THE EFFECTS OF NAPHTHALENE ON THE PHYSIOLOGY AND LIFE CYCLE OF *CHIRONOMUS ATTENUATUS* AND *TANYTARSUS DISSIMILIS*

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PREFACE

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CHAPTER I

INTRODUCTION

About 30,000 chemicals are in use today with 1000 to 2000 new chemicals produced yearly (Miller 1978). Many of these toxic chemicals including herbicides, pesticides, heavy metals, and organic compounds enter aquatic environments. Since this contamination is increasing and will remain a major water pollution problem in the future, it is necessary to detect and define the effects of these chemicals on aquatic biota.

Polycyclic (polynuclear) aromatic hydrocarbons (PAH) are condensed multi-ring structures that are acutely toxic to aquatic organisms and have chronic effects as possible carcinogens (Peirce 1961, Huggins and Yang 1962), mutagens (McCann et al. 1975), and teratogens (Rigdon and Rennels 1964). The chronic toxicological effects are generally attributed to the covalent binding of electrophilic metabolites of PAH to cellular macromolecules (Heidelberger 1976).

Low molecular weight PAH such as naphthalene occur in petroleum and are considered to be among the most acutely toxic (Anderson et al. 1974). However, acute toxicity data do not reflect adequately the potential impact of chronic low-level PAH contamination of aquatic biota. Chronic exposure to low concentrations of PAH in water, sediment, or food may produce subtle sublethal responses in aquatic organisms.

Freshwater contamination by petroleum products are generally much smaller in magnitude than in marine situations. Most sublethal PAH studies have been conducted using marine fish and invertebrates. Little information exists on the effects of PAH on freshwater invertebrates (Evans and Rice 1974, Moore and Dwyer 1974).

Benthic macroinvertebrates have often been used as indicators of water quality. Much work has been done on the effects of various toxicants on the species diversity and composition of benthic macroinvertebrate assemblages. Although the acute toxicity of certain chemicals has been determined for a few macroinvertebrate species, only recently has work been done on chronic effects. Chironomus, a dipteran larva, is an important member of most freshwater benthic assemblages and has a world-wide distribution. It is often extremely abundant in aquatic environments and is important in the aquatic food web. It has been reared in the laboratory and has a relatively short life cycle (Biever 1965, Credland 1973), thus allowing the determination of acute and chronic effects.

Sublethal concentrations of toxicants affect the physiology of organisms. Physiological studies range from the enzyme level through cells to whole organisms. Studies on whole organisms led to information about system changes, while enzyme and cellular studies help to elucidate the mode of action of toxicants.

An essential life process for an aquatic organism is the regulation of its salt and water balance. The two most critical problems of water balance in freshwater insects are loss of salts to a hypotonic environment and of water entering through the body surfance and feeding activities. Aquatic insects have various osmotic and ionic regulatory

mechanisms. Chironomus takes up ions through the anal papillae and midgut. Disruption of the regulatory mechanism leads to physiological changes, stress, and possible death. Some toxicants appear to alter cell membrane permeability causing changes in the ionic transport by interferring with enzymatic activity. Little information exists on the effect of PAH on osmotic and ionic regulation of freshwater organisms.

The rate of oxygen consumption is an indicator of metabolic rate and is affected by many environmental variables. Stresses placed on organisms should be reflected in alterations of metabolic rates. Changes in the rate of oxygen consumption due to toxicants has been used as an indicator of sublethal effects (Cairns 1966). The oxygen consumption of Chironomus attenuatus increased as the concentration of phenol increased (Cole and Wilhm 1972). Barry et al. (1978) reported that sublethal concentrations of benzene, xylene, and toluene resulted in increased oxygen consumption rates in the mosquito larvae of Aedes aegypti. Exposure of the mussel Mytilus californianus to benzene, toluene, and benzo(a)pyrene resulted in significant declines in the oxygen consumption rate (Sanbourin and Tullis 1981). Levitan and Taylor (1979) reported that 4 mg/l naphthalene resulted in significantly higher oxygen consumption rates in Fundulus heteroclitus.

Hematological analysis has been used to determine the sublethal effects of toxicants. In the northern puffer, Sphaeroides maculatus, endrin decreased normal hemoglobin concentration at 0.05 µg/l, while the hemoglobin increased at 0.1, 0.5, and 1.0 µg/l (Eisler and Edmunds 1966). Buckley et al. (1976) established a linear dose-response relationship between hemoglobin and total residual chlorine. McKim et al. (1970) concluded that short-term changes in blood parameters such as

hemoglobin concentration could be used to predict chronic effects.

Most chronic toxicity tests involve determining the effects of various reproductive parameters of Daphnia or the fathead minnow. Recently life cycle tests using chironomids has increased because of their importance in freshwater ecosystems, short life cycle time, and ease of rearing in the laboratory.

The objectives of the study were to determine (1) the acute effects of naphthalene on oxygen consumption, ionic regulation, hemoglobin concentration, and glycogen content of Chironomus attenuatus; (2) the chronic effects of naphthalene on larval survival, pupation times, adult emergence, and egg deposition of Tanytarsus dissimilis; (3) if Chironomus or Tanytarsus could be used to indicate polycyclic aromatic hydrocarbon contamination; and (4) the mode of action of polycyclic aromatic hydrocarbons on the ionic regulation of freshwater organisms.

CHAPTER II

REVIEW OF LITERATURE

Polycyclic Aromatic Hydrocarbons

The chemistry of polynuclear (polycylic) aromatic hydrocarbons (PAH) is well known. They are planar condensed multi-ring structures possessing little or no substitution by alkyl or polar groups (International Agency for Research on Cancer 1973). The condensed ring structure provides a high molecular stability that makes decomposition or disruption of the compound difficult. Generally the compounds are hydrophobic, insoluble in water, nonpolar, and nonionic (McGinnes and Snoeyink 1974). Benzo(a)pyrene (BaP) has a low vapor pressure and an octanol/water partition coefficient of 11,140 (Lu et al. 1977) suggesting that BaP has the potential to bioaccumulate in aquatic organisms.

Although some investigators suggest that BaP is insoluble in pure water (Borneff and Knerr 1960), others have reported the solubility of BaP as 0.01 µg/l (Andelman and Snodgrass 1974), 0.172 µg/l (Lu et al. 1977), and 3.0-4.0 µg/l (Davis et al. 1972). The solubility of PAH can be increased by stabilization in a colloidal suspension by adding surface active agents such as detergents (Klevens 1950). The addition of 10-50 mg/l of surfactants increased the solubility of BaP from two to ten times (Il'nitskii et al. 1971). Organic solvents in water, such as occur in industrial effluents, can also increase the solubility of PAH

(Suess 1970). In natural waters PAH may be solubilized by miscelles of lipids, peptides, or alkaloids (Radding et al. 1976).

The stability of PAH in water is influenced by oxygen and light. The photodegradation of BaP and 12 other PAH dissolved in cyclohexane and dichloromethane was studied after illumination with sunlight, fluorescent lamps, and an ultraviolect source (Kuratsune and Kirohata 1962). The rate of degradation was similar in sunlight and fluorescent-exposed solutions. The most light sensitive compounds were naphthalene, dimethylbenzanthracene, and BaP, while the other PAH compounds generally did not decompose. BaP decomposed at a much higher rate in an oxygen-saturated solution than in a nitrogen-saturated solution. The degradation of BaP sorbed onto surfaces of calcium carbonate in aqueous suspensions exposed to fluorescent light was studied to simulate BaP behavior in natural water (Suess 1967). Higher light intensity, oxygen concentration, and temperature accelerated BaP degradation, while pH had no effect.

Chemical degradation of PAH in the environment can take place through a variety of oxidative reactions resulting in quinones as primary products, with lesser amounts of diols, epoxides, and peroxides (Radding et al. 1976). In natural waters the principal oxidizing species are alkylperoxy and hydroperoxy radicals generated by the photolytic cleavage of trace carbonyl compounds or from enzymatic sources (Radding et al. 1976). Degradation products may be more resistent to degradation than the parent PAH, and their carcinogenicity is generally much less than that of the parent PAH (Radding et al. 1976).

PAH may be produced by a variety of industrial operations and the

combustion of fossil fuels. The production of PAH was greatest by an oxygen-deficient flame, temperatures in the range of 650-900°C, and fuels which were not highly oxidized (Baum 1978). Industrial operations which produce PAH are the production of acetylene from natural gas, the pyrolysis of kerosene to benzene, toluene and other organic solvents, gas production from petroleum, and oil refineries (Andelman and Snodgrass 1974). Coal, petroleum, coal tar, coal tar pitch, shale oil, and carbon black when processed by high temperature pyrolysis contains PAH (Cahnman 1955, Andelman and Suess 1970). PAH may be produced during incomplete combustion of wood, coal, oil, coal gas, and gasoline (Commins 1969). Bitumen and asphalt used to construct roads contained PAH (Borneff and Kunte 1965). BaP was isolated from cracked mineral oils (Cook et al. 1958), and concentrations of 0.4-1.6 mg/l of BaP were found in three crude oils (Graf and Winter 1968).

Soil from uncontaminated areas of the earth may have low concentrations of PAH, while concentrations of PAH in contaminated soils may reach high concentrations. After a survey of soils from remote European forests, Borneff (1964) concluded that the natural background level of BaP was 1-10 µg/kg in soil. BaP concentrations of 40-1300 µg/kg were found in soil distant from major highways or industry (Blumer 1961). High concentrations of BaP in soils from remote areas could be due to forest fires (Blumer 1976), atmosperic fall-out (Baum 1978), or biosynthesis by organisms (Suess 1976). Concentrations of BaP were 220,000 µg/kg near an oil refinery and major road (Shabad et al. 1971), and soil contaminated by coal tar pitch had a BaP concentration of 650,000 µg/kg (International Agency for Research on Cancer 1973). Soil near a petrochemical plant had 1.8-2.6 times more BaP 50 cm deep than on

the surface (Sukhoteplyi et al. 1968). Thus, PAH can accumulate in deep soil layers and possibly contaminate the groundwater.

The biosynthesis of PAH by a variety of organisms has been demonstrated. Bacteria, including Escherichia coli, grown on solid culture media free of PAH, synthesized BaP in amouints of 2-6 µg/kg (Knorr and Schenk 1968). Sterilized soils seeded with either Clostridium putride or E. coli contained significant quantities of BaP after 6 months (Mallet and Tissier 1969). PAH were found in a wide variety of plants (Guddal 1959, Grimmer 1966). Various plants and the freshwater alga Chlorella vulgaris synthesized several PAH (Borneff et al. 1968). Wheat and rye seedlings grown hydroponically in solution free of PAH had BaP concentrations of 10-20 µg/kg after 8 days of growth (Graf and Nowak 1966). The biosynthesis of PAH by organisms suggests that PAH may have always been present in the environment at low levels.

Ground water typically contains low concentrations of PAH and BaP probably derived through leaching from the upper soil layers. Total and carcinogenic PAH concentrations in groundwater were 0.045-0.51 and 0.001-0.081 µg/1, respectively (Borneff and Kunte 1964). The mean concentration of 15 groundwater samples was 0.06 µg/1 for six PAH compounds (Borneff and Kunte 1964). Untreated river water generally contains ten times more PAH than groundwater or treated river water (Acheson et al. 1976). Treated river water contained 0.025-0.234 and 0.007-0.054 µg/1 total and carcinogenic PAH, respectively (Borneff and Kunte 1964). The concentration of BaP in an unpolluted lake was 0.0013 µg/1 (Borneff and Kunte 1964), while in an uncontaminated mountain stream the concentration of carcinogenic PAH was 0.005 µg/1 (Borneff 1964).

