) estimated that over 30,000 chemical substances were in use in the world and that the figure was increasing by 1,000 to 2,000 per year. Of these substances, 1500 are estimated to be produced in excess of 500 metric tons per year, and 50 are estimated to be produced in excess of 1,000,000 metric tons per year. One million metric tons spread over the earth's land surface would average only 6.8 mg m^{-2}. However natural and anthropogenic mechanisms cause local concentrations

to be much higher, creating serious toxic problems in many cases. In other instances, protochemicals can be concentrated locally in air, soil or water as a result of man's activities, causing problems which were unknown at normal background levels in nature. In still other cases (e.g., the petroleum industry) the composition of waste products may be extremely complex and/or relatively uncharacterized, and they may vary widely over time, causing major problems in the evaluation of their toxicities (Horning, 1977).

Extensive knowledge of the effects of some toxicants on man has resulted from pharmacological and toxicological studies. Much less is known about the environmental transport and impacts of chemical substances on wildlife. Although concern for wildlife has been limited in the past, biologists now recognize the intimate connection between man and the rest of the biosphere and are concerned about significant disturbances to the ecosystem. Although accidental spills release chemicals to the environment, the principal means by which an ecosystem receives most contaminants (excluding biocides) is through disposal of used chemicals or waste products. Accidental spills draw the public's attention and concern, but the chronic, low-level exposures associated with normal waste disposal practices have a far greater impact on the environment (Butler, 1978). This is especially true for chemicals which tend to bioaccumulate. The aquatic environment ultimately receives the majority of the chemical wastes generated by man's activities, either directly by point source effluents, or indirectly through precipitation and runoff. Aquatic systems can assimilate wastes due to inherent chemical, physical and biological mechanisms, but when the assimilative capacity is exceeded, various components of the biota suffer adverse

effects, upsetting the structure and functioning of the ecosystem.

The comprehensive testing now employed by industry attempts to detect adverse effects of a chemical prior to its adoption for widespread use. Thus the primary role of the toxicologist is to predict impacts of chemical agents, based on extrapolation from limited knowledge of properties or from small, short-term experiments. The methods employed in toxicity tests can employ physicochemical analyses, biological assays, or combinations of both. No single criterion is adequate for the evaluation of toxicity, and there is little standardization of methods for the screening of wastes. Almost all indices of toxicity, however, require time-consuming tests which are inadequate when rapid decisions must be made prior to an effluent's discharge to the environment.

My purpose in the present study was to develop a rapid and sensitive screening method to detect potential toxic effects of industrial effluents before they are released to the receiving stream. Inhibition of the normal light-avoiding behavior of the easily-cultured aquatic crustacean Daphnia magna is used as an indication of toxic effect. This type of monitoring is not intended to define the toxicity of an effluent, but to detect effluents which show the potential for creating toxic conditions if released to the environment. More rigorous (and time consuming) biological and chemical analyses then could be used to evaluate more accurately the effluent's toxicity.

CHAPTER II

REVIEW OF THE LITERATURE

Biological Monitoring

Identification of the components of a mixture through chemical analyses, and reference to the toxicological literature on the compounds allow some assessment of an effluent's toxicity. However, these methods yield limited predictive information on the environmental consequences of waste discharge. Synergistic, antagonistic and novel interactions between the many components of a complex mixture render chemical analyses alone insufficient to adequately characterize the toxicological properties of many effluents. Other physicochemical factors such as pH, temperature, dissolved oxygen content and water hardness greatly affect the expression of toxicity (Cairns and Van der Schalie, 1980).

A more direct approach has been to use biological monitoring to detect the presence of harmful substances and to predict their effects on the biota. Organisms which respond consistently to toxicity-induced stress facilitate more reliable estimation of the impacts of pollution on entire ecosystems.

Among the various types of biological testing, measurements of reproductive inhibition often are considered the most informative, as they measure the process perhaps most vital to a gene-pool, and are affected by levels of toxicants which would not be detectable in acute tests (Sprague, 1976). Unfortunately, such tests, even with short-lived

species, require one to several weeks before the results are obtained. Because of the need for more rapid results, short-term acute bioassays are the most widely used technique. Thus, there is a great need for rapid, accurate tests (Horning, 1977).

Chemically and biologically induced changes in the characteristics of an effluent, and volatilization of toxic components necessitate rapid determinations of toxicity (Henderson and Tarzwell, 1957), and Cairns et al. (1977) discussed the need for miniaturized, in-plant monitoring. Characteristics of an effective early warning system include tests of short duration which require minimal quantities of toxic material and employ organisms for which extensive toxicological data exist (Gehrs, 1978). The organisms must be inexpensive, easily acquired, and ideally the system should be amenable to automation (Cairns and Van der Schalie, 1980). Ladd (1977) reported industries' desire for rapid, simplified, technician-level tests.

Types of Biological Monitoring in Use

The majority of industrial biological monitoring has been performed by outside consulting groups (Ladd, 1977). While static, acute testing can be performed at laboratories distant from effluent sources, the plant site is the only practical place to conduct flow-through tests which constantly monitor effluent quality.

Bioassays currently are performed with a diverse range of organisms, and at various levels of integration. Although investigations of such proximate factors as blood parameters and enzyme systems are suitable for determining mechanisms of effect, the significance of pollutant effects is best determined at higher levels of integration

(Sprague, 1976).

Bacteria frequently are utilized in biological monitoring. Probably the most common technique involves measuring respiration (oxygen consumption) differences between influent and effluent of a test chamber. A reduction in the difference between the two levels indicates toxic inhibition of bacterial respiration. With a similar system, Marouka (1978) utilized cultured mammalian cells for estimating the toxicity of organic pollutants in aquatic systems.

Many animal species have been used in biological monitoring. A few examples are: benthic macroinvertebrates (Burks and Wilhm, 1977), snails (Canton and Sloff, 1972), oysters (Conger et al., 1978), grass shrimp (Hall, Buikema and Cairns, 1978), and sand dollar (Nicol et al. 1977). Fish are widely used as bioassay species. Irwin (1965) evaluated 57 species of fish in an oil refinery waste bioassay.

Dehnert (1979) and Matthews and Myers (1976) have used bioassays with fish to evaluate toxicities of refinery wastewaters after various treatment processes. Dorris, Burks and Waller (1974) used Daphnia in bioassays of refinery wastewater, and found that volatile fractions were much more toxic than the nonvolatile fractions.

Tests of lethality have been the mainstay of toxicity testing in the past, but more emphasis now is being placed on non-lethal measurements. Experiments on reproduction in various organisms yield easily interpreted results. While reproductive tests with fish are lengthy, such tests with short-lived organisms such as Daphnia and algae yield good data in a more reasonable amount of time.

Cowell (1974) divides toxicity tests into two main categories: those performed to produce comparative rankings of toxic materials, and

those from which ecological predictions can be made. The literature on the toxicity of various substances to fish is extensive, including reviews by Ellis (1937), Doudoroff and Katz (1950), and Doudoroff and Katz (1953). Although some research (Wilson, 1974; Baker and Crapp, 1974) suggests that toxicity ranking of various substances does not differ significantly with different test organisms, the toxicological literature for any particular chemical will show a wide range of toxicities obtained by different investigators using different test species. Thus, the use of a single species for all bioassay work to be critically compared is preferable.

One factor that increases variability of test results involves variation in laboratory methods, such as whether or not test tanks are aerated, as aeration can cause changes in the toxicants. A 2-hour Daphnia behavioral assay would not require the aeration needed for a long-term reproductive assay with fish.

Behavioral Assays

Effects of pollution need not be lethal to an organism in order to be detrimental to natural systems. Sublethal effects, such as impaired reproductive capability or behavioral changes, can markedly upset the normal functioning of the aquatic community.

The behavior of an animal is one of many factors, including physiological, histological or metabolic parameters, which can be considered in a toxicity assay. However, as Scherer (1977) pointed out, a behavioral response is based on the integration of potentially all underlying physiological events, and changes or impairments that might be overlooked or difficult to measure at biochemical or physiological

levels can manifest themselves in behavioral output.

Intra- and interspecific interactions depend on perhaps the highest levels of integration, but are more difficult to standardize to reproducible laboratory procedures than are 'simple reflexes' (Scherer, 1977). Preference/avoidance responses of animals to toxicants have been a popular behavioral test, but an animal cannot be expected to avoid a man-made xenobiotic which has not been present throughout the evolutionary development of preference/avoidance reactions (e.g., man's inability to detect carbon monoxide). However, changes in preference/avoidance reactions to non-toxic agents such as temperature or light potentially provide useful indicators of toxicity.

Locomotion is critical to non-sedentary, aquatic animals for obtaining food, escaping predators and maintaining position against a current or against gravity. Swimming represents the integration of several important factors, including sensory perception and muscular activity. Scherer (1977) emphasizes locomotor behavior, including free and forced activity, drift, and preference/avoidance responses as being suitable for biological assays. For example, rheotaxis of fish has been used for bioassay purposes (Besch et al., 1976).

Various species of zooplankton were found to undergo daily vertical migrations by Weismann (1877) (cited in Hutchinson, 1967). Since that time, extensive work has been done on this subject (Cushing, 1951). Because these behaviors have adaptive significance, a disturbance causing the behavior to be altered will disrupt the normal functioning of the animal with deleterious results to its fitness and often to the ecosystem (Scherer, 1977). Vertical migration in Daphnia is dependent on the ability to perceive changes in light intensity and wavelength,

and on locomotor abilities.

Simonet et al. (1978) used inhibition of negative phototaxis of mosquito larvae as a measure of toxicity. The criterion of toxic effect was the inability of the larvae to migrate 30 cm in 60 seconds after 8 hours of exposure. Sheffrin et al. (1984) used the crawling and attachment behavior of plantigrades of the common mussel Mytilus edulis in tests of copper-contaminated sea water. Bruins et al. (1981) used negative phototaxis of Daphnia magna to measure copper stress and found that the phototactic response was significantly inhibited in animals exposed to 10 ug 1-1 Cu. By comparison, reproductive assays showed that chronic survivorship was reduced only at concentrations above 20 ug 1-1. Four species of freshwater zooplankton, Cyclops bicuspidatus, Diaptomus sicilis, Daphnia galeata, and Daphnia magna were used by Wilson (1980) in phototactic assays. He found that phototaxis is more sensitive than reproductive impairment when assaying the effects of lead and cadmium. These studies indicated that the hypolimnetic Diaptomus sicilis is more sensitive than the epilimnetic animals. Jones (1971) also utilized changes in phototactic responses, as well as heart rates of micro-crustaceans as indications of toxic effect.

Although the toxicological literature of the past decade reveals the scope of behavioral responses described in relation to environmental toxicants, very few behavioral tests with common procedures have achieved widespread use.

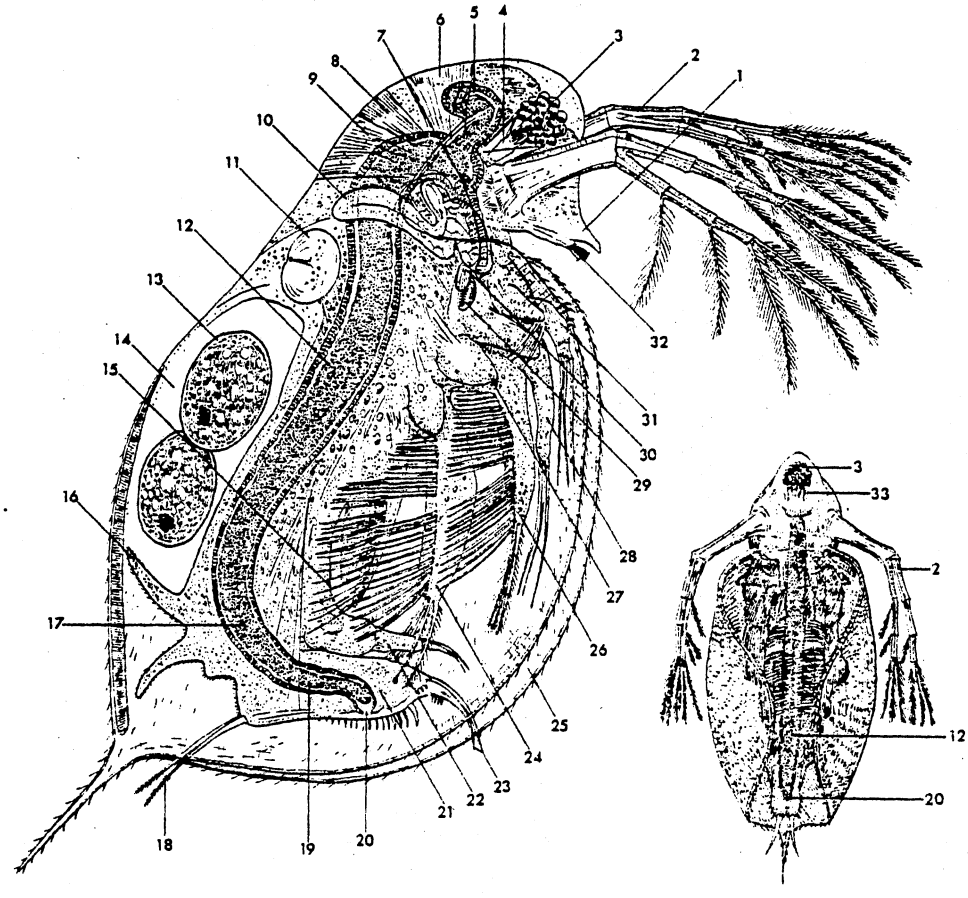
The Test Organism

The order Cladocera, to which Daphnia belongs, is one of the three major freshwater zooplankton groups and is of critical importance in the

aquatic food web. Together with the rotifers and copepods, cladocerans comprise a large proportion of the diets of both young and adult fish (Wetzel, 1975). The genus Daphnia includes approximately 30 species and is represented in most freshwater systems except rapid streams, brooks and grossly polluted waters (Hutchinson, 1967). D. magna, growing to over 3 mm in length, is the largest Daphnia species. It is typically a hardwater species and is so widely distributed as to be considered cosmopolitan.

Daphnia possesses a single compound eye which is controlled by three pairs of small muscles and is constantly moving in a rotating manner. The animals swim by movement of the very large second antennae activated by powerful muscles originating dorsally in the 'neck' region (Figure 1). Movements are usually vertical: the downstroke of the antennae propels the animal upward in short bursts, with slow sinking between antennal strokes. Complex movements of five pairs of setae-bearing, thoracic appendages produce a constant stream of water between the valves of the carapace. Algae, bacteria and other food particles filtered from the water are swept along a ventral food groove to mandibles which grind the food before ingestion. Oxygen and CO2 exchanges occur through the general body surface and through the leg surfaces and inner surfaces of the valves.

The lifespan of D. magna is highly dependent upon water temperature, and ranges from a few weeks to several months (MacArthur and Baillie, 1929). Reproduction in Daphnia is parthenogenetic under normal conditions, with only females present in the population. The eggs, after a single maturation division in the ovary, are released to the dorsally located brood chamber where they are easily visible with



1. Rostrum
2. Exopodite of antenna
3. Compound eye
4. Supraesophageal ganglion
5. Right midgut caecum
6. Antenna abductor muscle 1
7. Esophagus
8. Antenna abductor muscle 2
9. Antenna levator muscle
10. Shell gland
11. Heart
12. Intestine
13. Egg
14. Brood sac
15. Roof of food groove
16. Median dorsal process
17. Midgut, posterior portion
18. Caudal seta
19. Hindgut
20. Anus
21. Abreptor
22. Trunk appendage V, medial lobe
23. Caudal furca
24. Trunk appendage IV, endite
25. Carapace
26. Trunk appendage III, endite
27. Metepipodite
28. Trunk appendage II
29. 1st maxilla
30. Mandible
31. Trunk appendage I
32. Chemosensory setae
33. Eye muscle

Figure 1. Anatomy of Daphnia. (from Carolina Biological Supply).

the unaided eye. Young, similar in form to adults, are released approximately two days later.

Environmental variables indicative of impending uninhabitable conditions cause, by an unknown genetic mechanism, males to be produced from parthenogenetic eggs, and sexual reproduction follows. Rapid heating or chilling of the water, crowding or buildup of metabolic wastes may stimulate the change to sexual reproduction, resulting in the production of resting eggs able to withstand freezing and desiccation. In larger, more stable bodies of water reproduction may be parthenogenetic the year round.

Typical Culture Methods

As Daphnia are widely used in bioassays and other experimentation, and are commonly raised as fish food by hobbyists, it is surprising that little is known concerning their nutritional requirements (Frear and Boyd, 1967). Many laboratory culture methods have been described since Daphnia became widely used as experimental organisms. These methods range from preparations of very crude culture media described in older publications (Banta, 1921; Galtsoff et al., 1937; Hasler, 1937) to highly characterized synthetic media proposed in recent years (Freeman, 1953b; Freeman and Fowler, 1953; Frear and Boyd, 1967).

Many culture foods for Daphnia have been reported, including lettuce leaves and offal (Galtsoff et al., 1937), manure-soil infusions (Banta, 1921; Hasler, 1937), cottonseed (Chipman, 1934), wheat bran (Schluchter, 1937), and soybean meal (Viehoever, 1935). Several algae genera have been used as food for Daphnia, including Chlamydomonas (Murphy, 1970; Pacaud, in Hutchinson, 1967), and Chlorella (Tevlin,

1978). Pacaud also maintained Daphnia on a monoxenic culture of Gonium pectorale, while D'Agostino and Provasoli (1970) used a dixenic culture of Chlamydomonas and Scenedesmus.

A suspension of algae and Trout Chow fish culture food was used by Westlake et al. (1978) and several workers (Bond, 1934; Hasler, 1937; Whitman and Miller, 1982) have used dried brewer's yeast as the sole food source in Daphnia culture.

Because cladocera are very sensitive to water quality variables, the chemical characteristics of their culture water can significantly influence the general health of the animals. Frear and Boyd (1967) found laboratory-distilled water to be toxic to daphnids, probably due to metal ions from the piping used in the building. Anderson (1946) found that D. magna can survive at pH's between 6.0 and 9.5. Frear and Boyd (1967) found that a pH of 7.5 yielded the lowest mortality in cultures. They also noted that crowding of the test animals could cause a rapid shift in the pH of the culture water; 400 daphnids were able to raise the pH from 5.5 to 7.0 in 100 ml of a weakly buffered (200 ppm NaHCO3) solution in 24 hours.

Daphnia magna can survive for short periods at water temperatures as low as 0° C and as high as 39° C (Frear and Boyd, 1967) or 41° C (Brown, 1929). Whitman and Miller (1982) found that the phototactic response was greater and less variable in D. Magna cultured and tested at 25° C to 26° C than in animals cultured at lower temperatures.

Phototaxis

Numerous fresh water and marine zooplankton descend with the rising sun and ascend with declining sun, a phenomenon termed diurnal vertical migration. The magnitude of the migration may range from less than a meter in ponds to over 100 meters in the case of the copepod Calanus finmarchicus in the Atlantic Ocean (Ringelberg, 1964). Several chemical and physical factors are known to affect phototaxis. Concentration of CO_2 can affect the direction of phototaxis, as well as pH, temperature, and salinity (Loeb, 1908, in Ringleberg, 1964).

Early workers (Ewald, 1910; Rose, 1925; Russell, 1926, 1927) maintained that as long as daphnids remained in a preferred zone of light intensity their movements were in random directions. When the animals experience a light intensity above or below the optimum intensity range, they change their positions accordingly. This preferendum hypothesis has been rejected in recent years by the discovery that an essential component of the vertical migrations in Daphnia is movement toward a light source when intensity is decreased and movement away from a source of increasing light intensity (Harris and Mason, 1955). Similarly, Burris (1980) found changing light intensities to be responsible for vertical migrations of marine zooplankton.

Harris and Wolfe (1955) suggested that vertical movements are independent of the duration of the cycle and are related only to variation in overhead light intensity. They noted that in response to a photographic flash from the side, Daphnia rose towards the surface rather than in the direction of the light. They hypothesized that positive movement towards light (at least at low intensity) is purely

photokinetic in nature; the vertical rise is cued to gravity rather than the direction of the light. At higher intensities it was shown that a true positive phototaxis occurs. A change from white to blue light enhanced the photonegative movement while change from white to red light abolished the orientation reaction, leaving a response which was largely photokinetic.

Daphnids exhibit the dorsal light reaction (Radl, 1901; Ewald, 1910; von Buddenbrock, 1915). The animals swim with their dorsal side toward the light while maintaining a constant level in the water. Harris and Mason (1955) found that a change in light intensity produced a reorientation of the body axis and a consequent movement up or down in the water column (positive or negative phototaxis). Fraenkel and Gunn (1961) and Jander (1975) corroborated this observation, noting also that when not positively or negatively phototactic the daphnids always turned their dorsal surface toward the light, whatever the direction. Jander also noted that in Daphnia pulex, a species possessing an elongate eye, the vertex of the eye always faced the light, whatever the direction. Clarke (1932) found, however, that the difference in position of D. magna when positively or negatively phototactic is a result of a difference in the angle at which the antennae are held, and not a difference in the orientation of the body axis. The dorsal surface was turned toward the light under all circumstances.

