THE EFFECTS OF pH, PHENOL, AND SODIUM CHLORIDE ON THE BIOENERGETICS OF LABORATORY POPULATIONS OF *CHIRONOMUS ATTENUATUS*

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PREFACE

The objectives of this study were: (1) to measure the effects of pH, phenol, and NaCl on the percent survival of all life stages of Chironomus attenuatus, the caloric content of third and fourth instar larvae and adults, the lipid and protein-nitrogen content of fourth instar larvae, and the effects of interaction among the main treatment factors on the above responses, and (2) to determine if C. attenuatus was catabolizing phenol as an energy source.

Dr. Jerry L. Wilhm served as major adviser. Drs. Calvin G. Beames, Jr., Troy C. Dorris, William A. Drew, and Rudolph J. Miller served as members of the advisory committee and criticized the manuscript. Dr. George V. Odell provided advice and facilities for the metabolism studies. Drs. John R. Sauer and Dale M. Toetz provided facilities for the measurement of NaCl and protein-nitrogen, respectively. Dr. Ronald W. McNew and Mr. William D. Hahn provided assistance with the statistical design and analysis. Mr. Jeffrey H. Johnson and other members of the Reservoir Research Center helped during a critical period in the experiments. The assistance of all these people is appreciated.

Untold appreciation is expressed to my wife, Barbara, for her patience and assistance in collecting and analyzing the data and for typing the manuscript.

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CHAPTER I

INTRODUCTION

Oil pollution of aquatic ecosystems may result from drilling operations, refining processes, and transportation of oil or disposal of the used product. These procedures also result in introduction of extraneous materials such as salts, heavy metals, acids, and caustics. Oil is a mixture of many organic compounds from simple hydrocarbons such as methane to complex aromatic derivatives. Field studies have generally failed to elucidate the interactions of oil constituents on biological organisms. The complex materials in oil may have synergistic or antagonistic effects on the biota which are incapable of solution by field investigation.

Energy flow has been used in ecological studies as a means of measuring the effects of environmental factors on organisms, populations, or ecosystems. The transfer of energy follows the laws of thermodynamics and may be precisely defined (Wiegert 1964). The calorie is particularly useful for comparative studies since all forms of energy may be converted to heat (Phillipson 1966), and energy units are preferable over biomass units for studies of community metabolism (MacFadyen 1948). Biomass units are unsuitable because of variation in turnover rates among species (Teal 1957).

One of the first applications of the energy flow concept to aquatic ecosystems was made by Lindeman (1942). Studies have been made

on energy transfer through various levels in aquatic ecosystems (Coffman, Cummins, and Wuycheck 1971; Odum 1957; Odum and Odum 1955; Teal 1957, 1962; Tilly 1968; Welch 1968). Because of the complexity of ecosystem analysis, many of the energy transfers were based on assumptions. Several studies dealt with single populations in the field (Englemann 1961; Golley 1960; Golley and Gentry 1964; Hinton 1971; Lawton 1971 a, b; McNeill 1971; Paine 1971; Wiegert 1964) and in the laboratory (Clutter and Theilacker 1971; McDiffett 1970; Moshiri and Goldman 1969; Richman 1958; Schroeder 1969, 1971; Slobodkin 1959).

The caloric content of a wide variety of species has been calculated to provide data for energy flow studies (Comita and Schindler 1963; Golley 1961; Paine 1964; Slobodkin and Richman 1961). The range in the caloric content in life stages of the meadow spittlebug (Philaenus spumarius) was as broad as the range of the different species reported above (Wiegert 1965). The range in the caloric content of Chironomus attenuatus instars was similar to the range in Wiegert's study (Graham unpublished data). The precise energy budget of a population can not be constructed without knowledge of the environmental conditions surrounding the population and role of the different life stages within the population.

Microcosms have been used to investigate the effects of various factors on organisms under controlled environmental conditions. Microcosms were used to investigate community metabolism in freshwater (Beyers 1963). The effect of nitrate and phosphate addition on autotrophic production was determined in carboys under estuarine conditions (Abbott 1967, 1969). Since the effect of a single variable may be evaluated in a controlled environment, many toxicity studies have been

conducted in laboratory microcosms (Bell and Nebeker 1969; Jenkins 1964; Nebeker and Lemke 1968).

The physiological state of an organism affects its caloric content (Brody 1945). Physiological state is determined by the stage in the life cycle and environmental conditions. The chemical composition of Chlorella pyrenoidosa was affected by several environmental variables (Spoehr and Milner 1949). R-values, the degree of reduction of organic matter when compared to methane (100% reduction) and carbon dioxide (0% reduction), were used to indicate changes in chemical composition of C. pyrenoidosa. Since the R-value is proportional to the heat of combustion of the organic matter, it is an expression of energy content. R-values were correlated with changes in the carbon dioxide and oxygen content of the atmosphere, nutrient level of the medium, light intensity, and temperature. As R-values increased, a nearly linear increase occurred from 4.5 to 86.0% in the lipid content of C. pyrenoidosa while carbohydrate content decreased from 38 to 6% and protein content decreased from 58 to 9%.

Seasonal changes in the reproductive condition of the marine copepod, Calanus finmarchicus, resulted in changes in the caloric content (Comita, Marshall, and Orr 1966). Intraseasonal changes in the caloric content of some freshwater invertebrates has been noted (Wissing and Hasler 1971).

Toxicants also affect the physiology and, therefore, the caloric content of organisms. The effects of lead, nickel, and sodium pentachlorophenate (PCP) at different pH have been studied on the respiratory rate of tubificid worms (Whitley and Sikora 1970). The respiratory rate was inhibited by lead, decreased slightly by nickel,

and stimulated by PCP. Increases in phenol concentration (0-25 mg/1) resulted in increased respiratory rates in Chironomus attenuatus (Cole 1971). The caloric content of C. attenuatus was affected by the NaCl concentration of the media (Thornton and Sauer 1972).

Few studies have been made of energy flow in polluted ecosystems. Chironomid populations in an oil refinery effluent holding pond series reduced the caloric content of the final effluent by energy loss through adult emergence and through respiration (Tubb and Dorris 1965). Primary producers and decomposers were the dominant energetic components in a sewage waste stabilization lagoon (Kimerle and Anderson 1971). The chironomid population, Glyptotendipes barbipes, was a minor component in energy transfer through the lagoon (Kimerle and Anderson 1971). Carbon dioxide consumption was approximately four times greater than O_2 production in a sewage treatment lagoon (Verduin 1971). The greatest variations in the energy budget in five ecological zones of a polluted stream were associated with domestic and industrial pollution and the inflow of inorganic sediments (King and Ball 1967).

The purpose of the present study was to determine the effects of pH, phenol, and NaCl on Chironomus attenuatus (Walker) in laboratory microcosms designed to simulate an oil refinery effluent oxidation pond. To determine the effect of varying levels of these three variables, measurements were made on:

1) the survival of larval instars and adults;
2) the caloric content of third and fourth instars and adults;
3) the lipid and protein-nitrogen content of fourth instars;
4) the effect of interaction among the treatment variables;
5) the metabolism of phenol by C. attenuatus and bacteria.

CHAPTER II

MATERIALS AND METHODS

Stock Populations

The original laboratory population of Chironomus attenuatus was established with individuals collected from Skeleton Creek, Oklahoma, 7 miles below an input of domestic and industrial wastes (Wilhm and Dorris 1966).

Life Cycle

After mating, the adult female oviposits one egg mass on the surface of the water (Figure 1). Each egg mass contains an average of 412 eggs ensheathed in a gelatinous mass. The egg mass absorbs water and floats until the adhesive pedicel contacts a solid substrate and adheres. Development time of the eggs in the laboratory varies from 24 to 36 hr at 22 C. The first instar larvae initially feed on the gelatinous mass surrounding the eggs and later settle to the bottom. Cases are formed from salivary secretions, fine detrital particles, and sand grains. The first instar larvae molt to second instar larvae 2 to 4 days after hatching. The larvae increase in size through successive molts to fourth instar.

Chironomus larvae feed by spinning a net across the lumen of the tube and undulating to draw a current of water and suspended particles through the tube (Walshe 1947). Particles with a diameter greater than

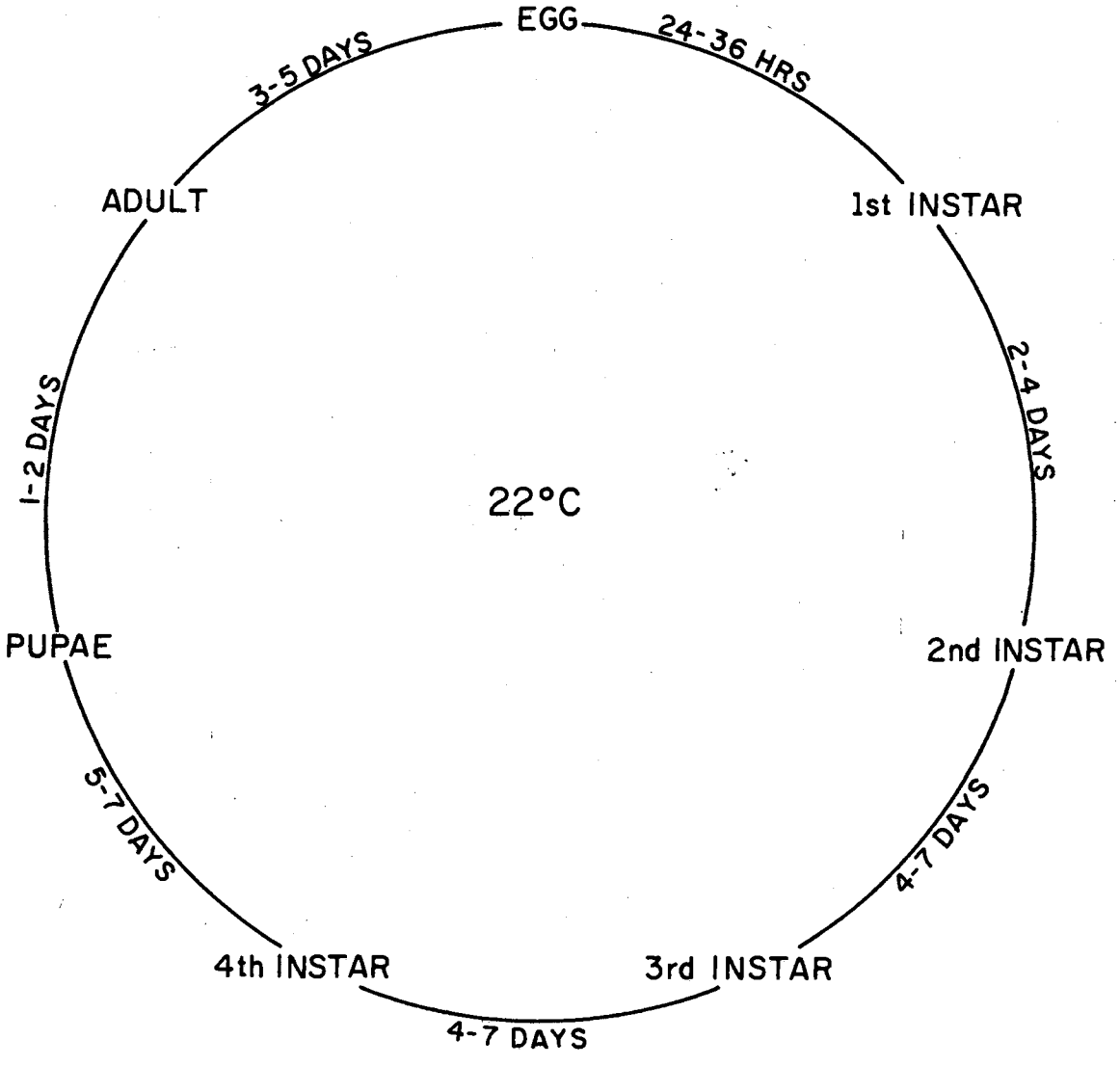

Figure 1. Life cycle of C. attenuatus

17μ are trapped by the net (Walshe 1947). The larvae eat the net and trapped particles, then spin another net and repeat the process. C. attenuatus larvae have been observed foraging on the bottom but were not observed spinning nets. This, however, does not preclude net spinning.

The second and third instar stages last from 4 to 7 days each. The fourth instar pupates after 5 to 7 days and emerges as an adult 1 to 2 days later. The sex ratio in the adult population is approximately 1:1 (4976 ♂♂: 5098 ♀♀). The adults do not feed and live only 3 to 5 days. Mating occurs during the first 2 days.

Laboratory Stock Population

The laboratory population was raised in galvanized metal trays, 88 x 56 x 18 cm, painted with a nontoxic epoxy paint to prevent entry of dissociated metal ions. Each tray was enclosed by a screened cage, 90 x 57 x 42 cm. A 40-watt light source in each cage was controlled by a timer set on an 8-hr light photoperiod. This photoperiod reduced algal growth but had no observable effect on C. attenuatus. Oxygen was continuously added to the water through a forced air system. Two grams of Hartz Mountain Dog Kisses were ground into a paste with water and fed to the larvae in each tray every 2 days (Biever 1965, 1971). The stock populations served as a source for egg masses used in the experiment. The trays were drained, cleaned, and repainted every 6 months. Adults and larvae from each tray were mixed with those from other trays to reduce inbreeding.

Experimental Design

Experimental subunits (48 Mason jars, 11 x 11 x 14 cm) contained 1 liter of treatment solution. The subunits were placed on racks in a water bath, 152 x 61 x 20 cm (Figure 2). Water was pumped from a center reservoir containing a Blue M Constant-Flow Portable Cooling Unit and continuously circulated around and under the units in the tank by a Little Giant submersible pump and a series of hoses. The temperature of the circulating water was maintained at 24 C (±0.5 C) by the cooling unit.

A bank of 40 General Electric Gro-Lux florescent lights provided a total illumination of 290 ft-c/cm^{-2} of water surface. The light bank was regulated by a timer set on a 12-hr photoperiod. A plywood cover was placed over the water bath to prevent heating the water and subunits were set in holes cut in the cover.

Each experiment consisted of 24 treatment combinations with a 3 x 4 x 2 factorial arrangement of treatments in a completely randomized design with two replications of each treatment. Treatment variables were pH at 6.2, 7.2, and 8.2; phenol at 0, 10, 20, and 30 mg/l; and NaCl at 0 and 600 mg/l.