Although raw sewage from domestic sources can contain significant levels of PAH (Borneff and Kunte 1965), the concentration generally increases as industrial contribution to the sewage increases (Wedgwood and Cooper 1955, 1956). Industrial effluents with high levels of PAH were reported from refineries, shale oil, chemical, plastic, dyestuff, and lime industries (Andelman and Suess 1970). Borneff and Kunte (1965) reported that during a heavy storm, levels of PAH in a sewage works increased more than 100-fold over a dry weather period and concluded that the increased PAH concentration was due to run-off from roadways.

The removal of PAH by water and sewage treatment processes has varying effectiveness. Removal of PAH during primary sedimentation varied from 20-80% (Borneff and Kunte 1967, Reichert et al. 1971). Percolating and activated sludge processes resulted in a 97-99% reduction of PAH (Reichert et al. 1971). The activated sludge process studied in the laboratory for 144 h did not show any significant ability to oxidize carcinogenic PAH (Lutin et al. 1965). Malaney et al. (1967) concluded that any removal of PAH by biological treatment processes was due to absorption of the PAH by activated sludge. Coagulation and flocculation of sewage achieved an average 90% removal of PAH (Harrison et al. 1975). Experiments using chlorine as an oxidizing agent indicated relatively slow removal of PAH, while treatment with gaseous chlorine dioxide resulted in a high percentage of PAH removed (Reichert 1968). Ozone had 99% removal of PAH (Borneff 1969, Burleson et al. 1979). Filtration through activated carbon is the most effective process for removal of PAH, and PAH were not found in activated carbon (Borneff and Fischer 1961) indicating that activated carbon is suitable for use in water treatment.

Concentrations of PAH in oil-contaminated sediment were from 600-1000-fold greater than in the overlying water (Herbes and Schwall 1978). Andelman and Suess (1970) reported that the BaP concentration in river sediments was 15,000 µg/1. The sediment below a coke by-product plant outfall ranged from 8,200-17,000 µg/kg of BaP (Fedorenko 1964). BaP was estimated to break down in 3.3 and 60 yr in oil-contaminated and uncontaminated sediments, respectively (Herbes and Schwall 1978). Microbial transformation of PAH in sediment is much less important for the larger PAH like BaP than smaller PAH in influencing the environmental transport of PAH (Herbes and Schwall 1978). The high concentrations of PAH in sediments suggests that absorption on particulate matter and sediment is a major process for removing these materials from the water (Radding et al. 1976).

Aquatic organisms concentrate PAH in their tissues. Mussels from Yaquina Bay, Oregon, had 5.7 µg/kg BaP (Mix and Schaffer 1979). Zechmeister and Koe (1952) showed that barnacles contained PAH including BaP. In a survey of PAH in marine tissues, BaP concentrations ranged from 0.2 µg/1 in oysters to 3 µg/1 in crabs (Pancirov and Brown 1977). The clam Rangia cuneata accumulated BaP approximately 200 times above the water concentration of 30.5 µg/1 after 24 h of exposure (Neff and Anderson 1975). Almost 75% of the BaP was located in the viscera including the digestive system, gonads, and heart. When returned to BaP free water, more than 70% of the BaP was released in 10 h.

The environmental fate of BaP was studied in a laboratory ecosystem using radiolabelled BaP (Lu et al. 1977). The organisms used in the microcosm were Oedogonium cariacum, Daphnia magna, Culex pipiens quinquefasciatus, Physa sp., and Gambusia affinis. The experiment

lasted 3 days at 26.7°C with 750 ft candle illumination. BaP was bioconcentrated and stored in substantial amounts in all organisms with the parent compound representing from 46% of the total radiolabelled carbon in Culex to 90% in Daphnia. The biomagnification factors were: Gambusia 930, Oedogonium 5258, Culex 11,536, Physa 82,231, and Daphnia 134,248. Physa had the lowest degradation of BaP, while Gambusia had complete degradation. BaP was degraded mainly by hydroxylation and conjugation to produce polar derivaties.

BaP has been indicated as a carcinogen, mutagen, and teratogen. Mice (Peirce 1961, Rigdon and Neal 1966), rats (Huggins and Yang 1962), and hamsters (Donetenwill and Mohr 1962) have been reported to develop cancer from doses of BaP. Mixtures of compounds which contain BaP have been associated with cancer in man (National Research Council 1977). BaP has been shown to be a mutagen by the Ames Salmonella/microsome test (McCann et al. 1975). It was demonstrated that BaP activated by microsomes entered cell nuclei and bound to DNA and RNA (Pezzuto et al. 1976). It is assumed that binding of PAH to DNA leads to carcinogenesis (Pitot and Heidelberger 1963). BaP was found to be teratogenic in rats (Rigdon and Rennels 1964).

Most investigators believe that BaP and other PAH are precarcinogens and require activation before they can bind to nuclear macromolecules. Activation can be produced by the aryl hydrocarbon hydroxylase (AHH) system which is also known as the mixed-function oxidase and benzo(a)pryene monooxygenase system. The principal role of these enzymes can convert PAH to active carcinogens (Anderson 1978). The initial action of AHH on PAH yields epoxides which undergo further reactions to yield dihydrodiol, phenolic, and diol-epoxide derivatives

(Brooks 1977). AHH is found in insects (Agosin and Perry 1974), microorganisms, fish, mammals (Brooks 1977), and Chironomus riparius (Estenik and Collins 1979).

The maximum permissable concentration (MPC) of PAH in drinking water has not been established, but several proposals have been made. Some investigators suggest that since BaP is a carcinogen, no safe concentration exists (Boyland 1958, Gerarde 1960). Borneff and Kunte (1969) suggested that water containing PAH in excess of 0.20 µg/l should be rejected for human consumption. Shabad (1971) administered a series of intragastric doses of BaP to rats to establish levels of BaP which do not produce cancer. On the basis of these data and taking into account the differences in body weight and organ surface areas of rats and humans, he calculated that the MPC for BaP should not exceed 0.0003 µg/l. Andelman and Suess (1970) suggested that the amount of carcinogens taken up by man in drinking water should not exceed 0.1% of the amount taken up from normal urban air. Using this concept they calculated that the MPC should be 0.017 µg/l. Based on an average daily consumption of 1.5 liters of drinking water, this calculated to 15 µg/l and about 1 mg in a lifetime. The World Health Organization (WHO 1970) proposed that the concentration of six specific PAH compounds should not exceed 0.2 µg/l in drinking water.

Life Cycle of Chironomus attenuatus

Chironomus has been reared successfully in the laboratory (Biever 1965, Credland 1973) and complete their life cycle in about 4 wk at 22°C (Thornton and Wilhm 1974). The life cycle of Chironomus consists of four stages: egg, larva, pupa, and adult. The eggs are laid in a

protective gelatinous matrix which absorbs water and sinks. Hatching occurs 24-36 h after the eggs are laid. The first instar is planktonic initially feeding on the gelatinous mass surrounding the eggs. The first instar larvae later settle to the bottom and build U-shaped tubes composed of substrate particles and detritus cemented by a salivary gland secretion. Chironomus feed by spinning a net across the opening of the tube and undulating to draw water and suspended particles through the tube (Walshe 1947a). The larvae eat the net and trapped particles and repeat the process. They have also been observed foraging for food on the substrate. During a period of about 2 wk, the larvae increase in size through successive molts to the fourth instar. After 5-7 days the fourth instar larvae pupate and emerge as adults 1-2 days later. The adults do not feed and only live a few days to a week during which mating occurs.

Life Cycle of Tanytarsus dissimilis

Tanytarsus is parthenogenetic and easily reared in the laboratory (Anderson 1980). The egg mass is cylindrical, up to 5 mm long, and contains 120 to 190 eggs arranged in a single row. The eggs complete development in about 3 days with the first instar larvae planktonic for about a day before settling to a substrate and building a tube. The tube consists of silk and debris woven together. At 22°C larval development is complete in about 2 wk. The pupal stage lasts about 2 days. The pupae leaves the tube, swims to the surface, and the adult emerges.

Physiology of Aquatic Insects

Osmotic and Ionic Regulation

The hemolymph of insects is approximately 90% water and contains inorganic ions, sugars, proteins, amino acids, and carboxylic acids (Chapman 1971). The major inorganic ions include sodium (Na^+), potassium (K^+), calcium (Ca^{++}), magnesium (Mg^{++}), chloride (Cl^-), and phosphorus (P). The composition of the hemolymph represents a steady state between influxes of salts, water, and organic materials which are obtained through the cuticle and alimentary canal and the loss of these materials by respiration, secretion, and excretion. The hemolymph functions in storage and transport of substances throughout the insect body.

The concentration of Na^+ and K^+ in the hemolymph is highly variable in insects. Before 1945 it was believed that K^+ was the dominant hemolymph cation in most insects. However, Bone (1945) in a survey of 28 insect species reported that the mole Na^+/K^+ ratios ranged from 25 to 0.1. In phytophagous Coleoptera, Hymenoptera, and Lepidoptera the ratio may be less than one. In carnivorous Coeloptera, Odonata, and Diptera the ratio may be much higher (Bone 1945). In aquatic insects studied by Sutcliffe (1962b), the Na^+ concentration was high (95-155 mM), while the K^+ concentration was often low (2-31 mM). In *Chironomus* larvae the Na^+ and K^+ concentrations were 92 and 8 mM (Bone 1945) and 104.3 and 2.1 mM (Florkin and Jeuniaux 1974), respectively. Fourth instar larvae of *Chironomus tentans* had Na^+ and K^+ concentrations of 93 and 5 mM, respectively (Wright 1975). *C. thummi* and *C. tentans* maintained nearly constant levels of Na^+ and K^+ throughout most of the fourth

instar (Firling 1970, Schin and Moore 1977). Significant changes in cation concentrations were reported near each ecdysial period (Schin and Moore 1977).

The concentration of Ca^{++} in insects is somewhat higher than in vertebrates but lower than in other invertebrates (Prosser 1973). Mg^{++} is higher in proportion to the other cations than in most other animals except marine invertebrates. The concentration of Mg^{++} in insect hemolymph is high enough to induce anesthesia in most non-marine animals (Buck 1953a). Though it is reasonable to assume that the Mg^{++} is mainly derived from chlorophyll (Metcalf 1945), there seems to be no clear correlation with diet. Chironomus had Ca^{++} and Mg^{++} concentrations of 10.5 and 14.6 mEq/l (Florkin and Jeuniaux 1974).

Hemolymph Cl^- is important in the cation-anion balance and is the most abundant inorganic anion in insect hemolymph. The Cl^- concentration in apterygotes and lower pterogotes is high, while in the advanced pterygotes it is usually less than 10% of the total osmolarity (Chapman 1971). Endopterygotes had higher Cl^- levels (75-127 mM) than exopterygotes (8-50 mM) (Sutcliffe 1962). Fourth instar larvae of C. tentans had a Cl^- concentration of 18 mM (Wright 1975).

Phosphorus in insect hemolymph occurs as acid-soluble (inorganic) phosphorus, nucleoprotein phosphorus, and lipid phosphorus. The concentration of P in insect hemolymph may be high, but aquatic insects generally have low levels. Total acid-soluble P in the pupae of Hyalophora cecropia was 26-44 mM (Wyatt 1961). The odonate larvae Aeschna grandis had a $H_2PO_4^-$ concentration of 4 mEq/l (Sutcliffe 1962). The contribution of P to the cation-anion balance is usually minor, but is higher than in most other invertebrates. Phosphorus may function as

part of a buffering system in the hemolymph of insects.

Various metallic trace elements have been found in insect hemolymph. The most common are copper (Babers 1938), a component of tyrosine, and iron which is present in the cytochromes and hemoglobin (Chapman 1971). Other elements found in insect hemolymph are zinc (Akao 1935, Levenbook 1950) and manganese (Bowen 1950).