More publications have addressed the spatial orientation of Daphnia than of any other crustacean genus (Jander, 1975). Studies on unrestrained daphnids have clearly shown the importance of both direction of light and of gravity for maintaining or changing body position (Schroder, 1956, in Jander, 1975). Daphnia, unlike some

crustaceans, does not possess statocysts for the detection of gravitational forces. Grosser, Baylor and Smith (1953) determined that gravity was sensed indirectly by an external mechanism, the sensation of sinking between swimming strokes due to the animal's density being greater than water. When placed in a medium with a specific gravity greater than themselves, the daphnids rose between swimming strokes, and their normal "color dances" (see below) were changed accordingly.

Harris and Mason (1955) also maintain that the light reaction is merely a photokinetic one; light stimulates movement in an unoriented position, with the upward movement being produced by the mechanical equilibrium of the body. The photokinetic response was related only to intensity and not to the direction of the light source. They maintained that at low intensities mere photokinesis (unoriented) mediates the response until a light intensity is reached where phototaxis (orientation) supervenes.

The phototactic response of Daphnia magna was shown long ago to be mediated by the compound eye (Hess, 1913; Radl, 1901; von Frisch and Kupelweiser, 1913). The question then arose as to whether photokinesis is also mediated by the compound eye. Harris and Mason (1955) have found good evidence that it is not. Viaud (cited in Harris and Mason, 1955, p.281) suggested that movement towards light is governed by a different photosensitive mechanism than is responsible for movement away from light. As proof, Harris and Mason (1955), in a delicate operation, removed the eye of D. magna and found that eyeless animals were much more sensitive to light than normal animals. They concluded that neither the compound eye nor the ocellus plays any part in photokinesis. The responsible mechanism remains unknown.

Light color is important in the life histories and ecology of cladocerans. In D. pulex moulting is stimulated and growth between molts is retarded when cultured under green wavelengths, while blue wavelengths increase the number of young per brood (Buikema, 1973).

Smith and Baylor (1953) studied the "color dances", behaviors found in many cladocerans. Under red light (>600 nm) the animals appear calm, dancing upright in the water with a small horizontal vector and a larger vertical vector, performing what is called the red dance. In the blue dance, which occurs in light of less than 500 nm, the animals are distinctly agitated and lean well forward in their dance, roaming about with a large horizontal vector. Moving the light source showed that the color dances are oriented by the direction of light and not by gravity. The largest vector in the blue dance is always oriented at right angles to the light source. The largest vector in the red dance is always oriented parallel to the light source. Under white light the dances are discrete; at any moment an individual is either red dancing or blue-dancing. Stearns (1975) corroborated the findings of Smith and Baylor, and found D. pulex to move vertically between 480 and 735 nm and horizontally at 440 nm.

The adaptive significance of the color dances lies in their function in guiding the cladocerans to areas of dense food. Since green phytoplankton filter out more of the shorter wavelengths, the total time spent red-dancing is greater in areas of phytoplankton. The small amount of horizontal movement keeps them in the good feeding areas. The blue dance, with its large amount of horizontal wandering, causes the animals to roam about to find areas of phytoplankton. Smith and Baylor demonstrated this function in the laboratory with green algae, finding

D. magna and Bosmina obtusirostris four times as dense beneath areas of phytoplankton as in clear water.

Temporal Variability in Phototaxis

Brock (1974) reported only two invertebrate species for which endogenous annual rhymicity is known. Although endogenous periodicities of any kind have been seldom described in planktonic crustaceans, Harris (1963) reported a circadian rhythm of 28 hr. in Daphnia magna, and, with a different parameter (threshold discrimination values for instantaneous decreases in light intensity) Ringelberg and Servaas (1971) found the same free-running period. Stearns (1975) also noted a circadian rhythm in D. pulex: total activity was low at night, increased to a peak in the morning, and declined through the afternoon.

Daphnia as a Test Organism

Daphnia magna is a much-used organism in aquatic toxicology, in both chronic and acute testing. The first insecticides measured by daphnid bioassay were pyrethrum (Belleuvre, 1938) and rotenone (Brown and Bell, 1943). Daphnia have been used for toxicity testing since 1929 (Buikema et al., 1976). Since then, insecticides (Wollerman and Putman, 1955; Sanders and Cope, 1966), heavy metals (Anderson, 1948), waste oil and emulsifiers (Dowden, 1962), and many other substances have been assayed. More recently Daphnia has been used in bioassays of oil refinery wastes (Dorris et al., 1974; Buikema et al., 1976).

Advantages of using Daphnia in experimentation include their small size, relatively short lifespans, and short generation time (the animals

maturing within the first week of life). They are easily cultured with various easily obtained foods. Daphnids can be raised individually or in mass cultures, and, most importantly, their parthenogenetic reproduction insures genetic uniformity within broods. Another factor making daphnids appropriate for toxicological experiments is that several species are important members of the aquatic community. The removal of daphnids from an ecosystem may greatly alter the food web and thus affect other aquatic species.

Leewangh (1978) discusses five specific applications of toxicity tests. These include screening tests to determine the acute toxicity of substances, tests to establish water quality criteria, effluent monitoring tests, legal tests and river monitoring tests. Daphnids are well-suited for all but river monitoring.

Maki (1979) compared the toxicity of several classes of test substances to D. magna with their toxicities to the fathead minnow, Pimephales promelas. Twenty-one-day chronic toxicity values for D. magna and P. promelas showed a strong correlation, indicating that daphnid bioassays could be used in place of the more bulky fish bioassays. Frear and Boyd (1967) bioassayed 56 different pesticides and found that, in all but a few cases, the relative toxicities with Daphnia were the same as reported for higher animals, i.e., the pesticides most toxic to Daphnia were the most toxic to higher animals.

Buikema et al., (1976) used D. pulex in an acute toxicity screening bioassay for oil refinery wastes discharged into fresh water. An arbitrary reference mixture was used which contained six common constituents of refinery wastewaters. Originally 15 invertebrate species and three fish species were tested, but D. pulex and D. magna were found

to be the most sensitive, inexpensive, and easy to maintain. After development, the bioassay was tested at six petroleum refineries by personnel trained in a two-day workshop. The data showed that the method was reproducible and that nonbiologist, refinery personnel could perform the bioassay.

Various sublethal tests using Daphnia have been devised or suggested. Reproductive bioassays measure the degree to which toxicants inhibit reproduction in test cultures. Willingham and Anderson (1966) suggested that differences in heartbeat in Daphnia may be valuable as an indicator of toxic effect. They also proposed the use of alteration of phototactic behavior of Daphnia and Artemia as indicators. Viehoever and Cohen (1938) noted that the normal swimming behavior of Daphnia is so characteristic that it may be an important toxicity symptom.

Canton and Adema (1978) found short-term toxicity tests with D. magna to be reasonably reproducible. Very little difference was found between the duplicates in a test. The sensitivities of D. pulex, D. cucullata and D. magna were approximately equal in short-term tests with 15 different chemicals. From these three species, D. magna was selected as the standard test organism because of its size and subsequent manageability. Furthermore, the Environmental Protection Agency chose D. magna as the invertebrate to be used in aquatic, ecological effects tests (Lentzen, 1978).

Whitman and Miller (1982) found that the phototactic behavior of D. magna could be altered by exposing the animals to various concentrations of naphthalene. A strong inverse correlation occurred between mean phototactic response and concentration of naphthalene in the test medium to which the animals were exposed for two hours prior to the test.

Effect of Naphthalene on Cellular Respiration

Polynuclear aromatic hydrocarbons generally occur in higher concentration in polluted waters. Thus these compounds may be useful as indicators of pollution (Andelman and Snodgrass, 1974).

Naphthalene is the simplest of the polycyclic hydrocarbons, consisting of two fused benzene rings. It is a common pyrolysis product of various organic compounds, and comprises a large portion of the water-soluble fraction of crude oil. Cladocerans bioaccumulate various polycyclic aromatic hydrocarbons. Southworth et al., (1978) found D. pulex, like most organisms, to concentrate these compounds in proportion to their octanol/water partition coefficients, an indication of the polarity of the compounds; Daphnia bioaccumulated naphthalene about 100-fold, while benz(a)anthracene was concentrated about 10,000-fold. Corner et al., (1976) found naphthalene to comprise 27.6% of the water-soluble fraction of crude oil.

The major site of energy transduction in eukaryotes is the mitochondrion. The generation of NADH and other electron-rich substrates in the Krebs cycle takes place in the mitochondrial matrix, and the subsequent oxidation of these substrates in the respiratory chain located in the mitochondrial membrane generates the ATP necessary for cellular metabolism. Any inhibition of these reactions will have detrimental effects on whole-cell function and on the organism.

Naphthalene inhibits the electron transport chain in mammalian cells cultured in vitro (Harmon and Sanborn, 1982) at concentrations as low as a few parts-per-million. Although Crider (1981) measured O_2 uptake in living Daphnia, the isolation of mitochondria from these

organisms has not been previously reported.

Many biological and environmental variables undoubtedly affect the rate of O_2 uptake by cladocerans. Obreshkove (1930) found that oxygen consumption decreases with the onset of senescence, and Schindler (1968) found that reproductive condition can affect oxygen uptake. Environmental variables affecting O_2 uptake are light intensity (Buikema, 1972), temperature (Obreshkove and Abramowitz, 1932), crowding (Zeiss, 1963), food type (Obreshkove and Banta, 1930), and O_2 content of water (Schindler, 1968).

Measurement of oxygen uptake in cladocerans has been determined by the micro Winkler technique (Zeiss, 1963), by respirometers (Obreshkove, 1930), and with the polarographic method (Crider, 1979; Hoshi and Inada, 1973). The polarographic method utilizes a platinum electrode to produce a current proportional to the oxygen content of the medium. As the current produced varies linearly with the O_2 content of the water (Kanwisler, 1959), calibration of the measuring device and measurement of changing O_2 content over time are easily accomplished.

CHAPTER III

METHODS

Objectives of Study

My primary objectives were to determine the major laboratory variables affecting the rate of negative phototaxis in D. magna and to establish culturing and testing protocols for maximizing phototactic response in control (unexposed) animals. Although the adaptive significance of vertical migration in the cladocera was considered, my primary goal was to exploit proximate environmental cues which maximize the utility of the toxicity test. The resulting culturing and testing protocols are aimed at producing a consistent, easily-measured response which can be utilized throughout the day and night, and throughout the year as an indication of unstressed animals.

Culture and testing parameters that affect the phototactic response in cladocerans were altered in different experimental groups of animals, and the rate and variability of response were measured. Only one parameter was varied at a time, with other conditions held constant for all groups in each experiment. In experiments comparing acclimation or test parameters all animals were taken from the same culture; in experiments examining the effects of culture variables, test animals were taken from cultures of similar metabolic condition and general health. Upon establishment of rigidly defined culturing and testing

protocols, the behavioral bioassay was performed concurrently with the commonly used 24- and 48-hour LC50 determinations and seven-day chronic life cycle tests. Three model compounds important in aquatic pollution were used. The sensitivities and variabilities of the alternate methods were then compared.

In addition, I examined the nature of the naphthalene-induced impairment of phototaxis in Daphnia. This included an examination of behaviors that might indicate impaired perception of light by intoxicated animals was examined, and experiments on how naphthalene acts on the mitochondrial electron transport chain. The latter effect could affect locomotor potential by interfering with energy production.

Culture Medium and General Culture Methods

The daphnia cultures were maintained in 160-liter glass and epoxy-painted wood tanks in the Oklahoma State University aquatic animal behavior research laboratory. Several culture media were tried, including aged tap water, natural pond and lake waters, dechlorinated tap water, and distilled water reconstituted with $NaHCO_3$ (200 mg/1), $CaCl_2$ (224 mg/1), and K_2SO_4 (26 mg/1) as specified by Frear and Boyd (1967). Initial difficulties were encountered in establishing successful cultures with all media. The most consistently successful cultures were obtained using dechlorinated or aged tap water. For ease of maintenance, dechlorinated tap water was adopted for use early in the study. Periodic analysis of the water performed by the Oklahoma State University Water Quality Research Laboratory verified the absence of significant changes in water quality parameters throughout the study. Results of a typical analysis are presented in Appendix A.

Three culture foods were tested: dried brewer's yeast; the green algae Selenastrum; and a mixture of the commercial fish food Trout Chow, and the commercial culture food Cerophyl, which consists of dried rye grass with added vitamins. The Trout Chow/Cerophyl culture food was prepared by stirring 1.5 g Cerophyl in 1 liter H_2O for one hour, after which 40 g Trout Chow were added and the mixture blended for one minute in a kitchen blender. The 160-liter cultures were fed from 20 to 50 ml daily. Due to the large numbers of daphnia required for the study, the culture of sufficient Selenastrum proved impractical. Brewer's yeast and the Trout Chow/Cerophyl mixture were therefore tested in an initial series of experiments to determine their effects on general culture health and the phototactic response. Based on these experiments, yeast was used as the exclusive food for the remainder of the study.

The amount of food and frequency of feeding were varied according to current population densities in the cultures. Food was added to the cultures when the medium appeared dilute, based on water clarity. At low concentrations of food particles the daphnia grazed on organisms growing on the sides of the culture tanks. An effort was made to maintain yeast concentrations in the culture sufficiently high to prevent grazing.

Thermostatically controlled, 100-watt aquarium heaters were used to maintain constant temperatures. Recording thermometers in the cultures verified the heaters' accuracies to within +/- 1 C^{o}. Aeration was provided by standard aquarium air pumps attached to diffusing air stones. The buildup of metabolic wastes in the cultures necessitated periodic dilution of the culture medium. At intervals which varied from several days to two weeks, depending on the population densities of the

cultures, approximately 80% of the culture water was siphoned away, including unconsumed food particles that had accumulated on the bottoms of the tanks. The tanks were then refilled to capacity with fresh dechlorinated tap water and the cultures provided with a greater than normal amount of food.

The major problem encountered in maintaining the cultures was the growth of an unidentified aquatic fungus which, if permitted to grow uninhibited, produced a toxin which killed the daphnia. Attempts at chemical inhibition of fungal growth failed due to the sensitivity of the daphnia to the fungicides used. The apparent source of the fungal contamination was the presence of many fish cultures in the aquatic behavior laboratory, and was especially problematical during the winter months when poor temperature control in the building caused water temperatures in unheated fish tanks to fall below 20^{o} C for extended periods. Heating of the daphnia cultures to 26^{o} C was usually successful in inhibiting the fungal growth.

Illumination

Because culture facilities were located in a room common to the culture of fish used for behavioral research limits were placed on the range of illumination possible for the daphnia cultures. Within a range, however, it was possible to vary the intensity of light incident upon the surfaces of the cultures. The overhead fluorescent lighting in the laboratory produced 14 to 70 foot-candles of illumination at the level of the tanks initially. By suspending additional fluorescent lamps over individual culture tanks light intensity could be increased; lower levels of light were produced by placing screens between the

culture area and the laboratory lights. The effects of culturing daphnia in total darkness were studied by constructing plywood panels to surround one of the tanks.

During the one- to two-hour acclimation period (after transfer of the daphnia from the cultures to the test tubes but before the phototaxis test) the animals were normally exposed to a light intensity of approximately 100 fc on the laboratory bench. The use of a light-tight enclosure, screens, or auxiliary lighting permitted the experimental determination of effects of various light intensities during the acclimation period.

A dimming control was added to the test apparatus to allow changes in intensities of light used to stimulate the phototactic response. The use of incandescent flood lamps ranging from 30 to 150 watts, in conjunction with the dimming control, allowed varying of the test light intensity without appreciable changes in color due to excessive dimming of the lamps. All light intensity measurements were made with a Gossen Luna Pro sbc light meter with diffuser in place.

Simulation of Crepuscular Periods

In preliminary experiments, animals were exposed to a 16-hr light, 8-hr dark photoperiod with a simple on/off transition between light and dark periods. The Daphnia displayed a high rate of negative phototaxis during the summer months, but response rates dropped somewhat unexpectedly during the winter months and many animals showed a reversed (positive) phototaxis. In retrospect it was seen that during the summer days, the automatically controlled lighting was turned off during the daylight hours, and because of poorly shuttered windows, the animals

were subjected to a decreasing intensity of light during the natural sunset. During the winter months, on the other hand, the lights were switched off after sundown so that the animals were exposed to a sudden transition from light to dark. To examine this phenomenon, a device was constructed which gradually brightened incandescent lamps at sunrise before switching on the fluorescent lamps, and gradually dimmed the incandescent lamps after switching off the fluorescent lamps at sunset.

The device consisted of a toggle-type dimming control activated by levers attached to a Paragon Model 4001-0, 24-hour automatic light timer (Figure 2). The device was constructed with provisions for altering the dimming and brightening rates of the incandescent lamps, and switched the fluorescent lamps on and off as well. The control was used during the final 16 months of the study. Phototactic responses of daphnia cultured under lighting regimes with and without crepuscular simulation were then compared. A summary of the two lighting regimes is provided in Table I.

Although not implemented during the course of the study, a more sophisticated, microprocessor-based lighting control, consisting of power control circuitry interfaced with the inexpensive Commodore VIC-20 microcomputer and controlling software, was developed. The system allows a much closer approximation of a natural photoenvironment in the laboratory by simulating natural intensity curves throughout the day, and by varying the daylength, if desired, automatically with the changing seasons. The electronic circuit diagram and controlling program are presented in Appendix B.

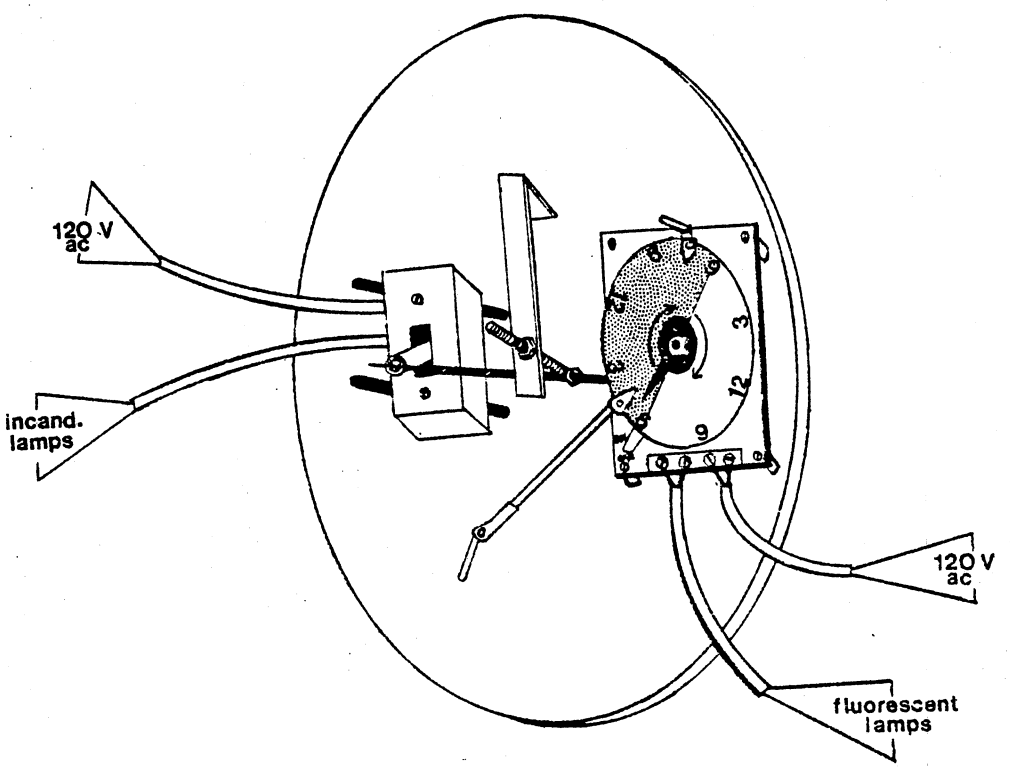


Figure 2. Mechanical Light Control Used to Gradually Dim and Brighten Laboratory Lighting at Sunset and Sunrise.