Each liter of treatment solution contained 0.01% modified Knop solution (Beers 1959) to provide nutrients, 28,571 units of buffered potassium penicillin G and 0.142 g of dihydrostreptomycin sulfate to control bacteria, 0.003 M phosphate buffer to maintain the appropriate pH level, 2 mg of methylene blue to control fungus, and the appropriate phenol and NaCl concentration. Deionized water was used as the carrier solution and pH was adjusted to 6.2 with 2N H_2SO_4 and to 8.2 with 2N KOH. Treatment combinations were assigned to experimental units by a

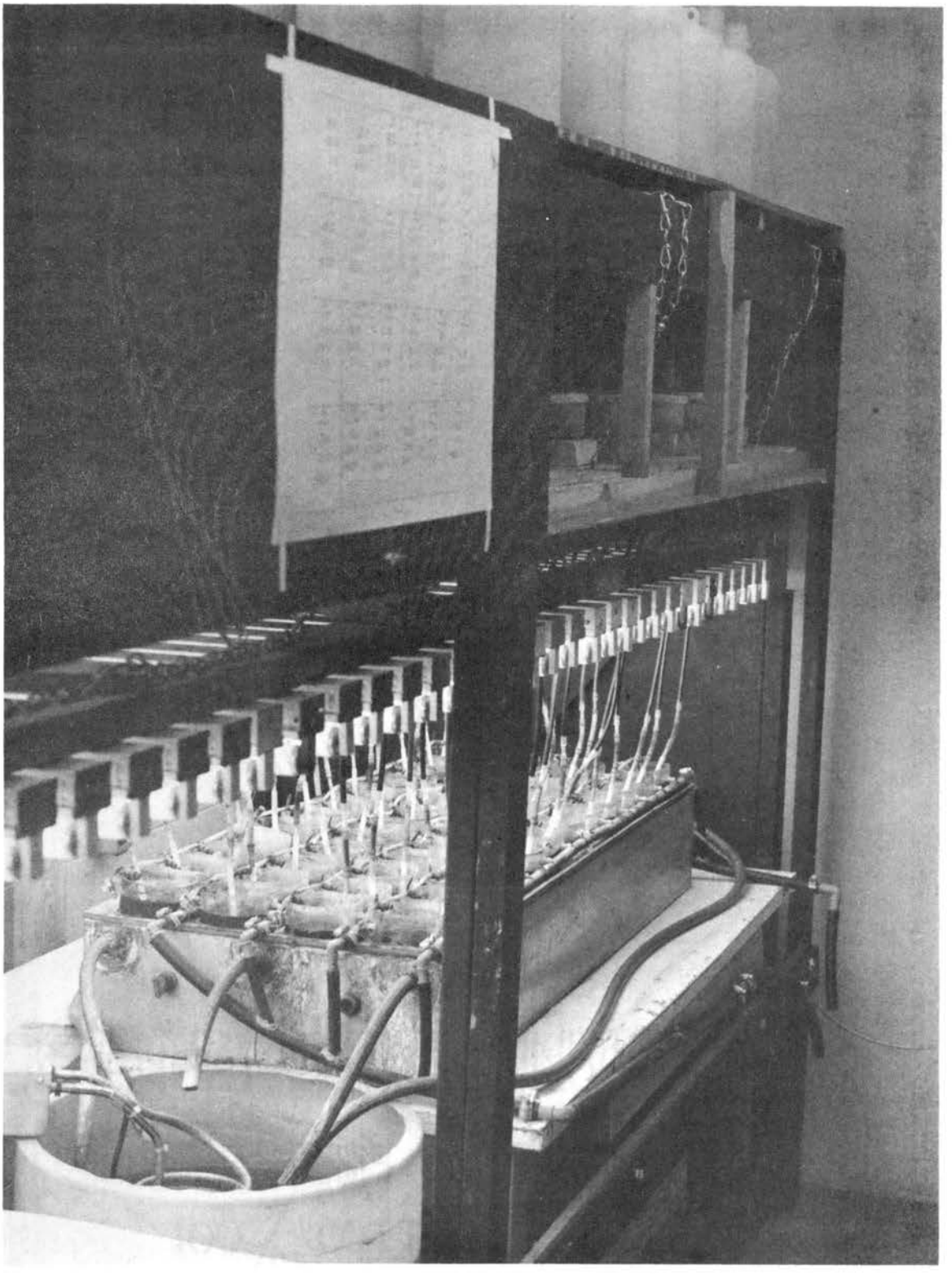

Figure 2. The continuous flow system

random selection procedure.

Egg masses used in the experiments were taken at random from the stock population and photographed in a Sedgwick-Rafter cell before being placed in a subunit. Preliminary experiments indicated that differences in larval survival depended upon the particular treatment combination. Because of differential mortality among treatment combinations, varying numbers of egg masses were added to each experimental unit to provide sufficient numbers of organisms for analysis. A direct census of the potential number of organisms per subunit was made by projecting the developed negatives of the egg masses onto a screen.

A continuous flow system similar to that of Cole (1971) maintained a constant level of the treatment factors (Figure 2). Twenty-four 7.6 liter polypropylene head tanks supplied two subunits each. Each head tank contained 7.5 liters of one of the 24 possible treatment combinations. Preliminary experiments indicated that the volume of flow from the head tanks was dependent upon the volume of fluid remaining in the head tank. A 0.47 liter polypropylene container was inserted between head tanks and subunits to provide constant gravity flow. Flow into the experimental subunits was regulated so that the entire water mass was replaced every 0.66 days. Each head tank was cleaned and replenished every 2 days to ensure a constant flow, maintain a constant treatment level, and prevent microbial use of the phenol and/or sodium chloride before the solution entered the units. Overflow tubes maintained 1 liter of water in the subunits. Silk bolting cloth across the mouth of each piece of tubing prevented loss of larvae by outflow.

Dissolved oxygen was maintained at a minimum of 8 mg/l (95% saturation at 24 C) by compressed air. Each subunit received 0.3 g of Dog

Kisses every 2 days during the experiment.

The effluent of each subunit was analyzed every 2 days for pH, phenol, and NaCl in the first experiment. A Beckman Zeromatic SS-3 pH Meter was used for pH determinations. Phenol concentrations were measured with a Beckman DB-G Grating Spectrophotometer (Martin et al. 1967). The concentration of NaCl was determined as the Na^+ and Cl^- concentration. The Na^+ concentration was determined with a Beckman 440 Atomic Absorption Spectrophotometer while Cl^- concentration was measured with a Fiske/Marius Micro Chlor-o-counter. Since pH and NaCl concentration remained constant throughout the course of the first experiment, later experiments were analyzed only once a week. Since phenol concentrations decreased after day 11 of the experiment, additional phenol was added daily to maintain the appropriate treatment level in the subunits.

Collection of Larvae for Determinations

Daily observations were made to determine the stage in the life cycle of the larvae after the egg masses were placed in the experimental units. From preliminary experiments it was found that the variation in number of eggs among egg masses from the same tray and egg masses from different trays was not significant over several months.

The experimental units were removed when the majority of larvae had reached a certain stage, i.e. first, second, third, or fourth instar, pupa, or adult. If only a portion of the larvae were removed at each life stage, the remaining larvae in the subunit after each sampling might have been injured resulting in erroneous mortality and caloric data. By sacrificing the entire unit at a given life stage, possible injury was avoided. Since the treatment affected the growth rate, each

unit was observed every day and the time required to reach a certain stage was recorded.

The head capsule width at the broadest point was used to determine the instar. The widths of the head capsules as determined by Graham (unpublished data) for different instars are:

first instar - .08 to .10 mm
second instar - .12 to .15 mm
third instar - .25 to .30 mm
fourth instar - .40 mm.

Determination of Caloric Content

Larvae and adults were placed in tared crucibles in a drying oven at 100 C for 24 hr. The crucibles were allowed to equilibrate at room temperature for 24 hr in a dessicator.

A Phillipson Oxygen Microbomb Calorimeter (Gentry and Wiegert Instrument, Inc.) connected to a Honeywell Electronik 194 Lab/Test Recorder with a 0.5 mV span was used for caloric content determinations. Ten samples of benzoic acid (6318 cal/g), ranging from 1 to 10 mg were burned every 2 weeks or after 30 determinations, whichever occurred first, in order to calculate a calorie/line factor.

Samples of organisms ranging from 5 to 10 mg were taken from the tared crucibles, pressed into a pellet, and weighed to the nearest 0.01 mg on a tared platinum disc. Samples were fired as directed in the manual for the Phillipson Microbomb Calorimeter. The caloric content was computed with the IBM 360-65 computer and recorded as calories/g and calories/g ash-free weight. Reweighing the platinum disc to determine the percent ash gives erroneous results since escaping gases remove ash from the disc as the bomb is unsealed (Cummins 1967). The bomb

combustion technique underestimates the ash content by 1.46% when compared with the muffle furnace technique which leads to an error of 1.56% in adjusting caloric values to an ash free basis (Reiners and Reiners 1972). Thus, percent ash and its weight were determined separately. Samples ranging from 5 to 10 mg were placed in tared crucibles, weighed, and combusted in a Thermolyne 1500 Electric Furnace for 1 hr at 600 C. The samples were placed in a dessicator for 24 hr, reweighed, and the percent ash determined.

Determination of Lipid and Protein

The methods of Folch et al. (1957) were used for the extraction of total lipids. Lipid extraction required 1 g wet weight of fourth instar larvae. Larvae were weighed in a tared sealed vial containing water to prevent dessication. Larvae were added to the vial until 1 g wet weight was obtained. The organisms were placed in a 0.45 μ pore size Millipore filter apparatus to remove excess water and aggregate the organisms and then placed in a cold (3 C) Potter-Elvehjem tissue grinder. A cold 2:1 chloroform-methanol mixture was added and the organisms homogenized in an ice bucket. The mixture was 20 parts of chloroform-methanol to 1 part tissue. The homogenate was allowed to settle for 2 hr at 3 C and filtered through fat-free filter paper. The supernatant was thoroughly washed in 0.2 its volume with 0.01% $MgCl_2$ solution and allowed to settle for an additional 2 hr at 3 C. The upper phase was removed by suction pipetting and the solution was placed in a freezer at -20 C to freeze remaining water. After freezing, the solution was filtered through fat-free paper to remove the ice crystals. The lipid extract was dried in a water bath to evaporate the chloroform and placed in a dessicator for

48 hr before weighing.

The protein content of the fourth instar larvae was determined as percent nitrogen in a Coleman Nitrogen Analyzer. Twenty mg of larvae as dry weight were placed in tared aluminum boats, sprinkled with a catalyst, and placed in a catalyst chamber. The meniscus level of the mercury bulb and barometric pressure were noted and the organic matter was fired. The resulting gases were passed through a solution of KOH to remove the CO_2 before entering the mercury bulb. The meniscus was rezeroed and the amount of nitrogen gas present was determined with corrections for change in barometric pressure.

Metabolism of Phenol

The metabolism of phenol was studied using 0.94 mg of uniformly labeled phenol-C^{14} (5 mCi/mM) diluted to 5 ml with ether. The radiochemical purity of the phenol-C^{14} was 97%.

The metabolism chamber (Figure 3) was a 500 ml filter flask connected in series with rubber tubing to four 75 ml test tubes (25 x 200 mm). Each metabolism chamber contained 50 g of washed ashed sand; 100 ml of the treatment combination pH 6.2, 20 mg/l phenol, 600 mg/l NaCl; and 0.2 ml of the phenol-C^{14} ether solution. Each test tube contained 20 ml of 1N NaOH to trap any $C^{14}O_2$ released from the metabolism chambers as $NaHC^{14}O_3$. Air was aspirated through 20 ml of 1N NaOH before entering the metabolism chamber. A 500 ml filter flask between the aspirator and the last test tube prevented NaOH or radioactive compounds from entering the aspirator.

Three metabolism chambers were set up for each experiment with three replications. Phenol-C^{14} was added, the units were sealed, and

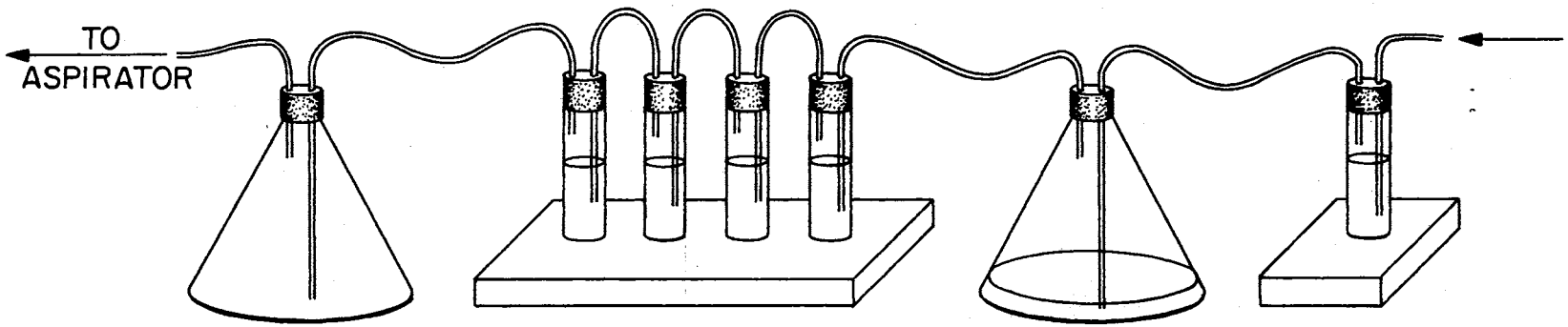

Figure 3. Metabolism chambers

allowed to bubble for 1 hr to remove the ether from solution. At the end of 1 hr, 100 fourth instar larvae were added to the first chamber, the second chamber was retained as a control, and the third chamber received 100 dead larvae and 0.5 wet weight of bacteria. The control was necessary for determinations of carry-over of phenol-C^{14} since phenol is a volatile compound. Fourth instar larvae were killed by 5 hr exposure to 5,000 mg/l of phenol.

The units operated for 48 hr with an additional 100 fourth instar larvae added after 24 hr to ensure adequate respiration. After 48 hr, the NaOH from the four test tubes was transferred to a 100 ml volumetric flask, diluted to 100 ml with distilled water, and thoroughly mixed. A 1 ml subsample was transferred to a 20 ml scintillation vial, neutralized with 0.8 ml of 1 N HCl, and 10 ml of Bray's scintillation cocktail added. Each subsample was replicated. A Packard Tri-Carb Scintillation Spectrophotometer was used to count the radioactivity. Three 1 min counts were conducted and converted to disintegrations per minute.

The NaOH was also analyzed for $C^{14}O_2$ content by adding three drops of 1M $BaCl_2$ to a 10 ml subsample of NaOH. The subsample was centrifuged to precipitate the $BaCO_3$. Two 1 ml subsamples of the supernatant were placed in 20 ml scintillation vials, neutralized with 0.8 ml of 1N HCl, and 10 ml of Bray's scintillation cocktail added. Counts were determined as above.

CHAPTER III

RESULTS

An uniformity trial was conducted to evaluate edge effects. An analysis of variance (Table 1) indicated no significant differences existed among subunits in an experimental unit or among experimental units.