The osmotic pressure of insect hemolymph is an estimate of the concentration of solutes in solution. It is quantified by evaporation against a standard salt solution in a capillary tube (Rouschal 1940) or by determining the freezing point depression (Ramsey 1949, Frick and Sauer 1973). The osmotic pressure is primarily due to electrolytes. Therefore, ionic regulation is important to maintain the osmotic pressure within tolerance limits.

In most animal phyla, the osmotic pressure of the body fluid is due to inorganic ions, principally Na^+ and Cl^- (Lockwood and Croughton 1959). In primitive pterygote insects the sum of Na^+, K^+, Ca^{++}, and Mg^{++} contributes to almost half of the osmotic molecules (Florkin and Jeuniaux 1974). In higher insects the importance of cations and Cl^- is less with organic molecules being the main osmotic effectors. The organic molecules are predominantly free amino acids which act as substrates in the citric acid cycle and to a lesser extent carboxylic and organic acids (Florkin and Jeuniaux 1974).

The hemolymph freezing point depression of insects is generally between -0.4 and -1.3°C (Buck 1953a) and tends to be lowest in freshwater insects (Stobbart and Shaw 1974). The mosquito larvae Culex pipiens and Aedes aegypti had hemolymph osmolarities of between 0.75 and 0.89 osmoles (Wigglesworth 1938). Various mosquito larvae had

osmolarities ranging from 0.4 to 0.8 osmoles (Winogradskaja 1936, Beadle 1939). The larvae of Chironomus attenuatus, Culex pipiens quinquefaciatus, and Aedes aegypti had hemolymph osmolarities of 0.345, 0.477, and 0.908 osmoles, respectively (Ramsey 1950, Frick and Sauer 1973). The hemolymph osmolarity increased from 0.271 to 0.304 osmoles during the fourth instar of A. aegypti (Richards and Meier 1974).

Freshwater insects have two major problems with salt and water regulation. Since the hemolymph is hypertonic to the water, a tendency exists for water to pass through the cuticle into the insect. Cuticle permeability varies in different aquatic organisms. Adult water beetles have relatively impermeable cuticles due to a hydrophobic wax monolayer. Aquatic larvae have no wax layer and thus have highly permeable cuticles. Also, freshwater insects tend to lose salts to the hypotonic environment which is compensation by the reabsorption of salts in the rectum and the active uptake of ions from the environment.

The osmoregulatory capacity of aquatic insects is usually assessed by acclimatizating groups of animals to the widest range of saline solutions which they will tolerate and then comparing the osmotic pressure of the medium and the hemolymph. Breakdown of regulation occurs when the hemolymph osmotic pressure is iso-osmotic with the medium. A. aegypti, C. pipiens, and C. thummi have been shown to have a high degree of regulation throughout a range of external salinities that exceeds typical freshwater limits (Stobbart and Shaw 1974). When the osmoregulation breaks down, the internal ion regulation fails and iso-osmotic conditions occur in the hemolymph and medium. The hemolymph of A. aegypti was iso-osmotic with the medium when the medium had an osmotic pressure of 0.350 osmoles (Richard and Meier 1974). The

regulatory capacity of the larvae of Limnephilus stigma and Anabolia nervosa was not so well developed (Shaw and Stobbart 1963), and the osmotic pressure of the hemolymph rose steadily until the iso-osmotic state was reached.

Although little is known about the osmoregulation of adult freshwater insects, some data are available on corixids. Claus (1937) showed that three species of Sigara (Corixia) have an active regulatory process which prevents the osmotic pressure of the blood to rise as rapidly as that of the environment. The regulatory curves of Cenocorixa bifida and C. expleta resemble those of Aedes, Culex, and Chironomus larvae. Some evidence exists that they can regulate up to a medium concentration of 2% NaCl (Stobbart and Shaw 1974). C. expleta tolerated a greater than twofold increase in the hemolymph osmotic pressure (Scudder et al. 1972).

To test the ability of an insect to regulate its hemolymph, the ionic composition of the external medium must be altered. The Na^+ regulation curve of A. aegypti resembles the curve of the hemolymph osmotic pressure. The hemolymph Na^+ concentration is kept below the external Na^+ concentration even at high external concentrations (Shaw and Stobbart 1963). The regulation of the hemolymph osmotic pressure depends on the regulation of the ionic fraction of the hemolymph. When A. aegypti were kept in freshwater the intestinal fluid was slightly hypotonic to the hemolymph, but its Na^+ concentration was only about one-half that of the hemolymph (Ramsey 1950). The Malpighian tubules contributed to the work of salt retention by excreting a fluid containing less salt than the hemolymph. The adult hemipterans Corsella edulis and Cenocorixa bifida showed a similar Na^+ regulation pattern

(Frick et al. 1972, Scudder et al. 1972).

The regulation of hemolymph K^+ was found to be efficient in the larvae of A. aegypti and Sialis lutaria and the adults of Cenocorixa bifida and C. expleta (Scudder et al. 1972). When A. aegypti were kept in distilled water, the hemolymph K^+ concentration was 3.1 mM. It rose to 4.2 mM/l at an external concentration of 1.7 mM KCl and 5.7 mM in an 85 mM solution (Ramsey 1953a). S. lutaria maintained its hemolymph K^+ concentration at 5 mM in external concentrations up to 34 mM KCl (Shaw 1955). The regulation of K^+ is probably independent of the regulation of Na^+ and Cl^- (Stobbart and Shaw 1974).

The regulation curves for Cl^- closely resembles those of Na^+ suggesting a similar regulation of these ions. However, at higher external concentrations, the hemolymph Cl^- is kept below external concentrations, while hemolymph Na^+ is kept above. Thus, some degree of independent regulation exists over part of the external range (Shaw and Stobbart 1963). When A. aegypti larvae were kept in chloride-free distilled water, the Cl^- concentration dropped from a normal 0.3% to 0.05%, while the osmotic pressure remained relatively constant. The loss of Cl^- was compensated by the liberation of nonchloride solutes, perhaps amino acids, into the hemolymph (Wigglesworth 1938). The mechanism restricted the variation in the hemolymph osmotic pressure to $\pm$15%, while the hemolymph Cl^- varied over a 12-fold range. Sutcliffe (1961) suggested that this mechanism operated in the brackish-water larva Limnephilus affinis. Thus, some aquatic organisms appear to regulate an osmotically-active nonelectrolyte fraction of the hemolymph.

Ion uptake from dilute water has been studied in various aquatic

organisms. The coleopteran larvae Helobes minuta and H. marginata absorb Cl^- ions primarily through the anal papillae and secondarily through the gut (Treherne 1954a). The odonate nymphs Libellula and Aeschna absorb Na^+ and Cl^- through the rectal respiratory chambers (Krough 1939). Larvae of the hellgrammite Sialis are unlike other aquatic organisms in that no special surface exists for ion absorption. However, due to the low permeability of the cuticle to ions, they can survive in distilled water for several weeks (Beadle and Shaw 1950). Shaw (1955b) reported that the gut wall is rather permeable to water and salts and that Na^+ could be actively transported. Various ephemeropteran larvae posses chloride cells in the tracheal gills which probably function in Na^+ and Cl^- uptake (Komnick and Abel 1971, Wichard and Komnick 1971). Similar cells have been found in the tracheal gills of the stonefly nymph Paragnetina media (Kapoor and Zachariah 1973).

The uptake mechanism in dipteran larvae has been studied intensively. Ramsey (1953a) and Stobbart (1960) have shown that Na^+ and Cl^- ions can be absorbed through the anal papillae against large concentration gradients. Na^+ and Cl^- ions can be absorbed through the anal papillae against large concentration gradients. Na^+ and Cl^- appears to be actively transported through separate mechanisms (Stobbart and Shaw 1974). Although there seems to be a separate uptake mechanism for K^+ (Trehern 1954b), uptake of K^+ in the absence of Cl^- has not been observed (Stobbart 1967). The uptake mechanism in Culex and Chironomus is located in the anal papillae (Koch 1938). In A. aegypti larvae the anal papillae probably function in the uptake of Na^+, K^+, and Cl^- (Ramsey 1953a; Stobbart 1960, 1967). The anal papillae are also responsible for the exchange of phosphate in A. aegypti (Hassett and

Jenkins 1951) and for the osmotic uptake of water (Wigglesworth 1933b). Except for Aedes detritus (Beadle 1939), an inverse relationship exists between environmental salinity and the size of dipteran anal papillae. In freshwater forms the anal papillae may be greatly enlarged, while they may be reduced or absent in saline water forms (Buck 1953b). The increased surface area presumably facilitates increased ion uptake from waters with low concentrations of ions.

The Malpighian tubule may also play a role in the osmotic and ionic regulation of aquatic insects. Ramsey (1953a,b) showed that the tubule fluid of A. aegypti reared in distilled water was slightly hypo-osmotic to the hemolymph. Ramsey (1953b) proposed that the fluid passing down the intestine is derived from the Malpighian tubules. The fluid excreted by the tubules has almost the same osmotic pressure as the hemolymph, but its Na^+ concentration may be one-half that of the hemolymph (Ramsey 1953b). Thus, the tubules can contribute to the work of salt retention by excreting a fluid containing less Na^+ than the hemolymph. When A. aegypti were reared in distilled water, the K^+ in the hemolymph was 3 mM and in the tubule fluid 88 mM (Ramsey 1953b). The high concentration of K^+ in the tubule indicates that active transport of K^+ must be occurring (Ramsey 1953a).

Despite increased ion secretion by the Malpighian tubules in response to increased hemolymph concentrations, osmotic and ionic regulation is largely effected by differential absorption in the rectum. The ionic composition of the excretory fluid can be changed dramatically by absorption by the rectal epithelium. In freshwater insects the rectal fluid is usually hypo-osmotic to the hemolymph with the major ions of the hemolymph occurring in low concentrations. The rectal

epithelium is capable of diluting the excretory fluid by absorbing ions and concentrating the fluid by absorbing water (Wigglesworth 1933b).

The osmoregulatory ability of aquatic invertebrates may be impaired by toxicants. The brown shrimp, Penaeus aztecus, exposed to polychlorinated biphenyls (PCB), showed a significant decrease in the total ion concentration of the blood (Nimmo and Bahner 1974). Exposure to sublethal and 96 h LC50 concentrations of PCB did not significantly alter hemolymph chloride and osmotic concentrations in adult grass shrimp, Palaemonetes pugio (Roesijadi et al. 1976b). However, juvenile shrimp showed disruption of hemolymph Cl^- regulation and large mortalities. Also, the PCB had no significant effect on the free amino acid level in the abdominal muscle of the grass shrimp, suggesting that PCB do not affect intracellular osmoregulation (Roesijadi et al. 1976a). Lewis and Lewis (1971) reported that 5 mg/l copper decreased the blood osmolarity of the channel catfish from 280 to 230 mOsm. However, when 235 mOsm NaCl was added to the medium, the blood osmolarity increased to 340 mOsm. Zinc had a similar but less dramatic effect. Evidently the metal ion interferred with the osmoregulation of the fish, but the additional NaCl in the external medium allowed for recovery of the lost salt. The pesticide endrin caused an accelerated transfer of Na^+, K^+, and Ca^{++} from the liver into the blood of the northern puffer (Eisler and Edmunds 1966). The exact cause of the toxic effect of mercury (Hg) is not known, but it has been shown that methyl-mercury and $HgCl_2$ inhibits ion transport in teleost fish (Renfro et al. 1974). The Na^+ uptake by the bladder and gills of freshwater fish was inhibited by methyl-mercury and $HgCl_2$ (Schmidt-Nielson 1974). $HgCl_2$ was more effective than methyl-mercury in inhibiting ion transport. Renfro et

al. (1974) postulated that Hg affects ionic regulation on the cellular level by interferring with Na^+,K^+ ATPase activity.