TABLE I

DETAILS OF LIGHT REGIMES USED IN CULTURE ROOM DURING EXPERIMENTS

Light Regime with Dawn & Dusk	On/Off Light Regime
5:30 am - incandescent lamps begin to brighten	6:00 am - all lamps on
6:15 am - incandescent lamps at full brightness and 1/2 fluorescent lamps switched on	
6:30 am - all fluorescent lamps on	
-FULL DAYLIGHT-	
9:30 pm - 1/2 fluorescent lamps switched off	10:00 pm - all lamps off
9:45 pm - all fluorescent lamps off - incandescent lamps begin to dim	
10:30 pm - incandescent lamps fully off	
-DARKNESS-	

Clone Cultures

The normally parthenogenetic reproduction of Daphnia enables a simple method of obtaining cultures of genetically homogeneous animals: a single individual is isolated and its progeny are used to establish the cultures. It was hoped that by selecting strongly responding animals to establish the clone cultures the populations would exhibit a higher and less variable rate of response than genetically heterogeneous cultures. Daphnia used to start the clone cultures were chosen by performing the normal phototaxis test described below, and selecting the most strongly responding individual (the first to the top) in each of the tubes. These animals were then placed in a single tube and the procedure was repeated. The daphnia selected from this second contest was then transferred to a 1-liter beaker and kept until several generations of young had been produced, at which time the entire population of the vessel, consisting of a few dozen to more than 100 animals, was introduced into a 160-liter aquarium.

The Phototaxis Test and Test Apparatus

The test chamber used in the study was the same as that designed and used by Whitman and Miller (1982). The box, measuring 122 cm in depth x 30 cm x 30 cm, was painted flat black inside and contained two shelves with 25-mm diameter holes for suspension of tubes containing the daphnia. The top shelf was approximately 190 mm above the lower shelf, and held the 200-mm test tube in a position to direct light unidirectionally into the tube from either an upper or lower incandescent flood lamp (Figure 3). Between the lamps and the shelves

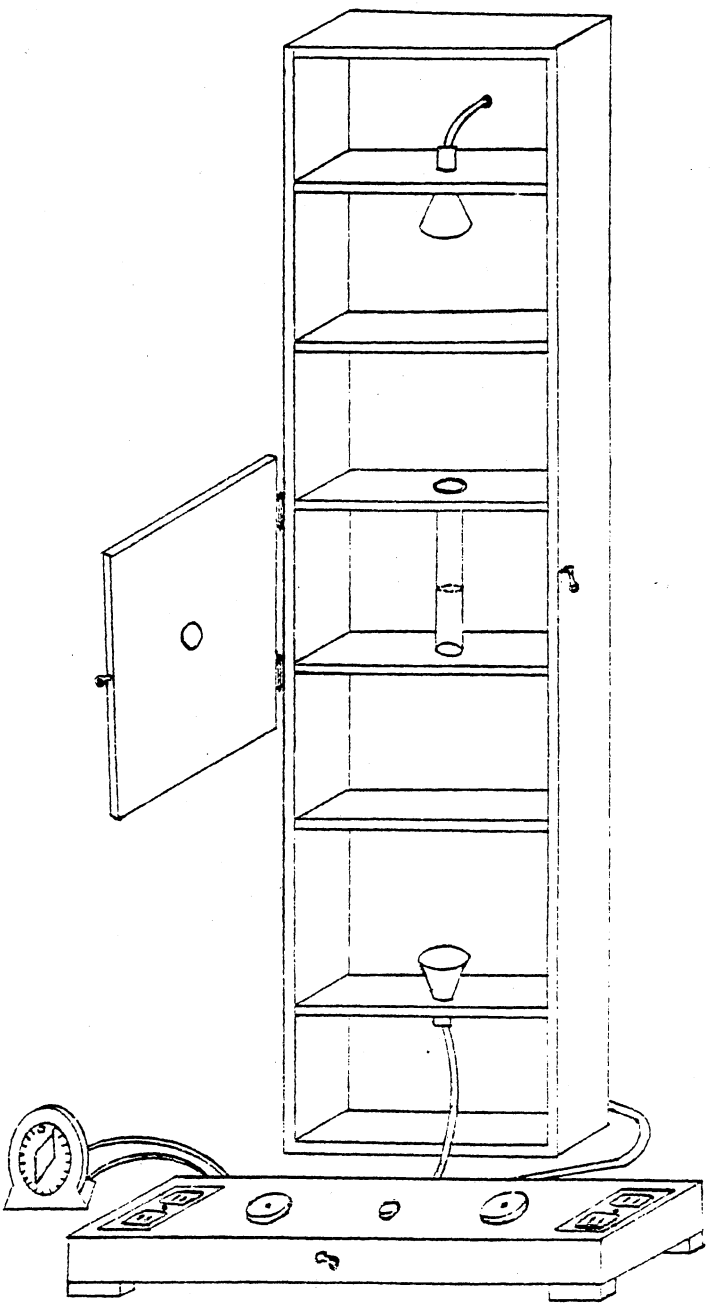


Figure 3. Test Apparatus Used in Phototaxis Experiments.

holding the test tube, sheets of 6.35-mm plate glass served as heat filters. A hinged door on the front of the box facilitated insertion and removal of the test tubes. Observations of the responses of the animals were made through a 25-mm hole in the door.

An electrical control panel was constructed and equipped with a 60-second light timer and three light dimmers which enabled light intensities of both lamps in the apparatus to be varied either individually or in concert. A single two-position toggle switch enabled changing the direction of the light stimulus by providing power to only one lamp in each position.

Preparation for the phototaxis bioassay was begun by transferring several hundred daphnia from the culture tanks to the 200-mm test tubes containing water from the same culture. A hand net with 2-mm mesh was first used to separate the larger instars to be used in the test from neonates and smaller instars. The net was supported in the culture tank with its edge projecting slightly above the water level for several minutes, enabling the smaller animals to escape by swimming through the mesh of the net. Twenty of the larger animals were then gently pipetted into each of the test tubes. Five tubes (100 animals) were normally assigned to each treatment. Any toxicant used in the test was added at this time, and the racks of test tubes were placed on the test bench for an acclimation/exposure period of two hours.

The phototaxis test was performed by transferring the tubes one at a time to the test apparatus and subjecting the daphnia to overhead light, from which normally responding animals swam to the bottom of the tube. After one minute the direction of light stimulus was reversed, and responding daphnia swam upwards in the tubes away from the light.

The animals were counted as they swam past a mark drawn midway up the tube, and the number of animals so responding within 30 seconds was recorded. Upon completion of the test the experimental animals were discarded and if a toxicant had been used, the waters from both control and treated vessels were analyzed for actual toxicant concentration using spectrophotofluorometry, ultraviolet spectrophotometry, or atomic absorption spectrophotometry (Christian, 1980).

Response Criterion

To ascertain the validity of the criterion for measuring phototactic response, separate experiments were performed in which the vertical distances moved by the animals were measured. Each specially calibrated test tube was prepared by placing marks at intervals of 20 mm along its length. Ten daphnia were placed in each tube, and the number of animals in each segment was recorded at the end of the test. Multiple tests were performed under laboratory lighting conditions which yielded low, moderate and high response rates, and the vertical distributions of the animals were plotted in order to examine the locations of the majority of responding and non-responding animals.

In addition, other stimuli were tested as elicitors of measurable behavioral responses in Daphnia which were thought to have possibilities for a toxicity assay. A test based on the positively phototactic response to a gradually dimming source of light from above was devised and examined. The animals were tested in the normal test apparatus, but during the 30-second response period, the overhead lamp was steadily dimmed to darkness. Because the daphnia were found to exhibit a substantial positively phototactic response when subjected to a

directional beam of red light, the standard phototaxis test was performed using a red lamp from above, and numbers of positively phototactic animals were recorded.

Toxicity levels necessary to inhibit the dorsal light response at various intensities of the light stimulus were determined. One humdred milliliters of culture water were placed in glass bottles and appropriate aliquots of naphthalene were added. The daphnia were then pipetted into the bottles and left for a 2-hour exposure period before being tested. The animals were then subjected to a light from beneath in a darkened room and examined for presence of the dorsal light reflex. After a 20-second period for adjusting to the light, each animal was observed for 10 seconds. The animal was designated as "responding" when it swam upside down for more than 90% of the 10-second interval. Other stimuli thought to be capable of stimulating directed movement in the animals were tested, including heat and electrical fields.

Diurnal Variability in Phototactic Response

Phototaxis tests were performed throughout the day and night to determine whether diurnal variability existed in the phototactic response. More frequent tests at shorter intervals were performed near and during the sunset and sunrise hours to detect any irregularities in response during these periods. Preliminary experiments showed that phototactic responses fell markedly as sunset began; in all further nighttime experiments the test animals were pipetted into the test tubes, placed in a lighted environmental chamber shortly before sunset, and kept there until testing. These daphnia were, in effect, exposed to an abnormally long daylength before being tested, but the effects of

sunset and darkness were eliminated. Thus, the animals were tested for the presence of a circadian periodicity affecting phototactic response, and for effects of an abnormally long daylength immediately prior to the test.

Due to the necessity of obtaining all daphnia for each experiment from the same culture (to eliminate variability among cultures) and because the large numbers of animals of the correct age class required for a complete 24-hour experiment were seldom available from one culture, each experiment spanned only a portion of the 24-hour light cycle. The time spans of the individual experiments overlapped times covered in other experiments so that trends in the rates of response could be verified.

Effects of Recent Feeding

A small, but consistent decrease in phototactic response was noted in daphnia after several days without feeding, and was undoubtedly due to reduced vigor of the animals from inadequate food; however markedly lower responses observed within a few hours of feeding were more difficult to explain. An initial hypothesis was that increased hemolymph glucose levels, or stimulation of stretch receptors in the gut due to its distension from food particles, may have mediated a depression in the phototactic response in nutritionally satisfied animals in order to reduce energy expenditure and the risk of predation during migrations to the well-lighted upper layers.

To test this hypothesis the daphnia were deprived of food for several days, then placed in bacteriologically filtered culture water to which were added fluorescently labelled, carboxylated, 0.91-micron

polystyrene beads (catalog # 15702, Polysciences, Inc., Warrington, PA). The daphnia readily ingested the beads, as evidenced by the strongly fluorescent gut readily seen in the animals. Any stretch receptors present in the gut would thereby be stimulated without providing nutritional substances to the animals. By comparing the phototactic responses of these animals with those of unfed 'control' animals and animals fed normally with yeast before the test, it was hoped that the effects of gut distension or nutrient input could be distinguished.

Toxicant Bioassays

Upon defining a standardized protocol for conducting the phototaxis bioassay, the technique was performed using animals exposed to three model compounds important in fossil-fuel-derived aquatic pollution. Daphnia were exposed for two hours to varying concentrations of two polycyclic aromatic hydrocarbons, naphthalene and phenanthrene, and one azaarene, acridine. The animals were cultured and the tests performed according to the guidelines summarized in Appendix A.

One hundred milligrams of naphthalene, acridine or phenanthrene (Aldrich Chemical Company) were dissolved in 100 ml 95% ethanol, and appropriate aliquots were added to water from the Daphnia cultures to produce concentrations ranging from 0.1 to 2.0 mg l^{-1} (phenanthrene), 0.25 to 4.0 mg l^{-1} (naphthalene), or 0.25 to 8.0 mg l^{-1} (acridine). The solutions were poured into the large test tubes and 20 daphnia were pipetted into the tubes; the tubes were then sealed with parafilm and placed in a water bath held at two to three centigrade degrees above the temperature of the culture. Tubes containing control animals received aliquots of ethanol equivalent to that used as carrier in the highest

toxicant concentration in the bioassay (2000 or 4000 mg l^{-1}).

EC50 values (concentration at which response is observed in 50% of test animals) obtained with the phototaxis bioassay were then compared with published LC50 (lethal concentration for 50% of test animals) values obtained in 24-hr or 48-hr bioassays of lethality.

Measurement of Cellular Respiration

After several attempts at isolation of mitochondria, a satisfactory method, similar to that used by Harmon and Crane (1973) for the isolation of mitochondria from beef heart, was achieved. With a hand net having 2-mm mesh, five to twenty grams (wet weight) live daphnia were removed from the culture water. This method ensured that only the larger adult instars were taken so that a uniform age class could be obtained. The animals were then placed on a preweighed weighing paper and blotted dry. After weighing, the daphnia were placed in cold (4°C) homogenization buffer of 0.25 M sucrose and 0.01 M Trizma-HCl, brought to pH 7.4 with acetic acid or Trizma base. Five milliliters buffer were used per gram of daphnia prepared. From this point on, all solutions and glassware were kept cold (<4°C) to preserve mitochondrial activity. The daphnia and buffer were then placed in a Potter Elvehjen glass and teflon tissue homogenizer (a few grams of daphnia at a time) and homogenized by drawing the piston of the homogenizer up and down 10 times. The homogenate was centrifuged at 500 x G for 10 minutes in a refrigerated centrifuge (4°C). The pellet was discarded and the supernatant fluid containing the mitochondria was filtered through four layers of cheesecloth and centrifuged at 30,000 x G for 30 minutes to pellet the mitochondria. With the glass-teflon

homogenizer, the resulting pellet was then resuspended in 50 ml of homogenization buffer, and again centrifuged at 30,000 x G for 30 minutes.

Samples of the homogenized daphnia, and of each supernatant fraction and pellet, were saved, and with the modified Biuret protein assay (Yonetani, 1961) protein content was determined. A standard curve, using hyaluronidase or B-galactosidase was run each day an isolation was performed. A summary of the final procedure used to isolate active mitochondria from daphnia is shown in Figure 4.

Pellet P3 and supernatant S2 (Figure 4) were examined for mitochondrial activity with a succinate dehydrogenase activity assay. 2,6-dichlorophenol-indophenol (DCPIP) was substituted as the final electron acceptor, in place of oxygen. When oxidized, DCPIP absorbs strongly at 600 nm, but becomes clear as it is reduced, enabling the speed of reaction to be determined spectrophotometrically. A Bausch & Lomb Spectronic 20 spectrophotometer was used to measure absorbance.

Oxygen consumption in isolated mitochondria was determined polarographically. The purified mitochondria were suspended in the homogenization buffer, protein concentration of the mixture was determined, and the mixture was diluted to a protein concentration of 4.8 mg/ml. The oxygen monitoring system consisted of a YSI Model 5331 oxygen electrode fitted into a Gilson reaction chamber which held a total volume of 1.8 ml. A Johnson Foundation oxygen electrode amplifier was used to boost the signal from the electrode to drive a chart recorder. Although the reaction chamber contained a water jacket designed for continuous flow, it was not connected to a water bath, but instead was filled with water and all reactions were run at room

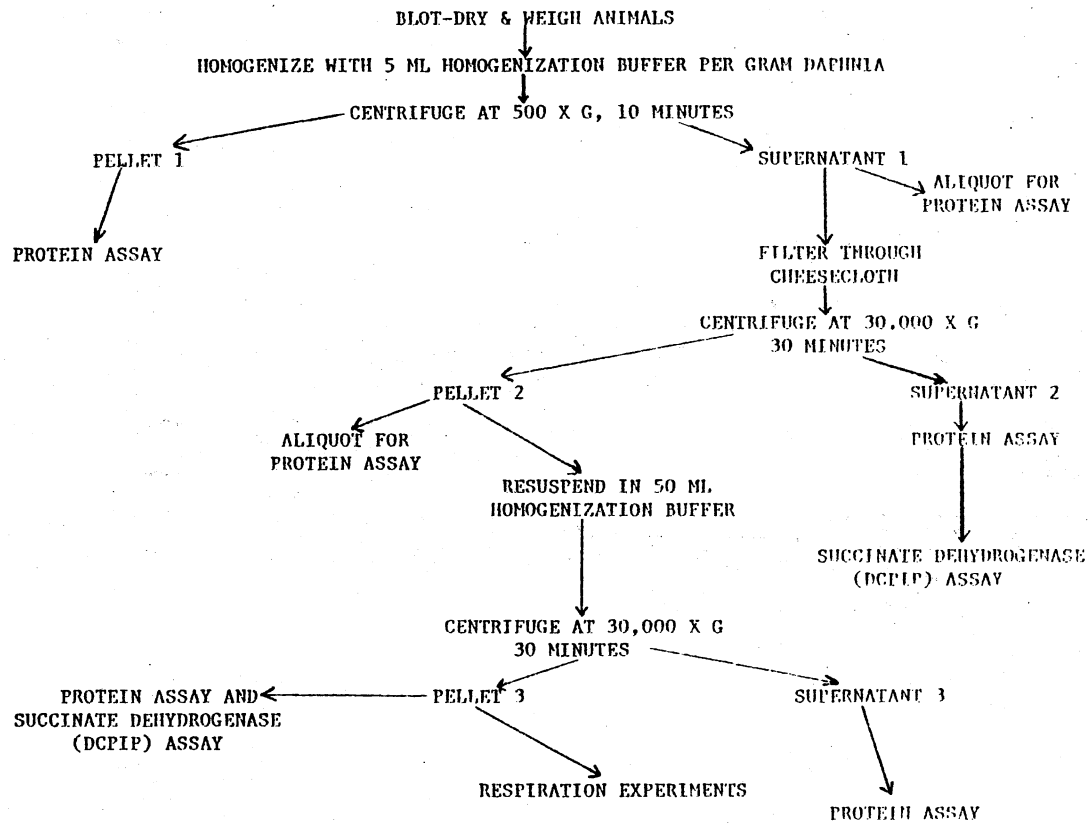


Figure 4. Procedure Used to Isolate Active Mitochondria from D. magna for Use in Respiration Experiments.

temperature (25^{o} +/- $1C^{o}$). A small magnetic stir bar circulated the mixture in the reaction chamber to obtain a linear tracing on the chart paper.

The apparatus was calibrated with 0.1 M KCl solution, which holds 294 nmoles O_2/ml at 25o C. After calibration and between successive assays, the reaction chamber was rinsed thoroughly with distilled water to remove all traces of substances from previous runs. If a toxicant had been used, the chamber was rinsed with 95% ethanol before the distilled water rinse.

To the chamber were added assay buffer (0.5 M sodium phosphate, an equimolar mixture of monobasic and dibasic forms), distilled water, and the mitochondria suspension. When a smooth pen tracing was obtained, with very little slope, due to absence of substrate, the substrate was added to the chamber. Addition of substrate caused a steady drop of the pen as oxygen was removed from the solution. After several minutes, during which an accurate slope of the tracing was established, exogenous cytochrome C was added to the chamber. This caused an increase in the rate of O_2 consumption due to the fact that some of the cytochrome C in the mitochondrial membrane had been removed during isolation and thus a portion of the electron transport chains were incomplete. The added cytochrome C took its place in the deficient chains, restoring their function. By comparing O_2 consumption rates before and after the addition of exogenous cytochrome C, the percentage of incomplete chains resulting from isolation procedures could be determined.

Finally, naphthalene was added to the chamber and the effect on O_2 consumption was evidenced by a change in the slope of the pen tracing. The order of addition of the various components of the reaction mixture

and their compositions and amounts are presented in Table 2.

For comparative purposes, an identical experiment was performed on mitochondria from beef heart. The mitochondria were isolated, using the procedure of Harmon and Crane (1973). The final mitochondrial fraction was diluted to 4.8 mg/ml protein; i.e., the concentration used in the Daphnia experiments.

TABLE II

REAGENTS USED IN MITOCHONDIRIAL RESPIRATION ASSAY
(LISTED IN ORDER OF ADDITION TO CHAMBER

volume (ml)	
0.30	0.05 M Sodium Phosphate Buffer
1.25	H_2O
0.05	substrate (1.0 M sodium succinate or NADH)
0.02	cytochrome C (10 mg/ml)
0.05	naphthalene (1.08 mg/ml in ethanol) - or 95% EtOH control

Statistical Methods

Differences in phototactic response of daphnia subjected to varying culture or testing conditions were analyzed with Student's t test. Variations in metabolic condition of cultures and, in some experiments, order of testing individual groups of animals, were found to affect phototactic responses; therefore, experimental units (tubes of 10 or 20 animals) were compared only with other units treated identically, and the procedure for paired experiments was used (Steel and Torrie, 1960).

In toxicant bioassays, EC50 values were determined by the Litchfield-Wilcoxon probit method for dose-effect experiments (Litchfield and Wilcoxon, 1949).

RESULTS

Response Criterion

The experiments performed to determine the vertical distributions of responding and non-responding animals verified the validity of the response criterion chosen for the bioassay. Distributions of daphnia in the 200-mm tubes at the end of the tests can be seen in Figure 5. The mean distance travelled by the 414 negatively phototactic animals in the experiment was 170 mm, while the 376 animals which did not meet the response criterion travelled a mean distance of 22 mm. The difference between means is significant at the .0005 level ($t=85.40$, $df=788$).

Attempts to evoke a consistent response by using either heat or weak electrical stimulation were unsuccessful. No apparent directed movements were produced by either of these stimuli. The use of a decreasing overhead light intensity or an overhead red light of constant intensity also produced poor responses. Less than half the response was obtained with these methods as compared with concurrent controls tests using the original bioassay technique.

Daphnia tenaciously maintained the dorsal light reflex while intoxicated with naphthalene. Of 80 daphnia exposed to naphthalene concentrations up to 1.24 mg l^{-1} all animals exhibited the reflex until intoxicated to such an extent that convulsive behavior interfered with normal swimming (Table 3). Under conditions of excessive intoxication (above 1.24 mg l^{-1} naphthalene) the animals turned rapid somersaults and exhibited no phototactic response.

