RESEARCH PROJECT TECHNICAL COMPLETION REPORT
OWRR PROJECT NO. A-032-OKLA.

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

Submitted to

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by

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PREFACE

This study represents a continuation of "Comparison of energy flow parameters of midge populations in biological oxidation ponds", which was described in Research Project Technical Completion Report, OWRR Project No. A-018-Oklahoma. The initial study involved exposing Chironomus attenuatus larvae to phenol concentrations of 0, 3, 8, 11, 16, and 22 mg/1 of phenol and analyzing for oven-dry weight, ash-free weight, caloric content, and oxygen uptake. Caloric content per gram in larvae increased with phenol level and linear regression equations were determined. Caloric content was also measured in C. attenuatus larvae collected at different distances below an industrial and domestic sewage outfall. Caloric content per gram in larvae decreased with distance downstream. Linear regression equations of oxygen uptake were adjusted for phenol level, pH, and oxygen concentration. The effect of salt on survival was determined by exposing larvae to six NaCl concentrations between 0 and 342 mM/1 for four time periods. Concentration of sodium and chlorine in the haemolymph was also measured. Voyle Graham and Stephen Cole performed much of the work on calorie content and oxygen uptake, respectively. Kent Thornton studied the effect of salt in collaboration with Dr. John Sauer.

Most of the work in the present study was performed by Kent Thornton. Considerably more data and a thorough discussion of results appear in his completed dissertation.

The original title of this proposal was "A pilot study of the effects of mercury and other environmental factors on bioenergetics, meristic parameters, and frequency of chromosomal aberrations in

Chironomus attenuatus Walker". However, due to difficulties involved in analyzing for mercury and the short duration of the project, the study was altered to investigate "The effects of pH, phenol, and sodium chloride on the bioenergetics of laboratory populations of *Chironomus attenuatus*".

ABSTRACT

Continuous flow, laboratory microcosms were used to measure the effects of pH, phenol, and NaCl on the survival of the 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 interaction among the various responses. Each experiment consisted of 24 treatment combinations with a 3 x 4 x 2 factorial arrangement in a completely randomized design with two replications of each treatment. Treatment variables were pH at 6.2, 7.2, 8.2; phenol at 0, 10, 20, and 30 mg/l; and NaCl at 0 and 600 mg/l.

pH had a significant effect on the survival of all life stages of C. attenuatus. Increasing phenol levels resulted in a nearly linear increase in caloric content. Sodium chloride affected the lipid content of fourth instar larvae. The lipid content was higher with NaCl present in the media than without NaCl. Interaction had a significant effect on all responses except survival of third and fourth instar larvae.

INTRODUCTION

Energy flow has been used in ecological studies as a means of measuring the effects of environmental factors on organisms, populations, or ecosystems. 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). Caloric content is influenced by the stage in the life cycle of an organism (Wiegert 1965) and by environmental conditions (Spoehr and Milner 1949). Toxicants also affect the physiological state and therefore the caloric content of organisms (Whitley and Sikora 1970). Few studies have been made of energy flow in polluted ecosystems.

Microcosms have been used to investigate the effects of various factors on organisms under controlled environmental conditions (Beyers 1963). 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). Oil is a mixture of many organic compounds from simple hydrocarbons such as methane to complex aromatic derivatives. The complex materials in oil may have synergistic or antagonistic effects on the biota which are incapable of solution by field investigation.

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. 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.

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). The laboratory stock populations were maintained in a laboratory system described by Thornton (1973). Organisms were fed Hartz Mountain Dog Kisses ground into a paste.

Laboratory populations complete their life cycle in 3 to 5 weeks at 22 C. Females oviposits one egg mass containing an average of 412 eggs after mating. 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 a 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 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 accurs during the first 2 days.

Experimental Design

Experimental subunits (48 Mason jars, 11 x 11 x 14 cm) containing 1 liter of treatment solution were placed on racks in a water bath, 152 x 61 x 20 cm. A Little Giant submersible pump and a series of hoses provided interchange of water between the water bath and a reservoir. The circulating water was maintained at 24 C (+0.5 C) by a Blue M Constant-Flow Portable Cooling Unit located in the reservoir. Forty General Electric Gro-Lux florescent lights regulated by a timer set on a 12-hr photoperiod provided a total illumination of 290 ft-c/cm^2 of water surface. Subunits were set in holes in a plywood cover placed over the water bath to prevent heating the water.