Oxygen consumption

The rate of oxygen consumption in insects is influenced by temperature, season, time of day, activity, stage of life cycle, and genetic background (Prosser 1973). In general, an increase in temperature increases the metabolic rate as indicated by the increased oxygen consumption (Chapman 1971). Chironomus, Glyptochironomus, Polypedilum, Stictochironomus showed significant differences in oxygen consumption due to temperature. Chironomus had oxygen consumption rates of 0.40, 0.78, and 1.29 µl O_2 consumed/mg dry weight/h at 5, 10, and 15°C, respectively (McFarlane and McLusky 1972). Chironomus riparius consumed oxygen 2.6 times faster at 20°C than at 10°C (Edwards 1958).

Data on temporal variation of oxygen consumption by aquatic insects are contradictory. C. riparius had a higher oxygen consumption at 20°C in the summer than in the autumn (Edwards 1958). However, four species of chironomids had no significant differences in oxygen consumption due to seasonal effects or acclimation (McFarlane and McLusky 1972). Knight and Simmons (1975) reported seasonal effects in the megalopteran larva Corydalus cornutus, an inhabitant of summer-warm streams; however, Nigronia serricornis, an inhabitant of summer-cold streams, showed no seasonal effects. Time of day had a significant effect on the oxygen consumption of the mosquito larva Culex pipiens (Buffington 1968). Oxygen consumption was lowest near dawn with peaks during mid-morning and late afternoon. The plecopteran nymph Paragnetina media had significantly higher oxygen consumption in the dark than in the light

(Kapoor 1972). Chironomid larvae from lotic habitats had higher oxygen consumption rates than chironomids from lentic habitats (Walshe 1948).

The expression of respiratory rates has received much attention. Since the prime consumer of oxygen is tissue protoplasm, which is largely protein, Keister and Buck (1974) suggested that respiration is best expressed as volume of gas exchanged per unit protein nitrogen content. Many investigators believe that oxygen consumption is proportional to either estimated surface area or body weight (Zeuthen 1953). Since tissues vary in water content more than solid matter content, tissue dry weight is widely used for expressing respiratory rate (Keister and Buck 1974). In general, the total oxygen consumed is higher in larger than smaller animals (Prosser 1973). However, Keister and Buck (1974) suggested that weight could reflect changes in body water and gut content which are not involved in oxygen consumption. Oxygen consumption by C. riparius was not proportional to the estimated surface area, but varied by the 0.7 power of the dry weight (Edwards 1958). Erman and Helm (1970) suggested that body length was perferrable to other body size measurements for estimating oxygen consumption in chironomids. Although no consensus exists on how best to express respiratory rates, most investigators prefer wet or dry weight.

The specific control of oxygen consumption rates is unclear. Prosser (1973) stated that generally the activity of oxidative exzymes per unit of tissue weight is higher in tissues from small animals than in those from large animals and concluded that there must be a subtle control system relating the metabolic need of the whole organism to enzyme activity in cell masses. Chapman (1971) suggested that in some cases cellular metabolism in insects is controlled partially by hormones

and the structural organization of the cell.

Changes in physiology and behavior may serve as indicators of sublethal effects, which may be quantified by changes in activity, respiration rates, and respiratory movements (Spraque 1971). Many investigators have measured changes in oxygen consumption as a response to toxicants. Cairns (1966) claimed that measuring oxygen consumption was one of the best methods of sublethal bioassay.

The effect of sublethal concentrations of toxicants on oxygen consumption of aquatic organism has received much study. When four species of freshwater fish were exposed to hydrogen sulfide, the respiratory rate increased to compensate for decreased aerating surface (Belding 1929). Whitley and Sikora (1970) exposed tubificid worms to sublethal concentrations of three chemicals and reported that oxygen consumption was unchanged by nickel, inhibited by lead, and stimulated by pentachlorophenol. Sublethal concentrations of Hg affected the oxygen consumption of the larvae of the fiddler crab Uca pugilator (DeCoursey and Vernberg 1972). In acute toxicity tests, 0.018 and 1.8 µg/l Hg increased the oxygen consumption above untreated controls. Crawling and respiratory rates were used as indices of sublethal effects of oil and a dispersant on an intertidal snail Littorina littorea (Hargrave and Newcombe 1973). The oil increased respiration 34% and crawling 28% above untreated controls, while the dispersant decreased respiration 36% and crawling 43%. The organophosphate pesticide Dibrom increased oxygen consumption in stonefly and hellgrammite larvae and decreased oxygen consumption in the golden shiner (Maki et al. 1973). Klewoski and Zvirgzds (1971) examined the effect of the herbicide 2,4 dichlorophenoxyacetic acid (2,4-D) on the cladoceran Simocephalus

vetulus. They reported an increase in the respiration rate at the low concentrations and postulated that the toxicant acted as an inhibitor of the cellular respiratory chain. Respiratory changes by the sunfish Lepomis macrochirus were analyzed following short term exposure to 3 mg/l 2,4-D or 2,4,5 trichlorophenoxyacetic acid (Sigmon 1979). The respiration rate was not significantly altered by the herbicides at 20, 25, or 30°C. Exposure of Crangon franciscorum to five concentrations of kelthane depressed the respiration rate below control levels (Sharp et al. 1978).

The respiratory rate of chironomids has been used as an indicator of sublethal effects. Cole and Wilhm (1973) reported that oxygen consumption of C. attenuatus generally increased as the concentration of phenol increased from 0 to 22.4 mg/l. The effect of the lampricides 3-trifluoromethyl-4-nitrophenol (TFM) and 5,2'-dichloro-4-'nitrosalicylanilide (Bayer 73) on the oxygen consumption of C. tentans was studied (Kawatski et al. 1974). Chironomids exposed to sublethal concentrations of TFM exhibited a significant increase in oxygen consumption over controls. Bayer 73 concentrations between 0.75 and 1.00 mg/l stimulated oxygen consumption and concentrations greater than 1.20 mg/l inhibited oxygen consumption. The authors postulated that both lampricides acted as uncouplers of oxidative phosphorylation.

Hemoglobin

Lankester (1868) was the first to show that hemoglobin occurred in Chironomus larvae. Since then the botfly larva and backswimmers among the insects have been found to possess hemoglobin. The pigment first appears in the second instar of Chironomus and may constitute 90% of the

hemolymph protein in the fourth instar (English 1969). Changes in concentration of hemoglobin has been shown to occur during development (Laufer and Pluhowich 1971) and has been attributed to the activity of several gene loci at different stages of development (Manwell 1966). The concentration of hemoglobin varies in different species (Wigglesworth 1972). In some chironomid species the concentration of hemoglobin is about 25-30% of the concentration in human blood. In the C. plumosus group and Tanypodinae hemoglobin composes 14.5% and 10-14%, respectively, of the concentration in human blood. Synthesis of hemoglobin by Chironomus occurs primarily in the subepidermal fat body (Schin et al. 1977). The visceral fat body is involved with the uptake, storage, and eventual degradation of hemoglobin. After molting to the adult, 95% of the hemoglobin may be degraded (Schin et al. 1974).

The characteristics of chironomid hemoglobin have received much study. Chironomus hemoglobin has a molecular weight of about 32,000 (Shrivastaba and Loughton 1970), which is one-half the molecular weight of vertebrate hemoglobin. Unlike most organisms that possess hemoglobin, Chironomus displays hemoglobin polymorphism. C. plumosus has at least ten forms of hemoglobin (Manwell 1966), while C. thummi has 14 types (Bergstrom et al. 1976). Some forms of hemoglobin appear to be species specific (Thompson and English 1966) and stage specific (Schin et al. 1977).

Considerable attention has been given to the synthesis of hemoglobin by invertebrates due to low concentrations of dissolved oxygen in the environment. Fox (1949) was the first to show that the brine shrimp Artemia salina synthesized hemoglobin in response to low dissolved oxygen conditions. When the oxygen concentration decreased

from 90 to 17% saturation, Artemia showed a three-fold increase in hemoglobin. When they were returned to well-aerated water, the hemoglobin concentration decreased to normal in 3 wk (Gilchrist 1954). Artemia kept in a medium of 10% oxygen saturated showed a 1.5-2.8 times increase in hemoglobin (Bowen et al. 1969). Chironomus synthesized more hemoglobin in response to conditions of low dissolved oxygen (Fox 1955).

The function of chironomid hemoglobin has received much study. Although Lankester (1868) observed that chironomids occurred in situations where oxygen at times may be low or absent, he did not postulate its function. Other investigators observed that the larvae would swim upward at hight into well-aerated water suggesting that hemoglobin has a storage function (Walshe 1949). Walshe (1947a,b; 1949) demonstrated that hemoglobin functions in both transport and storage of oxygen. The normal tube activity of chironomids consists of alternating periods of filter-feeding, respiratory activity, and rest. Walshe (1949) determined that only 9 min of oxygen could be stored. In the usual habitat of chironomid larvae, oxygen may be absent for months (Birge and Juday 1911). In anoxic environments chironomids quickly use the store of oxygen and are forced to rely on anaerobic respiration. Under laboratory conditions larvae have survived for 3-120 days (Ssinitza 1937, Lindemann 1942). Hemoglobin is nonfunctional in well-aerated water, because it is completely reduced (Ewer 1942).

Laboratory experiments by Harnisch (1936) on C. thummi have shown that the hemoglobin functions in oxygen transport at low oxygen pressures. Ewer (1942) demonstrated in C. plumosus that hemoglobin only functions below 3 ml/l (44% saturation) at 17°C. Chironomus has a much

higher affinity for oxygen than vertebrate hemoglobin. It is half-saturated at tensions of less than 1 mm of oxygen, while vertebrate hemoglobin is half-saturated at 27 mm (Chapman 1971). The high affinity for oxygen allows chironomids to pick up oxygen from an environment which is low in dissolved oxygen.

Although the effect of oxygen on hemoglobin synthesis is well known, the effect of other factors is not well studied. Increased levels of iron, vitamin B_{12}, and temperature were shown to increase the concentration of hemoglobin in Daphnia (Fox and Phear 1953). In studies on Artemia salina, Gilchrist (1954) found that increased salinity caused increased hemoglobin concentrations, but Heip et al. (1978) reported that salinity had no effect. In the northern puffer the normal hemoglobin concentration decreased at a concentration of 0.05 µg/l endrin, while hemoglobin increased at 0.1, 0.5, and 1.0 µg/l (Eisler and Edmunds 1966).

Hemotogical analysis can be used to determine the incipient lethal concentration of a toxicant (McLeay 1973). Buckley at al. (1976) assayed hemoglobin content and other blood parameters in coho salmon (Oncorhychus kisutch) exposed to residual chlorine for 12 wk. They established a linear dose-response relationship between hemoglobin and total residual chlorine. Exposure of brook trout (Salvelinus fontainalis) to copper concentrations of 67.5-69.2 µg/l resulted in a significant increase in hemoglobin (McKim et al. 1970). They concluded that short-term changes in blood parameters such as hemoglobin concentration could be used to predict chronic effects.

Glycogen

Anaerobic metabolism usually involves the breakdown of a carbohydrate to pyruvic acid and then to lactic acid without oxygen. It appears that during anaerobiosis chironomids may use proteins, fats, and carbohydrates. Harnisch (1938) reported an increase of protein in C. thummi following anaerobiosis. Some fatty acids formed during anaerobiosis can be converted into fat and stored in the fat body serving to reduce the oxygen debt of the organism (Harnisch 1939, Hers 1942).

Glycogen may be converted anaerobically to lactic acid with the release of energy. The necessary enzymes, a glycolytic enzyme system and lactic dehydrogenase, has been found in C. plumosus (Augenfeld and Ness 1961). Augenfeld (1967) reported that the glycogen content in C. plumosus larvae declined during long anoxic periods in the hypolimnion of a lake. The glycogen content did not change significantly in organisms from the oxygenated epilimnion.