Survival

First Instar

The survival of the first instar larvae (Figure 4) was significantly affected by the three main treatments - pH, phenol, and NaCl (Table 2). Newman-Keuls multiple range test for differences among means indicated a significant difference between survival at pH 8.2 and survival at the other two pH levels (Table 3) but no difference between the means at pH 6.2 and 7.2 ($P < .05$). Survival at 10 mg/l phenol was significantly higher than at 0 or 30 mg/l phenol but not different from survival at 20 mg/l phenol ($P < .05$). Survival at 20 mg/l phenol was also significantly higher than at 30 mg/l phenol ($P < .05$). Mean survival was significantly higher with NaCl than without NaCl ($P < .05$).

Survival was affected by the interaction between phenol and NaCl (Table 2). If the treatment effects were additive with no interaction, survival with NaCl would be greater than without NaCl and survival in 10 mg/l phenol would be higher than survival in the other phenol levels

TABLE 1. AN ANALYSIS OF VARIANCE (AOV) OF THE UNIFORMITY TRIAL

Source	d.f.	S.S.	M.S.	F_{cal}	F_{tab}
Total	47	1769.85			
Rows	3	122.80	40.93	0.81	3.01
Columns	5	336.13	67.23	1.33	2.62
R x C	15	99.88	6.66	--	--
Error	24	1211.04	50.46	--	--

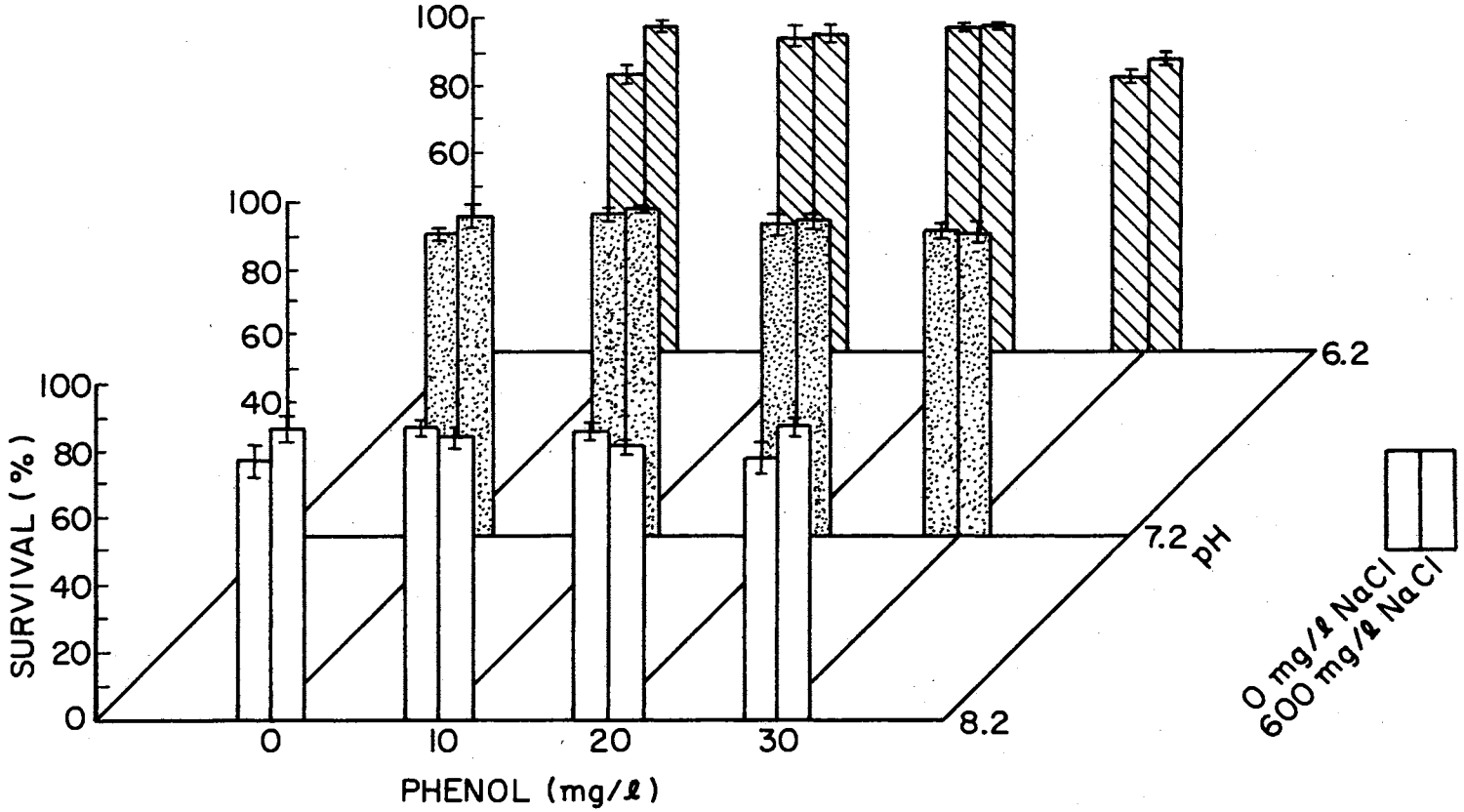

Figure 4. The effect of varying levels of pH, phenol, and NaCl on the percent survival of first instar larvae

TABLE 2. A SUMMARY OF THE AOV's COMPUTED FOR THE 3 x 4 x 2 FACTORIAL ARRANGEMENT OF TREATMENTS IN A COMPLETELY RANDOMIZED DESIGN

Treatments	Percent Survival				Caloric Content		Lipid	Nitrogen
	1	2	3-4	A	3	4	4	4
pH	***	***	***	***	0	0	0	0
Phenol	***	***	0	0	0	***	0	0
NaCl	***	***	**	0	0	0	**	0
pH x NaCl	0	0	0	0	*	0	**	0
pH x Phenol	0	***	0	0	0	0	0	**
Phenol x NaCl	**	**	0	0	0	***	0	0
pH x Phenol x NaCl	0	***	0	**	*	0	0	**

Significance level: * P < .10, ** P < .05, *** P < .01
1,2,3,4 = Instar stages, A = Adult

TABLE 3. A SUMMARY OF MAIN TREATMENT EFFECT MEANS

Variable	Level	Percent Survival				Calories/ash free g x 10^3	Lipid (mg/g)
		1	2	3-4	Adult	4	4
pH	6.2	92.84	64.56	49.20	35.61	*	*
	7.2	94.83	69.46	53.97	17.07	*	*
	8.2	84.80	45.35	26.77	0.92	*	*
Phenol (mg/1)	0	89.26	54.97	*	*	5.91	*
	10	93.46	65.06	*	*	5.92	*
	20	92.61	64.37	*	*	6.07	*
	30	87.95	55.27	*	*	6.19	*
NaCl (mg/1)	0	89.19	54.00	37.10	*	*	21.62
	600	92.45	65.58	49.52	*	*	24.86

* = No significant difference among means
1,2,3,4 = Instar stages, A = Adult

since a significant difference existed among these levels. However, Newman-Keuls test indicated only two survival means were significantly lower ($P < .05$) than the other means: $a_{i,0,0}$ and $a_{i,30,0}$ ($a_{i,j,k}$, where i = pH, j = mg/l phenol, k = mg/l NaCl). The other means were not significantly different. The interaction between phenol and NaCl resulted in similar survival at all but two levels (Figure 5).

Second Instar

Percent survival of second instar larvae was affected by all three treatments (Table 2). Percent survival was greater at pH 7.2 (Table 3) and survival was generally higher in units receiving NaCl (Figure 6). Newman-Keuls test indicated a significant difference among survival at the three pH levels and two NaCl levels ($P < .05$). Survival was significantly higher in 10 and 20 mg/l phenol than in the other two levels of phenol ($P < .05$).

Survival was affected by the interaction between pH and phenol (Table 2). If the main treatment effects were additive without interaction, the highest survival should have occurred at $a_{7.2,10,k}$ and $a_{7.2,20,k}$. However, the highest survival occurred at $a_{6.2,10,20,k}$ and $a_{7.2,10,k}$ (Figure 7). Newman-Keuls test indicated these means were similar to those predicted by the main treatment effects with additivity.

Survival was affected by the interaction between phenol and NaCl (Table 2). If treatment effects were additive, without interaction, survival should be higher at $a_{i,10,20,600}$. Survival was similar at all but two levels of phenol x NaCl (Figure 8). Newman-Keuls test indicated lower survival at $a_{i,0,0}$ and $a_{i,30,0}$ than at other levels ($P < .05$). Means were also similar, with two exceptions, in the interaction between

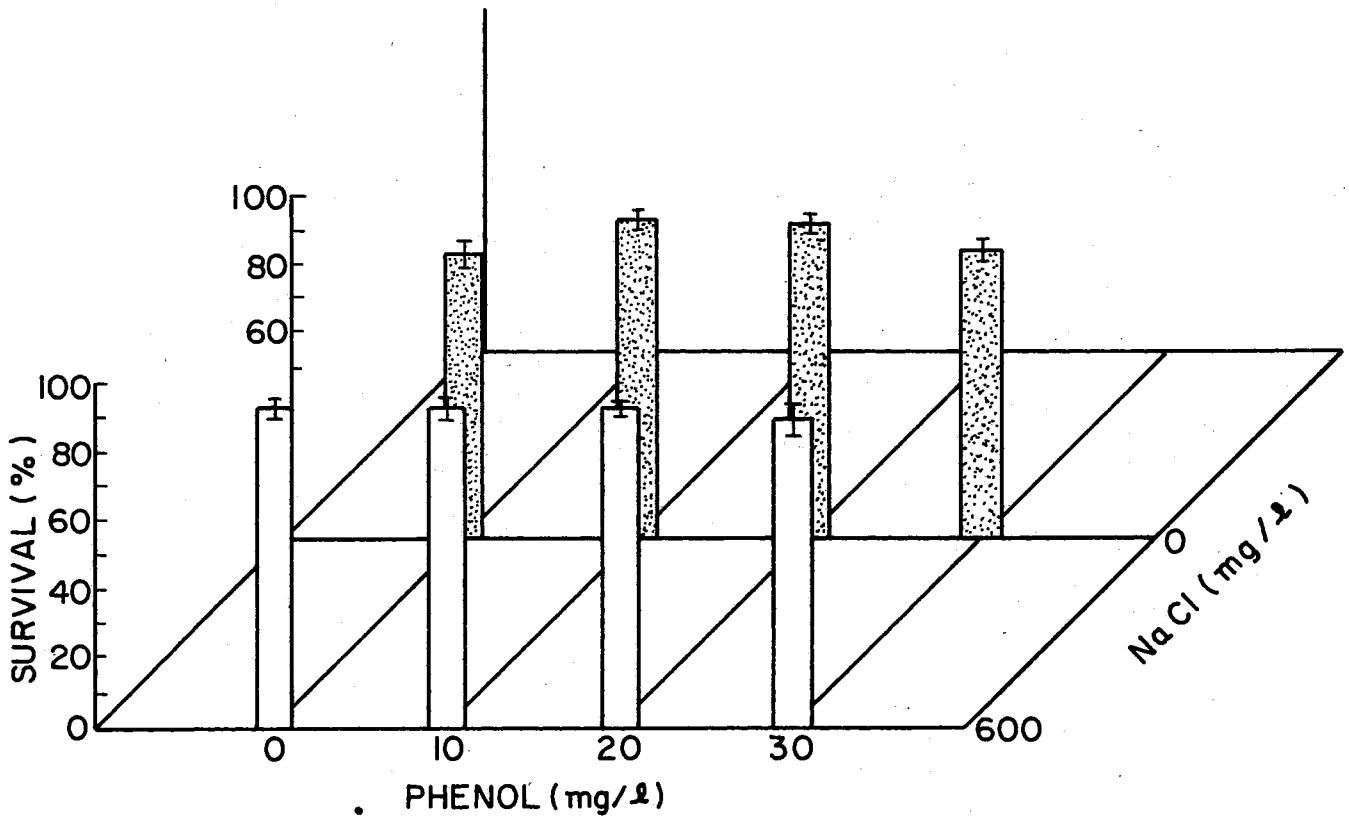

Figure 5. The effect of interaction between phenol and NaCl on percent survival of first instar larvae

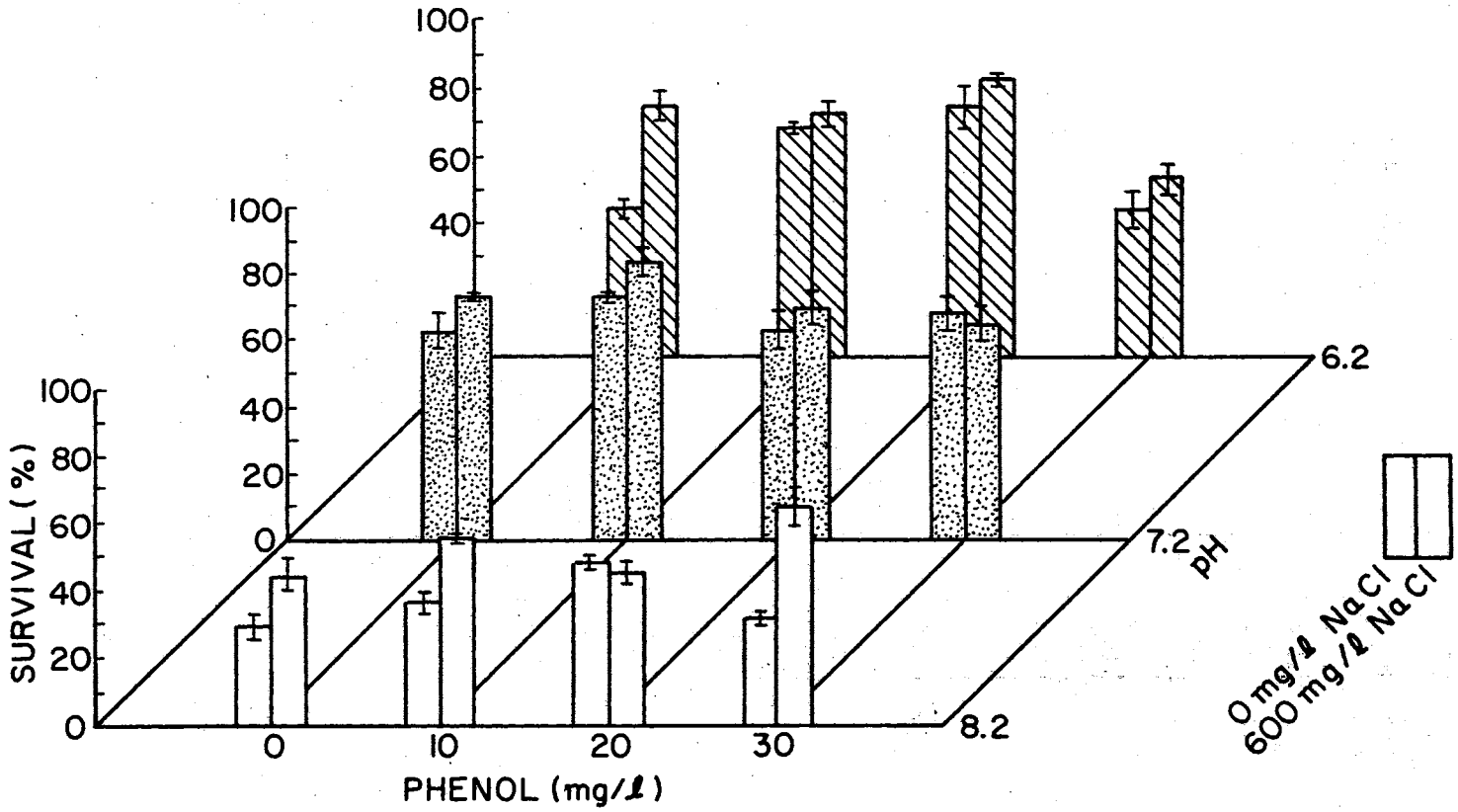

Figure 6. The effect of varying levels of pH, phenol, and NaCl on the percent survival of second instar larvae