A continuous flow system maintained a constant level of the treatment factors. Twenty-four 7.6 liter polypropylene head tanks each contained 7.5 liters of one of the 24 possible treatment combinations. Head tanks were 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. Each head tank supplied two subunits. A 0.47 liter polypropylene container was inserted between head tanks and subunits to provide constant gravity flow. The entire water mass in the subunits was replaced every 0.66 days. Overflow tubes containing silk bolting cloth across the mouth to prevent the loss of larvae by outflow maintained 1 liter of water in the subunits. Dissolved oxygen was maintained in the units at a minimum of 8 mg/l (95% saturation at 24 C) by compressed air.

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/1; and NaCl at 0 and 6000 mg/1. 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 random selection procedure. Each subunit received 0.3 g of Dog Kisses every 2 days during the experiment.

Egg masses used in the experiments were taken at random from the stock population, photographed in a Sedgwick-Rafter cell, and placed in a subunit. Since differential mortality existed among treatment combinations, varying number 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.

The effluent of each subunit was analyzed every 2 days for pH, phenol, and NaCl in the first experiment. pH was measured with a Beckman Zeromatic SS-3 pH Meter, phenol with a Beckman DB-G Grating Spectrophotometer (Martin et al. 1967), the Na^+ concentration with a Beckman 440

by the method described by Thornton (1973). 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 dried organisms ranged from 5 to 10 mg. The caloric content was computed with the IBM 360-65 computer and recorded as calories/g and calories/g ash-free weight. Ash-free weight was determined by combusting samples in a Thermolyne 1500 Electric Furnace for 1 hr at 600 C.

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. The protein content of the fourth instar larvae was determined as percent nitrogen in a Coleman Nitrogen Analyzer by the method described by the Coleman Nitrogen Analyzer manual. Each sample required 20 mg of larvae as dry weight.

RESULTS

Survival

First Instar

The survival of the first instar larvae was significantly affected by the three main treatments - pH, phenol, and NaCl (Table 1). Survival was affected by the interaction between phenol and NaCl. Newman-Keuls multiple range test for differences among means indicated a significantly lower survival at pH 8.2 than at the other two pH levels (Table 2), 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 no 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 ($P < .05$).

Second Instar

Percent survival of second instar larvae was affected by all three treatments (Table 1). Survival was affected by the interaction between pH and phenol, phenol and NaCl, and between the three factors. Percent survival was greater at pH 7.2 (Table 2) and survival was generally higher in units receiving NaCl. 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 ($P < .05$).

TABLE 1. 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 2. 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	5.99*	23.76*
	7.2	94.83	69.46	53.97	17.07	5.93*	23.14*
	8.2	84.80	45.35	26.77	0.92	6.03*	22.84*
Phenol (mg/1)	0	89.26	54.97	38.71*	10.41*	5.91	22.29*
	10	93.46	65.06	49.44*	15.15*	5.92	21.36*
	20	92.61	64.37	48.91*	18.65*	6.07	25.40*
	30	87.95	55.27	36.18*	15.91*	6.19	23.62*
NaCl (mg/1)	0	89.19	54.00	37.10	15.59*	5.94*	21.62
	600	92.45	65.58	49.52	14.44*	6.04*	24.86

* No significant difference among means of different levels for a particular variable
1, 2, 3, 4 = Instar stages, A = Adult

Third and Fourth Instar

Survival of third and fourth instar larvae was affected by pH and NaCl (Table 1). Newman-Keuls test indicated the means at pH 6.2 and 7.2 (Table 2) were significantly higher than the mean at pH 8.2 ($P < .05$), but not significanly different from each other. Survival was not affected by phenol ($P < .05$). A significantly higher survival occurred with NaCl.

Adults

The percent survival to the adult stage was affected by but not significanly influenced by phenol and NaCl (Table 1). A significant three factor interaction indicated survival to the adult stage was affected by the particular combination of the three factors. As the pH increased, survival decreased substantially (Table 2). Newman-Keuls test indicated a significant difference among survival means 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.

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 1). Since main treatment effects were not significant, these

data were not included on Table 2. 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 trend without NaCl was for higher caloric means at pH 6.2 and 8.2 with lower caloric values at pH 7.2.

Fourth Instar

Phenol affected the caloric content of fourth instar larvae (Table 1). The caloric content was also affected by the interaction between phenol and NaCl. Newman-Keuls test indicated a significant difference among the means for caloric content (Table 2) 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.

Lipid and Nitrogen

The lipid content of fourth instar larvae was affected by NaCl (Table 1). The lipid content of the larvae was also affected by the interaction between pH and NaCl. Newman-Keuls test indicated significantly higher lipid means (Table 2) with NaCl ($P < .05$).

Nitrogen content of fourth instar larvae was affected by the interaction between pH and phenol and the three factor interaction (Table 1).

DISCUSSION

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).

Chironomus attenuatus has little direct effect on the degradation of phenolic compounds (Thornton 1973). 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 (Kimerly 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 mocrobes from the water.

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