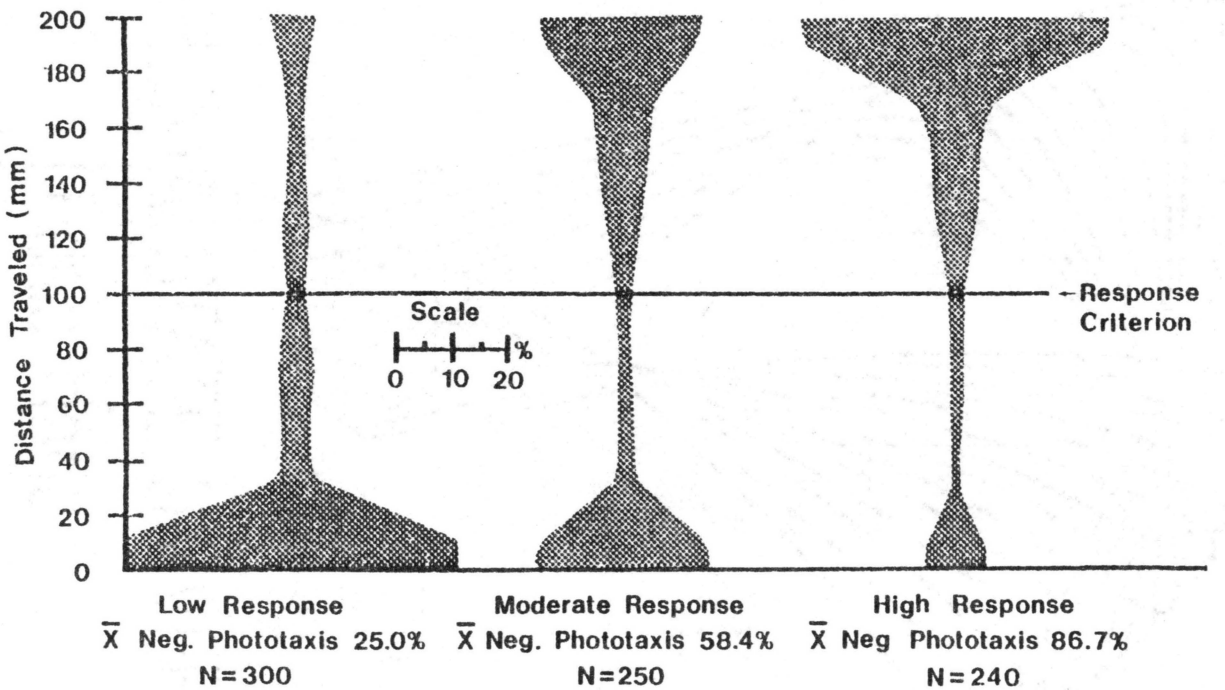


Figure 5. Vertical Distributions of 790 D. magna in 200-mm Tubes at End of Phototaxis Test

TABLE III

NUMBERS OF D. MAGNA EXHIBITING DORSAL LIGHT RESPONSE UNDER VARYING DEGREES OF INTOXICATION WITH NAPHTHALENE

Experimental Group	n	animals exhibiting D.L.R.	animals not exhibiting D.L.R.	%
control	20	20	0	100
0.44 mg l^{-1}	20	17	3	85
0.78 mg l^{-1}	20	18	2	90
1.24 mg l^{-1}	20	12	8	60

Culture Food and Feeding

A total of 1280 animals were tested during 11 experiments to compare the phototactic responses of animals fed dried brewer's yeast with those fed with the Trout Chow/Cerophyl mixture. The results were analyzed by the procedure for paired experiments (Steel and Torrie, 1960, p. 78). Yeast-fed animals showed a mean response 32% greater than animals fed Trout Chow/Cerophyl (Fig. 6). The difference in response was significant at the .001 level ($t=7.87$, $df=31$).

Four experiments were performed to compare the effect of time of feeding relative to the phototaxis test. Two hundred daphnia fed 24 hr prior to the start of the phototaxis test exhibited a response rate of 84.5% while an equal number of animals fed 4 days prior to the test

showed 70.0% response. Animals fed 3 hr before the test showed 25.5% response (Figure 7). Statistically significant differences were found, using Student's t test, between the 24-hr animals and the 4-day animals ($t=3.29$, $df=9$, $p<.01$), between the 4-day group and the 3-hr group ($t=6.91$, $df=12$, $p<.001$), and between the 24-hr animals and 3-hour animals ($t=7.95$, $df=9$, $p<.001$).

Effect of Recent Feeding

In experiments performed to determine the cause of decreased phototactic response in recently fed animals, both the daphnia fed yeast and those fed polystyrene beads exhibited significantly lower responses (67.2% and 63.3%, respectively) than control animals (85.0%), but no statistically significant difference in response was found between yeast-fed animals and those fed polystyrene beads.

Effects of Light Intensity

Culture Light Intensity

Daphnia cultured with an incident light intensity of 14 foot-candles at the surface of the culture displayed higher rates of negative phototaxis (65.8%) than animals cultured in tanks with a surface light intensity of 70 foot candles (34.3%) (Figure 8). The difference in means was significant at the .001 level ($t=6.97$, $df=19$).

Difficulties occurred in attempts to maintain daphnia cultures in darkness. Fungal growth in the tanks apparently released a toxin which killed the animals. Only one phototaxis experiment was performed with daphnia cultured in darkness for one week; only 11% of the animals exhibited a negative phototaxis. Several tests were performed with

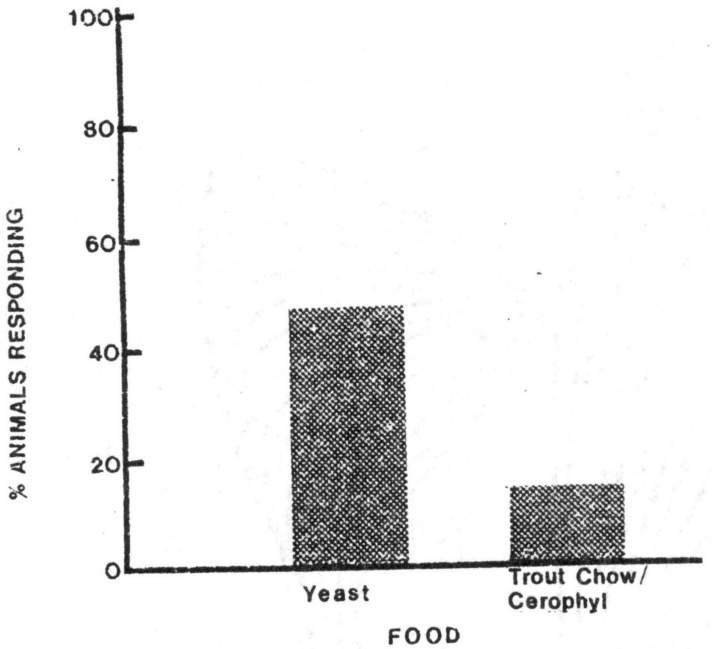


Figure 6. Phototactic Responses of D. magna Fed Yeast or Trout Chow.

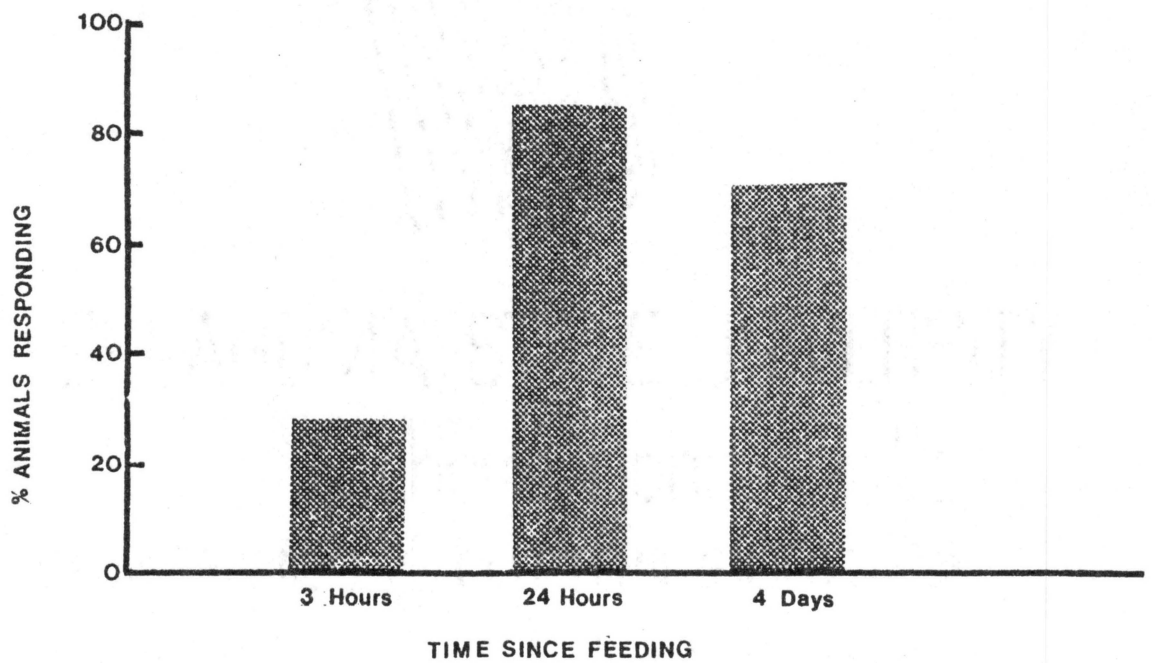


Figure 7. Phototactic Responses of D. magna Fed 3 Hours, 24 Hours, or 4 Days Prior to Test.

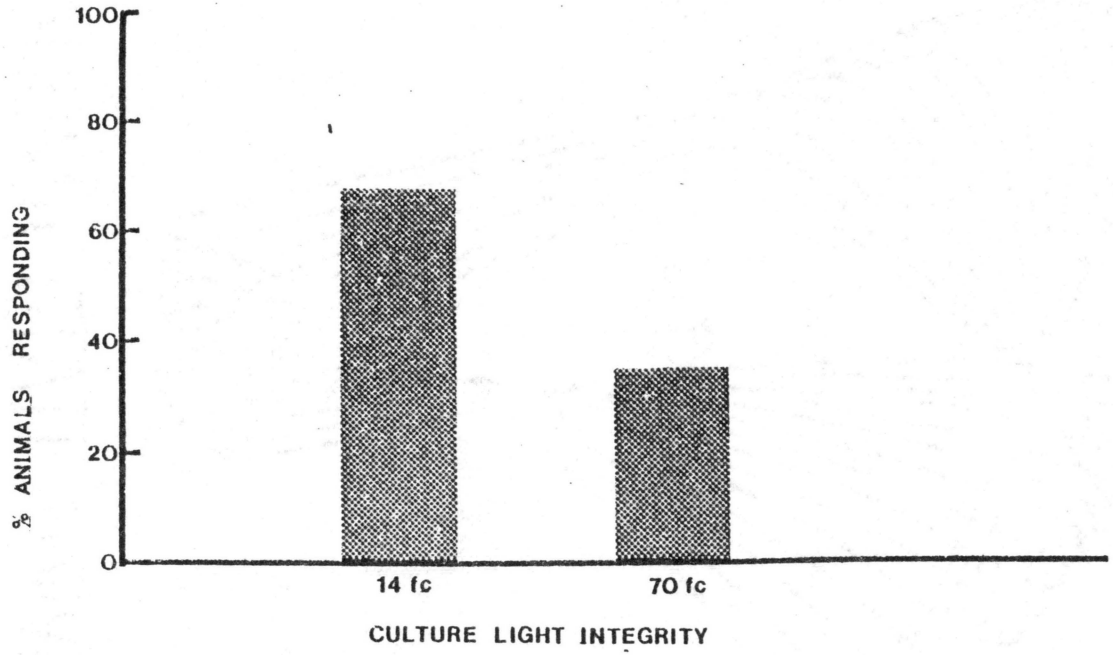


Figure 8. Phototactic Responses of D. magna Cultured Under a Light Intensity of 14 or 70 Foot-Candles at the Water Surface

animals cultured under light intensities greater than 70 foot-candles. Although a valid statistical comparison of responses of these animals with those of animals cultured under lower light intensities is impossible due to differences in other laboratory variables affecting phototactic response, all daphnia cultured at intensities between 70 and 2000 foot-candles showed greatly reduced response rates.

In all experiments comparing effects of culture light intensity, acclimation light intensity and test light intensity were held constant (100 fc and 500 fc, respectively).

Light Intensity During Acclimation

In three experiments performed to test the effect of light intensity during acclimation, a total of 1040 daphnia were exposed to light intensities ranging from darkness to 3000 foot-candles for the 2-hour period between their transfer from the cultures to the test tubes and the time they were tested. In each experiment all animals tested were taken from the same culture and the results analyzed by the procedure for paired experiments to eliminate variables other than acclimation light intensity from affecting the comparison. In each case, animals acclimated under the higher light intensities performed better than those acclimated at the lower intensity (Figure 9). All differences in response were significant at the .05 level.

Test Light Intensity

Whitman and Miller (1982) performed phototaxis experiments on D. magna at light intensities from 15 fc to 500 fc. To determine effects

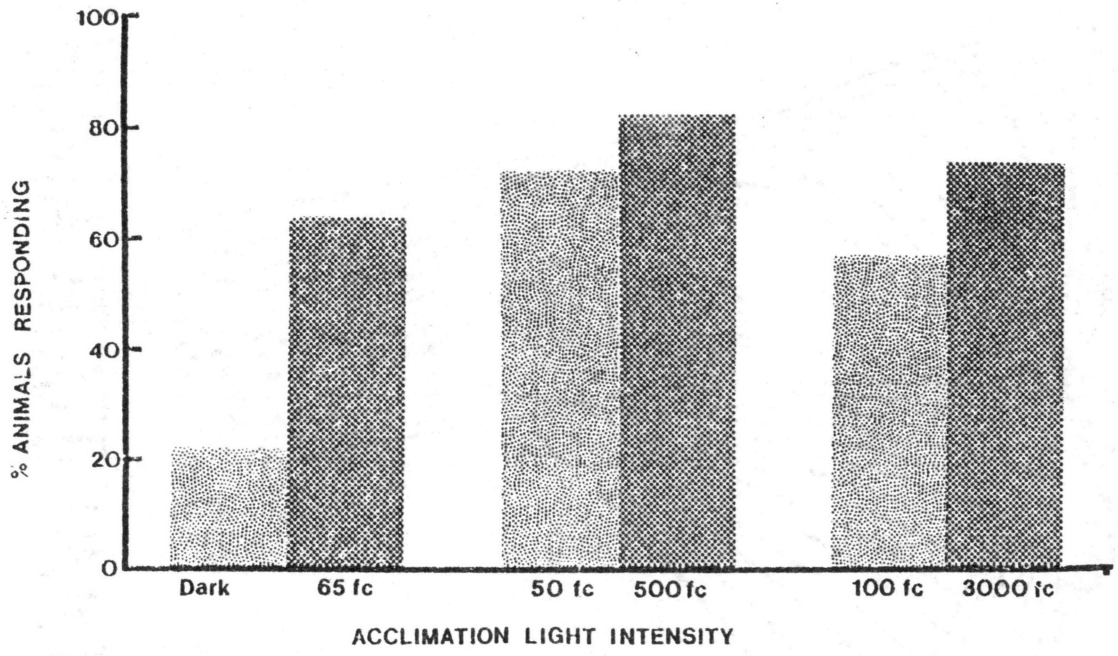


Figure 9. Phototactic Responses of D. magna Acclimated 2 Hours Under Different Light Intensities.

of higher intensities in the phototaxis test, higher wattage bulbs were used during seven experiments to produce test intensities up to 4000 fc. In each of the experiments, during which 1040 daphnia were tested at intensities greater than 500 fc, control animals were tested at an intensity of 500 fc. In each case animals exposed to 500 fc stimulus showed statistically higher (Students t, $p<.05$) responses than animals tested with a stimulus of greater intensity (Figure 10). No significant regression occurred between phototactic response and light intensity above 500 fc.

Effects of Crepuscular Simulation

The phototactic responses of daphnia differed greatly when cultured with and without gradual changes between daytime illumination and darkness. The dawn/dusk simulator was installed during January and phototactic rates immediately increased dramatically (Fig. 11). Near the end of March the lighting in the laboratory was returned to an instantaneous on/off regime, and the response rates fell quickly. Upon recommencement of the crepuscular periods phototactic rates again increased greatly. The difference in response at each lighting transition was significant at the .005 level. All experiments were begun at 11:30 am (5 1/2 hours after 'sunrise'), and all animals were last fed 24 hr prior to the experiments. Water temperatures of the cultures used in this series of experiments ranged from 19° to 23° C, but response rates showed no significant correlation with these temperatures.

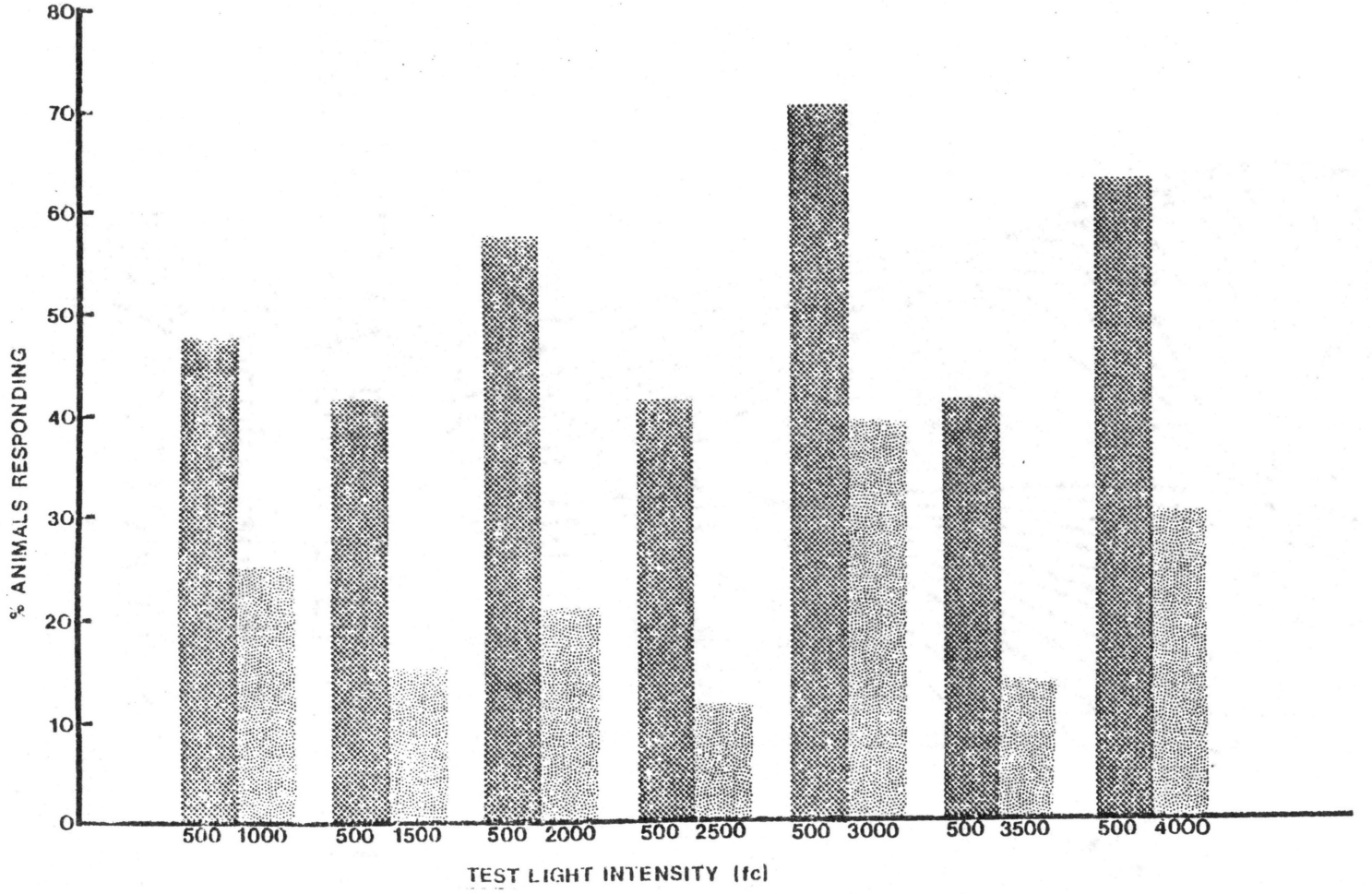


Figure 10. Phototactic Responses of D. magna Evoked by Different Light Intensities