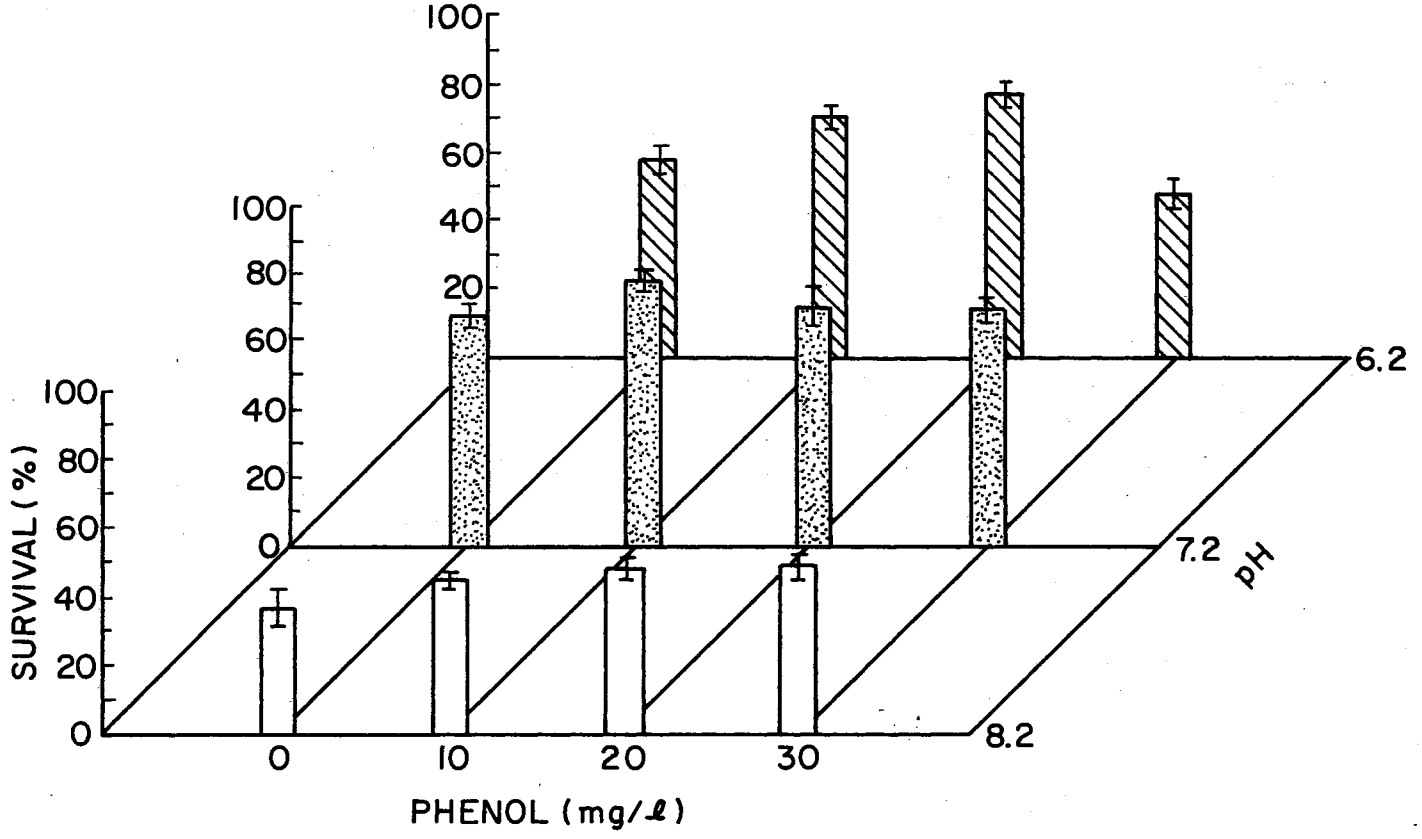

Figure 7. The effect of the pH x phenol interaction on the percent survival of second instar larvae

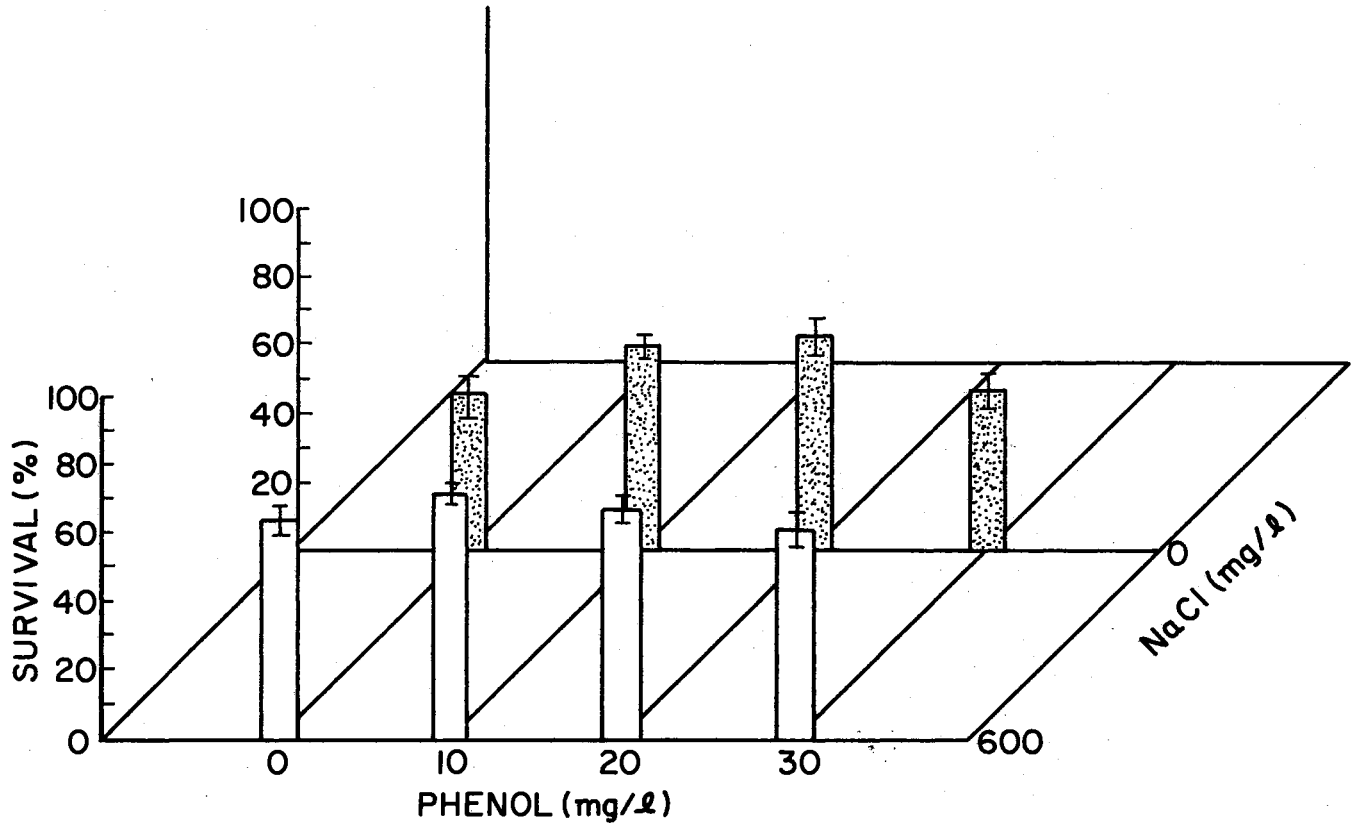

Figure 8. The effect of the interaction between phenol and NaCl on percent survival of second instar larvae

phenol and NaCl for second instar.

The three factor interaction indicated percent survival depended upon the particular combination of pH, phenol, and NaCl (Figure 6).

Third and Fourth Instar

Survival of third and fourth instar larvae was affected by pH and NaCl (Table 2). The larvae had higher survival in the lower two pH's. With the exception of two means, survival was also higher if units received NaCl (Figure 9). Newman-Keuls test indicated the means at pH 6.2 and 7.2 (Table 3) were significantly higher than the mean at pH 8.2 ($P < .05$). The trend was higher survival in pH 7.2 but no significant difference existed between survival at pH 6.2 and 7.2. A significantly higher survival occurred with NaCl ($P < .05$).

Adults

The percent survival to the adult stage was affected primarily by pH (Table 2). As the pH increased, survival decreased substantially (Figure 10). Newman-Keuls test indicated a significant difference among survival means (Table 3) at all three pH levels ($P < .05$). Many adults had undergone eclosion but were found dead in the units. These were included in survival values since it was not possible to determine if death resulted from the treatment effect, the small air space between the screened top and the water, the lack of emergent structures for adhering, or a combination of the above.

A significant three factor interaction indicated survival to the adult stage was affected by the particular combination of the three treatment factors (Figure 10).

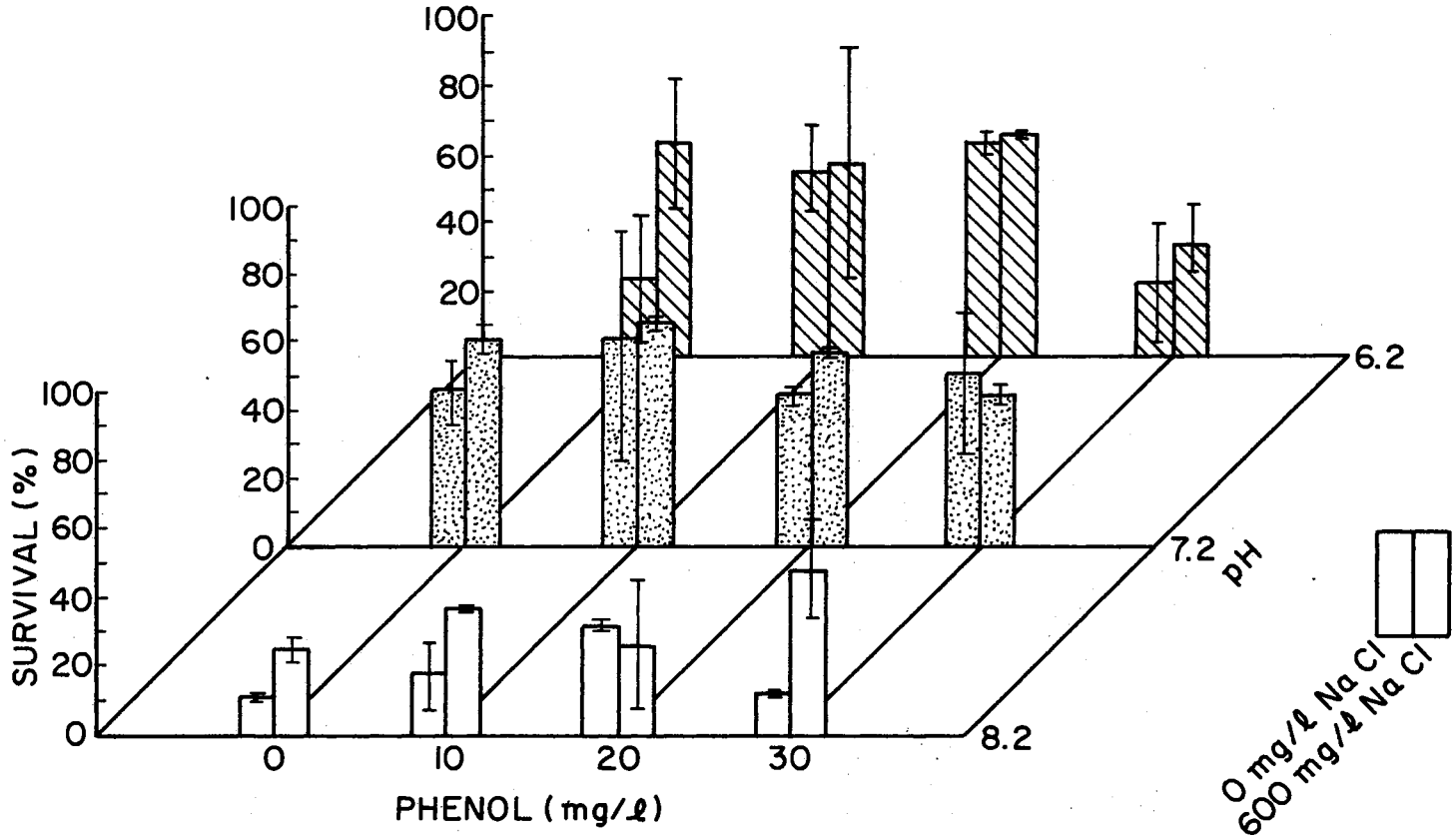

Figure 9. Percent survival of third and fourth instar larvae showing the effect of varying levels of pH, phenol, and NaCl