CHAPTER III

MATERIALS AND METHODS

Cultures

The original laboratory population of Chironomus attenuatus was established from larvae collected from Stillwater Creek, Oklahoma below a sewage plant outfall. The laboratory population was raised in galvanized metal trays, 88 x 56 x 18 cm, lined with a sheet of polyethylene to prevent entry of dissociated metal ions (Thornton and Wilhm 1974). Each tray was enclosed by a screen cage, 90 x 57 x 42 cm. The substrate consisted of about 2 cm of ashed sand. Approximately 5 cm of aged tap water was added to each cage. A paste of Hartz Mountain Dog Kisses was added twice a week as food (Biever 1965, 1971). Lighting was provided by a 15-watt incadescent bulb on a 16 h light photoperiod. Oxygen was added continuously by a forced air system.

The laboratory population of Tanytarsus dissimilis was established from eggs obtained from the Environmental Protection Agency at Duluth, Minnesota. Organisms were raised in 19 and 38 liter glass aquaria fitted with glass covers. Each aquarium contained 6 liters of aged tap water receiving aeration from an air pump. Lighting was provided by two fluorescent lamps on a 16 h light photoperiod. Food consisted of 10 g trout chow and 0.5 g cerophyl in 300 ml distilled deionized water. Food was added at a rate of 2 ml/l every other day (Anderson 1980).

Experimental Design

Short-term experiments were conducted to determine LC50 values and physiological responses of chironomids to naphthalene. Only fourth instar larvae were used. Experimental subunits were 2000 ml pyrex beakers containing 1 liter of treatment solution. Because of the limited solubility of naphthalene in water, ethanol was used as a carrier. Nominal naphthalene concentrations were 1, 5, 10, and 12 mg/l.

Life cycle experiments were conducted using a continuous flow system similar to the one recommended by the EPA (1978). Five naphthalene concentrations and a control were replicated with dimethylformamide used as a carrier. Test chambers were all glass (10 x 12 x 23 cm) and were covered by a glass plate. To initiate the experiment, adults from the stock tanks were isolated for egg deposition in cottoned-stoppered vials. The following day egg masses were cut into strings of 20 eggs and placed randomly in the subunits. Daily measurements and activities are shown in Table I. The test chambers were analyzed daily for temperature, dissolved oxygen (Yellow Springs Instrument Model 54A Oxygen Meter), and naphthalene concentration (Aminco-Bowman spectrophotofluorometer). The dilution water was analyzed every 2 days for pH (Beckman Zeromatic pH Meter), conductivity (YSI Model 33 Conductivity Meter), and total hardness and alkalinity (titrimetrically, EPA 1979). The test was terminated after all larvae had pupated and emerged.

Oxygen Consumption

Oxygen consumption measurements were taken on a Gilson Differential Respirometer following techniques of Umbreit et al. (1972).

TABLE I

DAILY ACTIVITIES DURING LIFE CYCLE TEST
WITH TANYTARSUS DISSIMILIS

Day	Activity
1	Isolate adults in vials for egg deposition
2	Add 20 eggs/unit
4	Observe for hatching; start feeding
5	Determine hatching success
7	Count larvae
9	Count larvae
11	Count larvae
13	Count larvae; observe for pupation
14	Count pupae; isolate adults for egg deposition
15	Same as Day 14 until all larvae have pupated

Fifteen fourth instar larvae from the subunits were placed into 15 ml reaction flasks containing 5 ml of treatment water. Carbon dioxide was absorbed by 0.2 ml of 10% KOH on a piece of Sargent-Welch No. 501 filter paper in the center well. The flasks were then attached to the respirometer with the water bath at 22°C ($\pm$ 0.02°C). At least 30 min were allowed for equilibration of the system.

All measurements were made over 5 h at the same time of day to minimize the influence of any circadian rhythm (Buffington 1968)j. Lighting variations were minimized by keeping the flasks in darkness during measurement. A control flask with 5 ml of water was run simultaneously to correct for oxygen uptake by any factor other than the larvae. After the oxygen consumption measurements were made, the larvae were dried at 100°C for 24 h and weighed on a Mettler H16 Analytical Balance. The oxygen consumption values were corrected to standard temperature and pressure and expressed as µl O_2 consumed/mg dry weight/h.

Hemolymph Ion Analysis

Hemolymph samples for ion analysis were collected after the larvae were dried by Kimwipes and fixed with masking tape to a microscope slide. Under a dissecting microscope, hemolymph samples were obtained from a dorsal puncture made with an insect pin in the mesothorax. The hemolymph was collected in disposable glass pipettes and was discarded if contaminated by other larval tissues.

Measurements of sodium (Na^+) and potassium (K^+) were made on individual larvae. The samples (1.0 µl) were transferred into 1 ml of double-distilled water for analysis on a Beckman 440 or Perkin-Elmer

5000 Atomic Absorption Spectrophotometer. The chloride (Cl^-) measurements were made withiout dilution with a Fiske Marius Micro Chlor-o-counter using a pooled hemolymph sample from several larvae.

Hemoglobin Concentration

The measurement of hemoglobin was performed by the cyanide method (Briegel et al. 1979). An 8 1 hemoloymph sample was obtained with a micropipette from several larvae and reacted with 1 ml of Drabkin's reagent for 10 min. The absorbance at 540 nm was read from a Bausch and Lomb Spectronic 20 or a Beckman DU spectrophotometer. The hemoglobin concentration was obtained from a regression line constructed from a calibration curve determined with three Hycel certified human hemoglobin standards.

Glycogen Concentration

Total body glycogen content was determined by homogenizing a group of 10 larvae with 1 ml distilled water. The homogenate was added to a test tube containing 4 ml of 0.2% anthrone reagent and placed in a boiling water bath for 10 min. The samples were cooled and the absorbance read at 620 nm from a Beckman DU Spectrophotometer. The concentration was determined from a regression line constructed from glycogen standards (Sigma, oyster type III). The results were expressed as mg glycogen/100 mg dry weight.

Inhibition of Na^+,K^+ ATPase

The inhibition of sodium, potassium adenosine triphosphotase (Na^+,K^+ ATPase) by naphthalene and other PAH was determined using

cultured HEp-2 cells by a method modified from Adolfsen and Moudrianalis (1971). The cells were suspended in growth medium and sonicated for 30 sec at full power using a Branson Model 185 Sonifier. The ATPase activity was assayed in a 1 ml reaction system containing 100 mM NaCl, 25 mM KCl, 28 mM $MgCl_2$, and 3 mM ATP in 300 mM Tris buffer (pH 7.6). After an incubation period of 30 min with 10 mg/l PAH, 100 1 of sonicated cells were added to the incubation mixture to start the reaction. The reaction was run at 37°C and terminated after 10 min with 1 ml cold 10% trichloroacetic acid. Inorganic phosphate was assayed by the addition of 2 ml of pre-mixed 1% ammonium molybdate in 0.5 N H_2SO_4 and 5% $FeSO_4$ in 0.5 N H_2SO_4. The tubes were then incubated at room temperature for 10 min and the absorbance read at 660 nm from a Beckman DU Spectrophotometer. The treatment data were compared to control data to calculate per cent inhibition.

Ion Leakage Experiments

A series of experiments were conducted to determine if naphthalene and other PAH disrupt membrane integrity. Phospholipid liposomes were used as model membranes and were made by adding 200 mg asolectin (95% purified soy phosphatide, Associate Concentrates) to 10 ml of 0.05 M sodium or potassium buffer. The mixture was then vortexed at high speed for 1 min and incubated at 37°C for 1 min. After three repetitions, the mixture was centrifuged three times for 10 min each at 18,500 rpm in a Sorvall RC2-B Centrifuge. Between spins the pellet was washed with a 0.02 M Tris-SO_4 buffer (pH 7.4).

The last pellet was suspended in 5 ml of buffer and diluted with 75 ml double-distilled water. The mixture was divided into two 40 ml

aliquots. One aliquot was exposed to 1% ethanol as a control and the other to a PAH. External ion concentration was monitored at 10, 30, and 60 min with an Orion Ionanlyzer Model 407A. After 60 min 10% Triton X-100 was used to disrupt the liposomes to determine the internal concentration of the ion.

Alteration of Hemoglobin Structure

Two experiments were conducted to determine if naphthalene alters the molecular structure of human hemoglobin. The assumption was that a change in molecular structure results in a change in the visible spectrum of the molecule. Spectra were obtained using a Cary 14 Recording Spectrophotometer scanning from 500 to 380 nm. In the first experiment, 1 mg/ml type IV human hemoglobin (Sigma) was exposed to naphthalene concentrations of 1, 5, 10, 12, and 15 mg/l. After incubation at room temperature for 1, 2, and 4 h oxyhemoglobin and reduced hemoglobin scans were compared to ethanol controls. Sodium dithionite was used to reduce the hemoglobin. The second experiment was similar to the first except that the incubation solution was reacted with Drabkin's reagent and then analyzed. Spectra were obtained from scans of 650-350 nm. The hemoglobin was exposed to 5, 10, and 15 mg/l naphthalene with an ethanol control for the same time intervals as in the first experiment.

CHAPTER IV

RESULTS

Acute Experiments

Physicochemical Conditions

The physicochemical conditions of the test water were maintained as constant as possible. Temperature ranged from 19-22°C. Dissolved oxygen (DO) at the start of the experiments ranged from 7.5-8.8 mg/l. DO levels decreased during the exposure period, but usually the decrease was less than 1.0 mg/l. The pH ranged from 7.9-8.3 with diel variation usually less than 0.2. Conductivity varied from 340-440 µmhos/cm with diel changes less than 30 µmhos/cm.