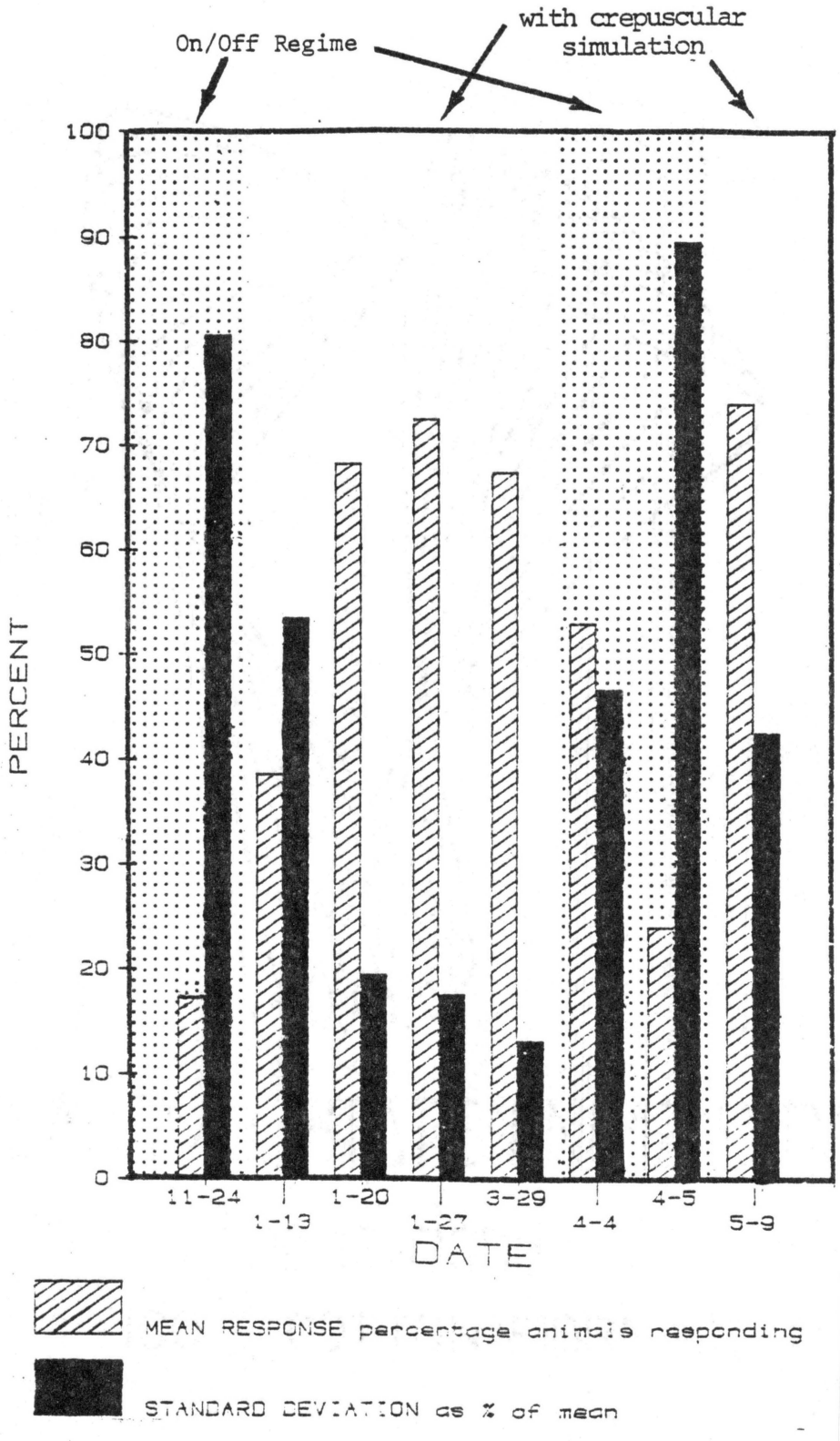


Figure 11. Phototactic Responses of D. magna Cultured With and Without Crepuscular Photoenvironment.

Diurnal Variability in Phototactic Response

Phototactic response was greatest at mid-day, slowly decreasing through the evening hours and throughout the night, and rising again in the morning (Fig. 12). Because animals tested at night were retained in a lighted environment until the time of the test, the absence of a marked change in phototactic response suggests that any endogenous circadian periodicity of phototactic response in D. magna does not interfere with the efficacy of the test, if the animals are not subjected to the stimulus of decreasing light intensity at sunset.

Toxicant Bioassays

EC50 values and their 95% confidence limits (in parentheses) obtained for phenanthrene, naphthalene and acridine are 0.39 mg l^{-1} (0.20 - 0.79), 0.69 mg l^{-1} (0.37 - 1.28), and 1.66 mg l^{-1} (0.73 - 3.80), respectively. Figures 13, 14 and 15 summarize actual phototactic responses of animals exposed 2 hours to concentrations of phenanthrene (0.1 to 1.0 mg l^{-1}), acridine (0.5 to 4.0 mg l^{-1}), and naphthalene (0.25 to 1.50 mg l^{-1}). The graphs were compiled from four experiments with each compound, and plotted responses are not corrected for control responses.

Figures 16, 17, and 18 display plots of probit (decrement from control responses) obtained by the Litchfield-Wilcoxon procedure. Both sets of graphs are based on experimental populations of 660, 820, and 620 daphnia for phenanthrene, acridine and naphthalene, respectively. Ultraviolet spectrophotometric determinations of toxicant concentrations documented accuracy of dilutions used in the experiments, but because

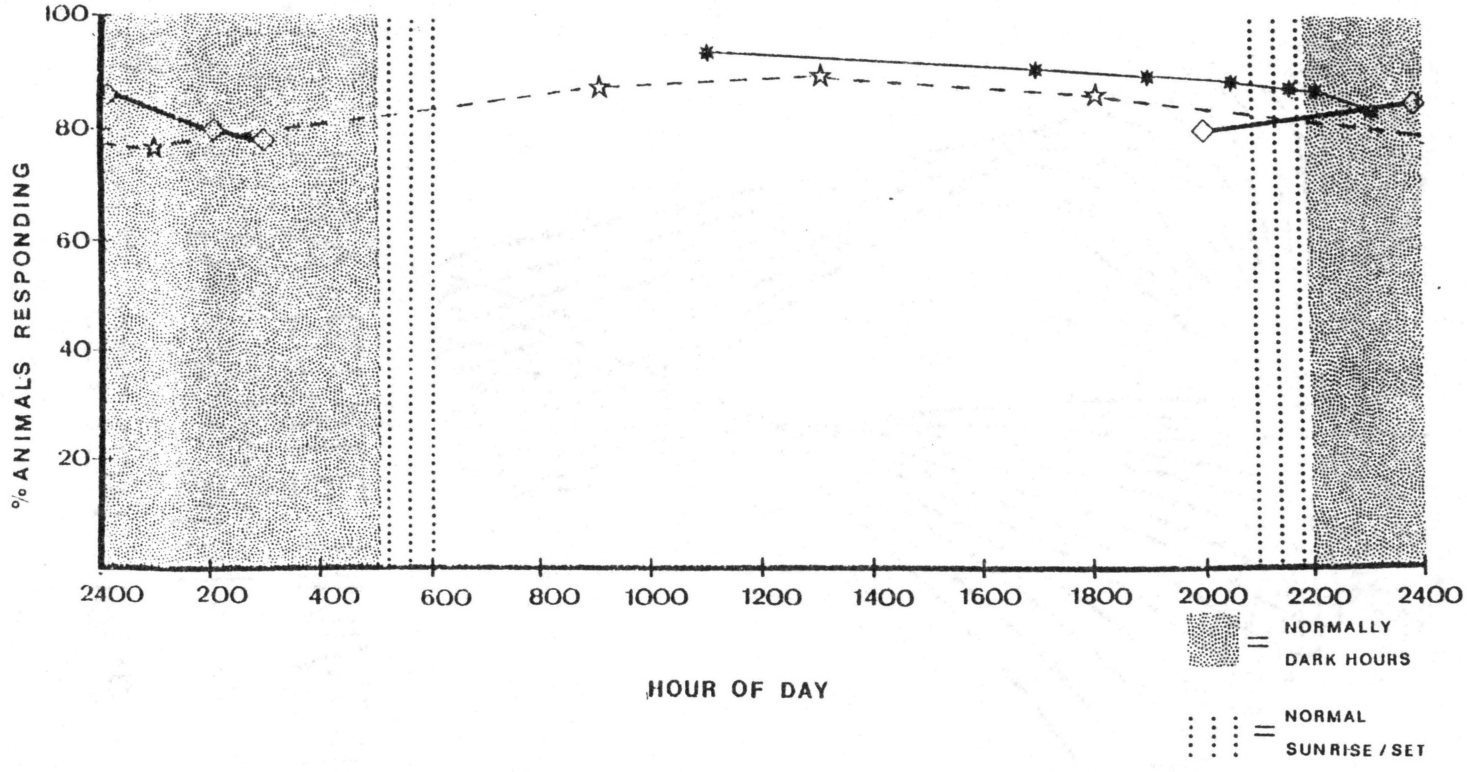


Figure 12. Diurnal Variability of Phototactic Response in Daphnia magna.

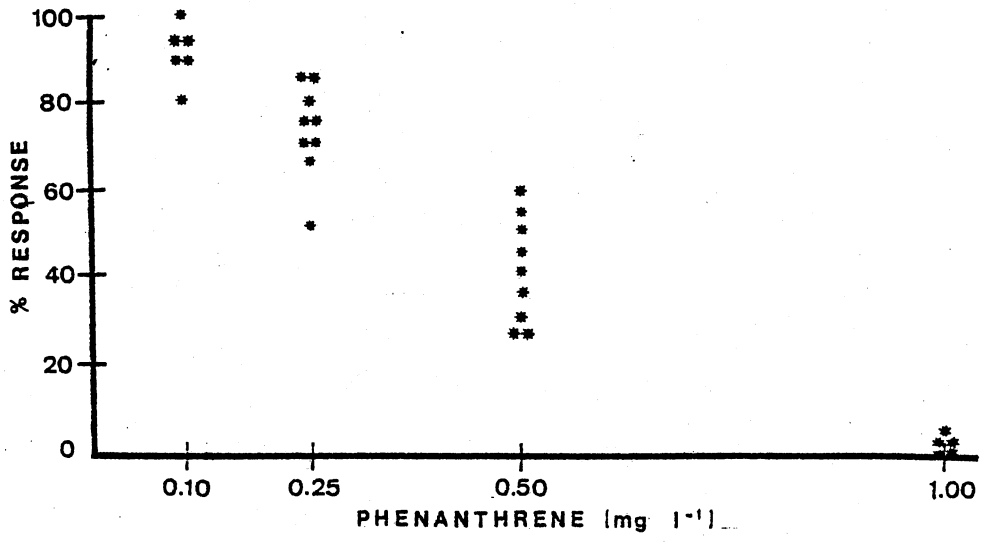


Figure 13. Effect of Two Hour Exposure to Phenanthrene on Phototactic Response of D. magna.

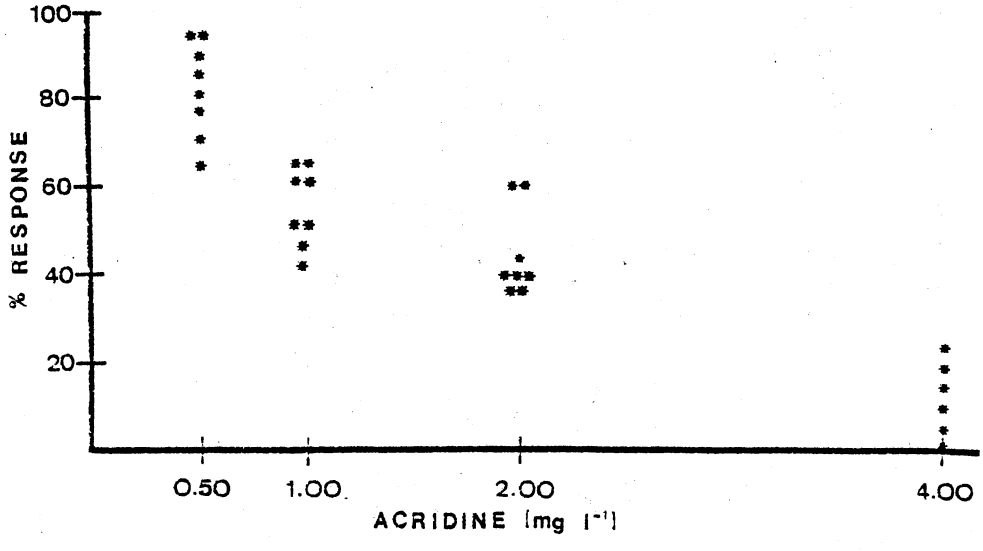


Figure 14. Effect of Two Hour Exposure to Acridine on Phototactic Response of D. magna.

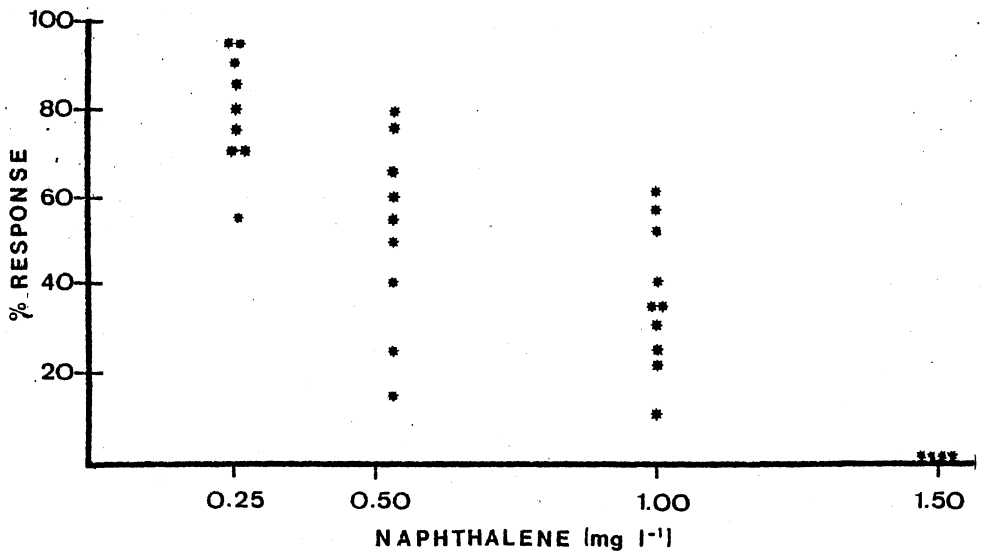


Figure 15. Effect of Two Hour Exposure to Naphthalene on Phototactic Response of D. magna.

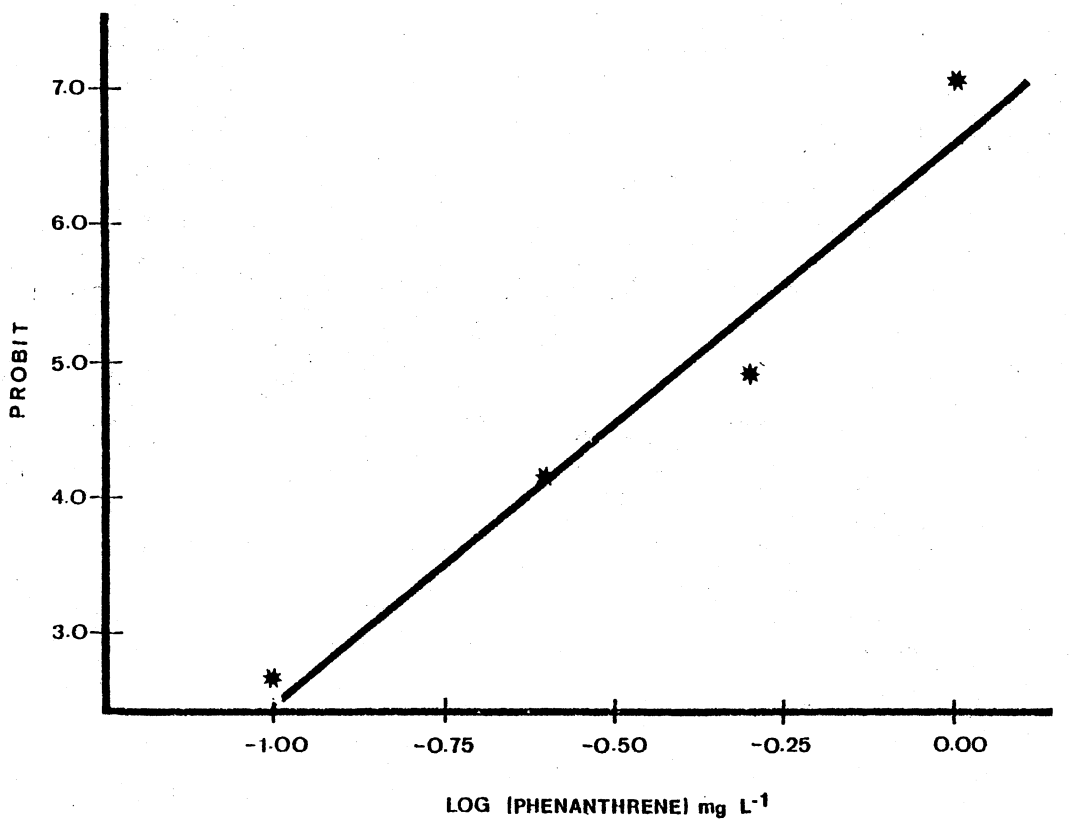


Figure 16. Plot of Log Concentration vs Probit (Decrement in Phototactic Response) of D. magna Exposed 2 Hours to Phenanthrene.

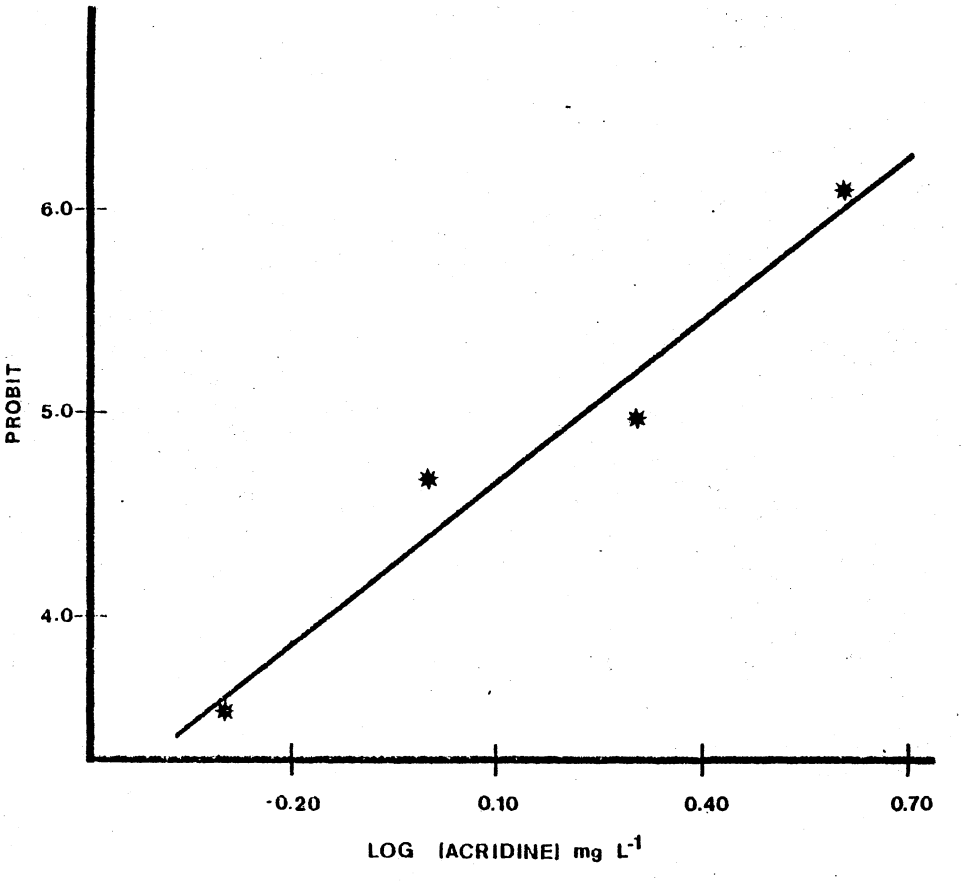


Figure 17. Plot of Log Concentration vs Probit (Decrement in Phototactic Response) of D. magna Exposed 2 Hours to Acridine.