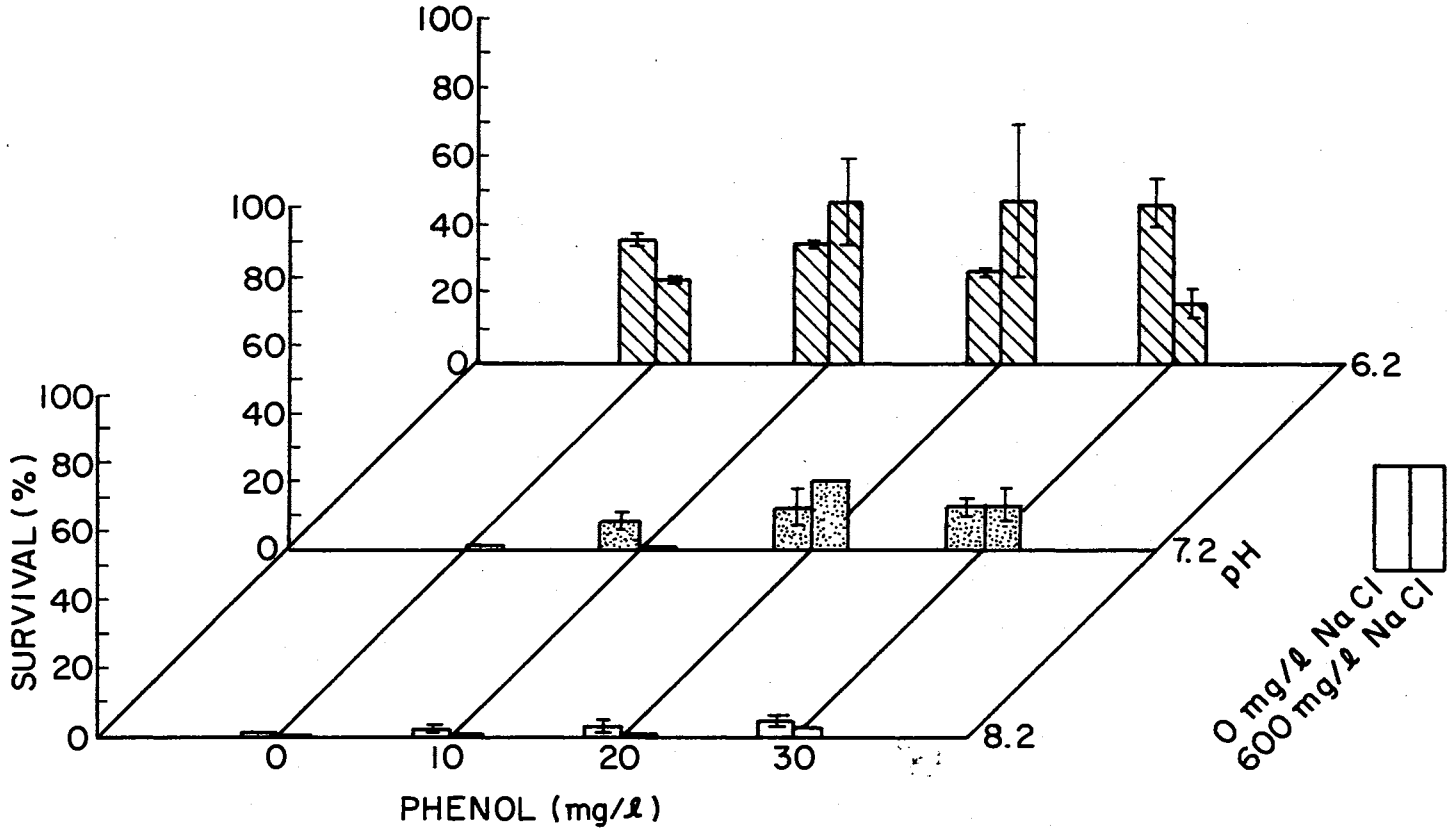

Figure 10. The effect of varying levels of pH, phenol, and NaCl on percent survival to the adult stage

Summary of Survival

Survival was significantly affected by pH at all life stages (Figure 11). Maximum mortality occurred between the first and second instar larvae at all pH levels and during metamorphosis at pH 7.2 and 8.2. Mortality at pH 6.2 was nearly constant after the second instar larvae stage. Survival was higher during the first and second instar larvae stage at the intermediate phenol levels and when NaCl was present. Interaction among treatment variables was significant for all life stages except third and fourth instar larvae. The interaction between phenol and NaCl resulted in similar survival means among the first and second instar larvae. Survival means were higher when both phenol and NaCl were present than when either was present without the other.

Caloric Content

Third Instar

The caloric content of the third instar larvae was affected by the interaction between pH and NaCl and the three factor interaction (Table 2). The main treatment effects were not significant.

The effects of the three factor interaction were quite variable (Figure 12). Caloric content at pH 8.2 was higher without NaCl than with NaCl. No apparent trend existed in the caloric content at pH 6.2 or 7.2

The caloric content was also affected by the two factor interaction between pH and NaCl (Figure 13). The trend without NaCl was for higher caloric means at pH 6.2 and 8.2 with lower caloric values at pH 7.2.

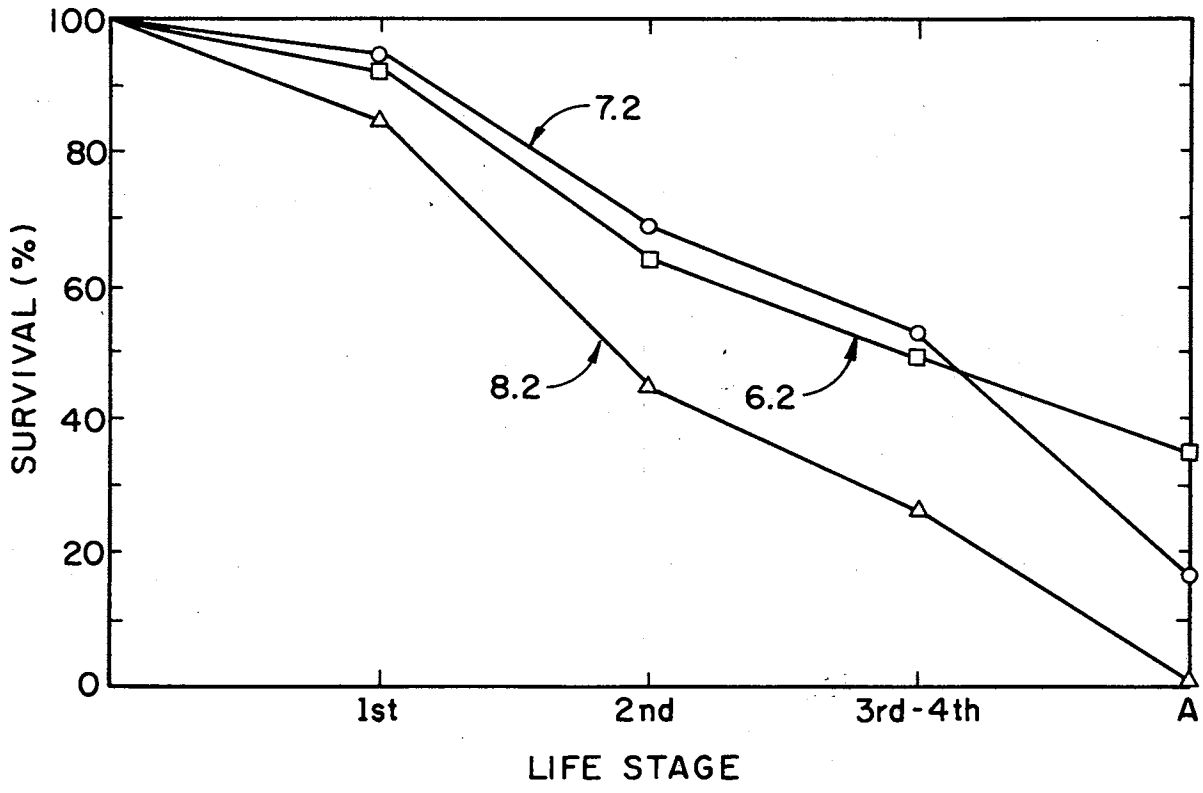

Figure 11. Survival means of the different life stages at the three pH levels

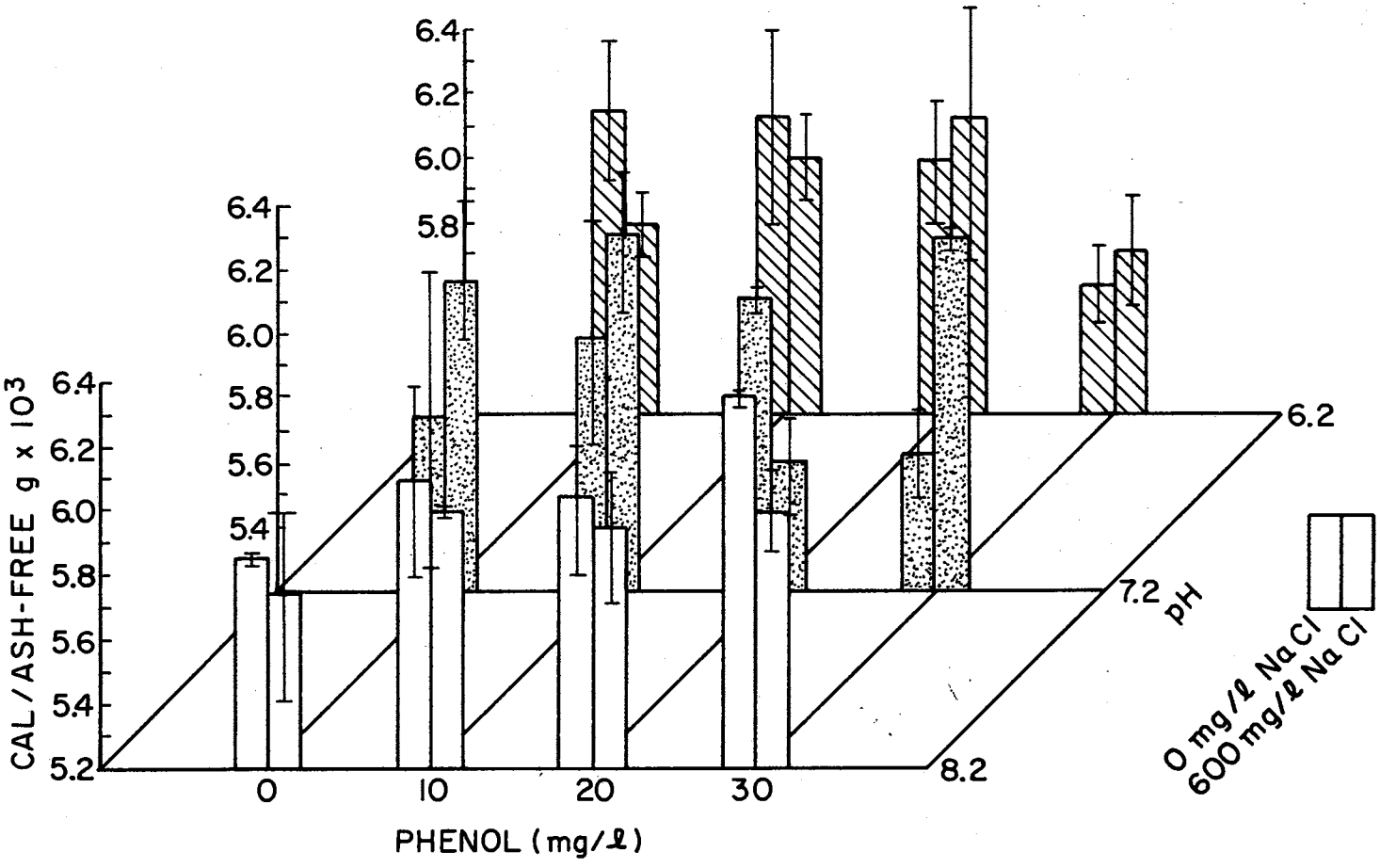

Figure 12. The effect of varying levels of pH, phenol, and NaCl on the caloric content of third instar larvae

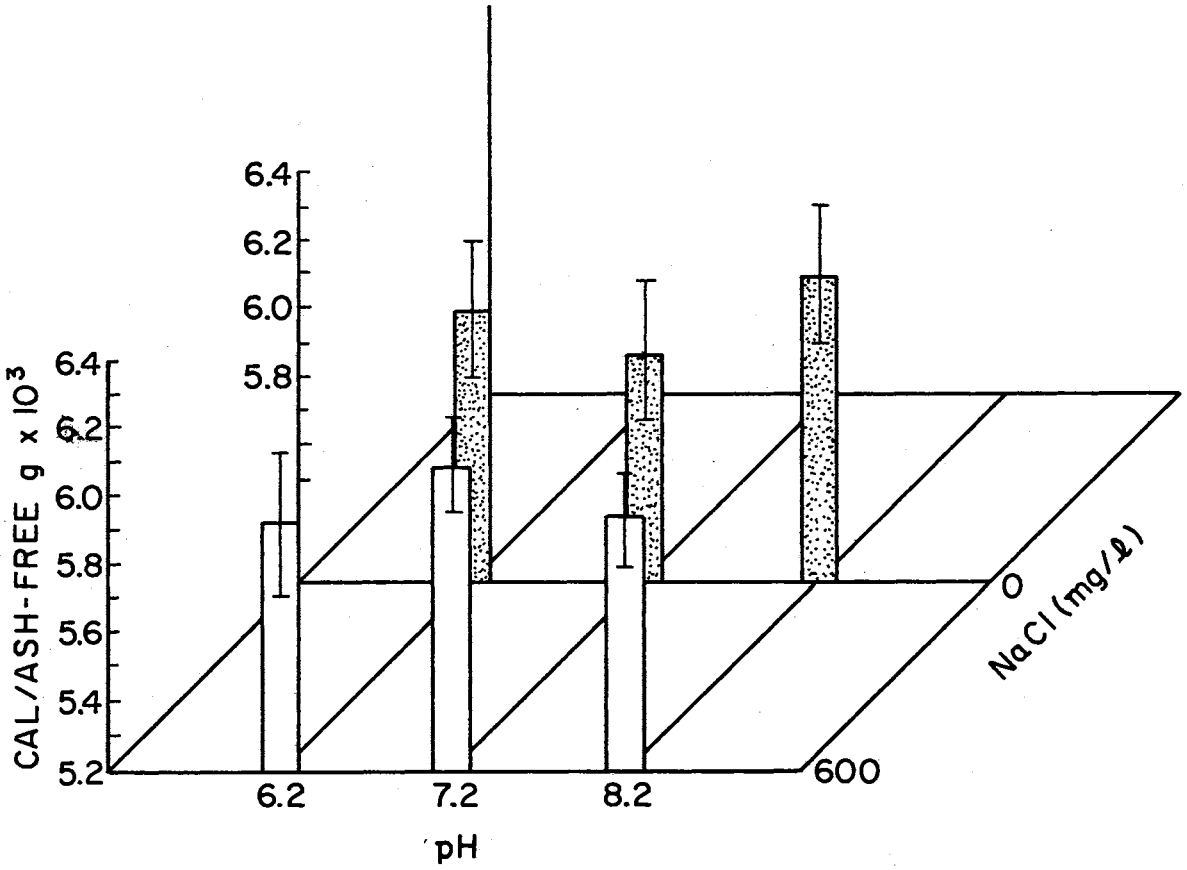

Figure 13. The effect of the pH x NaCl interaction on the caloric content of third instar larvae

Fourth Instar

Phenol affected the caloric content of fourth instar larvae (Table 2). Newman-Keuls test indicated a significant difference among the means for caloric content (Table 3) at 30 mg/l phenol and the means at 0 and 10 mg/l phenol ($P < .05$). The means for caloric content at 20 mg/l phenol were not significantly different from the other means (Figure 14, 15).

The caloric content was affected by the interaction between phenol and NaCl (Table 2). If the phenol treatment effects had been additive with no interaction, the caloric content should have increased from 0 to 30 mg/l phenol. However, only three means were significantly lower than the other means (Figure 16). Newman-Keuls test indicated all means were significantly greater than the mean at $a_{i,10,0}$ ($P < .05$). The caloric content at $a_{i,30,0}$ was also significantly higher than the means at $a_{i,0,0}$ and $a_{i,0,600}$ ($P < .05$). The other means of caloric content were not significantly different. The trend in caloric content without NaCl was a decrease in caloric content to 10 mg/l phenol and then an increase to 30 mg/l phenol. The trend with NaCl was reversed with an increase in caloric content to 10 mg/l phenol and then a decrease to 30 mg/l phenol.