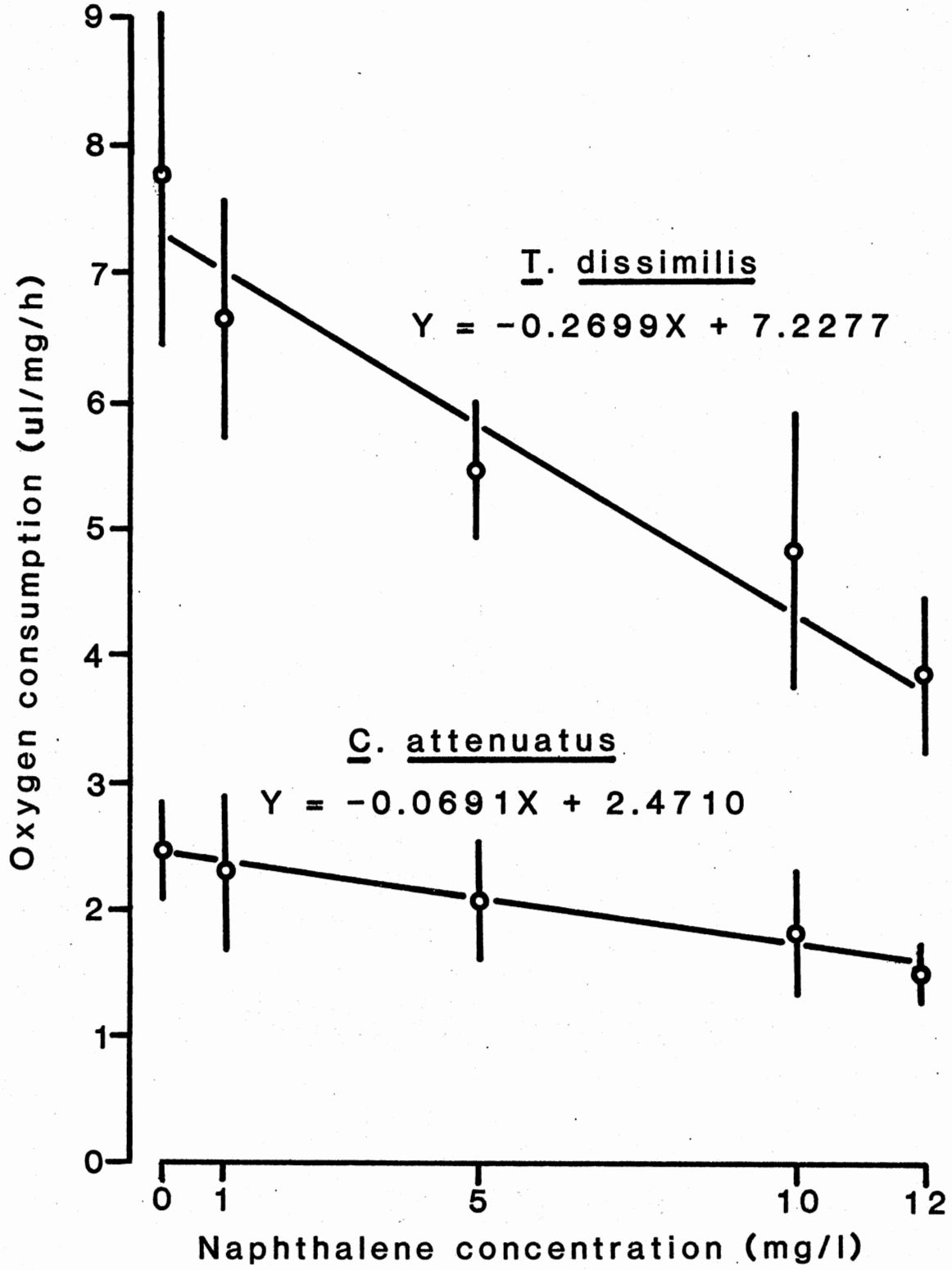
LC50

Chironomus attenuatus exposed to naphthalene for 24 h had LC50 values of 13.9 and 13.3 mg/l when analyzed by linear regression. Probit analysis (Barr et al. 1979) resulted in values of 13.1 and 13.0 mg/l. Tanytarsus dissimilis had 48 h LC50 values of 13.7 and 12.2 mg/l by linear regression and 20.7 and 12.6 mg/l by probit analysis.

Oxygen Consumption

The oxygen consumption of Chironomus attenuatus was depressed by a 1 h exposure to naphthalene (Figure 1, Appendix Table X). Oxygen

Figure 1. Oxygen Consumption of *Chironomus attenuatus* and *Tanytarsus dissimilis* Exposed to Naphthalene for One Hour ($\bar{x} \pm s$).


T. dissimilis
Y = -0.2699X + 7.2277
C. attenuatus
Y = -0.0691X + 2.4710
Oxygen consumption (ul/mg/h)
Naphthalene concentration (mg/l)
0
1
2
3
4
5
6
7
8
9
0
1
5
10
12

consumption decreased from 2.52 to 1.66 µl/mg/h at 0 and 12 mg/l naphthalene, respectively. A regression line fit to the data resulted in the following equation: $Y = -0.0691X + 2.4710$. The correlation coefficient, $r = -0.57$, was highly significant (OSL < 0.01). An one-way ANOVA with unequal sample size was calculated (Sokal and Rohlf 1981). Oxygen consumption among naphthalene concentrations was significant (OSL < .005).

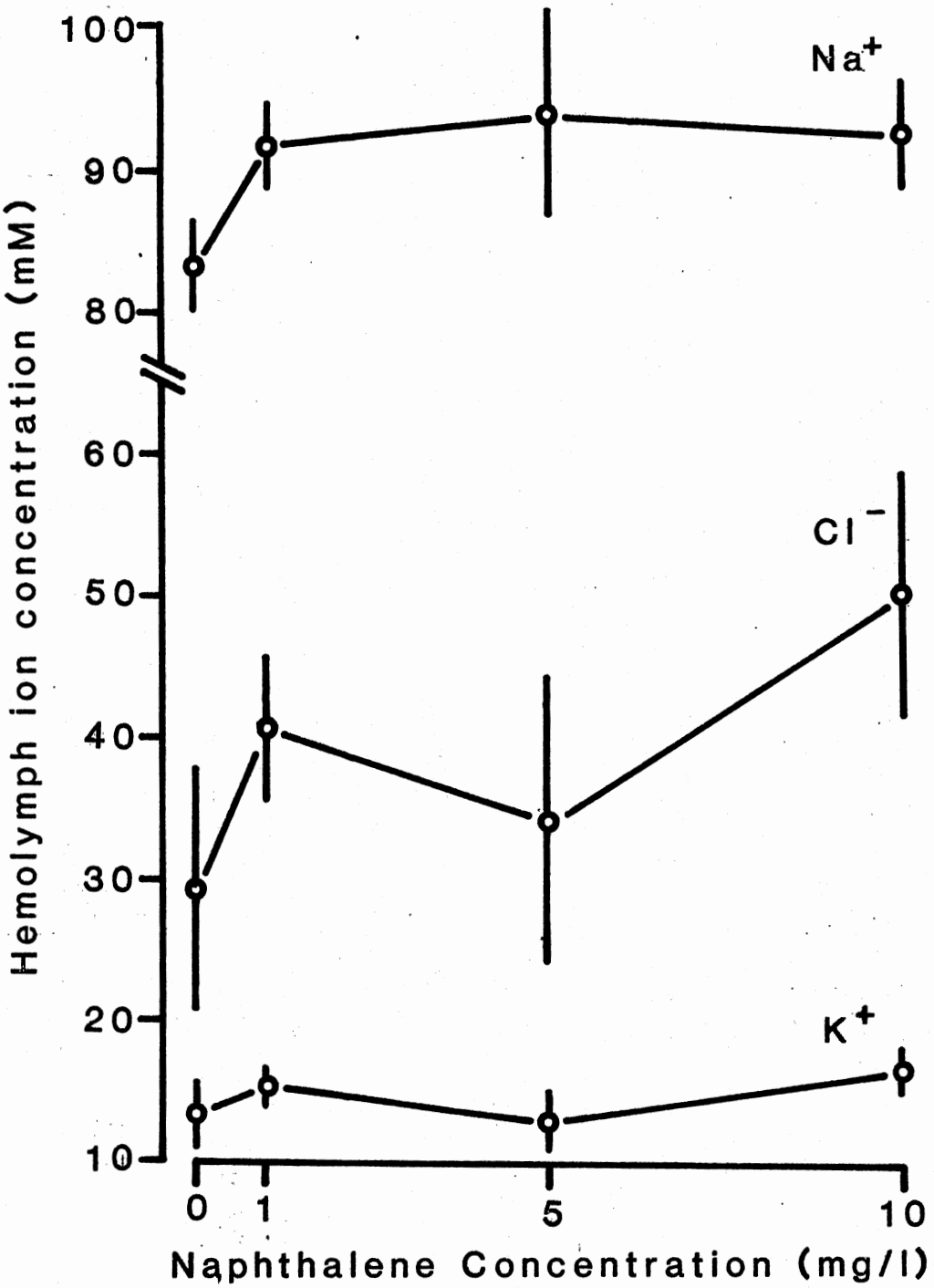
The oxygen consumption of Tanytarsus dissimilis followed the same trend as Chironomus (Figure 1, Appendix A Table XI). Oxygen consumption decreased from 7.70 to 3.90 µl/mg/h at 0 and 12 mg/l, respectively. The calculated regression line was $Y = -0.2699X + 7.2277$ ($r = -0.81$, OSL < 0.01). The slope was about four times greater than the slope of the Chironomus line. An one-way ANOVA with equal sample size showed that oxygen consumption among treatment groups was highly significant (OSL < 0.001).

Hemolymph Ion Analysis

Hemolymph ion analysis was performed after Chironomus was exposed to naphthalene for 1, 2, and 4 h. ANOVAs for Na^+, K^+, and Cl^- showed that variation among time periods and the naphthalene concentration-time interaction were not significant (OSL > 0.10). Thus, values at the three time periods are presented as means in Figure 2.

Hemolymph sodium (Na^+) concentration increased after exposure to naphthalene (Figure 2, Appendix Table XII) with means ranging from 83.8 mM at 0 mg/l to 94.3 mM at 10 mg/l. Mean Na^+ concentration increased between 1 and 5 mg/l with subsequent change slight. A two-way ANOVA with unequal sample size resulted in a highly significant (OSL < 0.001) effect due to naphthalene. A SNK test revealed that each

Figure 2. Variation of the Hemolymph Concentration of Na^+, K^+, and Cl^- in Chironomus attenuatus Exposed to Naphthalene ($\bar{x} \pm s$).


100
90
80
60
50
40
30
20
10
Hemolymph ion concentration (mM)
Na+
Cl−
K+
0
1
5
10
Naphthalene Concentration (mg/l)

treatment group was significantly different from the control (OSL < 0.05), but no comparison among treatment groups was significant.

Naphthalene exposed Chironomus also had elevated levels of potassium (K^+) in the hemolymph (Figure 2, Appendix Table XII). Means ranged from 13.3 to 17.1 mM for the control and 10 mg/l groups, respectively. A two-way ANOVA resulted in a significant naphthalene effect (OSL < 0.01). A SNK test had two significant comparisons: control versus 10 mg/l and 5 mg/l versus 10 mg/l.

Chloride (Cl^-) concentrations were elevated by exposure to naphthalene (Figure 2, Appendix Table XII). The variation in Cl^- with increasing naphthalene concentration was similar to K^+. An ANOVA resulted in a highly significant effect due to naphthalene (OSL < 0.005) with all treatment means except at 5 mg/l significantly different than the control (SNK, OSL < 0.05).

Hemoglobin Concentration

Human hemoglobin was used as the standard, so the values obtained can not be considered absolute. After 4 h exposure to naphthalene, the hemoglobin content of Chironomus attenuatus increased above the control at 1 and 5 mg/l and decreased at 10 and 12 mg/l naphthalene (Table II). However, an one-way ANOVA showed no significant differences exist among naphthalene concentrations (OSL > 0.10).

Experiments were conducted to determine if naphthalene changed the molecular structure of hemoglobin by measuring changes in the peak and trough heights of spectra after 1, 2, and 4 h exposure. Variation over the three time periods was not significant, thus the values are presented as means in Table III. Naphthalene caused a decrease in peak

TABLE II

HEMOGLOBIN CONCENTRATION OF *CHIRONOMUS ATTENUATUS* AFTER EXPOSURE TO NAPHTHALENE FOR FOUR HOURS

Naphthalene Concentration (mg/l)	$\bar{x} \pm s$ (n) (g/100 ml)[1]	Difference From Control (%)
0	3.23 ± 0.35 (20)	
1	3.40 ± 0.28 (5)	+5.2
5	3.36 ± 0.41 (5)	+4.7
10	3.01 ± 0.37 (4)	-6.8
12	2.90 ± 0.18 (5)	-10.2

[1]Based on human hemoglobin standards and may not be absolute.

TABLE III

THE EFFECT OF NAPHTHALENE ON THE MOLECULAR STRUCTURE OF HEMOGLOBIN AS DETERMINED BY MEASUREMENTS OF PEAKS AND TROUGHS OF SPECTRA SCANS FROM 500 TO 380 NM ($\bar{x} \pm s$)

Naphthalene Concentration (mg/l)	Oxyhemoglobin Peak (mm)	Reduced Hemoglobin	
		Peak (mm)	Trough (mm)
0	112 (0.0)	122 (8.5)	71 (4.2)
5	91 (3.8)	99 (12.2)	60 (6.1)
10	90 (2.4)	101 (4.7)	57 (2.3)
12	103 (3.6)	109 (24.1)	72 (6.6)
15	84 (1.5)	90 (5.3)	54 (4.9)

height of oxyhemoglobin from 112 mm at 0 mg/l to 84 mm at 15 mg/l. The peak heights of reduced hemoglobin showed a similar trend. Except at 12 mg/l a similar pattern occurred in the trough height of reduced hemoglobin. The seocnd experiment was like the first except Drabkin's reagent was added. No differences existed in peak heights due to naphthalene (Table IV). Heights of the control peaks decreased from 8 to 0 mm over the 4 h exposure period. The 5 mg/l treatment group showed a similar trend, while the 10 and 15 mg/l groups did not decrease.