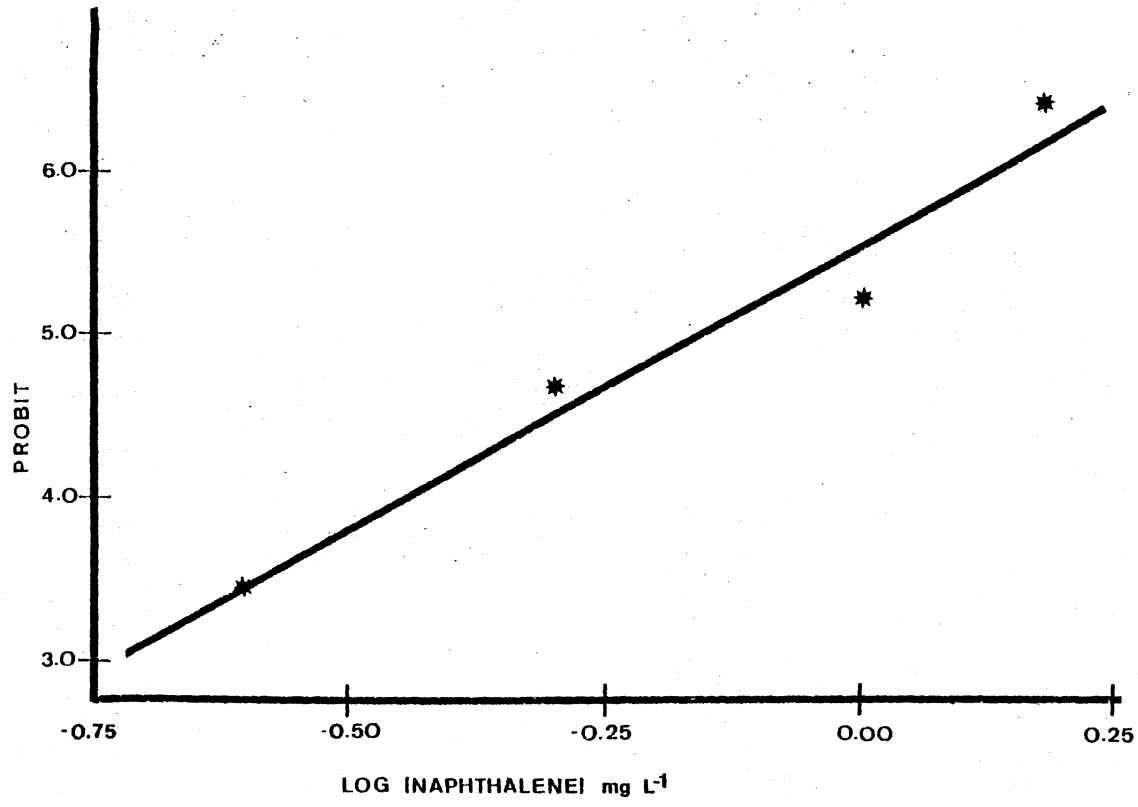


Figure 18. Plot of Log Concentration vs Probit (Decrement in Phototacitc Response) of D. magna Exposed 2 Hours to Naphthalene.

data from several experiments were combined, intended concentration values were used to construct the concentration-response curves.

Effect of Naphthalene on Cellular Respiration

The wet weight of an average adult instar Daphnia magna was approximately 3.25 mg. The dry weight was 0.32 mg, or approximately 9.8% of the wet weight.

The initial isolation attempt demonstrated that approximately one-third of the succinate dehydrogenase activity was still present in the supernatant fraction after 15,000 X G for 15 minutes (Table 4). Thus, at the end of the procedure, after two such centrifugations, fewer than half of the original mitochondria were present in the final pellet. The estimated weight of mitochondrial pellet P3 (see flow chart in methods section) was 150 mg, or 4.41% of the wet weight of the daphnia. The speed and duration of centrifugations were changed accordingly to increase yield of mitochondria. Relative centrifugal force was increased to 30,000 x G (the maximum possible using the Beckman J21-B rotor) and the duration of the spin was increased to 30 minutes.

In a subsequent preparation of mitochondria from a large (10.0 g) number of daphnia, results of the Biuret protein assay showed the daphnia to have an estimated 198 mg protein, or approximately 10% of their dry weight. The estimated protein content and percentage recovery for each fraction containing the majority of mitochondria at each step are given in Table V.

TABLE IV

SUCCINATE DEHYDROGENASE ACTIVITY IN FRACTIONS FROM CENTRIFUGATION OF DAPHNIA MAGNA HOMOGENATE (DCPIP used as an artificial electron acceptor; initial isolation method)

	A600(initial)	A600(15 min)	A600/min/mg
Fraction S2	0.510	0.300	9.33×10^{-5}
Fraction P3 (run#1)	0.390	0.171	2.05×10^{-4}
(run#2)	0.390	0.159	1.94×10^{-4}

From the data presented in Tables 4 and 5 it can be seen that an estimated 1.6% of the total dry weight of the daphnia was recovered as mitochondrial protein in the final pellet.

Comparable assays performed at 25°C with daphnia mitochondria and beef heart mitochondria using NADH as substrate produced significantly different rates of O_2 consumption. Four determinations with daphnia mitochondria produced a mean oxygen comsumption of 17.98 nmoles/mg/minute (range 13.98 to 21.15 nmoles/mg/minute). A single experiment with mitochondria from beef heart produced an oxygen consumption of 98.96 nmoles/mg/minute, or 5.5 times the rate observed in Daphnia.

TABLE V

ESTIMATED PROTEIN CONTENTS OF FRACTIONS FROM CENTRIFUGATION OF DAPHNIA MAGNA HOMOGENATE
(Biuret protein assay; final isolation method)

Fraction	Total Protein	% Original Protein
Homogenate	298.6 g	100.0
Supernatant 1	138.5 g	48.4
Pellet 2	46.3 g	15.5
Pellet 3	16.0 g	5.4

Estimates of the amount of cytochrome C lost during the isolation procedure (i.e., percentage of incomplete electron transport chains before addition of exogenous cytochrome C) ranged from 37% for the first isolation to 61% for the final method. An estimated 52% of the cytochrome C was lost during isolation of the beef heart mitochondria.

When succinate was used as substrate for the electron transport chain, naphthalene (30 mg/l final concentration) caused a mean increase of 13.5% in oxygen uptake in the daphnia mitochondria. In contrast naphthalene at 30 mg/l produced a mean decrease of 47.9% in oxygen uptake when NADH was the substrate (Appendix C). A control run with only the ethanol carrier as a toxicant before adding naphthalene produced a 31% decrease in respiration. However, the subsequent addition of naphthalene produced a further 67% decrease in O_2 uptake (a

reduction of 78% from the initial rate). The experiment with beef heart mitochondria revealed a 47% inhibition of O_2 uptake caused by naphthalene at 30 mg/l, when NADH was used as substrate.

CHAPTER V

DISCUSSION

Response Criterion

Previous work by Whitman and Miller (1982) demonstrated the inhibition of phototaxis to be an indication of toxic effect in Daphnia. My data further indicate that the response criterion used by Whitman and Miller (1982) is a reliable measure of the phototactic locomotor abilities of the animals. Individual animals usually exhibited an all-or-none response, either rising to the top of the tube or remaining at the bottom, with few animals showing partial response (Figure 5).

Response in only a portion of the population indicates variation in phototactic tendencies of individual animals. This variability may be due to inherent genetic differences or perhaps to non-genetic (e.g., nutritional state or general health) parameters. The methods developed in this study (Appendix D) produce consistent responses, with low variance among replicates. Further improvement in phototactic response and consistency can easily be obtained by establishing cultures of animals cloned from a single parthenogenetic female.

None of the alternate stimuli I examined produced consistent, measurable responses in Daphnia. Thus, the phototaxis test as described by Whitman and Miller (1982) appears the easiest and most reliable indicator of toxic stress. Because highly intoxicated daphnia retain the dorsal light response, I infer that impairment of neither perceptual

nor orientational abilities are responsible for reduced phototaxis in intoxicated animals; the toxic condition probably impairs locomotor abilities by disrupting energy-producing metabolic pathways.

Culture Food and Feeding

Based on experimental comparisons of the effects of different food types and convenience of feeding, dried brewer's yeast was chosen for use in culture of Daphnia for the phototaxis bioassay. The only significant problems encountered in maintaining healthy cultures resulted from the growth of aquatic fungi and probably was aggravated by the reservoir of fungal species in fish cultures in the same laboratory. Fungal growth would depend greatly on the food type used, and excessive problems may necessitate using an alternate culture food. However, the fungal species I encountered were adequately controlled through careful control of water temperatures.

A decrement in phototactic response, probably resulting from starvation effects, occurred in daphnia which had not been fed for 4 days prior to the phototaxis test. Many animals initially swam to the top of the tubes, then sank to the bottom, apparently exhausted, during the 30-second test and were not counted as responding.

In experiments with recently fed daphnia, water in the test tubes contained a large amount of suspended particulate matter due to uneaten food and fecal products. Although the stretching of the gut by ingested food particles may serve to reduce phototactic drive in recently-fed daphnia, I hypothesize that the particles reflected light, causing the daphnia to perceive multidirectional light from reflection of particles near the compound eye. This phenomenon was particularly evident in

animals fed fluorescently tagged polystyrene beads, which were highly reflective of visible light. An earlier hypothesis that the enhanced metabolic state in recently fed animals reduced the phototactic response is tentatively rejected.

The majority of particulate matter appears to result from excretory products. Filtering the test medium prior to the phototaxis test would probably be ineffective unless performed immediately prior to testing each tube, as the test medium rapidly becomes suffused with particles voided from the well-packed alimentary canals of recently fed daphnia. Because of these phenomena, the testing protocol should include a standardized feeding schedule, with test animals being fed approximately 24 hr prior to the phototaxis test.

Laboratory Photoenvironment

Daphnia exhibited greater negatively phototactic responses when cultured at low light levels, and when exposed to higher light levels during the 2-hour acclimation/exposure period. The magnitude of light intensity difference between the culture and acclimation environments may be determinant of phototactic rates, although specific experiments were not performed to test this hypothesis. Based on data from experiments on test light intensity and on findings by Whitman and Miller (1982), highest phototactic responses should be obtained in the 250 to 500 fc range.

The most significant discovery concerning the laboratory photoenvironment was that phototaxis in Daphnia is greatly reduced when the animals are deprived of slowly changing light intensities between the light and dark phases of the photoperiod. The stimulus for vertical

migration of Daphnia in the natural environment is known to be changing light intensity (Harris and Mason, 1955), and it is now apparent that previous exposure to such twilight phenomena is necessary for strong light responses.

Transition from the natural photoenvironment to laboratory lighting regimes is likely to have behavioral or physiological effects on many animals now used for behavioral research. Variables of illumination in the laboratory may simulate those of a particular time of day or season, so that effects of experimental manipulations may be affected accordingly (Rusak and Zucker, 1975). The sudden on/off transitions between daylight and dark provided by normal automatic lighting controls are poor approximations of the gradually changing intensities under natural conditions.

Species may be physiologically adapted to be active at a particular optimum level of illumination (Kavanau, 1962), which may be a moderate twilight intensity, rather than the brightest or darkest part of the day-night cycle (Rusak and Zucker, 1975). Some species are active exclusively during crepuscular periods, and their behaviors obviously would be greatly altered by the unnatural lighting regime. Allen (1980) states that twilight may be especially critical to fish and amphibians, as up to 30 minutes may be required for intraocular adjustments to daytime or nighttime.

A circadian organization of learning occurs in bees (von Frisch, 1971), in birds (Hoffman, 1965; Kramer,1952), and in rats (Holloway and Wansley, 1973). Synchronization of enzymatic activity and hormonal functions often depend on precise temporal patterns of secretion (Rusak and Zucker, 1975) which may be synchronized by photoenvironmental

changes. Responses to some drugs are dependent on temporal relationships to the light cycle (Scheving et al., 1968). Visual perception and associated behaviors vary seasonally (Fibiger and Campbell, 1971). The sun-compass of bees and birds (von Frisch, 1971; Hoffman, 1965), affected by both time of day and season, may be critical to navigation during migration.

Neglect of laboratory variables affecting biorhythmicity may significantly affect the results of many experiments. Knowledge of these variables often has been lacking or ignored in psychological and biological research (Rusak and Zucker, 1975,p.156].

Until recent technology resulted in the availability of inexpensive microcomputers, control of complex environmental changes, such as daily and seasonal fluctuations in photoenvironmental parameters, was a formidable endeavor which was rarely attempted. The great variability of photoenvironments in different habitats (e.g., desert, forest floor, aquatic environments) and at different latitudes (magnitude of daylength differences in different seasons) make simulation of natural lighting conditions a complicated task.

While the majority of automatic lighting controls reported in the literature served only to produce steady dimming and brightening of incandescent lamps at sunrise and sunset, close approximations of actual intensity curves throughout the day and an automatically changing daylength throughout the year are possible with computer control. By relatively simple changes in programming, the light output can be customized for different latitudes and habitat types.

A lighting system such as described above has been developed, based on the inexpensive (<$100) Commodore VIC-20 microcomputer. The control

software creates a natural curve of light intensity throughout the day, and an automatically changing photoperiod throughout the year, producing sunset and sunrise at correct times according to the user's latitude. Circuit diagrams, details of construction, and controlling software are presented in Appendix B.

Diurnal Variability in Phototactic Response

Results of the experiments examining diurnal variability in phototactic response suggest that vertical migrations in Daphnia are initiated by light stimuli and not by an intrinsic circadian rhythm entrained by photoperiodic events. No significant change in responsiveness to light was observed at any particular part of the photoperiodic cycle, unless the animals were exposed to a changing light stimulus. Nighttime patterns of response to the light in the test apparatus changed markedly when the animals were exposed to dimming light at sunset; however, if the animals were exposed to constant light conditions, response rates remained high during the normal sunset period, decreasing only slightly throughout the night.

Because no marked change in phototactic behavior occurs at any time of the photoperiodic cycle, the efficacy of the behavioral bioassay should not be hampered by the necessity of maintaining several cultures on staggered photoperiods.

Toxicant Bioassays

EC50 values obtained with the phototaxis bioassay technique are lower than any published values for 24-hour or 48-hour LC50

determinations with cladocerans. Confidence limits obtained with the phototaxis bioassay are comparable with values published for acute lethal bioassays.

The EC50 value obtained for phenanthrene, 0.39 mg l^{-1} (0.20 - 0.79), is considerably below the lowest reported value of 0.70 mg l^{-1} (0.59 - 0.84) for a 48-hour LC50 Daphnia bioassay (Milleman, 1984). Geiger et al. (1980) reported that the 48-hour LC50 value for phenanthrene was in excess of a saturated solution in pure water (> 1 mg l^{-1}).

The EC50 value for naphthalene in the phototaxis bioassay, 0.69 mg l^{-1} (0.37 - 1.28), is also significantly lower than any published acute lethal determinations with cladocerans. Milleman (1984) reported a 48-hour LC50 value of 2.16 mg l^{-1} (1.79 - 2.56) for naphthalene, while LeBlanc (1980) found LC50 values of 17.0 mg l^{-1} and 8.6 mg l^{-1} in 24-hour and 48-hour determinations, respectively. The United States Environmental Protection Agency reported a 48 hour LC50 value of 7.2 mg l^{-1} (USEPA, 1980). A comparison of reported LC50 values for phenanthrene, naphthalene, and acridine with those obtained with the phototaxis bioassay is presented graphically in Figure 19.

While the phototaxis bioassay appears to be slightly more sensitive to acridine than results reported by Milleman's (1984) studies with cladocerans, overlapping fiducial limits prevent stating the superiority of either technique. The EC50 value obtained by the phototaxis technique, 1.66 mg l^{-1} (0.73 - 3.80), is only slightly lower than Milleman's (1984) 2.05 mg l^{-1} (1.75 - 2.40) 48-hour LC50 value. The 24-hour LC50 value of 2.9 mg l^{-1} (Southworth, 1978), and 48-hour LC50 value value of 2.3 mg l^{-1} (Parkhurst, 1981) are also only slightly

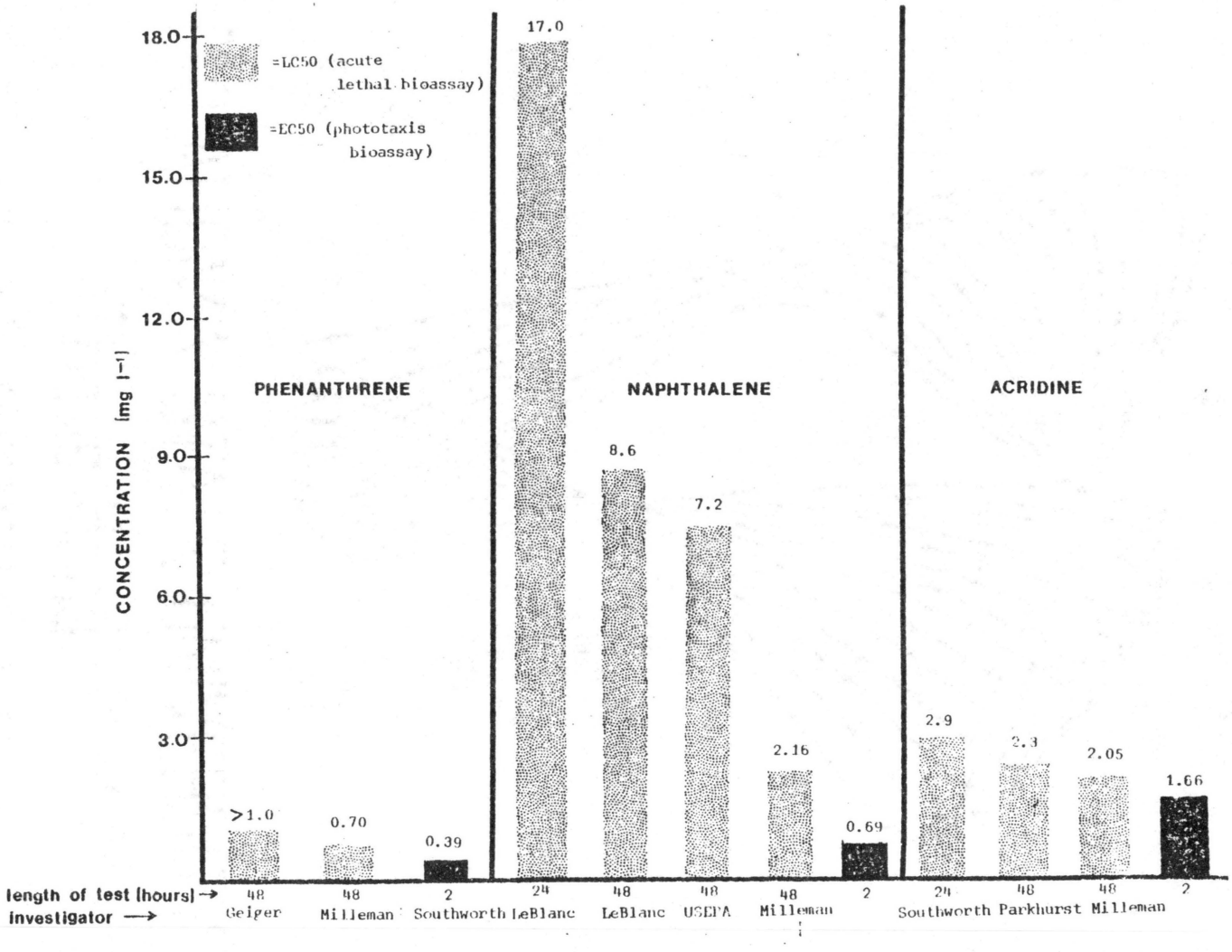


Figure 19. Comparison of Reported LC50 Values for Phenanthrene, Naphthalene and Acridine with EC50 Values Obtained in Phototaxis Bioassay.

higher than that obtained with the phototaxis bioassay.

The three compounds tested in the bioassay were chosen because of their importance in aquatic pollution associated with production and use of fossil fuels and because they are toxic at low concentrations. All three compounds have relatively high octanol/water partition coefficients, which estimate the rate of uptake by the biota. The two-hour phototaxis bioassay should be particularly useful in detecting conditions in which a significant portion of the toxicity is contributed by similar compounds.

Because a decrement in phototactic response can be detected at a degree of intoxication far below that necessary to produce the response criterion (=death) used in acute-lethal bioassays, the test should be amenable to many types of aquatic monitoring. To detect the presence of toxicity produced by compounds which are bioaccumulated less rapidly, the exposure time may have to be increased. However, the phototaxis test will probably continue to produce more rapid and sensitive determinations than can be obtained when death is the response criterion. Possible exceptions may include toxicants posessing an abnormal time-response curve due to their actions on biological membranes (e.g., many metals). In these instances, a decrement in phototactic response may be delayed until changes in membrane qualities permit the rapid uptake of toxicants, with death ensuing quickly.

Effect of Naphthalene on Mitochondrial Respiration

As cytochrome C is an extrinsic protein of the mitochondrial membrane and is easily dislodged from the membrane by mechanical jarring or by salt contamination, the additional handling necessary to increase

the yield of mitochondria undoubtedly caused significant losses of the protein in the final isolation procedure.