Adult

No definitive statement can be made about the effect of the treatments on the adult caloric content (Table 4). Analysis of adult survival in several units was not possible due to the small sample size. The trend was an increase in caloric content with an increase in phenol concentration at pH 6.2 and 7.2. The caloric content of the adults was,

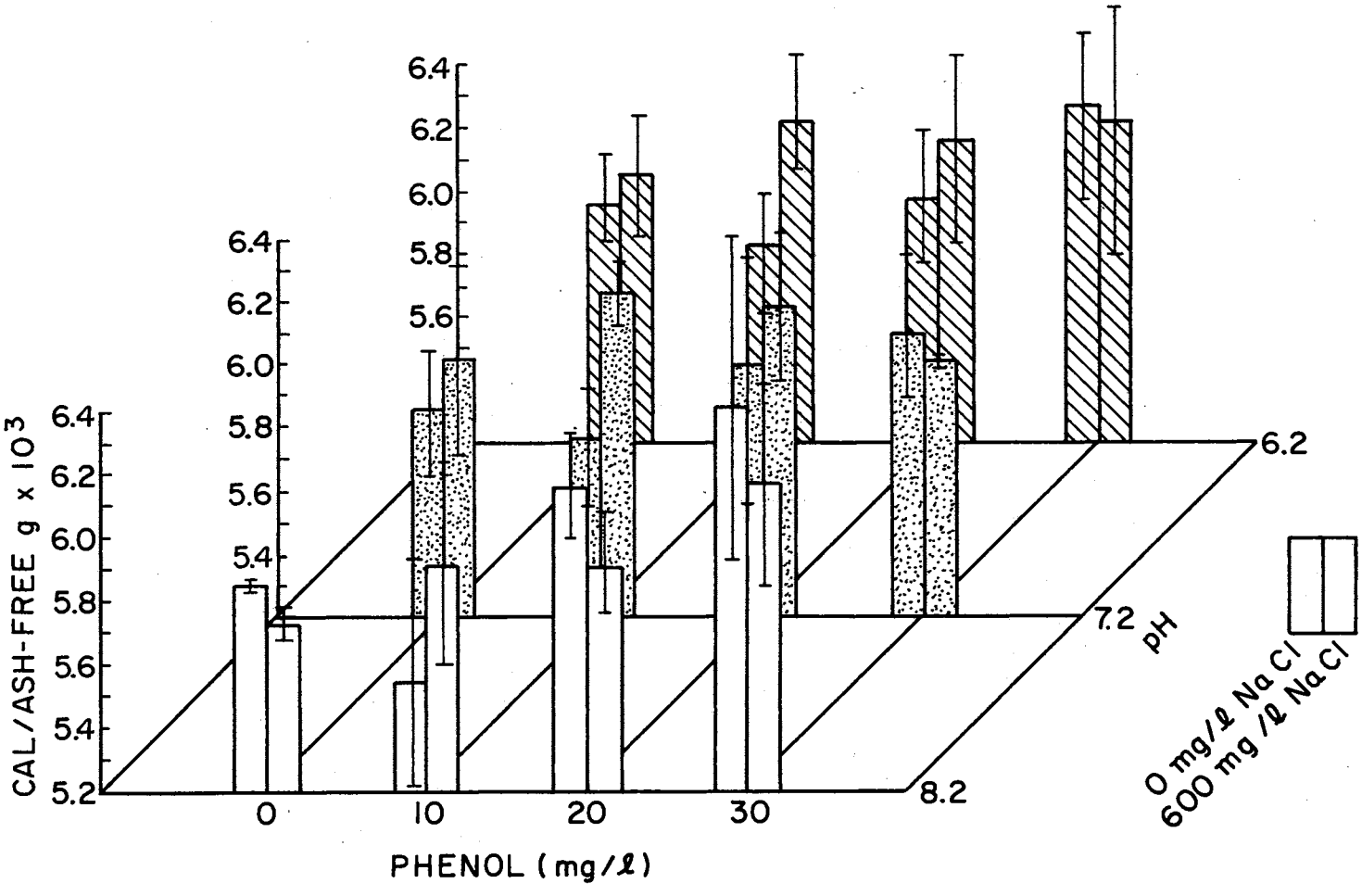

Figure 14. The effect of varying levels of pH, phenol, and NaCl on the caloric content of fourth instar larvae

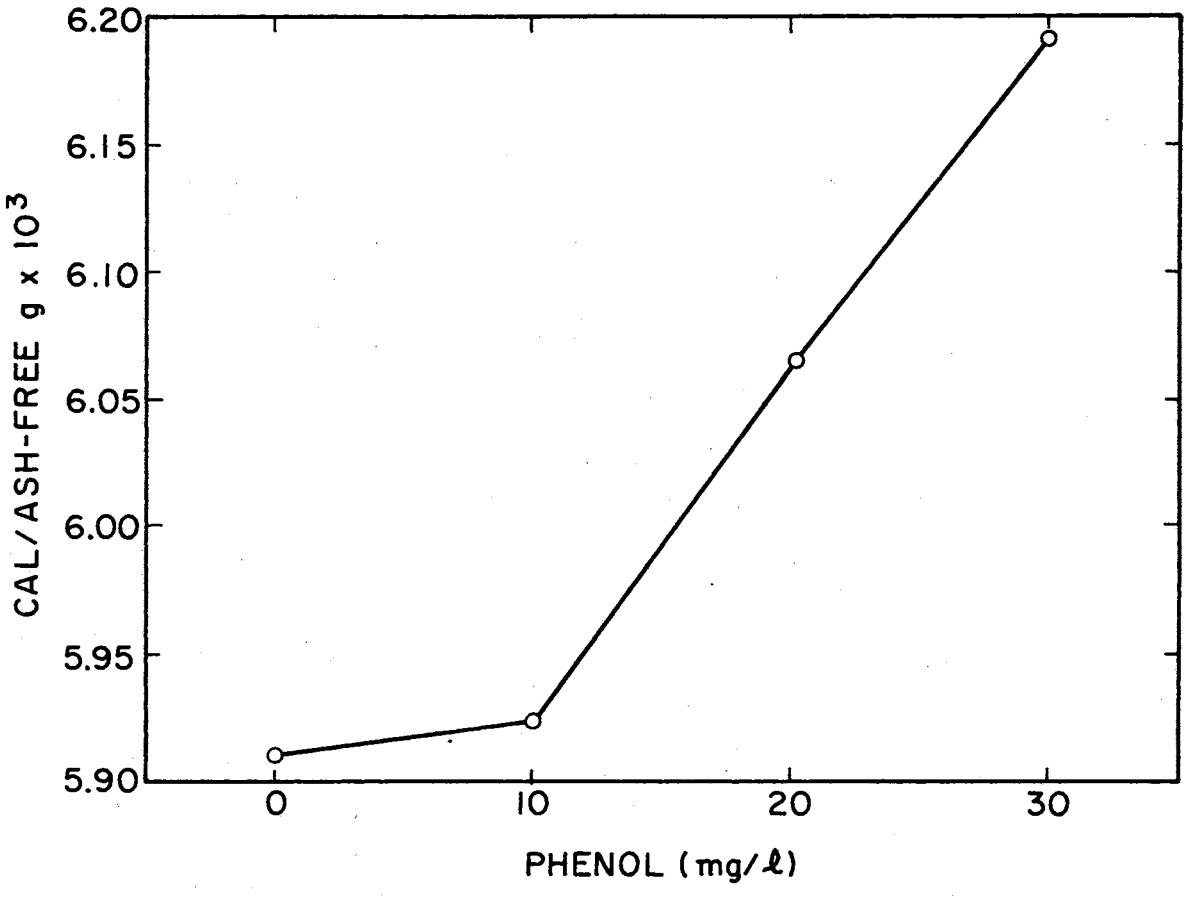

Figure 15. Caloric content means of fourth instar larvae at the four phenol levels

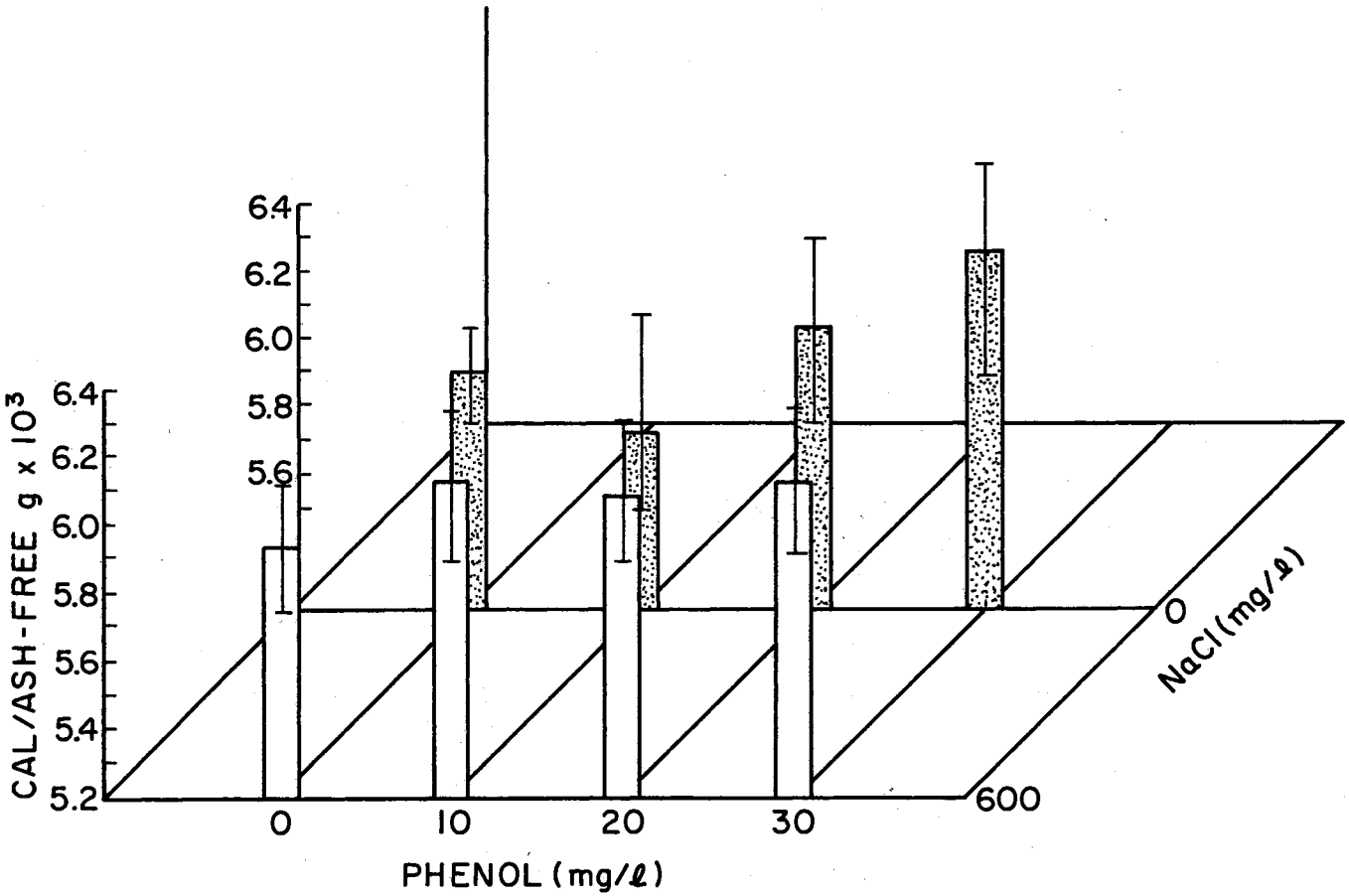

Figure 16. The effect of the interaction between phenol and NaCl on the caloric content of fourth instar larvae

TABLE 4. THE EFFECT OF VARYING LEVELS OF pH, PHENOL, AND NACL ON THE CALORIC CONTENT OF ADULTS

Phenol	pH 6.2		pH 7.2		pH 8.2	
mg/l	0 mg/l NaCl	600 mg/l NaCl	0 mg/l NaCl	600 mg/l NaCl	0 mg/l NaCl	600 mg/l NaCl
0	5.80	5.52	--	--	--	--
10	6.33	6.00	5.80	--	--	--
20	6.49	6.57	5.63	5.67	5.40	--
30	6.46	6.32	6.17	5.90	5.67	--

in general, higher without NaCl than with NaCl.

Lipid and Nitrogen

The lipid content of fourth instar larvae was affected by NaCl (Table 2). NaCl had a large effect on lipid content at pH 8.2 and less effect at pH 6.2 and 7.2 (Figure 17). Newman-Keuls test indicated significantly higher lipid means (Table 3) with NaCl ($P < .05$).

The lipid content of the larvae was also affected by the interaction between pH and NaCl (Table 2). If the treatment effects were additive, without interaction, lipid means should be higher with NaCl. However, little difference existed among means with the two factor interaction (Figure 18). Newman-Keuls test indicated a significant difference only between the lipid means with and without NaCl at pH 8.2 ($P < .05$).

Nitrogen content of fourth instar larvae was affected by the interaction between pH and phenol and the three factor interaction (Table 2). Nitrogen values were quite similar in response to the three factor interaction (Figure 19). The two factor interaction also resulted in similar nitrogen means (Figure 20). Newman-Keuls test indicated only two nitrogen means were significantly different: $a_{7.2,0,k}$ was higher than $a_{7.2,10,k}$ ($P < .05$). The other means were not significantly different.