Glycogen Concentration

The total body glycogen content of Chironomus attenuatus ranged from 9.24 to 13.94 mg/100 mg. Glycogen concentration did not vary significantly with naphthalene or time of exposure (Table V). The Variances within groups were too large to detect differences among groups or over time (OSL > 0.10). Over all time periods the 5 mg/l group was lowest in glycogen content than the other concentrations. The highest glycogen content always occurred after 2 h exposure.

Mode of Action

Four polycyclic aromatic hydrocarbons (naphthalene, 1-naphthol, phenanthrene, and chrysene) were tested for ion leakage and inhibition of Na^+,K^+ ATPase to formulate a general model for the mode of action of PAH. None of the PAH tested caused significant leakage of Na^+ or K^+ from the lipid vesicles when compared to the ethanol control (Tables VI and VII). Leakage of Na^+ and K^+ occurred over the 1 h exposure period, but the rate of leakage between the ethanol control and treatment groups was always similar.

TABLE IV

THE EFFECT OF NAPHTHALENE ON THE MOLECULAR STRUCTURE OF HEMOGLOBIN AFTER REACTION WITH DRABKIN'S REAGENT AS MEASURED BY PEAK HEIGHT (MM) AT 555 NM

Naphthalene Concentration (mg/l)	Time (h)			
	0	1	2	4
0	8	6	1	0
5		5	4	1
10		5	2	5
15		6	1	5

TABLE V

THE EFFECT OF NAPHTHALENE ON THE GLYCOGEN CONTENT (mg/100 mg) OF CHIRONOMUS ATTENUATUS ($\bar{x} \pm s$, n)

Naphthalene Concentration (mg/l)	Time (h)		
	1	2	4
0	11.37 + 3.34 (12)	12.89 + 4.34 (12)	11.04 + 3.57 (12)
1	10.35 + 2.76 (4)	13.94 + 2.65 (4)	13.68 + 2.40 (4)
5	9.62 + 3.22 (4)	10.19 + 2.24 (4)	9.24 + 2.51 (4)
10	11.79 + 2.94 (4)	12.27 + 6.14 (4)	11.88 + 6.68 (4)

TABLE VI

THE CONCENTRATION OF SODIUM (mM) IN SOLUTION AFTER EXPOSURE OF LIPSOMES TO POLYCYCLIC AROMATIC HYDROCARBONS[1]

Exposure Time (min)	ETOH	Naphthalene	ETOH	Naphthol	ETOH	Phenanthrene	ETOH	Chrysene
0	100.0	100.0	193.5	126.0	85.0	74.0	87.0	80
10	126.5	124.0	215	150	106.5	96.0	98.5	97
30	126.5	124.0	217	150	115.0	104.5	98.5	97
60	128.5	126.0	226	158.5	115.0	113.0	98.5	97
Triton[2]	204.5	200.5	306.5	232.5	195.5	195.5	139.5	139.5

[1]Mean of two independent experiments.

[2]Concentration of ion in solution after dissolution of liposomes.

TABLE VII

THE CONCENTRATION OF POTASSIUM (mM) IN SOLUTION AFTER EXPOSURE OF LIPSOMES TO POLYCYCLIC AROMATIC HYDROCARBONS[1]

Exposure Time (min)	ETOH	Naphthalene	ETOH	Naphthol	ETOH	Phenanthrene	ETOH	Chrysene
0	166	128	34	35	136	64	34	32
10	182	131	38	38	144	66	36	34
30	184	133	41	41	145	67	39	36
60	201	136	42	42	155	69	42	40
Triton[2]	276	165	49	49	212	82	56	54

[1]Mean of two independent experiments.

[2]Concentration of ion in solution after dissolution of liposomes.

The same four PAH tested in the ion leakage experiments were tested for their effectiveness of inhibiting the ion transport enzyme Na^+,K^+ ATPase. The enzyme system was exposed to 10 mg/l of each PAH for 30 min. An one-way ANOVA showed that all PAH caused inhibition of the enzyme (OSL < 0.05) with naphthalene causing the greatest inhibition (54%) and chrysene the least (4%) (Table VIII). A t-test with H_o: $\bar{x} = 0$ resulted in naphthalene, 1-naphthol, and phenanthrene being significantly different from zero. Since inhibition decreased as the molecular weight increased, a regression line was calculated. The line obtained was $Y = -0.487X + 118.053$ with $r = -0.919$ (OSL < 0.01).

Life Cycle Experiments

Tanytarsus dissimilis was exposed to naphthalene for its entire life cycle. To initiate the experiments 20 eggs were added to each unit. Hatching success was high in the controls of both experiments except for unit 1 of the first experiment (Table IX). Naphthalene caused a decrease in the hatching success with means of 88.3 and 63.3% in the control and combined treatment groups, respectively. Highest larval mortality occurred the first 24 h following hatching with the loss of 60% of the larvae in some units. If the larvae survived the first 24 to 48 h after hatching, they generally survived to pupation. Naphthalene affected the ability of the larvae to pupate to the adult. Over both experiments pupation to the adult averaged 78% in the control units and 57% over all the treatment groups.

The time to the first pupation in each unit and the mean time to pupation was also measured (Table IX). The time to the first pupation did not differ significantly between the control (17.3 days) and the

TABLE VIII

THE INHIBITION OF NA^+,K^+ ATPASE EXPOSED TO 10 mg/l OF POLYCYCLIC AROMATIC HYDROCARBONS FOR 30 MINUTES

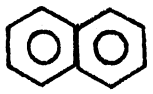
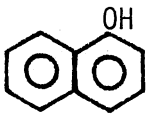
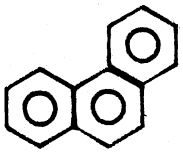
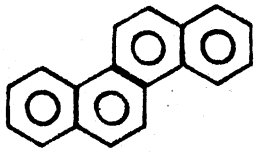
	Compound			
	Naphthalene	1-Naphthol	Phenanthrene	Chrysene
Structure				
Molecular weight	128.19	144.19	178.24	228.30
% Inhibition				
Experiment I	58	50	50	0
Experiment II	50	42	25	8

TABLE IX

THE EFFECTS OF NAPHTHALENE ON THE LIFE CYCLE OF *TANYTARSUS DISSIMILIS*

Parameter	Experiment	Unit 1	2	3	4	5	6	7	8	9	10	11	12
Naphthalene Concentration (mg/l)	I	0.00	0.00	0.05	0.05	0.14	0.14	0.14	0.23	0.29	0.32	0.35	0.34
	II	0.00	0.00	0.07	0.07	0.14	0.13	0.12	0.24	0.26	0.37	0.47	0.37
Hatching (%)	I	0	95	90	80	75	95	90	85	75	75	70	80
	II	90	80	55	50	65	0	70	55	75	75	80	45
No. of pupae	I	0	3	4	1	2	9	7	8	6	2	0	8
	II	17	5	9	2	3	2	10	9	11	4	10	9
No. of adults	I	0	2	3	1	2	6	7	2	2	2	0	2
	II	15	4	5	1	2	0	7	5	8	3	8	4
Time to 1st pupation (days)	I	-	21	23	26	21	19	18	21	21	21	-	21
	II	14	17	14	18	20	15	14	14	15	14	15	14
Mean time to pupation (days)	I	-	22.7	23.2	26.0	21.0	21.9	20.1	24.0	22.8	21.5	-	23.4
	II	16.1	18.8	17.0	18.0	20.0	16.5	17.9	18.4	18.8	16.9	16.9	16.7
No. of adults with eggs	I	0	2	0	0	0	4	7	0	2	1	0	2
	II	9	4	5	1	2	0	6	4	5	3	8	3
No. of eggs in 1st egg mass	I	0	205	0	0	0	389	833	0	272	91	0	138
	II	763	357	488	110	195	0	504	373	492	367	695	275

TABLE IX. CONTINUED.

Parameter	Experiment	Unit 1	2	3	4	5	6	7	8	9	10	11	12
Eggs/adult	I	0	103	0	0	0	97	109	0	136	91	0	69
	II	85	89	98	110	98	0	84	93	98	122	87	91
No. of eggs in 2nd egg masses	I	0	0	0	0	0	0	0	0	0	0	0	16
	II	0	46	14	0	0	0	0	41	22	26	88	75
Eggs/adult	I	0	0	0	0	0	0	0	0	0	0	0	16
	II	0	23	14	0	0	0	0	21	22	26	29	38

treatment groups (17.5 days). The mean time to pupation for each unit was similar in the control (20.2 days) and treatment groups (19.9 days).

The number of adults producing eggs, the total number of eggs produced in the first and second egg masses, and the mean number of eggs/adult were also measured (Table IX). In both experiments the number of adults producing eggs averaged 86.7% in the controls and 71.8% in the treatment groups. The total number of eggs and the number of eggs in the first egg mass/adult was not significantly different in the control and treatment groups. The mean number of eggs in the first mass was 92 and 99 in the control and treatment groups, respectively. In adults producing second egg masses, the control (23 eggs/adult) and the treatment groups (25 eggs/adult) were similar.

CHAPTER V

DISCUSSION

Acute Experiments

Chironomus and *Tanytarsus* had similar LC50 values of about 13 mg/l. Other freshwater animals tested with naphthalene have been *Daphnia*, the bluegill *Lepomis macrochirus*, and the African clawed toad *Xenopus laevis*. *Daphnia magna* had a 24 h LC50 of 6.6 mg/l (Crider et al. 1982) and 17.0 mg/l (LeBlanc 1980), and a 48 h LC50 of 9.3 mg/l (E. Stebler, personal communication). The bluegill 48 h LC50 was 7.0 mg/l (E. Stebler, personal communication). Three week old *Xenopus* larvae had a 24 h LC50 of 7.0 mg/l (Edmisten 1981).

LC50 values similar to the present study have been reported for a variety of marine organisms. The polychaete *Neanthes arenaceodentata* (Rossi and Neff 1978), the grass shrimp *Palaemonetes pugio* (Neff et al. 1976), and the spot shrimp *Pandalus platyceros* (Sanborn and Malins 1977) are more sensitive to naphthalene than *Chironomus* and *Tanytarsus*. The mosquito fish (Wallen et al. 1957) and the pacific oyster (LeGore 1974) are more resistant.

The oxygen consumption rates found in this study, 1.66 to 7.70 µl/mg/h, are similar to values reported for other chironomids. In laboratory studies *Chironomus tentans* had a rate of 2.34 µl/mg dry wt/h at 22°C (Kawatski et al. 1974). At 21°C, *C. attenuatus* had an oxygen uptake rate of 1.60 µl/mg ash free wt/h (Cole and Wilhm 1973). In a

field study Chironomus riparius and C. tentans had rates of 1.0-9.9 and 2.0 µl/mg/h, respectively (Barker 1980).

No consistent pattern of response of aquatic organisms to naphthalene appears to exist. Pink salmon fry exposed to 0.76 mg/l naphthalene showed a 400% increase in oxygen consumption (Thomas and Rice 1978). Fundulus heteroclitus exposed to 4 mg/l naphthalene also exhibited elevated oxygen uptake rates (Levitan and Taylor 1979). The present study is in agreement with two studies using Daphnia and one using cultured mammalian cells. Using Daphnia pulex Geiger and Buikema (1981) reported that 0.3 and 0.6 mg/l naphthalene depressed oxygen uptake by 30 and 34%, respectively. D. magna exhibited a 10 and 25% decrease at 1 and 8 mg/l naphthalene, respectively (Crider et al. 1982). Harmon et al. (1981) found that oxygen consumption of cultured Vero cells was inhibited 50% by 15 mg/l naphthalene.