Rates of oxygen consumption observed in Daphnia mitochondria before and after exposure to naphthalene indicate that NADH-driven oxidative respiration is inhibited significantly by naphthalene at 30 mg l^{-1}, while succinate-driven respiration is not. Additionally, naphthalene appears to stimulate oxidative respiration in the succinate-driven reactions. These results are corroborated by the findings of Harmon and Sanborn (1982), who obtained the same responses in mitochondria isolated from beef heart. Because the inhibitory effect is manifested only in NADH-driven reactions, I conclude that the toxic action of naphthalene takes place at the NADH dehydrogenase step of the respiratory chain. As the pathway between coenzyme Q and the final reduction of O_2 is uninhibited, the portion of the NADH pathway before coenzyme Q is the only possible site of inhibition.

A possible explanation of the enhancement of O_2 uptake in succinate-driven respiration is that naphthalene may act as an uncoupler of oxidative phosphorylation, thereby allowing the transport of electrons down the chain without the normally requisite phosphorylation of ADP to ATP. If the concentration of ADP in the reaction chamber were limiting, and if naphthalene acts like known uncouplers such as 2,4-dinitrophenol or dicoumarol, a partial uncoupling of substrate phosphorylation would result in an increase in O_2 uptake.

It should be noted that the concentrations of naphthalene used in these experiments were in excess of the LC50 values of naphthalene in D. magna. LeBlanc (1980) reported a 48-hr LC50 (median lethal concentration) of 8.6 mg l^{-1} for naphthalene in D. magna, while Crider

(1981) reported 3.4 mg l^{-1} as a value for the same determination. Crider also noted that at 5.0 mg l^{-1} naphthalene, the second antennae of the animals became inactive, causing them to sink to the bottom.

Crider (1981) found a mean O_2 consumption rate of 44 nmoles $animal^{-1}$ $hour^{-1}$ in control animals, while respiration in animals exposed to 1 mg l^{-1} and 8 mg l^{-1} for 24 hours was inhibited 10.2% and 25.1%, respectively. These rates may be due to a higher effective naphthalene concentration inside the organism due to the bioaccumulation of naphthalene over the 24-hour period.

The control experiments reported herein yielded an O_2 consumption rate of 5.4 nmoles per animal-equivalent per hour for NADH-fed reactions and 14.3 nmoles per animal-equivalent per hour, for succinate-fed reactions. These values were determined by calculating the O_2 uptake rate accomplished by the portion of mitochondria in the final pellet which would have resulted from one daphnia. These rates seem realistic in comparison with respiration rates for whole animals determined by Crider (1981), but direct comparison is not possible due to the unknown yield of the isolation methods, and unknown proportions of substrates used in respiration in whole animals.

The deleterious effect of naphthalene on energy producing pathways, together with the retention of orientational abilities in naphthalene-intoxicated daphnia strongly suggest that reduced phototactic response in intoxicated daphnia may be a result of energetic debilitation, rather than of disruption of perceptual or neuromuscular interactions.

Conclusion

The demand for rapid, sublethal, biological assays allowing predictions of ecosystem effects has arisen in recent years (Geiger et al., 1978), and leaders in environmental agencies and in industry have frequently expressed their need for such techniques.

The results of any single test are inadequate for extrapolation to natural conditions; a conservative approach to risk assessment requires a battery of tests with organisms from different trophic levels (Milleman, 1984). Individual tests can, however, be used to make predictions about ecosystem effects, and if implemented, the phototaxis bioassay developed in this study should allow more rapid and sensitive detection of toxic conditions.

LITERATURE CITED

Allen, D. M. 1980. A device for providing gradual transition between light and dark periods in the animal room. Lab. Anim. Sci. 30: 252-254.

Anderson, B. G. 1948. The apparent thresholds of toxicity of Daphnia magna for chlorides of various metals when added to Lake Erie water. Trans. Am. Fish. Soc. 78: 96-113.

Baker, J. M. and G. B. Crapp. 1974. Toxicity tests for predicting the ecological effects of oil and emulsifier pollution on littoral communities. p. 23-40. In: Ecological Aspects of Toxicity Testing of Oils and Dispersants. Ed. by L. R. Beynon and E. B. Cowell. John Wiley & Sons, New York. 149pp.

Banta, A. M. 1921. A convenient culture medium for daphnids. Science 53: 557.

Belleuvre, G. 1938. Physiological action of pyrethrins on some invertebrates. Ann. Physiol. Physicochim. Biol. 14: 717.

Besch, W. K., A. Kemball, K. Meyer-Waarden, and B. Scharf. 1976. A biological monitoring system employing rheotaxis of fish. p. 56-74. In: Cairns, J., K. L. Dickson and G. F. Westlake. Biological Monitoring of Water and Effluent Quality. Amer. Soc. Test. Mat. Publ. STP 607.

Bond, R. M. 1934. A culture medium for Daphnia. Science 79: 60.

Brown, L. A. 1929. The natural history of cladocerans in relation to temperature. I. Distribution and temperature limits for vital activities. Amer. Nat. 63: 248-264.

Brown, C. J. D. and R. C. Bell. 1943. An experiment in the use of Derris root (rotenone) on the fish and fish-food organisms of the Third System Lake. Trans. Amer. Fish. Soc. 72: 177.

Bruins, R .J. F., A. L. Flickinger, and R. W. Winner. 1981. Personal Communication. Dept. Zoology, Miami Univ., Oxford, Ohio.

Buikema, A. L., D. R. Lee and J. Cairns. 1976. A screening bioassay using Daphnia pulex for refinery wastes discharged into fresh water. J. Test. and Eval. 4(2): 119-125.

Burks, S. L. and J. L. Wilhm. 1977. Bioassays with a natural asemblage of benthic macroinvertebrates. p. 127-136. In: F. S. Major and J. L. Hamelink [Eds.]. Aquatic Toxicology and Hazard Evaluation. Amer. Soc. Test Mat. publ. STP 634. Philadelphia.

Butler, G. C., ed. 1978. Principles of Ecotoxicology. John Wiley and Sons. New York. 350pp.

Cairns, J. 1965. Biological concepts and industrial waste disposal problems. p. 49. In: Proc. 20th Ind. Waste Conf., Purdue Univ.

Cairns, J., K. L. Dickson and G. F. Westlake, eds. 1977. Biological monitoring of water and effluent quality. Amer. Soc. Test. Mat. publ. STP 607.

Cairns, J. and W. H. van der Schalie. 1980. Review paper. Biological monitoring. Part 1 - Early warning systems. Water Research 14: 1179-1196.

Canton, J. H. and D. M. M. Adema. 1978. Reproductbility of short-term and reproduction toxicity experiments with Daphnia magna and comparison of the sensitivities of Daphnia magna with Daphnia pulex and Daphnia longispina in short-term experiments. Hydrobiologia 59 (2): 135-140.

Canton, J. H. and W. Sloff. 1972. The usefulness of Lymnaea stagnalis as a biological indicator in toxicological bioassays (model substance α-HCH). Water Research 11: 117-121.

Chipman, A., Jr. 1934. A new culture medium for cladocerans. Science 79: 59.

Conger, K. A. et al., 1978. Shell growth of unfed oysters in the laboratory: A sublethal bioassay system for pollutants. Life Sci. 22: 245-254.

Corner, R. P. Harris, C. C. Kilvington and S. C. M. O'Hara. 1976. Petroleum compounds in the marine food web: short-term experiments of the fate of naphthalene in Calanus. J. Mar. Biol. Ass. U. K. 56: 121-133.

Cowell, E. B. 1974. A critical examination of present practice. p. 97-104. In: L. R. Beynon and E. B. Cowell [Eds.] Ecological Aspects of Toxicity Testing of Oils and Dispersants. John Wiley and Sons, New York. 149pp.

Crider, J. Y. 1981. The effects of naphthalene on the hemoglobin concentration and oxygen consumption of Daphnia magna. M.S. thesis, Oklahoma State University. 67pp.

Cushing, D. H. 1951. The vertical migration of plankton crustacea. Biol. Rev. 26: 158-192.

D'Agostino, A. S. and L. Provasoli. 1970. Dixenic culture of Daphnia magna straus. Biol. Bull. 139: 485-494.

Dehnert, J. F. 1979. Case history, the use of powdered activated carbon with a biodisc-filtration process for treatment of refinery wastes. In: Activated Carbon Treatment of Industrial Wastewaters. Industrial Sources Section, Source Management Board. R.S. Kerr Environ. Research Lab. Ada, OK.

Dorris, T. C., S. L. Burks and G. R. Waller. 1974. Effects of residual toxins in oil refinery effluents on aquatic organisms. Technical completion report to U.S.D.I., Office of Water Resources Research. NTIS OK-B023.

Doudoroff, P. and M. Katz. 1950. Critical review of the literature on the toxicity of industrial wastes and their components to fish. I. Alkalies, acids and inorganic gasses. Sew. Ind. Wastes 22: 11.

Doudoroff, P. and M. Katz 1953. Critical review of the literature on the toxicity of industrial wastes and their components to fish. II. The metals as salts. Sew. Ind. Wastes 25: 802-839.

Dowden, B. F. 1962. Toxicity of commercial waste oil emulsifiers to Daphnia magna. J. Water Pollut. Control Fed. 34 (10): 1010-1014.

Ellis, M. M. 1937. Detection and measurement of stream pollution. Bull. Bureau Fisheries 48: 365-437.

Ewald, W. F. 1910. Uber Orientierun, lokomotion und lichtreaktionen einiger cladoceran und deren bedeutung fur die theorie der tropismen. Biol. Zbl. 30: 1-16, 49-63, 379-384, 385-399.

Fibiger, H. C. and B. A. Campbell. 1971. The effect of para-chlorophenylalanine on spontaneous locomotor activity in the rat. Neuropharmacology 10: 25-32.

Frear, D. E. H. and J. E. Boyd. 1967. Use of Daphnia magna for the microbioassay of of pesticides. I. Development of standardized techniques for rearing Daphnia and preparation of dosage-mortality curves for pesticides J. Econ. Entomol. 60: 1228-1236.

Freeman, L. 1953. Toxicity thresholds of certain sodium sulfonates for Daphnia magna. Sew. Ind. Wastes 25: 1331-1335.

Freeman, L. and I. Fowler. 1953. Toxicity of combinations of certain inorganic compounds to Daphnia magna straus. Sew. Ind. Wastes 25: 1191-1195.

Galtsoff, P. S., F. E. Lutz, P. S. Welch and J. G. Needham, 1937. Culture methods for invertebrate animals. Dover, New York. 590pp.

Gehrs, C. W., B. R. Parkhurst and D. S. Shriner. 1978. Environmental testing. p. 319-330. In: Proc. of the Symposium on Application of Short-term Bioassays in the Fractionation and Analysis of Complex Environmental Mixtures. Williamsburg, Virginia, Feb. 21-23, 1978. Plenum Press, New York. 588pp.

Geiger, J. G., A. L. Buikema, Jr. and J. Cairns, Jr. 1980. A Tentative Seven-day Test for Predicting Effects of Stress on Populations of Daphnia pulex. Amer. Soc. Test. Mat. STP 707: 13-26.

Goodman, G. T. 1974. How do chemical substances affect the environment? Proc. R. Soc. Lond. B. 185: 127-148.

Grosser, B. I., E. R. Baylor and F. E. Smith. 1953. Analysis of geotactic responses in Daphnia magna. Ecology 34: 804-805.

Hall, L., Jr., A. L. Buikema and J. Cairns, Jr. 1978. The effects of a simulated refinery effluent on the grass shrimp Palamemonetes pugio. Arch. Environ. Contam. Toxicol. 7: 23-25.

Harmon, H. J. and F. L. Crane. 1973. Topography of the cristae membrane as elucidated by a new inhibitor, trifluorofuryl-butanedione. Biochem. Biophys. Res. Comm. 55(1): 169-173.

Harmon, H. J. and M. R. Sanborn. 1982. Effect of naphthalene on respiration in heart mitochondria and intact cultured cells. Environ. Research 29: 160-173.

Harris, J. E. 1963. The role of endogenous rhythms in vertical migration. J. Mar. Biol. Ass. UK 43: 153-166.

Harris, J. E. and P. Mason. 1955. A laboratory study of vertical migration. Proc. R. Soc. Ser. B Biol. 145: 280-290.

Harris, J. E. and U. K. Wolfe. 1955. A laboratory study of vertical migration. Proc. R. Soc. Lond. B 144: 329-354.

Hasler, A. D. 1937. Methods for culturing Daphnia. p. 214-215 In: R. S. Galtsoff, P. E. Lutz, P. S. Welch and J. G. Needham [Eds.]. Culture Methods for Invertebrate Animals. Dover, New York. 590pp.

Henderson, C. and C. M. Tarzwell. 1957. Bioassays for control of industrial effluents. Sew. Ind. Wastes 29 (2): 1002-1017.

Hess, C. 1913. In: Winterstein, Handbuch der Vergleichenden Physiologie, 4. Berlin: Gustav Fischer.

Hoffman, K. 1965. Clock mechanisms in celestial orientation of animals. p. 426-441 In: J. Aschoff [Ed.]. Circadian Clocks. Amsterdam: North-Holland. 479pp.

Holloway, F. A. and R. A. Wansley. 1973. Multiphasic retention deficits at periodic intervals after passive avoidance learning. Science 180: 208-210.

Horning, W. B. 1977. Research related to biological evaluation of complex wastes. p. 191-198. In: J. Cairns, Jr., K. L. Dickson, and G. F. Westlake [Eds.]. Biological Monitoring of Water and Effluent Quality. Amer. Soc. Test. Mat. Publ. STP 607.

Hutchinson, G. E. 1967. A Treatise on Limnology. Vol. II. Introduction to Lake Biology and the Limnoplankton. John Wiley and Sons, New York. 1115pp.

Irwin, W. H. 1965. Fifty-seven species of fish in oil-refinery waste bioassay. Trans. 13th N. Amer. Wildl. and Nat. Resourc. Conf. p. 89-99.

Jander, R. 1975. Interaction of light and gravity orientation in Daphnia pulex. In: H. Schone [Ed.]. International Symposium on spatial Orientation, Cologna, 1973. Mechanisms of Spatial Perception & Orientation. (d/Stuttgart: Fischer, 1975) p. 174-184.

Jones, H. R. 1971. Environmental control in the organic and petrochemical industries. Noyes Data Corp.:Park Ridge, N.J. 257pp.

Kavanau, J. L. 1962. Twilight transitions and biological rhythmicity. Nature 194: 1293-1295.

Kramer, C. 1952. Experiments on bird orientation. Ibis 94: 265-285.

Ladd, E. C. 1977. An industrial view of biological monitoring. In: J. Cairns, K. L. Dickson, and G. F. Westlake [Eds.]. Biological Monitoring of Water and Effluent Quality. Amer. Soc. Test. Mat. publ. STP 607.

LeBlanc, G. A. 1980. Acute toxicity of priority pollutants to the water flea (Daphnia magna). Bull. Environ. Contam. Toxicol. 24: 684-691.

Leewangh, P. 1978. Toxicity tests with daphnids: its application in the management of water quality. Hydrobiology 59: 145-148.

Lentzen, D. E., D. E. Wagoner, E. D. Estes and W. F. Gutknecht. 1978. IERL-RTP procedures manual: Level I Environmental Assessment. Second Edition.

Litchfield, J. T. and F. Wilcoxon. 1949. A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exper. Therapeutics 96: 99-113.

Loeb, J. 1908. Uber heliotropismus und die periodischen tiefenbewegung en pelagischer tiere. Biol. Zbl. 28: 732-736.

MacArthur, J. W. and W. H. T. Baillie. 1929. Metabolic activity and duration of life. I. Influence of temperature on longevity in Daphnia magna. J. Exp. Zool. 53: 221-242.

Maki, A. W. 1979. Correlations between Daphnia magna and fathead minnow Pimephales promelas chronic toxicity values for several classes of test substances. J. Fish. Res. Board Can. 36: (4): 411-421.

Marouka, S. 1978. Estimation of toxicity using cultured mammalian cells of the organic pollutants recovered from Lake Biwa. Water Research 12: 371-375.

Milleman, R. E., W. J. Birge, J. A. Black, R. M. Cushman, K. L. Daniels, P. J. Franco, J. M. Giddings, J. F. McCarthy, and A. J. Stewert. 1984. Comparative acute toxicity to aquatic organisms of components of coal-derived synthetic fuels. Trans. Amer. Fish. Soc. 113: 74-85.

Murphy, J. S. 1970. A general method for the monoxenic cultivation of the daphnidae. Biol. Bull. 139: 321-332.

Nicol, J. A. C., W. H. Donahue, R. T. Wang and K. Winters. 1977. Chemical composition and effects of water extracts of petroleum on eggs of the sand dollar Melitta quinquiesperforata. Marine Biology 40: 309-316.

Obreshkove, V. 1930. Oxygen consumption in the developmental stages of a cladoceran. Physiol. Zool. 3: 271-282.

Obreshkove, V. and A. Abramowitz. 1932. Temperature characteristics for oxygen consumption of a cladoceran. J. Cell. Comp. Physiol. 2: 133-139.

Pacaud, A. 1939. Contribution a l'ecologie des cladoceres. Bull. Biol. Fr. Belg., suppl 25: 260pp.

Parkhurst, B. R., A. S. Bradshaw, J. L. Forte, and G. P. Wright. 1981. The chronic toxicity to Daphnia magna of acridine, a representative azaarene present in synthetic fossil fuel products and wastewaters. Environmental Pollution Series A. Ecological and Biological 24: 21-30.

Ringelberg, J. 1964. The positively phototactic reaction of Daphnia magna straus: a contribution to the understanding of diurnal vertical migration. Neth. J. Sea Res. 2, 3: 319-406.

Ringelberg, J. and H. Servaas. 1971. A circadian rhythm in Daphnia magna. Oecologia 6: 284-292.

Rose, M. 1925. Contribution a l'etude de la biologie du plankton: le probleme des migrations verticales journalieres. Arch. Zool. Exper. et Gener. 64: 387-542.

Rusak, B. and I. Zucker. 1975. Biological rhythms and animal behavior. Ann. Rev. Psychol. 27: 137-171.

Russell, F. S. 1926. The vertical distribution of the marine macroplankton. IV. The apparent importance of light intensity as a controlling factor in the behavior of certain species in the Plymouth area. J. Mar. Biol. Ass. U. K. 14: 415-440.

Russell, F. S. 1927. The vertical distribution of plankton in the sea. Biol. Rev. 2: 213-262.

Sanders, H. O. and O. B. Cope. 1966. Toxicities of several pesticides to two species of cladocerans. Trans. Amer. Fish. Soc. 95: 165-169.

Scherer, E. 1977. Behavioral assays - principles, results and problems. Proc. 3rd Aquatic Toxicity Workshop, Halifax, N.S., Report No. EPS-5AA-77-1. Halifax, Canada. p. 33-40.

Scheving, L. E., D. F. Vedral, and J. E. Pauly. 1968. Daily circadian rhythm in rats to D-amphetamine sulphate: Effect of blinding and continuous illumination on the rhythm. Nature 219: 621-622.

Schluchter, A. W. 1937. Daphnia culture. p. 215-216. In: P. S. Galtsoff, F. E. Lutz, P. S. Welch, and J. G. Needham [Eds.] Culture Methods for Invertebrate Animals. Dover, New York.

Schroder, W. 1956. Simultane photo- und geotaxis bei der verticalwanderung von Daphnia longispina O.F.M. und Bosmina coregonia Bair Naturwissenschaflen 43: 285.

Sheffrin, N. M. H., N. R. J. Fieller and E. E. Williams. A behavioural bioassay for impaired sea-water quality using the plantigrades of the common mussel Mytilus edulis L.: The response to copper. Aquatic Toxicology 5: 77-91.

Simonet, D. E., W. I. Knausenberger, L. H. Townsend, Jr., and E. C. Turner, Jr. 1978. A biomonitoring procedure utilizing negative phototaxis of first instar Aedes aegypti larvae. Arch. Environm. Contam Toxicol 7: 339-347.

Smith, F. E. and E. R. Baylor. 1953. Color responses in the cladocera and their ecological significance. Amer. Nat. 88: 832.

Southworth, G. R., J. J. Beauchamp and P. K. Schmieder. 1978. Bioaccumulation potential and acute toxicity of synthetic fuels effluents in freshwater biota: azaarenes. Environ. Sci. Technol. 12: 1062-1066.

Sprague, J. B. 1976. Current status of sublethal tests of pollutants on aquatic organisms. J. Fish. Res. Board Canada 33: 1988-1992.

Stearns, S. C. 1975. Light responses of Daphnia pulex. Limnol. Oceano. 20: 564-570.

Steel, R. G. D. and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill: New York. 481pp.

Tevlin, M. P. 1978. An improved experimental medium for freshwater toxicity studies using Daphnia magna. Water Res. 12 (11): 1017-1024.

USEPA (United States Environmental Protection Agency). 1978. In-depth studies on health and environmental impacts of selected water pollutants. USEPA contract 68-01-4646. Washington, D. C.