Metabolism of Phenol

Phenol was catabolized to CO_2 by *Pseudomonas* sp. but this catabolism did not occur in *Chironomus attenuatus* (Table 5). Orthogonal contrasts were used to compare the three means. No significant differ-

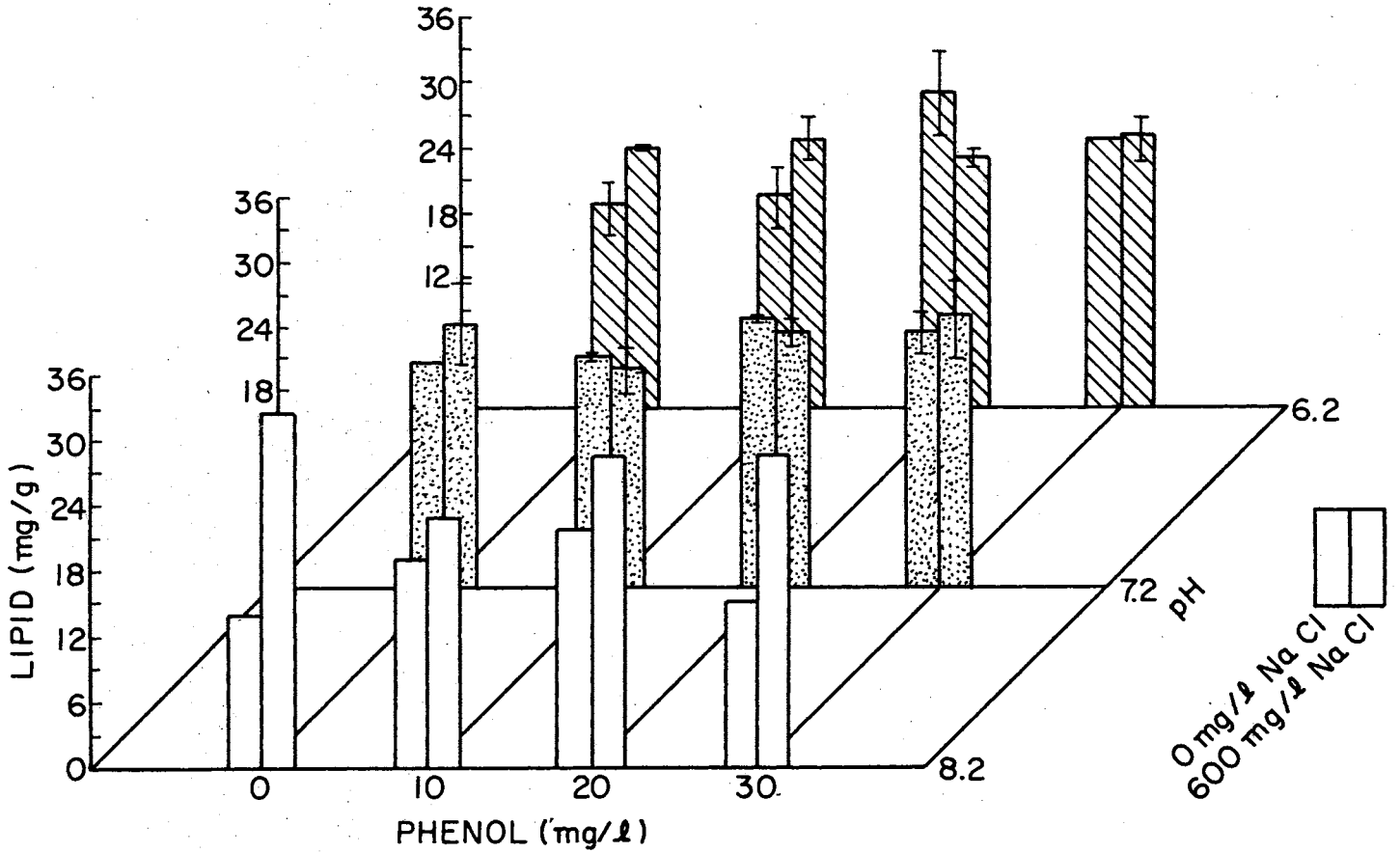

Figure 17. The effect of varying levels of pH, phenol, and NaCl on the lipid content of fourth instar larvae

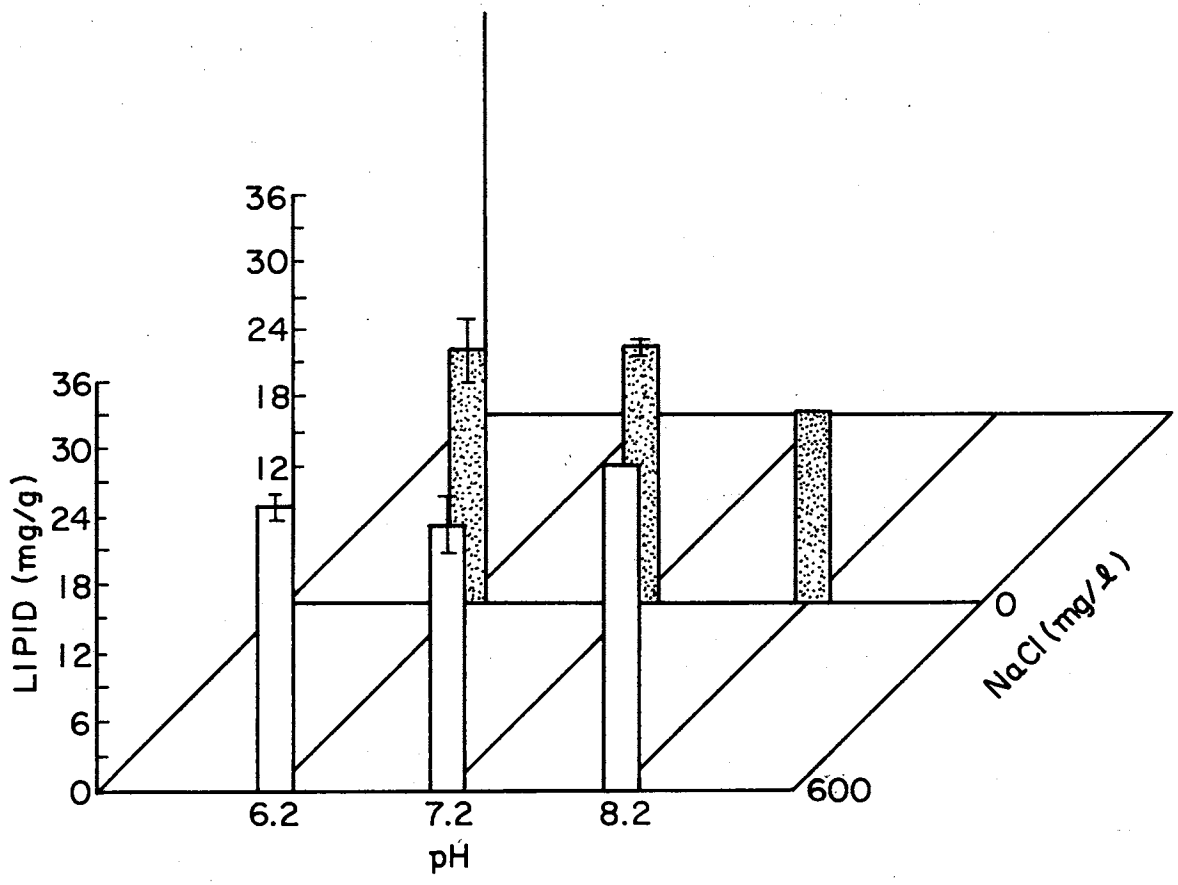

Figure 18. Lipid content of fourth instar larvae showing the effect of the pH x NaCl interaction

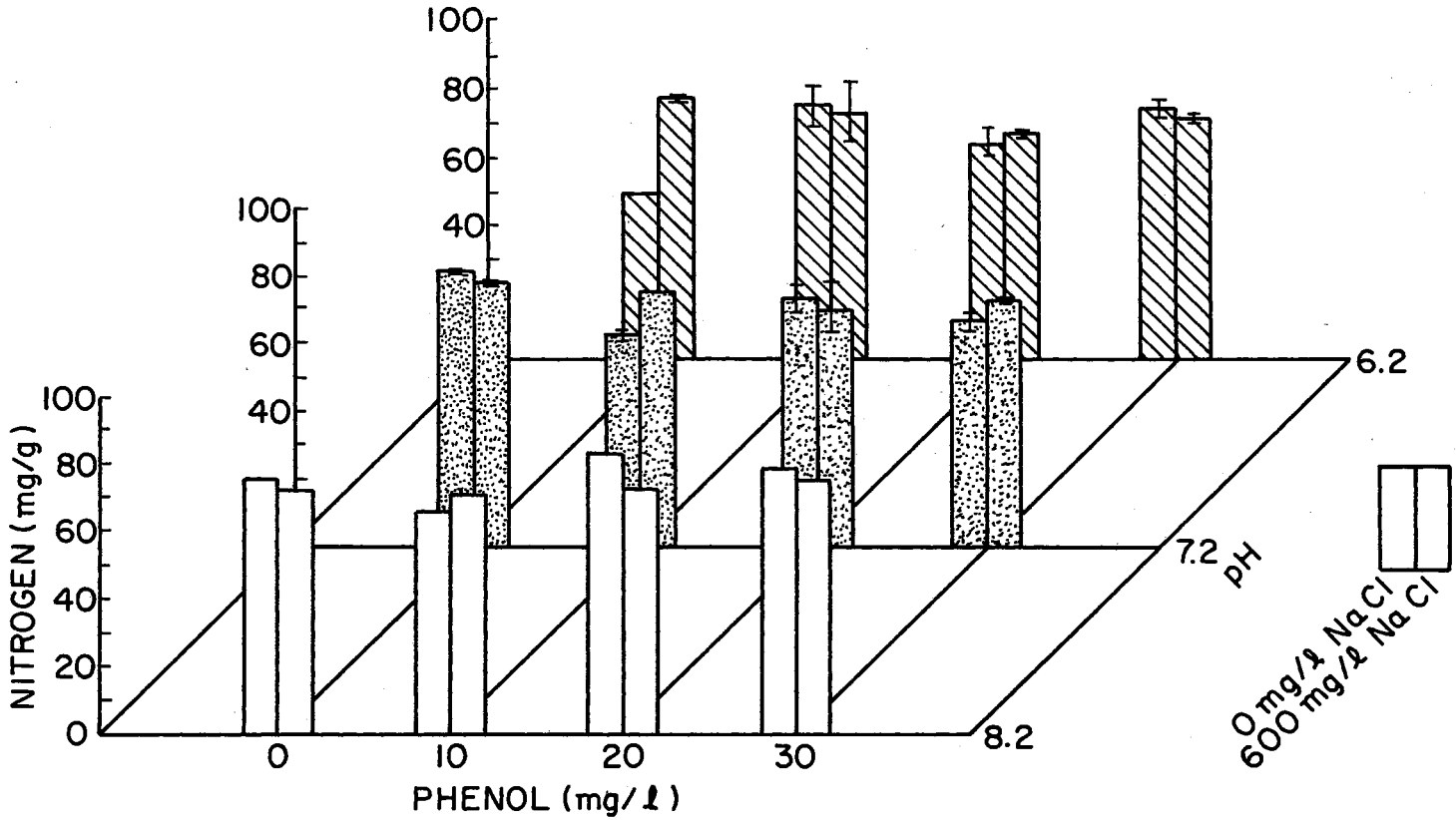

Figure 19. The effect of a three factor interaction among pH, phenol, and NaCl on the nitrogen content of fourth instar larvae

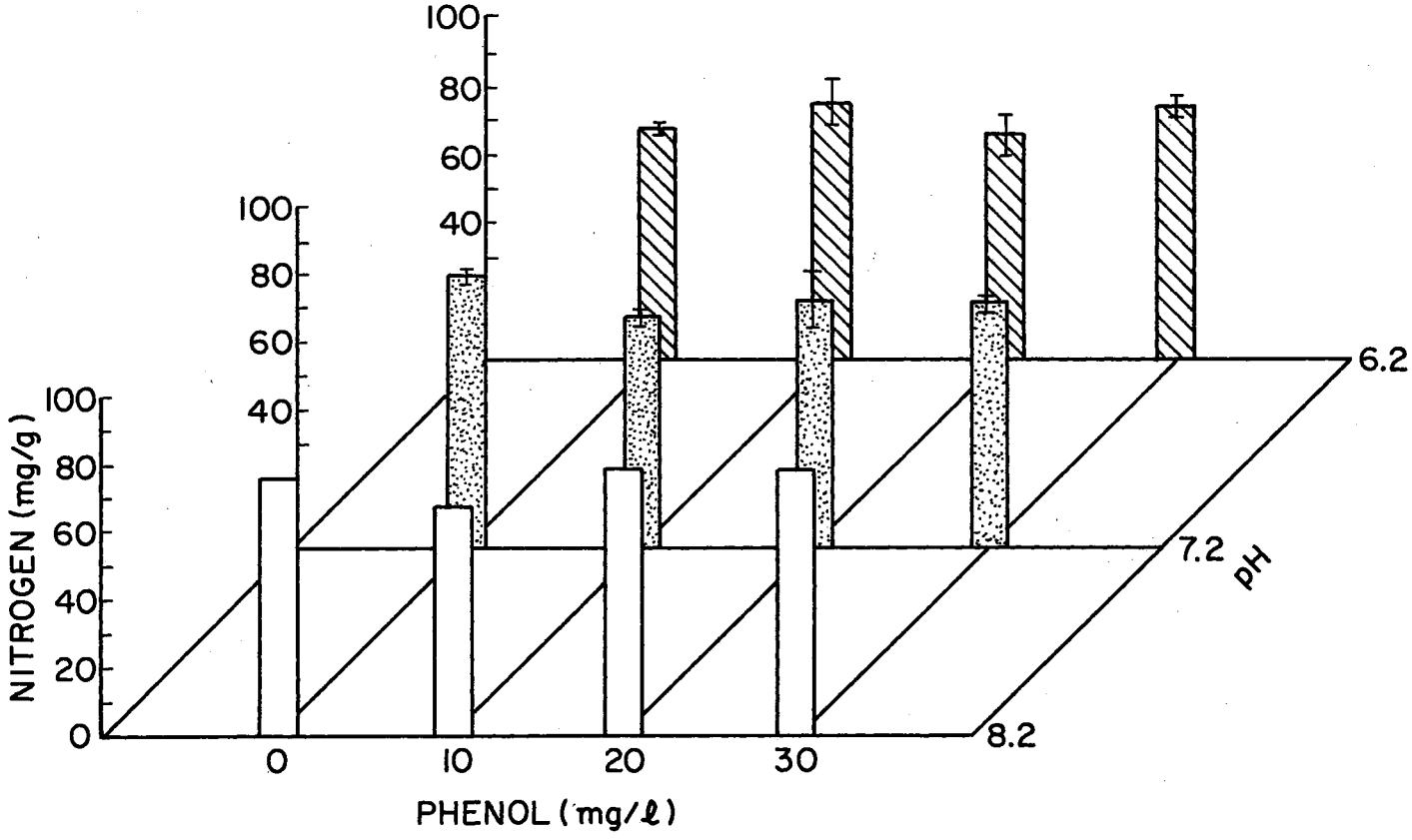

Figure 20. The effect of pH x phenol interaction on the nitrogen content of fourth instar larvae

TABLE 5. METABOLISM OF PHENOL-C^{14} BY *CHIRONOMUS ATTENUATUS* AND BACTERIA

Experimental Unit	Larvae	Control	*Pseudomonas* sp.
NaOH $\bar{x}$ dpm	21057	20387	25310*
$BaCl_2$ $\bar{x}$ dpm	4212*	5897*	3035*

* $P < .05$

ence existed between the radioactivity for the larvae and the control indicating the radioactivity was due to carry-over of phenol-C^{14} and not $C^{14}O_2$. The bacterial radioactivity, however, was significantly higher than either the control or the larvae, ($P < .05$), indicating the presence of both phenol-C^{14} from carry-over and $C^{14}O_2$ from the catabolism of phenol by the bacteria.