The immediate inhibitory effect of naphthalene on the activity of chironomids suggests a possible anesthetic action of the chemical on the nervous system. Many theories exist on how compounds cause narcosis, but all attempt to explain why the number of neurons firing per unit time is less than normal (Cohen and Dripps 1965). If the Na^+,K^+ ATPase pump essential to the proper function of neurons is inhibited, the neuron could become depolarized resulting in blocking of nerve conduction. With fewer neurons firing less ATP is needed and less oxygen is used. Naphthalene inhibits Na^+,K^+ ATPase (Table VIII), which could explain the decrease in the activity and oxygen consumption of the organisms.

Another possible explanation for the lowered oxygen consumption is that naphthalene inhibits a portion of the electron transport system.

Other chemicals such as rotenone, antimycin A, carbon monoxide, and cyanide are inhibitors of the chain (Stryer 1975). Naphthalene was found to inhibit two enzymes (NADH oxidase and NADH cyt-c reductase) in the NADH dehydrogenase portion of the chain by 50 and 30% (Harmon et al. 1981). Mitochondia account for 98% of oxygen uptake in cultured Vero cells. Therefore, inhibition of the chain should cause large decreases in the amount of oxygen used.

The hemolymph ion concentrations of Na^+,K^+, and Cl^- ($\bar{x}$ = 83.8, 13.3, and 29.4 mM) found in this study are similar to published values. Chironomus tentans had Na^+ and K^+ concentrations of 93 and 5 mM, respectively (Wright 1975). C. riparius had Na^+ and K^+ concentrations of 115 and 7 mM (Barker 1980). Cl^- concentrations in C. tentans were 28 mM (Wright 1975) and 50 mM in C. riparius (Barker 1980).

The effect of aromatic hydrocarbons on ion regulation in bacteria, algae, and fish has been studied. The bacterium Pseudomonas aerugionsa exposed to benzene, cyclohexane, and phenol did not lose significant amounts of K^+ (Bernheim 1974). The green alga Ankistrodesmus falcatus exposed to near saturation concentrations of benzene for 24 h lost all cellular K^+ and Mn^{++} (Kauss and Hutchinson 1977/1978). The decreased blood Na^+ in rainbow trout exposed to crude oil was attributed to inhibition of Na^+,K^+ ATPase in the gills (McKeown and March 1978). Blood ion levels in Coho salmon exposed to cyclohexane, benzene, toluene, and xylene initially increased but returned to normal ranges in 24 h (Morrow et al. 1975). The return to normal values was attributed to the volatilization of the toxicants resulting in lower exposure concentrations.

Since the work of Van Overbeck and Blondeau (1954), most

investigators suggest that PAH partition into lipophilic membranes and disrupt membrane integrity resulting in ion leakage. Recently Kauss and Hutchinson (1977/1978) used that model to explain the loss of ions from green algae. However, Roubal (1974) and Roubal and Collier (1975) showed that relatively nontoxic hydrocarbons, such as hexane and heptane, penetrated into the hydrophobic interior of membranes. More toxic aromatic hydrocarbons, such as benzene and toluene, were bound to sites on the membrane surface. Binding of PAH to sites on the external surface of membranes could lead to changes in cellular activities by the inhibition of enzyme systems with receptors on the membrane surface. Experiments from the present work support the membrane surface model and are in disagreement with the membrane structure disruption model. No significant leakage of ions occurred from liposomes, while inhibition of the membrane bound Na^+,K^+ ATPase was significant.

Although the hemoglobin concentration found in Chironomus attenuatus ($\bar{x}$ = 3.23 g/100 ml) probably was not absolute, it was similar to concentrations reported for other chironomids. Fourth instar Chironomus tentans had hemoglobin concentrations of 3.7 (Tichy 1980) and 3.48 g/100 ml (Firling 1977). Chironomus thummi had a hemoglobin concentration of 25% (3.5 g/100 ml) of the value for human blood (Wigglesworth 1972).

Naphthalene causes a decrease in hemoglobin in a variety of organisms. Naphthalene ingestion causes hemolytic anemia in man (Mackell et al. 1955). Dogs that ingested naphthalene showed a decrease in hemoglobin (Zuelzer and Apt 1949). Exposure of Daphnia magna to naphthalene also resulted in significant decreases in hemoglobin (Crider et al. 1982).

Although the response to naphthalene is similar in these animals, the mode of action may be different. Mammals have hemoglobin in red blood cells, while Daphnia and Chironomus have hemoglobin free in the hemolymph. In mammals naphthalene causes fragmentation of the red blood cells and subsequent loss of the hemoglobin. In the invertebrates the decrease could be due to changes in the molecular structure of hemoglobin or inhibition of the synthesis of hemoglobin. Naphthalene affected the spectra of oxyhemoglobin, but did not alter the spectra when Drabkin's reagent was used (Tables III and IV). Hemoglobin in chironomids is synthesized by the subepidermal fat body (Bergtrom et al. 1976) into which the lipophilic naphthalene would partition. C. tentans can synthesize hemoglobin at a rate of 0.42 µg/h (Tichy 1980). At 12 mg/l naphthalene C. attenuatus lost 0.075 g hemoglobin/100 ml/h (Table V). Complete inhibition of hemoglobin synthesis does not account for the decrease in hemoglobin concentration.

Glycogen content of Chironomus attenuatus was similar to values reported for Chironomus tentans (Augenfeld 1967). Augenfeld reported that glycogen content declined during long periods in the hypolimnion of a lake. Glycogen content was not a good indicator of naphthalene stress. No significant decrease existed in glycogen due to naphthalene or over time. Perhaps naphthalene by causing narcosis and reduced activity indirectly caused less glycogen to be used for energy.

Life Cycle Experiments

It is desirable to hold the physicochemical parameters as constant as possible to minimize effects and to enable comparisons among different

studies of a similar nature. For example, changes in parameters such as pH and hardness affect the toxicity of both organic and inorganic compounds. The present experiments were conducted with hard water, and it has been shown that calcium decreases the toxicity of certain substances (Maki and Bishop 1979).

Naphthalene in the test units decreased due to volatilization and photodegradation. Photooxidation of naphthalene to a variety of compounds such as diols and quinones occurs through many processes (Neff 1979). The necessary enzyme system for the degradation of PAH has been found in bacteria, fish, mammals (Brooks 1977) and Chironomus riparius (Estenik and Collins 1970). Bacteria can break down naphthalene by way of the metabolite 1,2-dihydro-1,2-dihydroxy-naphthalene (Lee and Anderson 1977). The importance of the many degradation products to the lethal and sublethal toxicity is not well known.

Insects are unique in contrast to other test animals, such as Daphnia and fish, in that their life cycle involves several developmental stages including adult emergence from the aquatic system. Previous life cycle studies with chironomids have ended with adult emergence (Derr and Zabik 1972, Sanders 1980). However, there have been no published accounts of chironomid tests where the reproductive capability of the organism has been used as an indicator of sublethal toxicity. In this respect Tanytarsus dissimilis is an ideal test animal because it reproduces parthenogenetically. Other advantages for using Tanytarsus are the easily identified stages, short life cycle time, and the ability to be reared in the laboratory.

Several life cycle and reproductive parameters were examined during this study. Hatching success was a good indicator of sublethal toxicity

with the treatment hatch rates lower than the control rates. Pupation and subsequent adult emergence requires a large supply of energy (Chapman 1971). If the critical amount of energy is not obtained, the animal may not be able to pupate. The pupation rate was higher in the control groups than in the combined rates of all treatment groups. The naphthalene could be interferring with the ability of the larvae to accumulate the necessary amounts of energy reserves.

The reproductive capacity of Tanytarsus was not significantly affected by naphthalene at the concentrations tested. The total number of eggs produced and the number of eggs/adult was similar in control and treatment groups. Exposure concentrations during the chronic experiments ranged from 20 to 200 times less than the acute LC50 values. No published results exist for comparison with this study. However, simultaneous work in this laboratory with Daphnia magna has been conducted (E. Stebler, personal communication). The same naphthalene concentrations caused minimal changes in the life cycle and reproductive parameters studied. Therefore, it is reasonable to conclude that the naphthalene concentrations tested were at or below the no effect level for the parameters measured.

CHAPTER VI

SUMMARY

1. LC50 values for both Chironomus attenuatus and Tanytarsus dissimilis were about 13 mg/l.

2. Oxygen consumption of both animals was inhibited by 1 h exposure to naphthalene. Decreased oxygen uptake was probably due to either an anesthetic action of the chemical on the nervous system or by inhibition of enzymes in the electron transport system.

3. Na^+, K^+, and Cl^- concentrations increased in the hemolymph of Chironomus following exposure to naphthalene.

4. Mode of action studies indicated that loss of ionic regulation was probably due to inhibition of Na^+,K^+ ATPase and not to a general disruption of membrane integrity.

5. Hemoglobin levels of Chironomus decreased at 10 and 12 mg/l naphthalene. Decreases in synthesis or changes in the molecular structure of hemoglobin could account for lower hemoglobin levels.

6. Total body glycogen did not vary significantly with time of exposure or naphthalene concentration.

7. Life cycle tests exposed Tanytarsus to a maximum of 0.5 mg/l naphthalene and showed that hatching success was greater in the controls than in the treatment groups. The highest larval mortality occurred within 24 h of hatching. Adult emergence was also lower in the treatment groups.

8. Time to the first pupation and the mean time to pupation was not affected. The number of eggs in the first and second egg masses per adult were similar in control and treatment groups.

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APPENDIX

TABLE X

OXYGEN CONSUMPTION OF *CHIRONOMUS ATTENUATUS* EXPOSED TO NAPHTHALENE FOR ONE HOUR

Naphthalene Concentration (mg/l)	$\bar{x} \pm s$ (n) (µl/mg/h)	% Decrease From Control
0	2.52 ± 0.44 (24)	
1	2.27 ± 0.67 (8)	10
5	2.04 ± 0.42 (8)	19
10	1.84 ± 0.51 (6)	27
12	1.66 ± 0.16 (6)	34

TABLE XI

OXYGEN CONSUMPTION OF TANYTARSUS DISSIMILIS
EXPOSED TO NAPHTHALENE FOR ONE HOUR

Naphthalene Concentration (mg/l)	$\bar{x} \pm s$ (n) (μl/mg/h)	% Decrease From Control
0	7.70 ± 1.38 (4)	
1	6.64 ± 0.94 (4)	14
5	5.47 ± 0.55 (4)	29
10	4.87 ± 1.19 (4)	38
12	3.90 + 0.67 (4)	49

TABLE XII

CONCENTRATION OF NA^+, K^+, AND Cl^- IN THE HEMOLYMPH OF *CHIRONOMUS ATTENUATUS* AFTER EXPOSURE TO NAPHTHALENE

Ion	Naphthalene Concentration (mg/l)	Exposure Time (h) 1	2	4	$\bar{x} \pm s$ (n)
Na^+	0	84.5	81.4	85.6	83.8 ± 4.6 (24)
	1	91.2	93.3	90.2	91.6 ± 3.2 (9)
	5	93.3	91.6	99.9	94.3 ± 7.6 (6)
	10	90.4	92.0	94.6	92.6 ± 3.9 (9)
K^+	0	12.4	13.8	13.6	13.3 ± 2.9 (26)
	1	14.7	15.3	15.7	15.2 ± 1.6 (8)
	5	12.7	12.9	13.9	13.2 ± 2.5 (8)
	10	17.3	17.0	17.0	17.1 ± 2.0 (9)
Cl^-	0	29.7	30.2	33.2	29.4 ± 9.2 (22)
	1	40.5	39.0	42.6	40.7 ± 5.2 (9)
	5	30.8	36.9	35.6	34.2 ± 10.3 (11)
	10	46.1	45.6	59.4	50.4 ± 8.3 (6)

VITA

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