Viehoever, A. 1935. Daphnia propagation for experimental use. Amer. Pharm. 107: 103-130.

Viehoever, A. and I. Cohen. 1938. The comparative physiological action of benzedrine (amphetamine) and derivatives on Daphnia magna. Amer. J. Pharmacol. 110: 526-532.

von Buddenbrock, W. 1915. Die tropismentheorie von J. Loeb ein versuch ihrer widerlegung. Biol. Zbl. 35: 481-506

von Frisch, K. 1971. Bees. Ithaca: Cornell Univ. Press. 1577pp.

von Frisch, K. and E. H. Kupelwieser. 1913. Uber den Einfluss der Lichtfarbe auf die phototaktischen Reaktionen niederer Krebse. Biol. Zbl. 33: 517-552.

Weismann, A. 1877. Das Tierleben im Bodensee. Schr. Gesch. Bodensees Umgebung 7: 1-31.

Westlake, G. F., D. W. Rowe, J. B. Sprague, T. A. Heming and I. T. Brown. 1978. Daphnia for superior sublethal testing. Proc 4th Annual Aquatic Toxicity Workshop. Fisheries and Marine Service Technical Report No. 818. 30pp.

Wetzel, R. G. 1975. Limnology. W. B. Saunders Co., Philadelphia. 743pp.

Whitman, L. J. and R. J. Miller. 1982. The phototactic behavior of Daphnia magna as an indicator of chronic toxicity. Proc. Okla. Acad. Sci. 62: 22-33.

Willingham, C. A. and K. J. Anderson. 1966. Use of microorganisms for detecting toxic materials in water. Part I. Wat. Sew. Works 113: 464-467.

Wilson, K. W. 1974. Toxicity testing for ranking oils and oil dispersants. In: L. R. Beynon and E. B. Cowell [Eds.]. Ecological aspects of toxicity testing of oils and dispersants. John Wiley and Sons, New York. 149pp.

Wilson, J. B. 1980. Personal Communication. Dept. of Zoology, Univ. of Guelph. Guelph, Ontario.

Wollerman, E. H. and L. S. Putman. 1955. Daphnids help to screen systemics. J. Econ. Entomol. 48: 759-760.

Yonetani, T. 1961. Studies on cytochrome oxidase III. Improved preparation and some properties. J. Biol. Chem. 236 (6): 1680-1688.

APPENDIX A

WATER QUALITY DATA FOR OKLAHOMA STATE UNIVERSITY

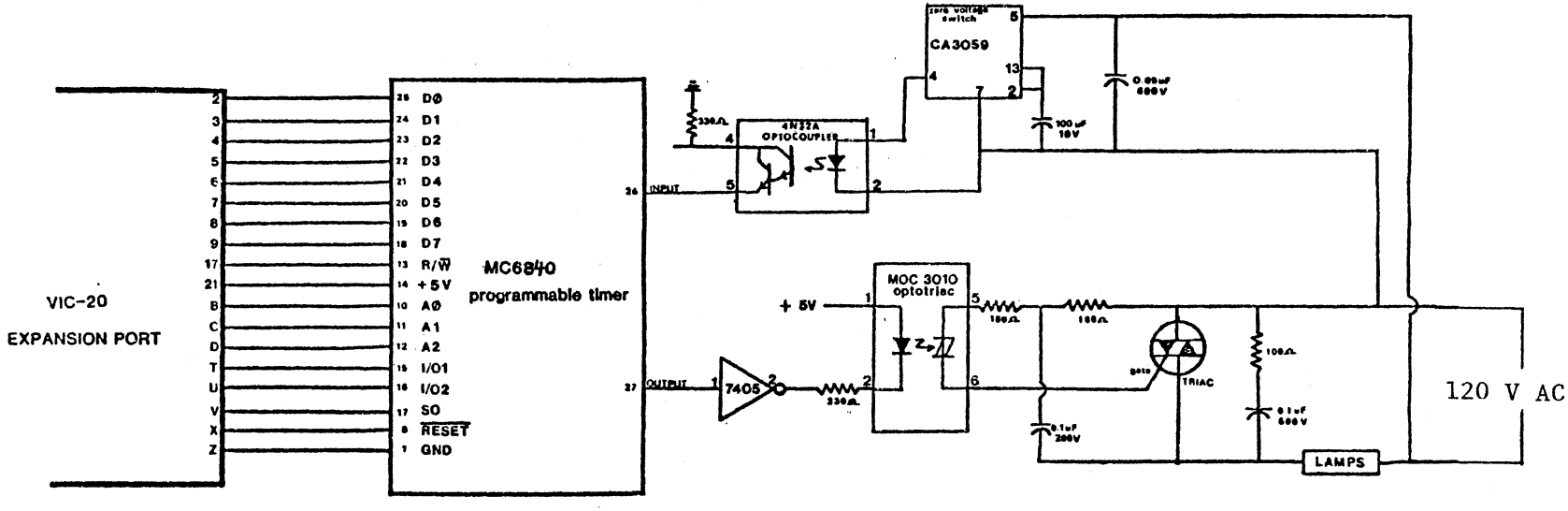
TAP WATER

WATER QUALITY DATA FOR OKLAHOMA STATE UNIVERSITY TAP WATER
JANUARY, 1982

Element	mg/l (ppm)
Sodium	23.73
Calcium	56.60
Magnesium	15.60
Potassium	5.14
Iron	0.000
Lead	0.007
Zinc	0.063
Copper	0.008
Chromium	0.000
Cadmium	0.001
Total Hardness	179 mg l-1
Alkalinity	135 mg l-1
Conductivity	426 uM cm-1
pH	7.6 - 8.1

APPENDIX B

POWER CONTROL CIRCUITRY AND BASIC PROGRAM TO SIMULATE NATURAL DIURNAL AND SEASONAL CHANGES IN LABORATORY PHOTOENVIRONMENT


VIC-20
EXPANSION PORT
MC6840
programmable timer
D0
D1
D2
D3
D4
D5
D6
D7
R/W
+5V
A0
A1
A2
I/O1
I/O2
SO
RESET
GND
INPUT
OUTPUT
4N33A
OPTOCOUPLER
CA3059
7405
MOC 3010
optotriac
+ 5V
TRIAC
LAMPS
120 V AC

The power control circuitry allows proportional AC phase control by controlling the time of firing of a triac which is optically coupled to the computer. The Motorola MC 6840 programmable timer integrated circuit provides varying delay times for the firing of the triac, according to data from the controlling program. Eight-bit resolution provides 256 levels of brightness.

The following BASIC program will run on the unexpanded Commodore VIC-20 microcomputer. The program is menu-driven, and will compute accurate sunrise and sunset times and daylength according to desired latitude and date, enabling the user to quickly determine these photoenvironmental parameters for any location between the equator and 60 degrees North Latitude.

When the computer is equipped with the preceding power control circuitry, the software will, after initial start-up data are entered, control incandescent laboratory lighting to produce crepuscular periods of slowly changing light intensity at sunset and sunrise, and will automatically advance or retard sunrise and sunset each day with the changing seasons. Times of sunrise and sunset are computed by trigonometric and fourth-degree polynomial functions, and are reported as Greenwich Mean Time. A static daylength option allows the user to specify sunrise and sunset times, which will remain unchanged until new values are entered.

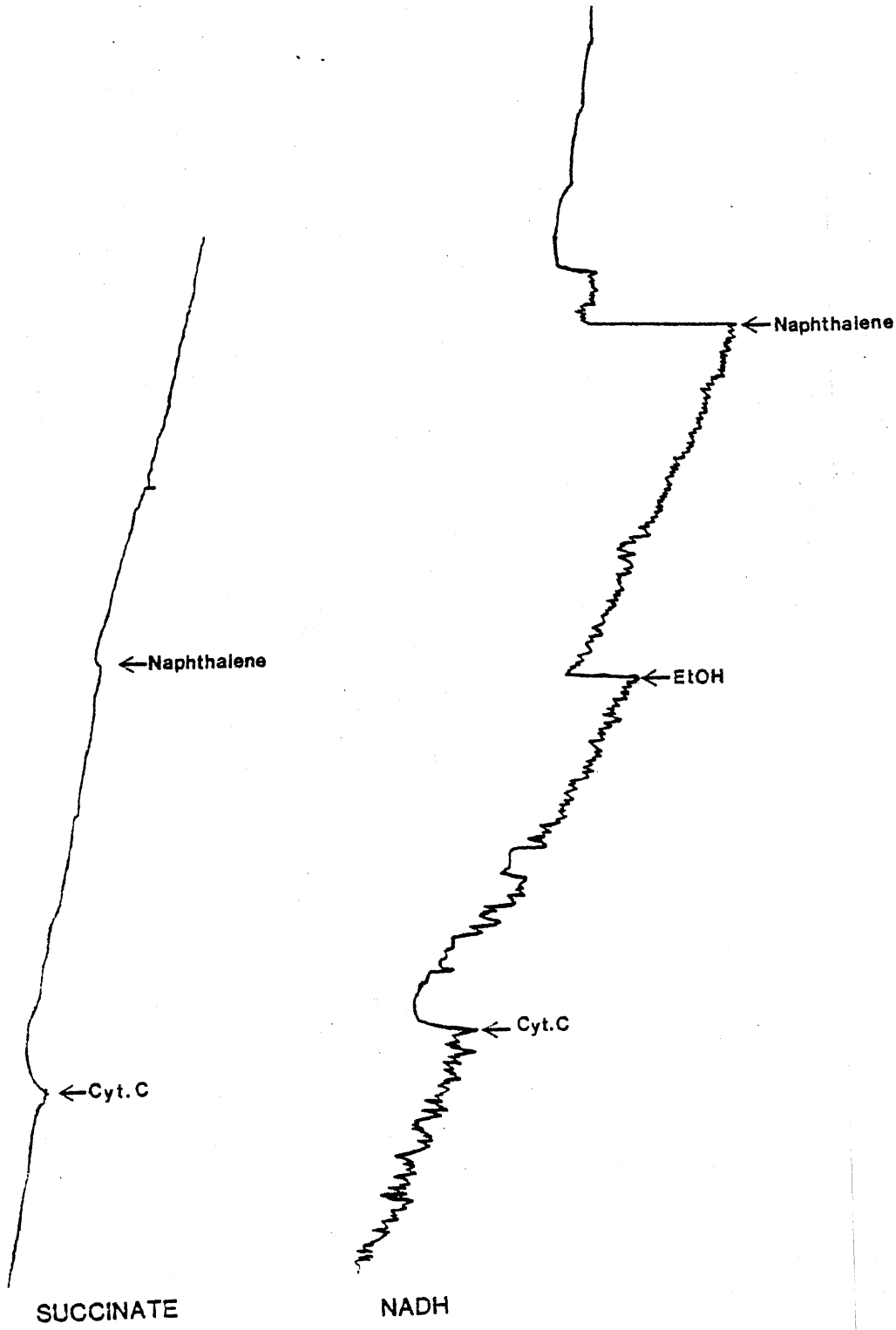
The circuit and program provided here should facilitate the study of the effects of crepuscular periods and of seasonal photoenvironmental changes on the physiology and behavior of many invertebrates and vertebrates.

```
2 PRINT"{SC}":PRINT"{CD}{CD}{CD}{CD}{CD}{CD}{CD}":PRINT"----------------------"
3 PRINT"       NATURAL{CD}":PRINT"{CR}  PHOTOENVIRONMENT{CD}":PRINT"      SIMULATOR"
4 PRINT"----------------------":PRINT"{CD}":PRINT"WRITTEN BY{CD}":PRINT"PAUL GROVER"
5 PRINT"DEPARTMENT OF ZOOLOGY":PRINT"OKLAHOMA STATE UNIV."
6 PRINT"STILLWATER, OK 74078"
7 PRINT"MARCH 1984"
8 FORI=1TO3000:NEXTI:PRINT"{SC}"
9 PRINT"{SC}":PRINT"{CD}{CD}":PRINTTAB(9)"MENU"
10 PRINT"{CD}{CD}{CD}C = CHECK SUNRISE &       SUNSET TIMES AND      DAYLENGTH"
11 PRINT"{CD}{CD}{CD}V{CR}= START SEASONALLY      VARYING LIGHT         REGIME"
12 PRINT"{CD}{CD}{CD}S = START STATIC LIGHT    REGIME"
13 PRINT"{CD}{CD}{CD}YOUR CHOICE?"
14 GETC$:IFC$=""THEN14
15 IFC$="V"THENPRINT"{SC}":GOTO6000
16 IFC$="C"THENPRINT"{SC}":GOTO6000
17 IFC$="S"THENGOTO5050
100 POKE38913,183:POKE38912,182:POKE38914,0:POKE38915,29
101 IFTI<50THEN100
200 REM SUNSET ROUTINE
201 X=0
202 POKE38912,182:POKE38914,X:POKE38915,29
203 FORI=1TO18750:NEXT I
204 X=X+1
205 IFX=255THENGOTO300
206 GOTO202
300 IFTI>FSTHEN300
399 C$="R"
400 DS=DS+1
401 IFDS=366THENDS=1
402 IFC$="S"THENGOTO404
403 GOTO9000
404 IFTI<SBTHEN404
500 REM SUNRISE ROUTINE
501 X=255
502 POKE38913,183
503 POKE38912,182:POKE38914,X:POKE38915,29
504 FORI=1TO18750:NEXT I
505 IFX=0THENGOTO100
506 X=X-1
507 GOTO502
5050 PRINT"{SC}";"ENTER DESIRED SUNRISE"
5051 PRINT"  HHMMSS":INPUTTI$:FR=TI:PRINT
5052 PRINT"ENTER DESIRED SUNSET"
5053 PRINT"  HHMMSS":INPUTTI$:FS=TI:PRINT
5054 PRINT"ENTER TIME OF DAY":PRINT"{USE MILITARY TIME)":PRINT"ENTER 6 DIGITS"
5055 PRINT"  HHMMSS":INPUTTI$:PRINT
5056 IFTI$>"240000"THEN PRINT"TRY AGAIN!"
5057 PRINT "LIGHTING CONTROL HAS  STARTED"
5058 SB=FR-108000
5059 SD=FS-324000:GOTO100
```

```
6000 PRINT"ENTER DEGREES NORTH   LATITUDE":INPUTNL
6001 IFNL>90THENPRINT"TRY AGAIN!":GOTO6000
6002 PRINT:PRINT"ENTER MONTH"
6003 PRINT "(JAN=1, FEB=2, ETC)":INPUT M:PRINT
6004 IFM>12THENPRINT"TRY AGAIN!":GOTO6002
6005 PRINT:PRINT"ENTER DAY OF MONTH":INPUTD:PRINT
6006 IFD>31THENPRINT"TRY AGAIN!":GOTO6004
6007 PRINT"ENTER TIME OF DAY":PRINT"(USE MILITARY TIME)":PRINT"ENTER 6 DIGITS"
6008 PRINT"  HHMMSS":INPUTTI$
6009 IFTI$>"240000"THEN PRINT"TRY AGAIN!":GOTO6006
6010 T=0:FORN=1TOM:READMD:T=T+MD:NEXTN
6011 DATA 0,31,28,31,30,31,30,31,31,30,31,30
6012 DA=T+D:DS=DA+10
6013 IFDA>355THENDS=DA-355
6014 RESTORE
6015 GOTO9000
7000 PRINT:PRINT"DAY #";DS;"OF YEARLY    LIGHT CYCLE"
7001 ER=1296000:ES=3888000
7003 TM%=FR/3600
7004 TH=TM%/60
7005 TH%=TH:PRINT"----------------------"
7006 MI%=(TH-TH%)*60
7007 PRINT:PRINT"SUNRISE AT";TH%;":";MI%
7008 QM%=FS/3600
7009 QH=QM%/60
7010 QH%=QH
7011 QI%=(QH-QH%)*60
7012 PRINT:PRINT"SUNSET AT";QH%;":";QI%
7013 PRINT
7014 DR=FS-FR
7015 DM%=DR/3600
7016 DH=DM%/60:DH%=DH
7017 DI=(DH-DH%)*60:DI%=DI
7018 PRINT"DAYLENGTH IS";DH%;":";DI%
7019 PRINT"----------------------"
7020 PRINT:PRINT"ANOTHER DATE? (Y OR N)"
7021 GETU$:IFU$=""THEN7021
7022 IFU$="Y"THENGOTO6000
7023 IFU$<>"Y"THENGOTO9
7030 GETU$:IFU$=""THEN6055
7031 IFU$="Y"THENGOTO6000
7032 IFU$<>"Y"THENGOTO9
8000 PRINT:PRINT"DAY #";DS;"OF THE YEARLY LIGHT CYCLE"
8010 PRINT:PRINT"LIGHTING CONTROL HAS  STARTED":GOTO100
9000 LF=(NL^4*.10358)-(NL^3*9.4858)+(NL^2*354.697)+(NL*1409)
9001 ER=1296000:ES=3888000
9003 SR=ER+LF:SS=ES-LF
9004 ZF=2.445*NL^3-90.45*NL^2+7356*NL
9005 SC=ZF*(-COS(.0172142*DS)+1)
9006 FR=SR-SC
9007 FS=SS+SC
9009 SB=FR-108000
9010 SD=FS-324000
9011 IFC$="R"THENGOTO404
9012 IFC$="C"THENGOTO7000
9013 IFC$="V"THENGOTO8000
```

APPENDIX C

OXYGEN UPTAKE BY ISOLATED *DAPHNIA MAGNA* MITOCHONDRIA


Naphthalene
EtOH
Cyt.C
Naphthalene
Cyt.C
NADH
SUCCINATE

APPENDIX D

PROTOCOL FOR CULTURING *DAPHNIA* AND PERFORMING PHOTOTAXIS FOR MAXIMUM RESPONSE IN CONTROL ANIMALS

PROTOCOL FOR CULTURING *DAPHNIA MAGNA* AND PERFORMING PHOTOTAXIS BIOASSAY FOR MAXIMUM RESPONSE IN CONTROL ANIMALS

Animals should be cultured at low light levels (below 14 foot-candles at surface of cultures) in large (at least 120 liters) tanks, preferably with three opaque walls. Laboratory lighting should adhere to a 16 hr light / 8 hr dark photoperiod, and should include transitions of gradual dimming and brightening (accomplished by one of the methods discussed in the text).

Room temperature is preferably held at approximately 25° C, but if subject to significantly lower temperatures or large fluctuations, culture temperatures should be held constant by use of aquarium heaters (remove any indicator lamp present in heater to allow total darkness at night). If daphnia are cultured at temperatures higher than that of the room, the animals must be acclimated at the same temperature by use of a water bath or environmental chamber.

Daphnia are fed dried brewer's yeast daily or when the culture medium appears dilute, as determined by clarity. The cultures should be fed last between 24 and 48 hours prior to the start of the bioassay. If the culture or toxicant dilution water contains significant amounts of suspended solids, the medium may be filtered immediately prior to the phototaxis test.

During the 2 hour exposure/acclimation period the daphnia should be placed in an area of higher light intensity (greater than 500 foot-candles). If flood lamps or other heat producing light sources are

used, heat filters may be necessary to prevent overheating the experimental animals. Incandescent lamps in the test aparattus (Figure 3) should produce approximately 500 foot-candles of illumination at the top and bottom of the test tube as it is held in the chamber.

Maximum phototactic response in control animals is obtained at mid-day, with response rates decrementing through the evening and night time hours; however if strongly phototactic animals are maintained, response rates should remain sufficiently high (above 75%) throughout the day and night to make the test useful on a 24-hour basis. For night time testing, test animals must be removed to a lighted area before onset of sunset.

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: A BEHAVIORAL BIOASSAY FOR ACUTE TOXICITY OF WATER-BORNE POLLUTANTS

Major Field: Zoology

Biographical:

Personal Data: Born in Clarksville, Arkansas, February 15, 1955 to Paul and Ethel Grover.

Education: Received Bachelor of Science degree in Biology from Northeastern Oklahoma State University in 1976; received Master of Science degree in Wildlife Ecology from Oklahoma State University in 1979; attended Oklahoma State University in 1980-1981, and enrolled in doctoral program in Fall, 1981; completed requirements for Doctor of Philosophy degree at Oklahoma State University in July, 1981.

Professional Experience: Graduate research assistant, Oklahoma State University, Zoology Department, 1976-77; consultant, Office of Ecological Services, U.S. Fish and Wildlife Service, 1978; intern, National Audubon Society, 1979; laboratory researchtechnician, Oklahoma State University, Biochemistry Department 1980-81; graduate research assistant, Oklahoma State University, Zoology Department, 1981-82; graduate teaching assistant, Oklahoma State University, Zoology Department, 1982-83; research assistant, Oklahoma State University, Zoology Department, 1983-84.

Societies, Organizations: Member, Sigma Xi, Phi Kappa Phi, Oklahoma Academy of Science, Oklahoma Ornithological Society, Inland Bird Banding Association.