Orthogonal contrasts were also used to compare the radioactivity of the $BaCl_2$ supernatant (Table 5). The $C^{14}O_2$ was precipitated in replicate samples of NaOH to determine if phenol-C^{14} had been catabolized in the larvae metabolism chamber. Co-precipitation of phenol with $BaCl_2$ resulted in lower means than in the NaOH analysis. There was, however, a significant difference among all three means ($P < .05$). The difference between the bacteria and the control was 2862 dpm, while the difference between the larvae and the control was 1685 dpm indicating the catabolism of phenol-C^{14} in the larvae metabolism chamber. While the larvae were rinsed several times to remove the external bacteria, bacteria in the alimentary canal were not removed. The $C^{14}O_2$ released from the larvae metabolism chamber probably resulted from catabolism by bacteria entering the media through defecation from C. attenuatus.

CHAPTER IV

DISCUSSION

Oil Refinery Effluent Holding Ponds and the Microcosm Approach

Oil refinery effluent holding ponds are of varied types and designs with the ultimate objective being an increase in water quality during detention. The basic principle is sedimentation and purification through biological degradation and chemical oxidation. Biological processes are essential for the removal of phenolic compounds from water (Ettinger and Ruchhoft 1949).

A holding pond is a complex system dynamically. Laboratory microcosms have provided insight into the functioning of natural systems. The microcosm approach provides a clearly delineated system, precise environmental conditions maintained over time, manageable size, and replication of experiments (Gordon et al. 1969). However, results of laboratory studies must be applied cautiously to field situations. Laboratory microcosms were used to simulate an oil refinery effluent holding pond and determine the relationship between Chironomus attenuatus and three factors - pH, phenol, and NaCl.

pH

pH has been recognized as an important variable in biological studies from the molecular to the ecosystem level. Ecological studies

have considered the effect of low pH on organisms (Jewell 1922; Cowles and Schwitalla 1923; Lackey 1938; Bick, Hornuff, and Lambremont 1953; Harp and Campbell 1967; Bell and Nebeker 1969; Bell 1971; Hagstrum and Gunstream 1971). Few studies have been conducted on the effects of high pH.

Survival in the present study was higher for all instar larvae at pH 7.2 since the pH of Chironomus hemolymph is alkaline, ranging from pH 7.2 to 7.7 (Chapman 1969). During the larval instar stage, therefore, pH 7.2 was nearer the pH optimum of the hemolymph which resulted in higher survival.

The highest adult emergence occurred at pH 6.2 with a substantial decrease in emergence as the pH of the media increased. Nine species of aquatic insects exhibited less tolerance to low pH for adult emergence than for existence as larvae and nymphs (Bell 1971). Adult emergence was highest at pH 6.2 since the optimum hemolymph pH was probably nearer pH 6.2 during metamorphosis. In a holometabolous insect, the hemolymph pH decreases during metamorphosis due to the increase in metabolic end products (Giese 1968). The mean percent emergence for C. attenuatus at pH 7.2 and 8.2 was 17.07% and 0.92%, respectively. C. plumosus had a mean percent emergence of 15.3% at an average pH of 7.38 (Hilsenhoff 1966, 1967). The range of tolerance for adult emergence probably has an optimum between pH 6.2 and 7.2 with an upper limit near 8.2.

Critical periods in survival occur between the first and second instar larvae and during metamorphosis. Three midge species had 80 to 90% mortality between the first and second instar larvae (Tubb and Dorris 1965). Mortality was attributed to the lack of a suitable

substrate. The first instar larvae emerge from the gelatinous mass and settle to the bottom. During settling, the larvae are susceptible to predation as well as abrasion by the sediment. In the present study, predation was not a factor but abrasion was an important aspect since forced air was bubbled through the water to maintain oxygen concentration near saturation. The water was continuously mixing and the first instar larvae were subjected to buffeting and abrasion. Mortality decreased from second instar to fourth instar larvae. The shift in the optimum hemolymph pH was indicated by an increased mortality rate during metamorphosis at pH 7.2 and 8.2. The mortality rate for adult emergence at pH 6.2 was nearly the same as the mortality rate for second, third, and fourth instar larvae.

Metabolism of Phenol

Many microorganisms have the capacity to use phenol and phenolic derivatives as the carbon source in the anabolism of molecular and cellular constituents (Davis 1956; Nickerson 1956; Dagley, Evans, and Ribbons 1960; Harris and Ricketts 1962; Evans 1963; Wase and Hough 1966; Gibson 1968; Beveridge and Tall 1969). Microbial degradation of phenol generally occurs through the hydroxylation of phenol to catechol. The aromatic ring of catechol is cleaved and several subsequent reactions result in the formation of acetyl-CoA and succinate or pyruvate and other compounds of the tricarboxylic acid cycle such as fumarate (Dagley, Evans, and Ribbons 1960; Evans 1963; Gibson 1968). It was evident that bacteria were metabolizing phenol-C^{14} and producing $C^{14}O_2$ as the end product of this metabolism (Table 4).

While it is possible C. attenuatus might also metabolize phenol-C^{14}

to $C^{14}O_2$, it is not probable. Mechanisms of phenolic metabolism in insects are detoxication of the nocuous compound to a relatively innocuous compound and removal from the body (Myers and Smith 1954; Smith 1955; Kikal and Smith 1959; Gessner and Smith 1960; Smith and Turbert 1961; Dutton 1962; Dutton and Ko 1964; Smith and Turbert 1964). The detoxication mechanisms in locusts (Locusta migratoria, Schistocerca gregaria, Monadacris septemfasciata), cockroaches (Periplaneta americana), mealworm larvae (Tenebrio), stick insects (Carausius), and sphinx moth caterpillars (Sphnix smerinthus) are ethereal sulfate and gluoside conjugations (Smith 1955). Gluoside conjugation also occurs in the housefly, Musca domestica (Dutton and Ko 1964). Degradation of phenolic compounds to CO_2 has not been reported in any insect.

The results of the $BaCl_2$ experiment can probably be interpreted in terms of the bacteria released into the media through larvae defecation. With approximately 150 larvae surviving in each experiment, the quantity of bacteria released through defecation would probably be sufficient to account for the $C^{14}O_2$. The greatest quantity of $C^{14}O_2$ was released from the bacterial metabolism chamber (Table 4).

Phenol

Although the effect of phenol on the percent survival of first instar larvae was significant because of the small variances about the means, the range in survival of the first instar over the four phenol levels was only about 5%. No observable differences were noted in the development of C. attenuatus eggs to first instar when reared in 0, 15, and 30 mg/l phenol (Johnson and Eliot unpublished data). The gelatinous mass surrounding the egg may afford some protection from environmental

contaminants.

Survival of the second instar larvae at the intermediate phenol levels was similar and higher than survival at the other two phenol levels. Increased phenol level resulted in increased bacterial density. The bacteria may provide a more readily attainable food source for the larvae at this stage than the dog kisses. Second instar larvae are about 2 mm in length and not capable of strong undulations for drawing water and food into the tube. The bacteria adhere to the substrate and would be available for forage whereas the dog kisses remain as colloidal particles in solution. While bacteria were readily available at 30 mg/l phenol, the larvae's limit of tolerance to phenol was approached which resulted in lower survival. C. attenuatus populations are difficult to maintain above 25 mg/l phenol (Graham unpublished data).

A linear increase occurred in the caloric content of the fourth instar larvae at the higher phenol levels. This was probably the result of the larvae feeding on the associated bacterial population. The bacteria were filamentous, forming large aggregates in the higher phenol levels. Since the phenol was continuously renewed through flow into the units, these bacterial populations were maintained. Larvae were observed to build tubes out of the bacteria and to forage on the bacteria. With a readily available food supply, the larvae probably expended less energy in foraging at the higher phenol levels and more energy was available for storage as carbohydrates or lipids. The percent composition of a larva as exoskeleton decreases with an increase in carbohydrate or lipid storage products. The increase in cal/g was probably a result of this change in chemical composition.

Without antibiotics, a linear increase in the caloric content of

C. attenuatus occurred with increasing phenol levels possibly because other bacterial populations were established (Graham unpublished data). There was a decrease in the caloric content of midge populations as water quality improved downstream from an outfall of domestic and industrial waste (Graham unpublished data). Since natural water purification is primarily a microbial phenomenon (Hawkes 1963), those species tolerant of enriched systems will have an increased food supply in the form of organic matter and microbes.

The increase in caloric content of C. attenuatus with increasing phenol levels was expected to be a function of the lipid content of the larvae. However, neither lipid nor nitrogen values were affected by phenol. The increase in the caloric content of the larvae probably was a function of the carbohydrate content of the larvae. Microbial degradation of phenol results in end products of pyruvate and other intermediates in glucose synthesis. The bacteria probably were a source of carbohydrates for the larvae.

NaCl

The effect of NaCl on the survival of the larvae was probably the result of osmotic conditions. The optimum salinity for C. attenuatus was around 400 mg/l (0.4%) NaCl (Thornton and Sauer 1972). This salinity required a minimum expenditure of energy for the regulation of the NaCl level of the hemolymph. In the present study, larval survival was higher with NaCl than without NaCl. Larvae receiving NaCl probably had less osmotic stress in the regulation of the sodium level of the hemolymph. Larvae in units without NaCl expended more energy (Thornton and Sauer 1972) and were probably under a greater stress both

in the retention of hemolymph sodium and in the uptake of sodium from the environment.

The difference in the lipid level of the fourth instar larvae may be explained by the mechanism of Na^+ uptake. Chironomus thummi actively secreted sodium into the hemolymph through the anal papillae (Harnisch 1934). The same mechanism occurred in Aedes aegypti (Wigglesworth 1933, 1938). A similar mechanism probably occurs in Chironomus attenuatus (Thornton and Sauer 1972). The active uptake of sodium requires the expenditure of energy. This energy may be provided by the catabolism of lipids. Larvae reared in 600 mg/l NaCl would have to expend less energy for the secretion of sodium into the hemolymph than those larvae reared in an environment where sodium was present only as an impurity.

Interaction

Interaction among treatment factors affected every response except percent survival of third and fourth instar larvae. Many of the main effects would not have been declared significant if the interaction terms had been included in the error term.

The interaction between pH and phenol affected the percent survival of second instar larvae and the nitrogen content of fourth instar larvae. The higher survival of second instar at the two lower pH levels could be expected from the response of the larvae to the main treatment factors. The lower survival at $a_{6.2,30,k}$ and $a_{8.2,\text{all phenol levels},k}$ probably was the result of increased stress at the higher pH of 8.2 and the higher phenol level at pH 6.2. The effect of pH x phenol on nitrogen was relatively insignificant. Only two means were signifi-

cantly different indicating that protein was not affected by the treatment levels.

The pH x NaCl interaction affected the caloric content of third instar larvae and the lipid content of fourth instar larvae. The effect of interaction on the caloric content is difficult to explain. The effect of the interaction with NaCl present in the media was expected since NaCl reduces osmotic stress and pH 7.2 is near the optimum for larvae. The caloric content decreased on both sides of the optimum since more energy must be expended to maintain the physiological optimum. However, this trend reversed in the absence of NaCl. The lowest caloric content occurred at the optimum pH with an increase in caloric content near the extremes. The additional stress without NaCl probably resulted in the metabolism of lipids as the energy source for uptake of Na^+ from the dilute media.

The interaction between phenol and NaCl was significant for survival of first and second instar larvae and the caloric content of fourth instar larvae. At least one main treatment effect was significant in all cases and yet the interaction means were similar when both compounds were present in the media. The combination of phenol and NaCl appears to result in a form of antagonistic action which inhibits the full expression of either compound. Under additive conditions, significant responses to each would have been expected. A possible explanation might be a mode of action similar to competitive inhibition. Perhaps when both NaCl and phenol are present in the media, the compounds inhibit the uptake of each other. In all cases the effect of phenol or NaCl is more pronounced without the other present. This study indicates the interaction between NaCl and phenol exists but does

little to elucidate the mechanism of interaction.

It would be erroneous to conclude phenol had little effect on the caloric content of midges. Such a conclusion could be reached, however, if NaCl were present in sufficient concentration in the system. In oil producing areas, both phenol and NaCl are frequently found entering lakes and streams (Jenkins 1964). Further attention should be given to the analysis of interaction in attempting to elucidate the effects of environmental variables on community structure and function.

The Role of *C. attenuatus* in Oil Refinery Effluent Holding Ponds

Chironomus attenuatus has little direct effect on the degradation of phenolic compounds. Its role in the removal of energy from the system is probably minimal. The midge, *Glyptotendipes barbipes*, contributed little to the removal of energy from a sewage lagoon (Kimerle and Anderson 1971). *G. barbipes* removed only 0.5% of the net production in the secondary sewage lagoon in 1967 (Kimerle and Anderson 1971). It had been stated emergence of midges from an oil refinery effluent holding pond series removed a significant amount of energy from the system per year, equivalent to the combustion of 92.8 moles of glucose (Tubb and Dorris 1965). This statement is questioned since a comparison between the loss of energy through microbial degradation of phenol from the same pond series and emergence of midges yields the ratio: 6.34×10^4 cal/l/yr in microbial degradation of phenol to 1 cal/l/yr in adult midge emergence. Phenol is only one of a multitude of organic compounds being degraded in this pond series.

Chironomus attenuatus probably plays an indirect role in the

functioning of an oil refinery effluent holding pond similar to the role of Glyptotendipes barbipes in a waste stabilization lagoon (Kimerle and Anderson 1971) and a deposit feeding amphipod on benthic microflora (Hargrave 1970). C. attenuatus probably increases the zone of oxidation by burrowing into the substrate, macerating organic detrital material which results in greater surface area of the material for microbial activity, and removing microbes from the water.

CHAPTER V

SUMMARY

1. Continuous flow, laboratory microcosms were used to determine the relationship between Chironomus attenuatus and pH, phenol, and NaCl.
2. pH had a significant effect on the survival of all life stages of C. attenuatus. The critical periods in survival were between first and second instar larvae and during metamorphosis. Abrasion of the larvae resulted in increased mortality during the first and second instar larval stage while a shift in the pH optimum resulted in increased mortality at pH 7.2 and 8.2 during metamorphosis.
3. Phenol was catabolized to CO_2 by bacteria but not by C. attenuatus. Phenol had a significant effect on the caloric content of fourth instar larvae. Increased phenol levels resulted in a nearly linear increase in caloric content. This increase in caloric content probably was a result of increased bacterial density which provided a more readily available food supply for the larvae.
4. The lipid content was higher in fourth instar larvae with NaCl in the media than without NaCl. Lipids may be metabolized as an energy source for the uptake of NaCl when NaCl is present in low concentration in the media.
5. Interaction had a significant effect on all responses except survival of third and fourth instar larvae. The phenol x NaCl interaction resulted in similar means even though the main treatment

effects were significant.

6. The role of C. attenuatus populations in the functioning of oil refinery effluent holding ponds probably is minimal with respect to energy removal.

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